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HYPERINFLAMMATION IN CRITICALLY ILL

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Hyperinflammation in critically ill

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my aunt and godmother Kitte, who is my hero and was a real fighter to the very end. And to all those dear to me who are an important part of my life.

ABSTRACT

Background: A wide spectrum of inflammatory responses, with overlapping characteristics, is encountered in critically ill patients in intensive care. At one extreme, critically ill patients may develop the potentially fatal condition secondary hemophagocytic lymphohistiocytosis (sHLH), characterized by excessive inflammation (hyperinflammation), driven by a 'cytokine storm', and multiple organ failure. Infections, malignancies and autoimmune diseases are the most common conditions associated with the development of sHLH. Prompt diagnosis and early appropriate intervention is crucial to improve survival in sHLH. Corticosteroids are a cornerstone of HLH therapy. The addition of the cytotoxic drug etoposide has been instrumental in the successful treatment of primary HLH, and may reduce morbidity and mortality also in selected cases of sHLH. While it is established that defective lymphocyte cytotoxicity causes primary HLH , the cause of sHLH remains incompletely understood.

Aims: The overall aim of the thesis was to broaden our knowledge of hyperinflammation and HLH in critically ill, with a focus on intensive care, to better identify the critically ill patients with hyperinflammation that could benefit from anti-inflammatory therapy, in order to reduce morbidity and improve survival. We also aimed to investigate the role of cytotoxic lymphocytes, and possible genetic correlations, in the pathogenesis of hyperinflammation and sHLH in critically ill.

Methods: The studies included several critically ill patient cohorts in intensive care, some with extracorporeal membrane oxygenation (ECMO) support, with various underlying conditions.

Results: Secondary HLH was encountered in critically ill patients in intensive care with a high proportion of malignancies and immunosuppression, in influenza AH1N1 infected patients, in severe dengue, and in systemic autoimmune conditions. The mortality in sHLH in critically ill is high. HLH patients were generally younger, with fewer comorbidities and predominantly male. Hyperferritinemia, which correlated with elevated soluble IL-2R and CRP, and thrombocytopenia were identified in critically ill in ICU, and were more prominent in inflammatory responses such as sepsis. However, the highest median levels of ferritin, and soluble IL-2R, were observed in HLH patients, who also demonstrated other common manifestations of sHLH such as cytopenias, elevated liver function tests and triglycerides, hemophagocytosis in the bone marrow and splenomegaly. In global diseases, such as severe dengue and pandemic influenza AH1N1, we found that a proportion of patients do develop hyperinflammation, albeit not always recognized which therefore limits appropriate treatment. A subset of critically ill patients not meeting HLH criteria (i.e. with <5 of 8 diagnostic HLH-2004 criteria and a median HScore of 138), but with high SOFA score (median 16.5) and fatal outcome, had similar levels of ferritin to HLH patients and highly elevated liver tests, but HLH patients had a significantly higher ferritin/ALT ratio, a possible novel diagnostic aid for the diagnosis of HLH. A faster rate of increase of ferritin was associated with a higher risk of death. In critically ill in ICU and severe dengue, elevated AST and SOFA score were independent risk factors of mortality. Reduced absolute numbers of NK cells and CTLs, and reduced lymphocyte cytotoxicity, were observed in critically ill, more prominent in sHLH patients with hyperferritinemia, probably contributing to the development of sHLH. Rare variants in HLH-causing genes were identified in a few patients. With regard to specific treatment, a number of children with severe MAS-HLH despite conventional therapy responded promptly to the addition of moderately dosed etoposide.

