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GENETIC AND CELLULAR STUDIES OF FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

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ABSTRACT

Familial hemophagocytic lymphohistiocytosis (FHL) is a rare autosomal recessive and genetically heterogeneous disorder of immune dysregulation with an incidence of 1/50000 live births that is inevitably fatal without appropriate treatment. The disease is characterized by fever, hepatosplenomegaly, cytopenias, hyperferritinemia, hypertriglyceridemia, hypofibrinogenemia and, sometimes, hemophagocytosis in bone marrow and/or other organs such as liver, spleen or lymph nodes.

Three genes have so far been linked to the disease: *PRF1*, *UNC13D* and *STX11*. In this thesis the mutational spectrum and clinical implications of *UNC13D* and *STX11* mutations in a well characterized cohort of patients were studied. Moreover, functional cellular studies with focus on natural killer (NK) cell activity and cytotoxic lymphocyte degranulation was performed in patients with mutations affecting these three genes. In addition, genotype-phenotype correlations in a large cohort of patients was studied.

The frequency of bi-allelic *STX11* mutations in our cohort of *PRF1*-negative families was 14%. Some affected patients had a remarkably less severe disease course than most FHL patients, including long periods of remission without therapy. However, a few patients developed secondary MDS/AML. Although this could be attributed to the treatment provided including etoposide, it is also possible that mutations affecting cytotoxic functions may affect the surveillance of transformed cells (paper I).

The localization and function of the protein syntaxin-11 encoded by the gene *STX11* was previously unknown. We report that the protein is expressed in cytotoxic T cells as well as NK cells, and that NK cells from patients with biallelic *STX11* mutations fail to degranulate when encountering susceptible target cells. The same pattern is seen in patients carrying *UNC13D* mutations whereas patients carrying *PRF1* mutations have a normal degranulation pattern. Notably, when stimulated with IL-2, syntaxin-11 deficient cells regained their cytotoxic capacity and this was also observed in a patient carrying a bi-allelic *UNC13D* mutation (paper II).

We identified six different *UNC13D* mutations affecting altogether 9/38 individuals (24%) in 6/34 (18%) unrelated *PRF1/STX11*-negative families. Four novel mutations were revealed. The age at onset varied from birth to 14 years in the patients carrying bi-allelic *UNC13D* mutations, high-lighting that FHL should be considered not only in infants but also in adolescents, and possibly young adults, presenting with fever, splenomegaly, cytopenia, hyperferritinemia, and/or CNS symptoms (paper III).

Since hemophagocytic lymphohistiocytosis (HLH) is a heterogeneous disease with regard to genotype and phenotype, we studied 76 patients with HLH in order to search for genotype-phenotype correlations. Patients carrying *PRF1* mutations had significantly higher risk of early onset (age <6 months) compared with patients carrying mutations in *STX11* [adjusted odds ratio 8.23 (95% CI=1.20-56.40), p=0.035]. Moreover, patients with *STX11* mutations had a decreased risk of pathological CSF at diagnosis compared to patients without any known biallelic mutation [adjusted odds ratio 26.37 (95% CI=1.90-366.81), p=0.015] (paper IV).

Key words: Familial hemophagocytic lymphohistiocytosis, *PRF1*, *STX11, UNC13D*, degranulation, genotype, phenotype.

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. **Rudd E, Ericson KG, Zheng C, Uysal Z, Özkan A, Gürgey A, Fadeel B, Nordenskjöld M, and Henter J-I**
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II. **Bryceson YT, Rudd E, Zheng C, Edner J, Ma D, Wood SM, Bechensteen AG, Boelens JJ, Celkan T, Farah RA, Hultenby K, Winiarski J, Roche PA, Nordenskjöld M, Henter J-I, Long EO, and Ljunggren H-G**
Defective cytotoxic lymphocyte degranulation in syntaxin-11 deficient familial hemophagocytic lymphohistiocytosis 4 (FHL4) patients.
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III. **Rudd E, Bryceson YT, Zheng C, Edner J, Wood SM, Ramme KG, Gavhed S, Gürgey A, Hellebostad M, Bechensteen AG, Ljunggren H-G, Fadeel B, Nordenskjöld M, and Henter J-I**
Spectrum and clinical and functional implications of *UNC13D* mutations familial hemophagocytic lymphohistiocytosis.
J Med Genet. In press.

IV. **Horne AC, Ramme KG, Rudd E, Zheng C, Wali Y, al-Lamki Z, Gürgey A, Yalman N, Nordenskjöld M, and Henter J-I**
Characterization of *PRF1*, *STX11* and *UNC13D* genotype-phenotype correlations in familial hemophagocytic lymphohistiocytosis.
Manuscript. Submitted.

Papers I-III were reprinted with permission from the publishers.

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LIST OF ABBREVIATIONS

BMT	Bone marrow transplant
CHS	Chédiak-Higashi syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T-lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
FHL	Familial hemophagocytic lymphohistiocytosis
GS	Griscelli syndrome
GRZB	Granzyme B
HLH	Hemophagocytic lymphohistiocytosis
IAHS	Infection-associated hemophagocytic syndrome
IFN-γ	Interferon-gamma
IL	Interleukin
LCMV	Lymphocytic choriomeningitic virus
MAS	Macrophage activating syndrome
MAHS	Malignancy-associated hemophagocytic syndrome
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
NK	Natural killer
OMIM	Online Mendelian Inheritance in Man
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRF1	Perforin
RNA	Ribonucleic acid
SCT	Stem cell transplantation
SNARE	Soluble-N-ethylmaleimide-sensitive factor attachment protein
STX11	Syntaxin-11
TNF-α	Tumour necrosis factor-alpha
XIAP	X-linked inhibitor of apoptosis protein
XLP	X-linked lymphoproliferative syndrome

1 FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS (FHL)

1.1 HISTORICAL BACKGROUD

In 1952 James Farquhar and Albert Claireaux for the first time described a familial disorder they called Familial Hemophagocytic Reticulosis [Farquhar & Claireaux 1952]. In this first report they described a family in which two children suffered from fever, hepatosplenomegaly, and café-au-lait pigmentation of the skin. A few years later they described another child from the same family who also fell ill with the same condition [Farquhar & Claireaux 1958].

The difficulty of diagnosing a rare disease such as this is illustrated by the rarity of the reports in the early fifties and sixties. As the knowledge of the condition is increasing, the diagnosis is becoming more and more recognized, and from the early eighties over 500 reports have been published about this disease.

1.2 HEMOPHAGOCYTIC SYNDROMES

Hemophagocytic lymphohistiocytosis (HLH) includes the familial form, familial hemophagocytic lymphohistiocytosis (FHL), as well as secondary forms of HLH including infection-associated hemophagocytic syndrome (IAHS) and malignancy-associated hemophagocytic syndrome (MAHS) [Henter et al 1991b]. HLH is also sometimes seen in children with systemic onset juvenile rheumatoid arthritis (sJRA) and the condition is then called macrophage activating syndrome (MAS), which is also a form of secondary HLH [Ramanan & Baildam 2002].

The secondary forms occur in all age groups ranging from early childhood [Edner et al 2007] to adults [Takahashi et al 2001]. The secondary form is most commonly triggered by an infectious agent, but sometimes the triggering factor is unknown. The exact incidence of secondary HLH is unknown and one might expect a certain degree of under-diagnosis since the condition is not well

recognized and due to the fact that it often presents in patients with a severe condition that might “mask” the HLH.

In addition to FHL, other genetic immune deficiencies may be associated with hemophagocytic syndromes. Chédiak-Higashi syndrome (OMIM 214500) with a genetic defect located in the *LYST* gene results in symptoms such as oculocutaneous albinism and bruising [Janka 2007]. These children’s leukocytes show giant lysosomes as a result of deficient lysosome fusion with the plasma membrane. Griscelli syndrome type 2 (OMIM 607624) is a result of mutations in the *RAB27A* gene. Affected children display hypopigmentation and sometimes hemophagocytosis [Janka 2007]. Finally, X-linked lymphoproliferative syndrome (XLP) (OMIM 308240) is characterized by an increased sensibility to Epstein-Barr (EBV) virus infections. The symptoms include fulminant mononucleosis, an increased risk to develop lymphoma and a hemophagocytic syndrome [Janka 2007]. XLP is caused by mutations in the *SH2D1A* gene. Recently a second gene causing XLP has been identified, *XIAP,* encoding the protein X-linked inhibitor of apoptosis protein [Rigaud et al 2006].

1.3 CLINICAL PRESENTATION OF FHL

Familial hemophagocytic lymphohistiocytosis (FHL) is an autosomal recessive, genetically heterogeneous disorder of immune dysregulation with an incidence of 1/50000 live births [Henter et al 1991a]. The disease is characterized by fever, hepatosplenomegaly, cytopenias, hyperferritinemia, hypertriglyceridemia, hypofibrinogenemia and, sometimes, hemophagocytosis in bone marrow and/or other organs such as liver, spleen or lymph nodes [Henter et al 2004, Janka 2005, Filipovich 2006]. The symptoms are driven by a storm of inflammatory cytokines, fuelled by a massive proliferation of activated macrophages and lymphocytes. When assessed, the patients show high levels of various inflammatory cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and a number of interleukins (IL). Apart from this, the patients may also develop jaundice, skin rash and edema as well as neurological abnormalities ranging from irritability and hypotonia to seizures,

cranial nerve deficits, and ataxia [Henter et al 2004, Janka 2005, Filipovich 2006].

In a study of 23 children with FHL, neurological symptoms were reported in 15 patients [Henter & Nennesmo 1997b]. Post mortem neuropathological examination revealed a wide variety of different findings and macroscopically the most common finding was either edema or that the brain had a normal appearance. In some patients with a severe neurological presentation, destruction of the white matter was seen. When hemophagocytosis was present, it was mainly located in the meninges.