Conclusions: Secondary HLH can be identified in various conditions in critically ill patients, who should be monitored for signs of hyperinflammation and progressive organ failure for prompt HLH evaluation using currently available diagnostic tools, including ferritin and soluble IL-2R, and additional helpful parameters. Early diagnosis and appropriate HLH-directed therapy, including etoposide in selected cases, is likely beneficial to reduce morbidity and improve survival in sHLH

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LIST OF ABBREVIATIONS

AH1N1-HLH	influenza AH1N1-associated HLH
AKI	acute kidney injury
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AOSD	adult-onset Still's disease
ARDS	acute respiratory distress syndrome
AST	aspartate aminotransferase
BSA	body surface area
Chemo-HLH	HLH during chemotherapy
CHS	Chédiak-Higashi syndrome
CMV	cytomegalovirus
CNS	central nervous system
CNS-HLH	HLH of the central nervous system
CRP	C-reactive protein
CRRT	continuous renal replacement therapy
CSA	cyclosporine A
CSF	cerebrospinal fluid
CTL	cytotoxic T-lymphocytes
Dengue-HLH	dengue-associated HLH
EBV	Epstein-Barr Virus
EBV-HLH	Epstein-Barr-associated HLH
ECMO	extracorporeal membrane oxygenation
FHL	familial hemophagocytic lymphohistiocytosis
GGT	gamma-glutamyl transferase
GS-2	Griscelli syndrome type 2
HDB/DIC	hepatobiliary dysfunction and coagulopathy syndrome
HLH	hemophagocytic lymphohistiocytosis
HSCT	hematopoietic stem cell transplantation
HIV	human immunodeficiency virus
IA-HLH	infection-associated HLH

ICU	intensive care unit
IFN-γ	interferon-gamma
IL	interleukin
IQR	interquartile range
IVIG	intravenous immunoglobulins
LA-HLH	lymphoma-associated HLH
LDH	lactate dehydrogenase
LST	life support treatment
Mal-HLH	malignancy-associated HLH
MAS-HLH	macrophage activation syndrome-HLH
MODS	multiple organ dysfunction syndrome
MRI	magnetic resonance imaging
MT-HLH	malignancy-triggered HLH
NK cells	natural killer cells
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PID	primary immune deficiency
SD	severe dengue
sHLH	secondary HLH
sIL-2R	soluble IL-2 receptor (also known as sCD25)
SIRS	systemic inflammatory response syndrome
sJIA	systemic juvenile idiopathic arthritis
SLE	systemic lupus erythematosus
SOFA score	sequential organ failure assessment score
TNF-α	tumor necrosis factor alpha
ULN	upper limit of normal
XLP	X-linked lymphoproliferative syndrome
WHO	World Health Organization

1 INTRODUCTION

1.1 INFLAMMATION

1.1.1 *Calor, dolor, rubor et tumor* to hyperinflammation

Little did we know about inflammation back in the 1st century A.D. when Roman scholar Aulus Cornelius Celsus simply defined inflammation by its four classical signs: *calor, dolor, rubor et tumor* (heat, pain, redness and swelling). Since then, two millennia of medicine and science have vastly expanded our knowledge of inflammation past this simple, yet correct, definition. The acute inflammatory response is the body´s primary reaction to eliminate an unwanted harmful pathogen. Optimally, it is a carefully orchestrated response where it's termination is just as important as it's induction to maintain homeostasis. On the other hand, uncontrolled inflammation has been found to be the pathophysiological basis of many of our common global diseases and health issues today[1]. In some cases, the immune system will run amok, and this initially protective inflammatory response will develop into a rampant destructive cytokine storm and hyperinflammation[2,3].

1.2 HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS (HLH)

In 1952, physicians Farquhar and Claireaux described two siblings, born to the same parents, who both died in infancy in a similar clinical condition, characterized by persistent fever, cytopenias, hepatosplenomegaly with lymphocyte infiltration and hemophagocytosis in the spleen and lymph nodes[4]. They named it hemophagocytic reticulosis, now called hemophagocytic lymphohistiocytosis (HLH).

Hemophagocytic lymphohistiocytosis (HLH) is a highly fatal clinical condition of extreme hyperinflammation, driven by a cytokine storm caused by immune dysregulation and overactivation of the immune response[5-9]. HLH can be likened to a violently erupting volcano after massive pressure build-up. HLH occurs in a primary (genetic) form and a secondary (acquired) form, with a similar clinical presentation but with differences in their etiology, treatment and outcome. Primary HLH predominantly occurs in infancy or early childhood, while secondary HLH can present at any age, but is more common in adulthood.