Cerebrospinal fluid (CSF) analysis in the patients varied from moderate pleocytosis and elevated protein content to more pronounced alterations. However, in some children with central nervous system (CNS) disease, CSF analysis was normal [Henter & Nennesmo 1997b]. CNS disease as the only symptom at onset is rare, with only a few cases reported [Henter & Elinder 1992, Rostasy et al 2004]. The CNS symptoms in FHL may also sometimes mimic other disorders such as septic embolism to the brain [Turtzo et al 2007] or other neurological disorders [Feldmann et al 2005].

The symptoms of FHL can be tied directly to the over expression of cytokines. The fever is caused by high levels of inflammatory cytokines. The hypertriglyceridemia is caused by a decrease in lipoprotein lipase activity, a direct result of high levels of TNF-α which is known to lower the activity of the lipase [Henter et al 1991c]. Activated macrophages secrete ferritin but also plasminogen activator which leads to high levels of plasmin that cleaves fibrinogen. Cytopenias are attributed to high levels of TNF-α and IFN-γ, but also to hemophagocytosis. Since not all patients show signs of hemophagocytosis at onset, despite the presence of cytopenias, the hemophagocytosis may play a secondary role in the initial stage of the disease with regard to this finding. The neurological symptoms and the hepatosplenomegaly are most likely due to the infiltration of activated macrophages and lymphocytes in these organs [Henter & Nennesmo 1997b].

The majority of the children have their disease onset during infancy or early childhood [Henter et al 2002, Henter et al 2007]. Notably, there have also been some reports of onset in adolescence and early adulthood [Allen et al 2001, Ueda et al 2007, paper III]. The diagnosis of HLH is made in accordance with diagnostic guidelines set up by the Histiocyte Society (Table 1). These guidelines published in 1991 [Henter et al 1991b, Henter et al 1997a] are based on clinical, laboratory and histological findings. As the knowledge about the disease grew, and more patients were diagnosed, the guidelines were revised in 2004 [Henter et al 2007]. The HLH-2004 guidelines include the 1991 criteria, but also three new criteria; high ferritin, low or absent NK- cell activity and, finally, high levels of sIL-2 receptor (sCD25).

Table 1: *Diagnostic criteria for Hemophagocytic Lymphohistiocytosis as outlined in the HLH-2004 protocol*

A molecular diagnosis consistent with HLH
or
Diagnostic criteria for HLH fulfilled (5 out of 8 criteria below):
-Fever
-Splenomegaly
-Cytopenias (affecting ≥ 2 of 3 lineages in peripheral blood)
-Hemoglobin < 90 g/L (in infants < 4 weeks; hemoglobin < 100 g/L)
-Platelets < 100x10^9/L
-Neutrophils < 1.0x10^9/L
-Hypertriglyceridemia and/or hypofibrinogenemia
-Fasting triglycerides ≥ 3.0 mmol/L
-Fibrinogen ≤ 1.5 g/L
-Hemophagocytosis (in bone marrow or spleen or lymph nodes)
-Low or absent NK cell activity * (according to local laboratory reference)
-Ferritin (≥ 500 microgram/L) *
-Soluble CD25 (≥ 2400 U/ml) *

The first diagnostic guidelines for HLH were presented in 1991 and indicated five criteria [Henter et al 1991b]. They were revised in the HLH-2004 protocol to include three new criteria, and altogether five of the eight criteria are to be fulfilled [Henter et al 2007].

*Indicates new criteria for HLH-2004.

NK-cell activity in FHL patients can be subcategorized into four groups, 1,-2,-3, and 4, depending on the pattern of cytotoxicity, as proposed by Schneider and colleagues [Schneider et al 2002]. The groups are characterized by four distinct cellular defects. (i) Cells that regained their capacity to lyse cells after agglutination with phytohaemagglutinin (PHA) were named type-1 deficiency. (ii) Cells that regained function after stimulation with interleukin-2 (IL-2) were named type-2 deficiency. This phenomenon is seen in patients that carry *STX11* and in some patients that carry *UNC13D* mutations [paper II, paper III]. (iii) Cells that were able to regain function after prolonged stimulation were named type-4 deficiency and finally, (iiii) cells where no reconstitution was seen regardless of stimuli were named type-3 deficiency (Table 2).

Table 2: *Sub-typing of cellular defects in FHL*

NK cell cytotoxicity activity defect						
Sub types	**4h**			**16h**		
	Resting	*PHA*	*IL-2*	*Resting*	*PHA*	*IL-2*
Type-1	yes	no	yes	no/yes	no	yes
Type-2	yes	yes	no	yes	no/yes	no
Type-3	yes	yes	yes	yes	yes	yes
Type-4	yes	yes	yes	no/yes	no	no

PBL were used as effector cells, and K562 cells were used as targets.
PHA= phytohaemagglutinin
Adapted from Schneider et al 2002

1.4 TREATMENT OF FHL

Defective NK cell cytotoxicity and hypercytokinemia are important hallmarks of the disease [Henter et al 1991c]. FHL is typically fatal unless treated, and before the introduction of the international HLH-94 treatment protocol developed by the Histiocyte Society [Henter et al 1997a], the treatment of the children with HLH/FHL was up to the treating physician. Since there is no way to distinguish secondary HLH from FHL, the suggested treatment is identical for all patients with severe, persistent or relapsed HLH for the initial 8 weeks, as it has been shown that patients with secondary HLH also respond well to treatment with the HLH-94 protocol [Imashuku et al 1999]. After these initial 8 weeks, treatment is

stopped if the patient has achieved remission and there is no family history of HLH and no causative mutation revealed. If the patient relapses, he or she will go on to continuation therapy, while those who do not relapse are considered to have the secondary HLH and these patients are taken off therapy.

When the HLH-94 protocol was initiated it had been known that the use of epipodophyllotoxins (etoposide) in combination with steroids had a favorable effect on the outcome of the disease with a prolonged survival [Ambruso et al 1980, Fischer et al 1985, Henter et al 1991d]. In addition to the HLH-94 and HLH-2004 protocols there is one other treatment protocol presented, which is based on anti-thymocyteglobulin [Mahlaoui et al 2007]. The rationale for using etoposide in FHL is that this is a disorder characterized by deficient apoptosis triggering [Fadeel et al 1999] and etoposide is an excellent inducer of apoptosis [Walker et al 1991, Negri et al 1995]. Due to this, etoposide and dexamethasone were used upfront in HLH-94, in combination with Cyclosporin A (CSA) that was initiated after eight weeks. The use of dexamethasone was favored to prednisolone as dexamethasone passes better through the blood-brain barrier. In children with progressive CNS disease, intrathecal methotrexate was added. Even though the results using this protocol with regard to survival are favorable, it is not a cure for the disease. The only cure for FHL is hematopoetic stem cell transplant (SCT) which was introduced as a treatment for FHL in 1986 by Fischer and colleagues [Fischer et al 1986, Bolme et al 1995].

The follow-up of the HLH-94 protocol showed that even though survival had increased markedly, some patients died during the first two months of therapy. These patients had mainly died due to disease. Therefore, it was decided that the treatment intensity should be increased up-front, and in the HLH-2004 protocol CSA, an immunosuppressant drug that does not induce myelotoxicity like etoposide/tenoposide, is recommended from start of therapy [Henter et al 2007].

Neurological sequelae is a serious problem in patients with FHL and due to this there is also a recommendation in the HLH-2004 protocol to do brain MRI as

well as CSF analysis at onset and at signs of neurological symptoms in the patients to evaluate CNS-involvement.

Stem cell transplantation is still the only known cure for FHL to date, while patients with secondary HLH do not need SCT. Since these conditions can be difficult to distinguish from each other, sub-typing of natural killer cell cytotoxicity (Table 2) may provide a therapeutic guidance as to if the patients should go to SCT. In a large study of 65 patients with FHL, it was shown that no patients with NK-cell deficiency type 3 attained remission after stopping therapy without SCT, in contrast to patients with NK-cell deficiencies type 1, 2 and 4, where 45% attained remission without SCT [Horne et al 2005a]. These data suggest that patients can be evaluated with NK-cell activity as a step in the diagnosis and subsequent treatment of the HLH/FHL.

1.5 PROGNOSIS AND OUTCOME

In a retrospective study from 2002 [Henter et al 2002], 113 children, all diagnosed with HLH and treated with the HLH-94 protocol, were studied with regard to overall survival. The estimated 3-year probability of survival of all 113 children was 55% ± 9% (95% confidence interval). Overall, the 3-year probability of survival was significantly better in children at least 1 year old at onset (72% ± 13%), compared to children younger than 1 year old (42% ± 12%). Of the 113 children 63 (56%) were alive at the latest follow-up (median follow-up was 37.5 months). Forty of these children had undergone a SCT. Of the children that were deceased, 50% had undergone a SCT (25/50).

The 3-year probability of survival for patients that had undergone a SCT 1995-2000 was 64%, and the best outcome was seen in patients that were transplanted with matched related donors (MRD) (71 $\pm$ 18%) or matched unrelated donors (MUD) (70 $\pm$ 16%). Data from the HLH-94 protocol also suggested that a certain degree of disease activity at time for transplant should not exclude the patients from this treatment [Horne et al 2005b, Henter et al 2007]. The studies also show that a persistent disease activity at 2 months after

start of HLH treatment suggests a worse long-term prognosis [Horne et al 2005b]. As mentioned previously, CNS sequelae is a serious problem in patients with FHL. In a recent study the frequency of CNS involvement was found to be 63% when defined as neurological symptoms and/or abnormal CSF at diagnosis. In the cohort studied, which included 193 patients, altogether 15% had neurological sequelae at follow-up (median 5.3 years) and patients with abnormal CSF at diagnosis had a significantly increased mortality when compared to patients with a normal CSF at diagnosis [Horne et al 2007].

2 GENETICS OF FHL

2.1 GENERAL BACKGROUND

Deoxyribonucleic Acid or DNA constitute the building stones of all human life. It regulates what we are, from the inside and out. There are approximately 20,000-25,000 genes in the human genome, built up of some 3 billion base pairs.

The hunt for mutations giving rise to FHL started in the early fifties when Farquhar and Claireaux discussed the possibility that the disease was a hereditary disorder [Farquhar & Claireaux 1952], but at the time of their report the structure of DNA was not known, and the field of genetics was young. There would be a considerable amount of time before the report of the first FHL causing gene was published [Stepp et al 1999] even though treatment for the disease with the HLH-94 protocol had been in use for many years [Henter et al 1997a].

2.2 FHL DISEASE LOCI

With the help of linkage analysis, two FHL loci were identified in 1999 and for the first time, a gene defect could be linked to the disease. The two loci were located in the chromosome regions 9q21.1-22 (FHL1, MIM 267700) and 10q21-22 (FHL2, MIM 603553)**.** Whereas a gene accountable for the disease at the 9q21.1-22 locus so far has not has been identified [Ohadi et al 1999], loss-of-function mutations in the perforin (*PRF1*) gene located on chromosome 10q22 were revealed to cause FHL2 [Stepp et al 1999]. Notably, the *PRF1* gene had previously been predicted as a possible gene in FHL by Fadeel and colleagues [Fadeel et al 1999]. Subsequently, two additional loci on chromosome 17q25 and 6q24 have been associated with FHL. Mutations impeding the function of the Munc13-4 encoding gene *UNC13D* located on chromosome 17q25 cause FHL3 (MIM 608898) [Feldmann et al 2003] while loss-of-function mutations in

the syntaxin-11 (*STX11*) gene, located on chromosome 6q24, are associated with FHL4 (MIM603552) [zur Stadt et al 2005] (Table 3).

Table 3: *Chromosome location and genes currently known to cause inherited hemophagocytic syndromes (HLH)*

Disease	Chromosome	Gene	Reference
FHL 1	9q21.3-q22	Unknown	Ohadi et al 1999
FHL 2	10q22	*PRF1*	Stepp et al 1999
FHL 3	17q25.1	*UNC13D*	Feldmann et al 2003
FHL 4	6q24	*STX11*	zur Stadt et al 2004
Griscelli type II	15q21	*RAB27A*	Menasche et al 2000
Chédiak-Higashi	1q42.1-q42.2	*LYST*	Barbosa et al 1997
XLP	Xq25 Xq25	*SH2D1A* *XIAP*	Coffey et al 1998 Rigaud et al 2006

The perforin gene is transcribed by cytotoxic T cells and NK cells. These cytotoxic lymphocytes store perforin in secretory lysosomes, specialized granules that mediate cellular cytotoxicity. Perforin facilitates granzyme-mediated apoptosis of target cells [Voskoboinik et al 2006a]. Mutations in *PRF1* have been identified in approximately 20-50% of FHL patients, depending on the ethnic origin of the patients [Stepp et al 1999, Göransdotter et al 2001, Suga et al 2002, Molleran et al 2004] (Figure 1).

Munc13-4 is ubiquitously expressed and implicated in regulating membrane fusion events. Munc13-4 is required for the vesicle-plasma membrane fusion during exocytosis of perforin-containing granules by cytotoxic T cells and NK cells [Feldmann et al 2003]. Recent studies have shown that Munc13-4 has an intracellular distribution distinct from perforin- and granzyme-containing granules [Menager et al 2007]. It has been proposed that Munc13-4 may play two different roles in the cytolytic pathway; first assisting in the availability of a pool of late endosomal vesicles at the plasma membrane independently of Rab27a and, in a second step, interacting with Rab27a at the plasma membrane, there priming the vesicles for exocytosis [Menager et al 2007] (Figure 1).

Syntaxin-11 is a widely expressed member of the syntaxin family of proteins containing soluble N-ethylmaleimide-sensitive factor attachment protein receptor

(SNARE) domains. Syntaxin-11 is expressed in placenta, lung, heart, and lymphoid organs, such as the thymus, spleen, lymph nodes [Tang et al 1998, Prekeris et al 2000] and is abundant in T cells, NK cells, as well as macrophages [Prekeris et al 2000, paper II]. SNARE proteins mediate membrane fusion events via interaction between SNARE domains of proteins localized on opposite membranes. Syntaxin-11 has been ascribed a role in secretory lysosome exocytosis, as cytotoxic lymphocytes from patients with mutations in *STX11* demonstrate defective degranulation [paper II] (Figure 1).

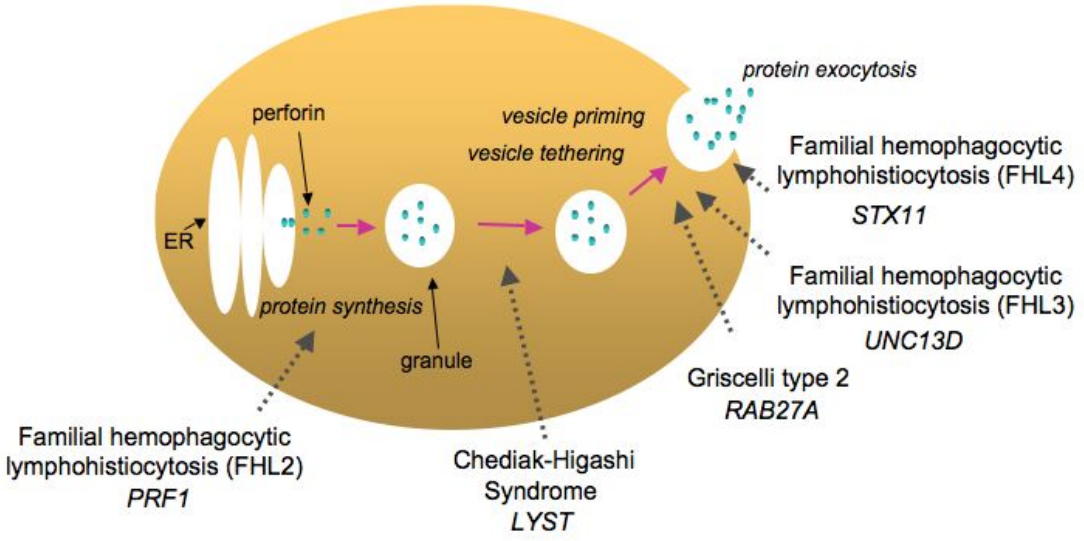

Figure 1: *Mechanisms of defective cellular cytotoxicity in hemophagocytic disorders.*

The genes known to be involved in hemophagocytic syndromes. *PRF1* encodes the protein perforin, defective in FHL2. *UNC13D* encodes the protein Munc13-4 defective in FHL3. *STX11* encodes the protein syntaxin-11 defective in FHL4. *RAB27A* is defective in Griscelli type 2, and *LYST* is defective in Chédiak Higashi syndrome.

Courtesy of Bengt Fadeel, Karolinska Institutet

These three genes (*PRF1*, *UNC13D, STX11*) account for up to 70% of the patients with FHL depending on ethnic origin and one can expect that one or more genes are involved in the pathology behind the disease. There have been

several attractive candidate genes investigated without finding any causative mutations in the genes studied. Granulysin and granzyme B are known to induce apoptosis in cells targeted by cytotoxic T lymphocytes [Kaspar et al 2001, Bolitho et al 2007]. In 2003, a cohort of 16 FHL patients were *PRF1* mutations had been excluded where studied with regard to these genes [Ericson et al 2003], but no mutations were found to segregate within the families studied and the data did not support mutations in these genes as being causative of FHL. Recently another candidate gene study has been done were *SRGN*, (serglysin) *AP3B1*, *ARF6* and *SH2D1A* were sequenced [Ma et al 2007]. Serglycin, encoded by the *SRGN* gene, is a glycoprotein. Granzyme B binds to serglycin to form a macromolecular complex entering into target cells [Raja et al 2002]. As a result, serglycin may be involved in the granule mediated apoptosis by acting as a carrier directing Granzyme B from cytotoxic granule to target cells. Arf6, coded by the *ARF6* gene, is a member of the ARF (ADP-ribosylation factor) family and plays a crucial role for cytotoxic granule secretion in human NK cells [Galandrini et al 2005]. *SH2D1A* encoding SAP (signaling lymphocyte activation molecule-associated protein) [Engel et al 2003] is mutated in XLP and it has been shown that a subpopulation of patients fulfilling the clinical criteria of HLH harbor *SH2D1A* mutations [Aricò et al 2001]. Hermansky-Pudlak syndrome type 2 (HPS2) is an autosomal recessive disease caused by a defect of the gene *AP3B1* coding for the beta subunit of the adaptor protein (AP)3 complex [Dell'Angelica et al 1999]. HPS2 patients present with a dramatic reduction of cytolytic activity of NK cells and CTLs. As a result of the cytotoxicity deficiency, HPS2 patients may share some clinical phenotypes with HLH [Enders et al 2006]. However, no bi-allelic mutations in the four genes studied were found [Ma et al 2007].

3 CYTOTOXICITY AND FHL

3.1 GENERAL BACKGROUND

The identification of mutations in *PRF1*, *UNC13D*, and *STX11,* in addition to studies of the biological function of their protein products, provide a compelling link between impaired lymphocyte cytoxicity and FHL. Cytotoxic T lymphocytes and NK cells play a crucial role in the surveillance of transformed cells and the detection of cells infected with intracellular pathogens. When this arm of the immune system encounters a cell that is transformed or infected with an intracellular pathogen, a massive reaction starts with production of cytokines, and a proliferation of different cell types to fight the intruder. The killing of the cell is mediated through the deposit of toxic granules containing proteases such as granzymes and the membrane disruptive protein perforin [Voskoboinik & Trapani 2006b], and through the death receptor ligand pathways.