The diagnosis of HLH relies on a set of criteria that are based on the clinical symptoms and laboratory findings of HLH, which are a direct reflection of the cytokine turmoil and inflammatory cell infiltration of involved organs[6]:

- Fever is caused by elevated levels of inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor-alpha (TNF-α).
- Cytopenias are predominantly a consequence of hematopoietic suppression from elevated levels of TNF-α and interferon-gamma (IFN-γ), and additionally, from hemophagocytosis.
- Ferritin is secreted by highly activated macrophages and upregulated transcription of ferritin by pro-inflammatory cytokines, such as IL-6, IL-8 and TNF-α.

- High levels of TNF-α and IFN-γ reduce the activity of lipoprotein lipase necessary for the degradation of triglycerides, causing elevated triglyceride levels.
- Activated macrophages secrete plasminogen activator, converting plasminogen to plasmin, that cleaves fibrin, which in turn stimulates conversion of fibrinogen to fibrin, causing hypofibrinogenemia.
- The α-chain of the soluble IL-2 receptor (sIL-2R, also known as sCD25) is secreted by activated lymphocytes.
- Infiltration of organs by activated lymphocytes causes splenomegaly and hepatomegaly. In the liver, infiltration of the portal tract results in obstructive jaundice with commonly observed elevated transaminases and bilirubin. Lymphocyte infiltration of the central nervous system (CNS) gives rise to neurological signs and symptoms.
- The cytokine storm leads to macrophage activation, causing hemophagocytosis, most typically observed in bone marrow, spleen, liver and lymph nodes.[10] It is frequently absent at diagnosis but may appear during the course of illness. Importantly, hemophagocytosis is not obligatory for the diagnosis of HLH. Furthermore, hemophagocytosis also occurs in a number of other critical and inflammation-driven conditions.

Table 1. HLH-2004 diagnostic criteria and HScore criteria[a]

HLH-2004 diagnostic criteria	**The HScore**	
1. A molecular diagnosis consistent with HLH *OR* *2. Diagnostic criteria for HLH fulfilled (≥5 of 8 criteria):*	*Parameter*	*Criteria scoring*[e]
- Fever	- Temperature (°C)	0 (<38.4), 33 (38.4–39.4), or 49 (>39.4)
- Splenomegaly	- Organomegaly	0 (no), 23 (hepato- or splenomegaly), or 38 (both)
- Cytopenias (≥ 2 of 3 lineages)[b]	- No. of cytopenias[c]	0 (1 lineage), 24 (2 lineages), or 34 (3 lineages)
- Ferritin ≥500 µg/L	- Ferritin (ng/ml)	0 (<2,000), 35 (2,000–6,000), or 50 (>6,000)
- Hypertriglyceridemia ≥3.0 mmol/L and/or hypofibrinogenemia ≤1.5 g/L	- Triglyceride (mmol/L) - Fibrinogen (g/L)	0 (<1.5), 44 (1.5–4), or 64 (>4) 0 (>2.5) or 30 (≤2.5)
- Hemophagocytosis	- Hemophagocytosis	0 (no) or 35 (yes)
- Soluble CD25 (soluble IL-2R) ≥2400 U/mL	- AST (IU/L)	0 (<30) or 19 (>30)
- Low or absent NK cell activity	- Known immunosuppression[d]	0 (no) or 18 (yes)

HLH, hemophagocytic lymphohistiocytosis; AST, aspartate aminotransferase; NK cell, natural killer cell
a**)** Adapted from Henter *et al* (2007)[11] and Fardet *et al* (2014)[12].
b) Defined as hemoglobin <90 g/L; platelets <100 x10^9/L, neutrophils <1.0 x10^9/L.
c) Defined as a hemoglobin level of ≤92 g/L and/or a leukocyte count ≤5 x10^9/L and/or a platelet count of ≤110 x10^9/L.
d) Human immunodeficiency virus positive or long-term immunosuppression (i.e. glucocorticoids, cyclosporine, azathioprine).
e) The total HScore estimates an individual's probability of having HLH. The best cut off value for HScore was 169 (sensitivity 93% and specificity 86%, and accurate classification of 90% of the patients).