3.2 PERFORIN DEPENDENT CYTOTOXICITY

Perforin (PRF) is a pore forming protein with a molecular weight of approximately 67kD and it is encoded by a single gene (*PRF1*) located on chromosome 10. For maturation of the protein, cleavage is needed since the protein is encoded in an immature precursor state. The membranolytic activity is exerted in the immunological synapse. Perforin activity is Ca^{2+} and pH-dependent and when stored in the effector cell, it is in an environment with low pH (<5) and low Ca^{2+} content. This is thought to protect the effector cell from "self-lysis". Upon stimulation, the cytotoxic lymphocyte granules containing perforin, granzymes and lysosomal proteins such as Lamp1 (CD107a) polarize towards the immunological synapse. Well in place, they fuse with the membrane and release their contents. In the extracellular compartment, where the free Ca^{2+} concentration is high and the pH is higher than in the granules, the perforin can exert its lytic activity toward the target cell [Voskoboinik & Trapani 2006b].

Perforin is needed for the delivery of granzymes but this delivery occurs has been debated. Initially, it was thought that perforin forms a pore that the granzymes passed through to reach the target cell and there induce apoptosis. Recent studies, however, have proposed an alternative model, as a complement to the initial "pore forming" model. In this model, the granzymes are thought to enter the target cell through pinocytosis, and then being released within the target cell through a perforin-dependent pore mechanism in the endosome [Bolitho et al 2007].

Granzyme B can induce apoptosis in two ways [Bolitho et al 2007], either through the cleavage of pro-caspase-3 to caspase-3, which in turn cleaves ICAD (Inhibitor of CAD) to release CAD (caspase activated DNase) that translocates into the nucleus of the target cell and induces apoptosis through DNA fragmentation. Alternatively, it induces apoptosis through the cleavage of Bid which in turn interacts with Bax and Bak on the mitochondrial membrane, resulting in a loss of membrane integrity, and the release of cytochrome c into the cytoplasm. Cytochrome c is a part of the apoptosome complex, which in turn activates pro-caspase 3 that mediates the cleavage of ICAD with the subsequent translocation of CAD to the nucleus. Granzymes A and M are thought to induce apoptosis in a caspase- independent manner [Waterhouse et al 2006, Bolitho et al 2007].

3.3 GRANULE EXOCYTOSIS

The fusion of the lytic granules with the plasma membrane is mediated through interaction of numerous proteins, among them proteins encoded by *UNC13D*, *STX11, RAB27A,* and *LYST* (Figure 1).

Munc13-4, encoded by the gene *UNC13D,* is required for the vesicle-plasma membrane fusion during exocytosis of perforin-containing granules by cytotoxic T cells and NK cells [Feldmann et al 2003], while syntaxin-11 has been ascribed a role in secretory lysosyme exocytosis [paper II]. Recent studies have shown that patients with mutations in either of these two genes have a defective

degranulation, using LAMP1 or CD107a as a marker, implying that Munc13-4 as well as syntaxin-11 are needed for the delivery of perforin and granzymes to the target cell [Marcenaro et al 2006, paper II]. When the first paper was published on *STX11*, syntaxin-11 was not found in cytotoxic lymphocytes but only in antigen presenting cells [zur Stadt et al 2005]. This rendered an array of different theories regarding the pathogenesis behind FHL 4. Syntaxin-11 had been proposed to have a regulatory role rather than being involved in the membrane fusion process [Valdez et al 1999] and due to the then known expression of syntaxin-11 in dendritic cells (DCs) [Prekeris et al 2000], we speculated that syntaxin-11 might be involved in the regulation of NK cells and CTLs by affecting the interaction of these cells with DCs [paper I]. Studies had demonstrated that NK cells participate directly in adaptive immune responses, mainly by interacting with DCs and such interactions can positively or negatively regulate DC activity [Moretta et al 2006]. Reciprocally, DCs regulate NK cell function [Raulet 2004]. Later on we demonstrated for the first time that syntaxin-11 is indeed expressed in NK cells and $CD8^+$ T cells, and that a loss of function in this gene impairs cytotoxic lymphocyte degranulation [paper II].

The protein Rab27a encoded by the *RAB27A* gene is crucial for vesicle exocytosis. Studies have shown that Rab27a interacts with Munc13-4 at the plasma membrane to mediate vesicle exocytosis from the cytotoxic lymphocyte [Menager et al 2007]. Mutations in this gene do not seem to affect the polarization of the vesicle, but the membrane docking of the vesicles [Stinchcombe et al 2001]. Patients with *RAB27A* mutations suffer not only from hemophagocytic syndrome, but also from fever, neutropenia, thrombocytopenia and partial albinism. This is due to the fact that the melanocytes also depend on Rab27a for vesicle exocytosis, but through a different mechanism, in which it interacts with Myosin Va and melanophyllin prior to exocytosis [Stinchcombe et al 2004].

The *LYST* gene encodes a 425 kDa protein needed for exocytosis, the exact function of which is unknown. Patients with a deficient protein fail to degranulate

and develop Chédiak-Higashi syndrome and they also have giant lysosomes located at the plasma membrane [Tchernev et al 2002].

3.4 CYTOKINE RESPONSES

When cytotoxic lymphocytes encounter an infected cell, they produce a vast array of inflammatory cytokines including IFN-γ, IL-2, IL-6, IL-10 and TNF-α. These cytokines will in turn activate the appropriate cells to fight off the intruder, and their abundance will give rise to the symptomatic picture seen in FHL, as described above.

It has been shown in perforin knockout mice infected with murine cytomegalovirus (MCMV) that perforin is important not only for the induction of apoptosis of the target cell but also for the down regulation of the immune response after the infection has been cleared [van Dommelen et al 2006]. In that study the knockout mice not only developed an HLH-like phenotype but they also had a higher frequency of NK cells, $CD8^+$ T cells, $CD11b^+$ cells and $CD11c^+$ cells, implying that perforin is needed to clear not only infected cells but also activated immune effector cells. Unlike previous studies [Jordan et al 2004], the main contributor to the HLH was found to be TNF-α, compared to mice infected with lymphocytic choriomeningitic virus (LCMV), where the main contributor to the HLH phenotype was found to be IFN-γ [Jordan et al 2004]. Which of these cytokines that play the most important roll in humans is unknown. Perhaps it is tied to the infectious agent as shown in mice [Crozat et al 2007] but further studies are warranted.

The disruption of this delicate machinery including proteins for membrane fusion and cytokines to activate the immune system will of course lead to devastating consequences, not only through the inability to eliminate intracellular pathogens or transformed cells, but also through the disruption of immune homeostasis.

4 AIMS OF THE STUDY

The ultimate aims of the study were to contribute to better diagnosis, and also better treatment, of patients with HLH and to provide novel insights to the biology of cytotoxic lymphocytes.

The specific aims of the study were:

- To determine the frequency and clinical implications of mutations in the *STX11* gene in patients with FHL.
- To study the effects of *STX11* gene mutations in cytotoxic cells from patients with FHL.
- To study the frequency and clinical implications of mutations in the *UNC13D* gene in patients with FHL.
- To study the correlation between genotype and phenotype in FHL patients/families.

5 PATIENTS AND METHODS

5.1 STUDY POPULATION

The patients studied were all referred to the Karolinska University Hospital for genetic studies. They all had familial disease or they fulfilled the diagnostic criteria set up by the Histiocyte Society in 1991 [Henter et al 1991b] or the revised diagnostic criteria set up in 2004 [Henter et al 2007]. For control samples, blood from healthy blood donors and healthy children was obtained after informed consent.

5.2 METHODS

5.2.1 Mutation detection of *PRF1, STX11* and *UNC13D* (papers I-IV)

Genomic DNA was isolated from peripheral blood or cultured fibroblasts according to standard procedures. Primers were designed for amplification and direct DNA sequencing of the coding region of the *PRF1*, *UNC13D* and the *STX11* genes. The sequencing was performed on ABI 310, 3130, or ABI 3730 Genetic Analyzers (Applied Biosystems, Foster City, CA), and analyzed either using SeqScape (Applied Biosystems) or by hand.

5.2.2 Cytotoxicity studies (papers II, III)

^{51}Cr labeled K562 target cells were incubated with peripheral blood lymphocytes (PBL) as effector cells. Standard 4-hour ^{51}Cr-release assay was modified in line with previous reports to include also prolonged incubation time of effector and target cells to 16 hours [Schneider et al 2002, Horne et al 2005a]. ^{51}Cr release was analyzed with a gamma-counter. In addition, lymphokine-activated killers (LAK) cells were generated by culturing peripheral blood mononuclear cells of patients in the presence of 400 IU/ml recombinant human interleukin (IL)-2 for 72-hours and thereafter assessed for cytotoxic activity as previously described by Schneider and co-workers [Schneider et al 2002].

5.2.3 Degranulation studies (paper II, III)

For quantification of secretory lysosome exocytosis, PBL were mixed with target cells and supplemented with 2.5 mg/ml of the indicated mAbs for stimulation, as previously described [Betts et al 2003]. Cells were incubated for 2 hours at 37°C 5% CO_2. Thereafter, the cells were spun down, resuspended in PBS, added 2% fetal bovine serum (FBS) and 2 mM EDTA and stained with anti-CD3-PerCP, anti-CD56-PE, and anti-CD107a-FITC mAbs (all BD Bioscience), followed by flow cytometric analysis. Data was analyzed with FlowJo software (TreeStar) as previously described [Betts et al 2003].