Altogether, the described criteria have formed the current HLH-2004 diagnostic criteria (Table 1), developed from the initial diagnostic criteria by the Histiocyte Society from 1991 and based on observations in children with mainly primary HLH[11,13]. Importantly, all criteria may not,

and do not have to be present at diagnosis, but commonly add up as the HLH aggravates. Although none of the criteria are specific for HLH, it is their combination, particularly of persistent fever, hepatosplenomegaly and cytopenias, and the unexpected disproportionate inflammation response and progress of these characteristics that should alert the physician with suspicion of HLH.

1.3 CLASSIFICATION OF HLH

A revised classification of histiocytoses and neoplasms of the macrophage-dendritic cell lineages has been proposed by Emile *et al* (Table 2)[14].

Table 2. Revised classification of histiocytoses and neoplasms of the macrophage-dendritic lineage

Langerhans histiocytoses (L group)
Langerhans cell histiocytosis (LCH)
Erdheim-Chester disease (ECD)
Mixed Erdheim-Chester disease and Langerhans cell histiocytosis
Cutaneous and mucocutaneous histiocytoses (C group)
Xanthogranuloma family
Non-Xanthogranuloma family
Rosai-Dorfman Disease (RDD) (R group)
Malignant histiocytoses (M group)
Primary malignant histicytoses
Secondary malignant histiocytoses
Hemophagocytic lymphohistiocytosis (HLH) and macrophage activation syndrome (H group)
Primary HLH: Mendelian inherited conditions leading to HLH
HLH associated with lymphocyte cytotoxicity defects
HLH associated with defect inflammasome activation
HLH associated with defined Mendelian disorders affecting inflammation
Familial (apparently Mendelian) HLH of unknown origin
Secondary HLH: apparently non-Mendelian HLH
Infection-associated HLH
Virus-associated HLH
Bacteria-associated HLH
Parasite-associated HLH
Fungal-associated HLH
Malignancy-associated HLH
Malignancy-triggered HLH
HLH occuring during chemotherapy
HLH associated with defined rheumatologic conditions (MAS or MAS-HLH)
Transplant-related HLH
HLH associated with iatrogenic immune activation
HLH associated with iatrogenic immune suppression
HLH associated with other apparently non-Mendelian condition
HLH of unknown/uncertain origin

Adapted from Emile *et al*, Blood, 2016[14].

1.4 PRIMARY HLH

In a Swedish population-based incidence study in 1991, FHL was reported to occur in 1 of 50,000 live born, or 1.2/1,000,000 children per year[15]. Primary HLH was initially believed to occur only in children, predominantly during infancy and early childhood, but as time and research have revealed the mysteries of HLH, a substantial number of patients with late-onset primary HLH caused by mutations in HLH-associated genes have been reported[16-18].