5.2.4 Clinical data collection (paper IV)

For the genotype-phenotype study, detailed clinical data of 76 patients from 65 distinct unrelated families were collected, either by retrospectively reviewing patient files and/or by a questionnaire sent out to the physicians treating the respective patients. Information was collected on clinical and laboratory findings at onset of disease, treatment, response to treatment, and long-term outcome. Regarding the other studies in this thesis, clinical data was obtained from the treating physician or from reviewing patient files.

5.2.5 Statistics (paper IV)

Differences in distribution were compared by using the Chi-square test, and when frequencies were small the two-tailed Fisher's exact test was used. Tests for associations between genotype and phenotype were performed by exact Pearson chi-square tests for r × c tables using PROC FREQ in the SAS software. Subsequently, multivariate analysis using logistic regression was performed with age less than six months at diagnosis, pathological CSF and jaundice as dependent variables. The covariates used were genetic mutation group and ethnicity. Logistic regression analyses were carried out using SPSS™ statistical software (version 11.5) (Chicago, IL, USA).

6 RESULTS

Table 4: *Summary of patients with mutations included in paper I, II and III.*

Patient	Gene	Mutation	Ethnicity	Paper
A:1	*STX11*	p.V124Fs	Turkey	I (A:1)
A:2	*STX11*	p.V124Fs	Turkey	I (A:2)
A:3	*STX11*	p.V124Fs	Turkey	I (A:3)
B:1	*STX11*	p.V124Fs	Turkey	I (B:1)
B:2	*STX11*	p.V124Fs	Turkey	I (B:2)
C:1	*STX11*	p.Q268X	Turkey	I (C:1)
D:1	*STX11*	p.Q268X	Turkey	I (D:1)
E:1	*STX11*	p.T37RFsX25*	Lebanon	II (4)
F:1	*STX11*	p.Q268X	Turkey	II (5)
G:1	*PRF1*	p.H222Q	Holland	II (1)
H:1	*PRF1*	p.E317R+D430Y*	Sweden	II (2)
I:1	*UNC13D*	p.R83X*	Holland	III (D:1)
J:1	*UNC13D*	c.2626-1G*	Pakistan	III (A:1)
J:2	*UNC13D*	c.2626-1G*	Pakistan	III (A:2)
K:1	*UNC13D*	p.R782SFsX11	Serbia	III (E:1)
L:1	*UNC13D*	p.R928P*	Turkey	III (C:1)
M:1	*UNC13D*	p.R214X	Turkey	III (B:1)
M:2	*UNC13D*	p.R214X	Turkey	III (B:2)
N:1	*UNC13D*	p.W382X*	Pakistan	II (3),III(F:1)

* *Indicates a novel mutation*

6.1 SPECTRUM OF *STX11* MUTATIONS IN FHL (PAPER 1)

In this first paper, we studied 34 HLH patients from 28 unrelated families in which mutations in *PRF1* had been excluded by DNA sequencing. In the families studied, at least one sibling fulfilled the diagnostic criteria for FHL developed by the Histiocyte Society in 1991 [Henter et al 1991b]. Four children with familial disease were diagnosed in an early stage without fulfilling the criteria, but in these families another child had been diagnosed that fulfilled the diagnostic criteria. In all, familial disease was demonstrated in 10 families. The main group of patients studied originated from Turkey (n=19).

The complete 861 base pair open reading frame of the gene was sequenced in all of the families studied. In a previous report [zur Stadt et al 2005], three different mutations in the *STX11* gene had been described. One was a deletion of AG and CGC at nucleotide positions 369_370 and 374_376, respectively, resulting in a frame shift and subsequently a premature Stop codon. A second mutation described was a nonsense mutation resulting in a change from Glutamine to a Stop at codon 268 and the third was a 19.2 kb genomic deletion affecting the entire *STX11* gene. The first two of these mutations were also present in our cohort studied. We did not find any patients carrying a large deletion.

Mutation spectrum

Mutations in the *STX11* gene were identified in four of the 28 families studied corresponding to 14% of all the non-*PRF1* families in our cohort. All of the families affected were of Turkish origin. We found no *STX11* gene mutations in patients of northern European descent.

One of the families reported, carrying the deletion of five base pairs, had in part been described previously [zur Stadt et al 2005]. We sequenced presumed carriers in this family and showed that the mutation co-segregated completely in a heterozygous state in obligate carriers available for analysis. We also found this mutation in another family of Turkish origin. Apart from this, sequencing also revealed the nonsense mutation described above in two families of Turkish origin. The different mutations are shown in table 4.

Clinical features

Clinical investigations of the families described in paper I showed that some of the patients carrying *STX11* mutations had a milder phenotype than most patients carrying *PRF1* mutations, with long periods of disease-free remission and later onset. On the other hand, two of the patients studied developed secondary myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML).

This could in part be attributed to the fact that the patients had received epipodophyllotoxin treatment. The standard treatment regimen for HLH includes etoposide, which is known for its risk to induce AML [Pui et al 1991, Sandoval et al 1993]. One of the patients was diagnosed before the HLH-94 protocol was taken into use, and this child received very high doses of both etoposide (6.9 g/m^2 i.v. and 13.6 g/m^2 per orally) and tenoposide (3.4 g/m^2 i.v.) [Henter et al 1993]. The second child was treated according to the HLH-94 protocol and received a cumulative dose of etoposide of 3.15 g/m^2. Notably, one cannot rule out the fact that mutations affecting cellular cytotoxicity pathways may result in an impairment of immune surveillance for transformed cells.

6.2 DEFECTIVE CYTOTOXIC LYMPHOCYTE DEGRANULATION IN SYNTAXIN-11 DEFICIENT FHL PATIENTS (PAPER II)

In the first paper to describe *STX11* gene mutations as being causative for FHL [zur Stadt et al 2005], the protein expressed by the gene was reported to be found in monocytes but not in cytotoxic T lymphocytes or NK cells. To determine the expression pattern of *STX11* in cytotoxic T lymphocytes, we purified and amplified RNA from unstimulated NK cells, $CD8^+$ T lymphocytes as well as the NK cell line NK92 by RT-PCR using primers specific for *STX11* and *UNC13D.* We found transcripts of the genes in the cell types studied, as well as a protein corresponding to the molecular mass of *STX11* (35-kDa) in unstimulated NK cells, $CD8^+$ T lymphocytes and the NK cell line NK92 by using Western blot.

To determine the function of *STX11* and the consequences of a loss of *STX11,* we used a previously developed assay [Betts et al 2003] to measure degranulation by assessing CD107a (LAMP-1) expression on the cell surface. CD107a is a transmembrane protein in cytotoxic granules. Upon degranulation this protein will be exposed at the cell surface, making it a good marker for degranulation. The surface CD107a can then be quantified by flow cytometry.

Mutation spectrum

We studied five patients with FHL, all fulfilling the HLH criteria, and all carrying mutations in the known FHL causing genes. Two of the patients carried mutations in their *PRF1* gene, one had a bi-allelic missense mutation (H222Q) and one a compound heterozygous missense mutation (E317R + D430Y). One of the patients carried a bi-allelic nonsense mutation in his *UNC13D* gene (W382X). Finally, we studied two patients with *STX11* mutations, one with a bi-allelic deletion of one nucleotide (T37FsX62) and another with a bi-allelic nonsense mutation (Q268X) (Table 4).

Degranulation and cytotoxicity

Degranulation studies showed that the patients carrying mutations in *STX11* or *UNC13D* had no or very limited CD107a expression on their NK-cells, indicating that these cells did not degranulate, while patients carrying mutations in *PRF1* showed a normal degranulation pattern as compared to healthy controls. All the patients studied expressed normal intracellular levels of CD107a. Flow cytometry revealed absent or very limited perforin expression in lymphocytes from the patients with *PRF1* mutations, but normal expression in the patients carrying *STX11* or *UNC13D* mutations. Western blot showed no or a truncated syntaxin-11 protein in the patients with *STX11* mutations.

As T cell dysfunction is viewed to cause FHL, we also studied degranulation by T cells. Compared to healthy adult donors, healthy infant donor blood showed markedly less CD107a surface expression. This can be explained by the fact that infants have reduced numbers of CD8+CD62L-CCR7 effector cells, in consistency with an immature immune system. Bearing this in mind, we suggest that degranulation studies in infant patients should be done on NK cells rather than T cells.

IL-2 is an important stimulator of the immune system. Due to this fact, we also studied the effects of IL-2 stimulation on cells from our patients. While there was no or little cytolytic activity in the patients with *PRF1* mutations, we noticed a

partial restoration of cytotoxicity in the patients with *UNC13D* or *STX11* gene mutations after stimulation with IL-2. In fact, some of the patients with *STX11* gene mutations developed cytotoxicity levels fully comparable to healthy, age matched donors, which might offer a partial explanation to the milder phenotype seen in some of these children.

Finally, to attain more insight into the functional defects resulting from *STX11* mutations, we studied other cellular functions in the cytotoxic lymphocytes. Our studies showed that cells with defective *STX11* were able to induce intracellular Ca^{2+} mobilization upon stimulation with IL-2. We also studied the polarization of perforin containing granules after stimulation and found that this was not affected, nor does syntaxin-11 co-localize with perforin. Taking these observations into account, we speculate that syntaxin-11 is an important factor in the late step of vesicle fusion or the docking of the vesicle on the plasma membrane.

In summary, in this paper we showed for the first time that the protein expressed by *STX11* is indeed expressed in cytotoxic T cells and NK cells. We also showed that a deficiency of the syntaxin-11 protein abrogates cytotoxic lymphocyte degranulation. Apart from this, we also reported a novel *STX11* gene mutation.

6.3 SPECTRUM OF *UNC13D* MUTATIONS IN FHL (PAPER III)

In this paper we studied the frequency of *UNC13D* mutations in a well-defined cohort of patients with HLH. In all we studied 38 patients from 34 families with HLH who all fulfilled the diagnostic criteria for HLH. Six different mutations were found in nine individuals, two previously described and four novel mutations (Table 4).

Mutation spectrum

The four novel mutations included two nonsense mutations, one splice mutation and one missense mutation. The nonsense mutations revealed were R83X located in exon 3 and W382X located in exon 13. Both of these mutations cause a predicted truncation of the protein resulting in a partial or complete loss of the region responsible for binding Rab27a and a complete loss of the region responsible for association with secretory granules. The splice acceptor mutation found is predicted to result in a loss of exon 28, located in the Munc13-homology domain (MHD) 2 region, which is involved in lytic granule targeting. Finally, the missense mutation was R928P, located in exon 29. This region is implicated in Ca^{2+} binding. Although this amino acid is not conserved, the change was not present in 118 alleles from healthy controls.

In two of the families studied we were able to confirm two previously described mutations [Yamamoto et al 2004, zur Stadt et al 2006], one nonsense mutation (R214X located in exon 3) and one deletion of four bases (R782SFsX11 in exon 24) resulting in a frame shift and premature stop codon. Apart from this, we found a nucleotide change previously described (A59T) [Santoro et al 2006] in a to date healthy 5-year-old sibling of one of our patients included in this study. This child has not displayed any signs of disease to date and carries the alteration in a bi-allelic state. NK cell analysis revealed decreased cytotoxicity but normal degranulation, suggesting that this change does not markedly affect secretory lysosome exocytosis. When studying healthy controls, we found this alteration in 1 of 118 alleles. Possibly, this is a disease-modifying, but not disease-causing, sequence variation. Nevertheless, we cannot exclude the possibility that this child may develop the disease later in life.

Of the patients carrying mutations, four were Pakistani, three were Turkish and two were European. Like in the studies of the *STX11* gene, we found no bi-allelic *UNC13D* mutations in the Nordic population.

Clinical features

The age of diagnosis ranged from 0 to 14 years of age. Six of the patients developed the disease before the age of 3 months, one child developed the disease at 3 years of age while the two patients carrying the splice mutation were 10 and 14 years old at onset, respectively. The median age at diagnosis was 69 days. Of the nine patients with mutations, three of nine (33%) developed CNS symptoms, whereas the remaining six did not.

Seven of the patients were treated with the HLH-94/HLH-2004 protocols, one received other therapy and one received no therapy at all in line with his parents wishes. At the time of the writing this thesis, six of the children are deceased, of whom five died without having a SCT, and three are alive (two are being prepared for SCT and one has undergone a SCT).

NK cell activity

NK cell activity was analyzed in four of the patients carrying *UNC13D* mutations; two with the splice mutation, one with the four base pair deletion (R782SFsX11), and one with a nonsense mutation (W382X) and was found to be decreased in all four. We also performed degranulation studies in two of the patients, one carrying the splice mutation and one carrying a nonsense mutation (W382X). These showed reduced degranulation. Interestingly, the degranulation defect was more severe in the child carrying the nonsense mutation with early onset than in the child with the splice mutation and adolescent onset. Of note, the NK cells that did degranulate from the child carrying the splice mutation did so with less intensity as compared to healthy controls, suggesting that fewer vesicles fused with the membrane.

6.4 GENOTYPE-PHENOTYPE CORRELATIONS IN HLH PATIENTS (PAPER IV)

In this study, 76 patients from 65 unrelated families originating from the Nordic countries, Turkey and the Middle East were included. All of the patients fulfilled

the HLH-2004 diagnostic criteria [Henter et al 2007], had a positive family history, or had a verified biallelic mutation in any of the three genes known to cause FHL.

Baseline characteristics

The median age at diagnosis was 198 days (range 18 days to 12 years). CNS involvement was found in 42/69 (61%) patients at the time of diagnosis. NK cell analysis had only been performed in 18 of the patients and was therefore not analyzed statistically.

The majority 50/76 (66%) of the patients had been treated with the HLH-94 protocol. Of the 26 remaining patients, six received treatment prior to the HLH-94 protocol, nine were treated with the HLH-2004 protocol, three received other treatment combinations, five died before treatment was started, and for three patients the parents declined therapy. Of these three, one died after 59 days and the other two were lost to follow-up.

At the last follow-up of all 76 patients, 31 were alive; the mean follow-up time from diagnosis was 3.9 years (range 53 days to 21 years). Forty-two patients were dead and three were lost to follow-up. SCT had been performed in 28 patients and 20 of these (71%) were alive.

Mutation spectrum

A molecular diagnosis was made in 33 of the 76 patients (43%) studied, corresponding to 24 of the 65 (37%) unrelated families. *PRF1* mutations were detected in 13/74 (18%) of the patients, *UNC13D* mutations in 6/61 (10%) and *STX11* mutations in 14/70 (20%) of the patients. In 27/60 (45%) patients, we did not find bi-allelic mutations in any of the three genes. In 16 patients, we did not have a sufficient amount of DNA to sequence all three genes and therefore these patients were not included in the genotype-phenotype correlation analysis. The different mutations identified are presented in Table 4.

Genotype-phenotype correlations

The patients were divided into four sub-groups, patients carrying *PRF1* mutations, *UNC13D* mutations, *STX11* mutations and patients not carrying bi-allelic mutations in any of these genes. Each group was then compared to the other groups to investigate if there were any phenotypic distinctions. We also subdivided the patients into ethnic groups (Middle East, Turkey and Nordic) to see whether there was any correlation to ethnicity.

We observed a higher incidence of *PRF1* mutations in patients originating from the Middle East as compared to the Nordic countries. We also observed a higher incidence of *STX11* mutations in patients originating from Turkey compared to the Nordic group. The patients with Nordic origin presented a higher incidence of no found mutation than the other groups.

The patients from the Middle East were younger at diagnosis compared to the Turkish patients. The median age at diagnosis of the patients studied was 2 months for patients with *PRF1* mutations, 14 months for patients with *UNC13D* mutations, 6 months for patients with *STX11* mutations and 5 months for patients not carrying bi-allelic mutations in any of the three genes.

Patients carrying *PRF1* mutations had a significantly increased risk of early onset as compared to patients carrying *STX11* mutations, this association remained after adjusting for ethnicity as a potential confounding factor. For the patients carrying *PRF1* mutations, the mean age at onset for the ones carrying nonsense mutations (n=4) was 5 months and the mean age for those carrying missense mutations (n=3) was 21 months.

Clinical symptoms and treatment response in relation to genotype

When comparing the different genotype groups with each other we observed a significant difference regarding history of familial disease ($p=0.027$), consanguinity ($p<0.001$) and ethnical origin ($p<0.001$). Apart from this we also observed significant differences regarding age under three months at onset

(p=0.040), age over six months at onset (p=0.055), jaundice (p=0.030) and pathological CSF (p=0.031). We did not observe differences regarding the presence of hepatosplenomegaly, edema, skin rash or ferritin at the time of diagnosis. In addition, comparing the four different genotype groups, we did not observe any difference in response to initial therapy (measured as if the patients were alive, dead or had inactive disease at 2 months after start of therapy).

CNS disease at diagnosis

CNS involvement was defined as abnormal neurological clinical examination and/or pathological CSF. Of the 33 patients carrying bi-allelic mutations, CNS disease was reported in 20. Of the patients carrying *PRF1* mutations, five of 13 had neurological symptoms. One had encephalopathy, one suffered from seizures, one had weakness of the left leg and balance difficulties, and for two of these patients the symptoms were not specified. Of the patients that carried *UNC13D* mutations, two of five patients were reported to have neurological symptoms at onset; one had microencephaly and mental retardation, and one had seizures. Of the patients with *STX11* mutations only 2 of 14 were reported to have neurological symptoms at onset, one with developmental delay and one with seizures. In the group with no bi-allelic mutations found, 11 of 26 had neurological symptoms at onset, the most common symptoms in this group being cranial nerve palsies, seizures and irritability.

To investigate the possible association between CNS disease and genotype, logistic regression analysis was performed for the four subgroups. Pathological CSF was the dependent variable. The unadjusted odds ratio showed an increased risk of pathological CSF for patients with *PRF1* mutations and patients with no bi-allelic mutation found in any of the genes compared to patients with *STX11* mutations. After adjusting for ethnicity as a potential confounding factor the association remained for patients with no mutations compared to those with *STX11* mutations.

7 DISCUSSION

Familial hemophagocytic lymphohistiocytosis is a rare autosomal recessive disease of immune dysregulation. In this thesis we have studied *STX11* and *UNC13D* mutations in a well defined cohort of patients as well as the effects of reduced expression or absence of the proteins encoded by *STX11* or *UNC13D* on a cellular level and finally, if there are any genotype-phenotype relations between the three genes known to cause FHL.

The *STX11* gene is a small gene consisting of 2 exons and only exon 2 is encoded for the 861 base pair open reading frame. The protein is widely expressed, especially in placenta, lung, heart and in the immune system where it is expressed in the thymus, spleen, lymph nodes, phagocytes, antigen-presenting cells as well as in cytotoxic lymphocytes [Prekeris et al 2000, paper II]. The syntaxin protein family is characterized by a carboxy-terminal hydrophobic transmembrane domain, thought to be the major driving force leading to SNARE-SNARE interactions *in vitro* and *in vivo* [Hong 2005]. However, the syntaxin-11 protein does not contain a hydrophobic sequence that is sufficiently long to function as a transmembrane anchor [Advani et al 1998, Tang et al 1998]. Despite this, the protein seems to play an important role in the process that leads to vesicle fusion with the plasma membrane as it has been shown that patients with *STX11* gene mutations fail to degranulate properly [paper II].