1.4.1 Genetic HLH

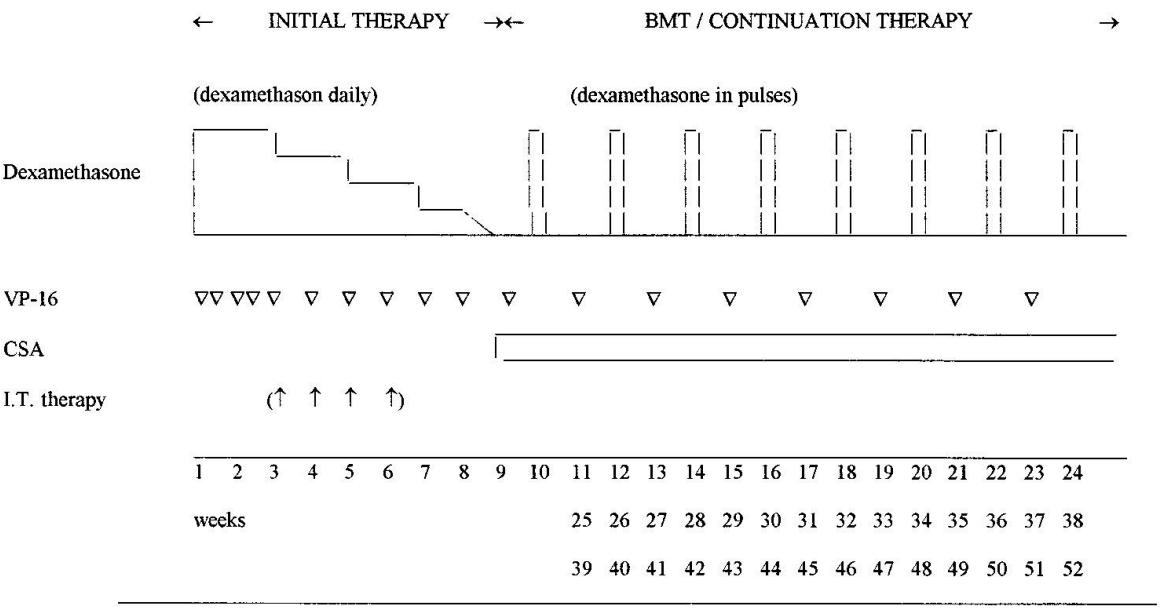
Primary HLH may be divided into familial HLH (FHL) and other primary immunodeficiency syndromes associated with development of HLH. FHL is an autosomal recessive disorder, with disease-causing mutations in genes involved in the granule-exocytosis pathway of cytotoxic lymphocytes, and where HLH is the main manifestation of the disease; four types (FHL 2-5) have been described, whereas FHL 1 has no defined mutation[6]. A number of primary immune deficiencies (PID) have been associated with HLH, but where HLH is only part of the clinical scenario of the syndrome, such as: Griscelli syndrome type 2 (GS-2), Chédiak-Higashi syndrome (CHS), Hermansky-Pudlak syndrome type 2 (HPS-2), X-linked lymphoproliferative syndromes (XLP1 and XLP2), severe combined immunodeficiency, *ITK*-deficiency, CD27-deficiency and chronic granulomatous disease[19,20]. HLH has also been associated with inborn errors of metabolism, such as lysinuric protein intolerance, Gaucher disease and Wolman disease[6,21]. Lymphocyte cytotoxicity function assays and genetic testing should be a part of the evaluation of a suspected primary HLH, along with searching for infectious triggers, but should not delay start of HLH-directed treatment.

1.4.2 Treatment of primary HLH

Without treatment primary HLH is almost invariably fatal. The immune dysregulation and hypercytokinemia with massive inflammation rapidly leads to a multiple organ dysfunction syndrome (MODS), turning the patient into a ticking time bomb. The purpose with all HLH-directed treatment is to calm the cytokine storm and minimize organ damage. In primary HLH it is a bridge-treatment to curative treatment with hematopoietic stem cell transplantation (HSCT)[22-33].

Successful treatment-induced remission of HLH was first seen with corticosteroids combined with chemotherapy (epipodophyllotoxin derivates) or anti-thymocyte globulin, but all FHL patients reactivated in their HLH until HSCT was added[34-37]. Prednisolone, teniposide and intrathecal methotrexate became the treatment backbone of the first Swedish treatment protocol (HLH-91)[38]. In 1994, the first international HLH protocol was developed (HLH-94) based on and resembling HLH-91[39]. A schematic overview of the HLH-94 treatment protocol is presented in Figure 1.

Figure 1. HLH-94 protocol schematic overview



This research was originally published in *Blood*. Henter *et al*. HLH-94: a treatment protocol for hemophagocytic lymphohistiocytosis. HLH study Group of the Histiocyte Society. *Blood.* 1997;28: 342-7. © the American Society of Hematology[39].