The *UNC13D* gene is a large gene consisting of 1091 residues encoding the protein MUNC13-4. The gene is ubiquitously expressed and implicated in regulating membrane fusion events. Munc13-4 is required for the vesicle-plasma membrane fusion during exocytosis of perforin-containing granules by cytotoxic T cells and NK cells [Feldmann et al 2003, Marcenaro et al 2006, paper II]. Recent studies have shown that Munc13-4 has an intracellular distribution distinct from perforin- and granzyme-containing granules [Menager et al 2007]. The gene consists of four distinct domains, two Munc13-homology domains (MHD1 and MHD2), as well as two C2 domains (C2A and C2B). The

MHD1 and MHD2 are thought to be important in granule targeting, while the C2 domains are implicated in Ca^{2+} and phospholipid binding of the membrane [Hong 2005]. Finally, the region between C2A and MHD1 is required for interaction with Rab27a [Hong 2005]. As seen in cytotoxic cells from *STX11* deficient patients, cytotoxic cells from some patients with *UNC13D* mutations also fail to degranulate upon stimulation [paper II, paper III].

There are to date numerous reports of the frequencies of the different mutations known to cause FHL, and there seems to be a difference in various ethnic groups [Göransdotter et al 2001, Suga et al 2002, Molleran et al 2004, zur Stadt et al 2006, Lee et al 2006] (Table 5). When studying the cohort in Stockholm, we found bi-allelic *STX11* gene mutations to be present in 14% of the families in the entire cohort. The mutations were only present in patients of Turkish or Middle Eastern origin, in line with other previous and later reports [Yamamoto et al 2005, zur Stadt et al 2006]. We did not find any *STX11* mutations in patients of European or Nordic origin. Similarly, no *STX11* mutations have been found in the Japanese population [Yamamoto et al 2005] (Table 5).

Table 5:*Spectrum of gene mutations in FHL*

Gene	Turkey	Northern Europe	Japan
PRF1	~45%	8-13%	30%
recurrent PRF1 mutation	c.1122G>A p.W374X	nd	c.1090_1091delCT p.L364EFsX83
STX11	14-20%	0	0
recurrent STX11 mutation	nd	nd	nd
UNC13D	19%	0-18%	37.5%
recurrent UNC13D mutation	nd	nd	nd
No mutation found	19%	70%-92%	nd

Adapted from Yamamoto et al 2004, Yamamoto et al 2005, zur Stadt et al 2006, Trizzino et al 2007, paper III, paper IV.

In studies regarding the *UNC13D* gene, we found a lower frequency than the ones reported from Italy [Santoro et al 2006] and Japan [Yamamoto et al 2004]. On the other hand, our findings were in line with reports from Germany [zur

Stadt et al 2006]. This could be explained by the fact that our cohort more ethnically resembles the German one. Another explanation for the lower frequency could be the limitations of conventional sequencing. There is always a risk that large heterozygous exon deletions might be overlooked, and as the cohort studied in Stockholm includes a large group of Western European children where consanguinity is rare, one might expect a larger percentage of compound heterozygous mutations than in the group of patients from the Middle East and Turkey where consanguineous marriages are more common. Another limitation in the sequencing of *UNC13D* is that we have only studied the exons and exon/intron boundaries and therefore may have missed splice-altering mutations that are located deep within the intronic sequences.

We have identified one novel *STX11* mutation and four novel *UNC13D* mutations (Table 4). The *STX11* mutation is a single nucleotide deletion resulting in a frame shift and a subsequent premature stop codon. Degranulation studies showed that lymphocytes from this patient along with lymphocytes from other patients carrying *STX11* mutations fail to degranulate properly upon stimulation, when using CD107a as a marker for degranulation. These data imply that the proteins encoded by the *STX11* gene play an important roll in the cellular machinery needed for vesicle release, on the other hand, when stimulated with IL-2 the cells from patients carrying *STX11* mutations showed a normal degranulation pattern as compared with healthy, age matched controls. This suggests that the system can be by-passed, and that there may be other proteins that can over-ride the syntaxin-11 protein in the degranulation process.

The four novel *UNC13D* mutations include one splice mutation, two nonsense mutations and one missense mutation. The splice mutation is located adjacent to exon 28 in the MHD2 region, a region important for granule targeting. Remarkably, the children carrying this mutation were aged 10 and 14 years at onset, and we speculate that this splice mutation might result in some residual function of the protein, which may explain why the two patients with this alteration both presented late. Of note, the NK cells from the patient with the

later onset (14 years) degranulated upon encounter with sensitive target cells, but did so with lower intensity than NK cells from healthy controls suggesting that fewer vesicles fused with the membrane. The two nonsense mutations R83X and W382X both predicted to result in a truncation of the protein, resulting in a loss of the granule-targeting domain of Munc13-4. NK analysis in the child carrying the W382X mutation showed very low NK cell cytolytic activity after 4 hours and after prolonged incubation to 16 hours, but the low NK cell activity was augmented by IL-2 stimulation similar to the findings in the children carrying *STX11* gene mutations. In this patient, Western blot revealed absence of Munc13-4 protein whereas syntaxin-11 protein was present. In addition, a novel bi-allelic missense mutation, R928P, was identified in two siblings. This mutation is located in the C2B domain of the gene and although Arginine928 is not a conserved peptide, we propose that a mutation to a Proline residue at this position plays a role in the FHL pathogenesis, since we did not find the mutation in 59 healthy controls and since an Arginine-to-Proline substitution may alter the protein structure significantly. Regrettably, functional studies to assess the impact of this mutation on lymphocyte cytotoxicity could not be performed since one of the children with this mutation is deceased and the other one has undergone a SCT.

There has been a previous report of a missense mutation located in exon 3, A59T [Santoro et al 2006]. We found this nucleotide alteration in a bi-allelic state in a to date healthy, 5-year-old sibling of one of our patients, and speculate that this alteration is a disease-modifying mutation as it had not been described in a single mutation state, only in combination with other bi-allelic mutations. Since there is a large spread in age at onset in children carrying *UNC13D* mutations, we cannot exclude that this individual will develop the disease later in life.

Perforin has been studied extensively over the last few years. Different animal models have shown its importance in clearing an infection but also its role in down-regulating the immune response after the infection has been cleared [van Dommelen et al 2006]. It has been postulated that the driving force behind the pathology of HLH in perforin knock-out mice infected with LCMV is IFN-γ,

produced by $CD8^+$ cells [Jordan et al 2004]. On the other hand, in knock-out mice infected with MCMV, the driving force behind the HLH phenotype seems to be primarily TNF-α [van Dommelen et al 2006].

When we studied blood samples from healthy infant donors, we noticed that NK cells were the major perforin-expressing lymphocyte subset and that fewer effector T cells were present. Notably, we did not see any perforin-positive T cells when examining cord blood from healthy donors, consistent with an immature immune system. This suggests that when the disease presents in early infancy, NK cells rather than T cells may play a more important role in the development of the disease in contrast to onset in early childhood or adolescence, when the immune system is more mature.

Degranulation studies can be used as a tool for diagnosis, and we have found several patients carrying *UNC13D* and *STX11* mutations that have showed a defective degranulation pattern. On the other hand, we also have a few patients with a defective degranulation pattern where no mutations in the *UNC13D* or *STX11* genes have been found, implicating defects in other, not yet identified genes regulating vesicle fusion or that there are mutations in the known genes that we have not detected.

When studying these children with *STX11* mutations, we also found that two out of six (33%) had developed a secondary MDS/AML. The treatment regimen for FHL includes etoposide (VP-16), an epipodophyllotoxin that may cause secondary hematological malignancies. The risk for this development is related to the amount of etoposide administered [Pui et al 1991]. In a recent report by Imashuku [Imashuku 2007], it was suggested that not only high dose etoposide administration give rise to secondary malignancies, but also low dose administration. In the HLH-94 and HLH-2004 protocols the dose of etoposide is 2850 mg/m^2 (week 1-26). However, even though epipodophyllotoxin administration might have influenced the development of MDS/AML, one cannot rule out the possibility that an impaired cytotoxic function and hence impaired surveillance for transformed cells could contribute to this MDS/AML

development. There are reports concerning lymphoma patients where heterozygous mutations in the *PRF1* gene have been found in up to 28% of the patients [Clementi et al 2005, Mehta et al 2006, Cannella et al 2007]. If there is indeed an increased risk of developing hematological malignancies when carrying bi-allelic or heterozygous mutations in the genes involved in the innate immune system, one might expect a higher frequency of hematological malignancies in the parents of these children. This has to our knowledge never been studied, so this question remains open to a certain extent. To our knowledge there has been no reports concerning patients with *UNC13D* mutations and malignancies, but as the *STX11* patients have a milder phenotype with a longer life expectancy, it is possible that children with bi-allelic *PRF1* and *UNC13D* mutations die before they develop a secondary malignancy.

Even though FHL is a genetically heterogeneous disease, the phenotypic presentation is remarkably homogeneous. To investigate genotype-phenotype correlations, we divided the patients into four subgroups; patients carrying *PRF1* mutations, patients carrying *STX11* mutations, patients carrying *UNC13D* mutations and, finally, patients not carrying any bi-allelic mutations in the known genes. Significant differences regarding familial history, consanguinity, ethnic origin, age at diagnosis lower than three months, age at diagnosis lower than six months, jaundice and pathological CSF were observed between the different groups. We found no difference regarding hepatomegaly, edema, skin rash or ferritin levels, nor did we find any difference regarding response to therapy two months after start of therapy. The mean age at diagnosis was 2.3 months for patients carrying *PRF1* mutations, 6.2 months for *STX11* mutations, 14.4 months for *UNC13D* mutations and 4.9 months for those where no bi-allelic mutations were identified. There was a significantly higher risk for early onset in patients with *PRF1* mutations as compared to those carrying *STX11* mutations, but we found no significant difference between the patients carrying *PRF1* gene mutations compared to the ones carrying *UNC13D* mutations. There are some reports of late onset in patients carrying *UNC13D* mutations as well as in patients carrying *PRF1* mutations [Allen et al 2001, Ueda et al 2007, paper III].