The Initial Therapy to achieve remission consists of high-dose corticosteroids, preferably dexamethasone for better CNS penetration, starting at 10 mg/body surface area(m2)/day, tapered during the first 8 weeks, and etoposide 150 mg/m2/dose, biweekly the first 2 weeks and then weekly. Intrathecal therapy with methotrexate is administered to patients with CNS symptoms. Subsequently, Continuation Therapy continues with the same drugs at 2-week intervals, and cyclosporine A (CSA), added to maintain remission by suppression of overactivated T-lymphocytes and reduction of elevated IFN-gamma levels, as a bridge to HSCT[5,40]. Similar to other conditions treated by HSCT, the survival in HLH is better for patients with non-active disease at HSCT[41]. With this protocol, 5-year overall survival of primary HLH successfully improved to 52%. In an attempt to reduce observed early mortality and neurological late-effects, a new international protocol HLH-2004 was established, where Initial Therapy was intensified with CSA already upfront, after its reported good effects on HLH, and corticosteroids were added to the intrathecal therapy[11,42]. However, early addition of CSA and addition of corticosteroids to intrathecal therapy did not significantly improve outcome in patients with HLH, nor significantly improve neurological late-effects[43]. Therefore, current recommendations are treatment according to the HLH-94 protocol, but using HLH-2004 diagnostic criteria[11,44].

HLH patients must be closely monitored since HLH can have an unexpected and aggressive course if unresponsive to therapy. Improvement of inflammatory markers, such as ferritin and sIL-2R, other HLH-associated parameters, and resolution of symptoms should be observed within 2-3 weeks from initiation of treatment for an acceptable therapy response[45]. A less than 50% decline in ferritin and persistent thrombocytopenia are poor prognostic indicators[46,47]. Recurrence of fever, increase of inflammatory markers and liver function tests should prompt

search for an HLH reactivation, that would require treatment intensification or corticosteroids and salvage therapy, or a secondary infection requiring other intervention[45].

Successful treatment of primary HLH has also been reported with a single-center French protocol, including corticosteroids and anti-thymocyte globulin, with similar survival rates[48]. Alemtuzumab (CD52 monoclonal antibody), followed by allogeneic HSCT, has proven successful as salvage therapy for refractory HLH[49]. Subsequently, Alemtuzumab, associated with corticosteroids and CSA, has become a promising first-line therapy for primary HLH[50]. Allogeneic HSCT with reduced-intensity conditioning (RIC) shows reduced transplant-related mortality compared to myeloablative conditioning (MAC) in children[51-54]. Donor chimerism levels as low as 10% without HLH recurrence have been reported, but a lowest donor chimerism level of >20-30% is recommended to protect against late reactivations[55].

1.4.3 CNS-HLH

Although the definition of central nervous system (CNS) involvement in HLH is not clearly defined, abnormal findings in cerebrospinal fluid (CSF) and on brain magnetic resonance imaging (MRI), and neurological symptoms are frequent in primary and secondary HLH[56-61]. HLH may even present as isolated CNS-HLH[62-64]. Patients can present with either abnormalities in CSF or MRI, or neurological symptoms, or with any combination of the mentioned parameters. In a large pediatric cohort of 193 patients with HLH (predominantly primary HLH), 63% of the children presented with either abnormalities in CSF (52%) or neurological symptoms (37%) or both[56]. Similar results were observed in a smaller cohort of children with only primary HLH[58]. In an adult sHLH cohort as many as 90% of patients presented CNS symptoms or signs, with CSF findnings in 39% and MRI changes in 52%[65]. Seizures, irritability, dizziness, impaired consciousness and meningismus are common neurological symptoms, but cranial nerve palsy, motor impairment and ataxia may also occur. CSF findings are commonly mild to moderate pleocytosis together with elevated protein, but either alone is observed in approximately 20% of cases[56,58,60]. Similar neurological symptoms and CSF findings are reported in sHLH, but at a lower frequency in a Chinese cohort compared to primary HLH[59,61]. MRI findings in primary HLH typically show bilateral, symmetric and periventricular white matter hyperintensities as fuzzy lesions in large areas, often sparing basal ganglia and brainstem. Secondary HLH appears to show more focal lesions and hyposignal intensities on T1-weighted sequences, and in adults with sHLH white mater and basal ganglia were most frequently involved[60,61,65]. Treatment recommendations are systemic treatment with corticosteroids (preferably dexamethasone with better CNS penetrance) and etoposide (HLH-94/HLH-2004) with intrathecal methotrexate and steroid therapy[64]. Although complete recovery is not uncommon, many suffer from permanent motor and cognitive deficits[41,43,56].