One explanation for this might be that the mutations that these children carry give rise to residual function of the respective protein.

The most important complication to FHL is CNS dysfunction. In a large study on 193 patients treated with to the HLH-94 protocol, clinical neurological abnormalities at diagnosis were reported in 72 patients (37%) [Horne et al 2007]. When studying the patients included in paper III, three of nine patients (33%) studied had CNS symptoms; however, since five of the six non-affected children died this percentage could be misleading. A higher frequency of patients with *UNC13D* mutations and CNS involvement has been reported from France (9 of 10 patients, 90%) and Italy (9 of 15 patients, 60%) [Feldmann et al 2003, Santoro et al 2006], whereas Japanese studies report 33% and 63% CNS involvement, respectively [Yamamoto et al 2004, Ishii et al 2005]. When studying the entire group of patients with *UNC13D*, *STX11*, *PRF1* or no found mutation, a higher risk of CNS involvement was observed in patients where no mutations was found compared to patients carrying *STX11* gene mutations. This suggests that these patients carry mutations in yet unknown genes that encode proteins important for immunological events important for surveillance of the CNS, or that these patients do indeed carry mutations in one of the known genes and that these mutations still are undetected. *PRF1* mutations were more frequent in patients originating from the Middle East, and *STX11* mutations were more frequent in patients originating from Turkey. The highest incidence of no detected bi-allelic mutations was found in the patients of Nordic origin.

To summarize, this thesis presents the spectrum and clinical implications of mutations of two of the three genes known to cause FHL, in a well characterized cohort of patients. Moreover, the thesis presents detailed functional studies of cytotoxicity and cytotoxic lymphocyte degranulation in FHL patients. In addition, a genotype-phenotype analysis has been performed in a large set of affected patients. A summary of all the mutations described in this thesis are presented in table 6.

Table 6: Spectrum of mutations in this thesis

Mutation	***Amino acid alteration***	***Paper***	***Reference (first report)***
PRF1:			
Nonsense			
	p.Y219X	IV	Stepp et al, 1999
	p.W374X	IV	Stepp et al, 1999
Missense			
	p.V50M	IV	Göransdotter Ericson et al, 2001
	p.P89T	IV	Al-Lamki et al, 2003
	p.I224N	IV	Göransdotter Ericson et al, 2001
	p.G149S	IV	Kogawa et al, 2002
	p.V38L	IV	Paper IV
	p.D430Y	II	Paper II
	p.H222Q	II	Molleran et al 2004
	p.E317R	II	Ueda et al 2003
In-frame deletions			
	p.ΔK285	IV	Göransdotter Ericson et al, 2001
	p.ΔK284-287	IV	Muralitheran et al, 2005
STX11:			
Nonsense			
	p.Q268X	I,II,IV	zur Stadt et al, 2005
Out of frame deletions			
	p.V124Fs	I,IV	zur Stadt et al, 2005
	p.T37RFsX25	II,IV	Paper II
UNC13D:			
Splice			
	c.2626-1G	III,IV	Paper III
Missense			
	p.A59T	IV	Santoro et al, 2006
	p.R928P	III,IV	Paper III
Nonsense			
	p.R214X p.W382X p.R83X	III,IV III III	Yamamoto et al, 2004 Paper III Paper III
Out of frame deletions			
	p.R782SFsX11	III	zur Stadt et al, 2006

8 CONCLUDING REMARKS

Even though more than 50 years have passed since FHL was first described, and even though we have come a long way regarding treatment and diagnosis of the disease, there are still many children that succumb from the disease and many for whom a molecular diagnosis never is made. In other words, improving the diagnostic tools with the ultimate aim to improve therapy is still an important task. The overall survival rate has risen from 0 to 50-60% with the use of the treatment protocols designed by the Histocyte Society in combination with SCT. The HLH protocol has proven highly successful in achieving symptomatic remission, allowing patients to proceed to SCT [Horne et al 2005b]. Notably, in order to facilitate the decision to go to transplant it is important with diagnostic tools indicating the need for SCT. Molecular diagnostics as well as the functional studies described in this thesis will facilitate such decisions, thus hopefully improving survival further. Moreover, early diagnosis will hopefully also reduce late sequelae in particular sequelae affecting the CNS.

In this thesis we have for the first time described the consequences of a loss of function of the syntaxin-11 protein in cytotoxic cells, and we have also presented a number of new mutations in the *UNC13D* and *STX11* genes. In addition we have reported that FHL can have a late onset, pointing to the fact that the disease should not only be considered in infants but also in young adults and adolescents.

The knowledge we obtain from degranulation studies and NK cell analysis can point us in the right direction as to which additional gene or genes may cause FHL. However, these results only provide a direction and not a definitive answer. The figure below (Figure 2) suggests a flow chart for genetic investigation in patients with suspected HLH. It is important to remember that a normal perforin expression using flow cytometry does no exclude *PRF1* mutations [Feldmann et al 2005]. Moreover, if the patients show signs of hypopigmentation or albinism, Griscelli syndrome type 2 or Chédiak-Higashi syndrome should be considered. In patients with secondary HLH, a normal

degranulation pattern as well as a lack of mutations are to be expected. Nevertheless, sequencing of the *UNC13D* gene has proven to be a difficult task and mutations in this large gene might be missed. Finally, NK cell analysis with sub-typing of the cellular defect might provide an insight to the diagnosis as secondary HLH patients so far have not been reported to have a type-3 deficiency. With our increased knowledge, genetic counseling can now be offered an increased number of parents of affected children. Moreover, if a molecular diagnosis can be made, there is also an opportunity for prenatal diagnosis.

To conclude, the present thesis has presented diagnostic tools of value for the diagnosis and treatment of affected children. Moreover it has provided novel insights into the field of immune regulation in humans. With regard to future studies I hope that the search for additional disease causing genes continues in order to improve diagnosis and treatment of FHL as well as of secondary forms of HLH. It will be very exciting to see in which way the studies of a rare inherited disease such as FHL may influence and improve the future medical care of a larger number of patients with secondary forms of HLH, as well as other diseases affecting the immune system. I hope to be part of that medical journey.

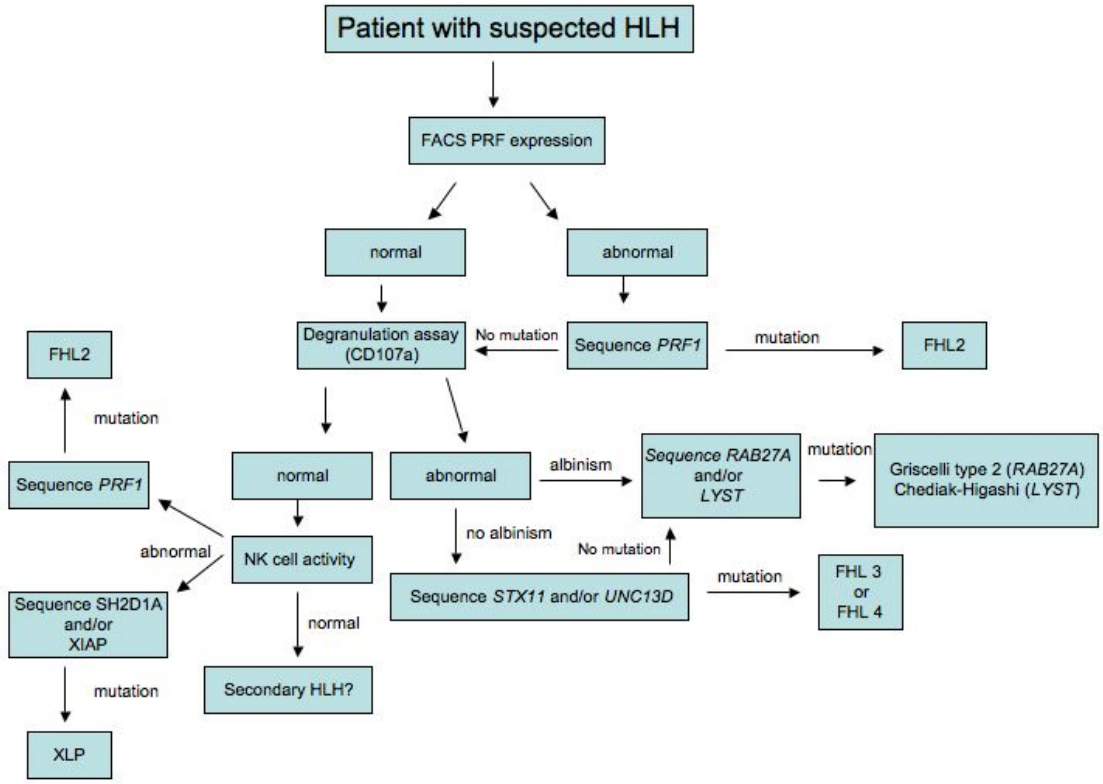

Figure 2: Suggested diagnostic flow chart for patients with suspected HLH. The patients included should fulfill the diagnostic criteria for HLH set up by the Histiocyte Society. A normal expression of perforin using flow cytometry does not exclude mutations in the *PRF1* gene [Feldmann et at 2005] and the gene should therefore be sequenced even if perforin expression is normal. Degranulation studies should be done when possible as well as NK cell activity. The absence of mutations in the known genes does not exclude the diagnosis.

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