1.4.4 Biology of primary HLH

Natural killer cells and cytotoxic T lymphocytes

Natural killer (NK, $CD3^{-}CD56^{+}$) cells and cytotoxic T-lymphocytes (CTL, $CD8^{+}$), known as cytotoxic lymphocytes, play a key role in maintaining immune homeostasis[66]. They recognize

and eliminate cells infected by viruses and other intracellular pathogens, tumor cells, and terminate the immune response, by elimination of antigen-presenting dendritic cells (DCs), once the immunological stimulus has been cleared. NK cells, part of the innate immune system, recognize the diseased cells in the absence of major histocompatibility complex (MHC) surface markers, through specific antigen receptors. Target cell contact activates the NK cell, releasing its preformed cytotoxic granule content to the target cell, allowing for early, rapid killing. CTLs are activated by specific antigen recognition through MHC on the target cell surface. The T-cell receptor activation causes cytokine-induced (eg by IL-2, IL-6) expression of cytotoxic granules, released at the next antigen encounter, and proliferation of antigen-specific CTLs, allowing for late, massive target cell killing[66]. Cytotoxic lymphocytes have secretory granules that contain proteases, such as granzyme A and B, that cause apoptosis when released into the target cell through a perforin-mediated immunological synapse formed upon cell contact[9].

Genetics of primary HLH

Disease-causing mutations in genes involved in the trafficking or exocytosis of granules, or perforin-formation in the perforin-dependent granule exocytosis pathway, have been identified for FHL 2-5 and in GS-2, CHS and HPS-2[6,9,67]. The specific gene involved for FHL-1 remains unknown. Mutations in the *PRF1*-gene encoding the pore-forming protein perforin cause FHL-2, whilst FHL 3-5 are caused by mutations in genes *UNC13-D*, *STX-11* and *STXBP2*, encoding for the proteins Munc13-4, Syntaxin 11 and Munc 18-2, respectively[68-72]. The PID syndromes GS-2, CHS and HPS-2 have disease-causing mutations in *RAB27A*, *LYST* and *AP3B1*[19,73]. XLP 1 and XLP 2 on the other hand are caused by mutations in *SH2D1A* (encoding SAP) and *BIRC4* (encoding XIAP) involved in the normal immune function of T cells, predisposing affected individuals to severe Epstein-Barr Virus (EBV) infections with associated HLH, but also to a variety of conditions including hypogammaglobulinemia, lymphoma (XLP1) and chronic hemorrhagic colitis (XLP2)[74-78]. The importance of perforin and lymphocyte cytotoxicity in tumor surveillance has been demonstrated in perforin-deficient mice, and also seems supported in human studies reporting increased occurrence of malignancies in patients carrying heterozygous mutations in HLH-associated genes[79-82].

The genetic defects in perforin or in the granule exocytosis pathway in NK cells and CTLs cause decreased intracellular protein expression or defect degranulation and cytolytic activity, which is measured with lymphocyte function assays and used as a diagnostic tool for primary HLH. These methods are described further in the *Patients, Methods and Materials* section.

Hypercytokinemia in HLH

In an immune competent individual activated NK-cells and CTLs will secrete pro-inflammatory cytokines such as IL-2 and IFN-γ, promoting activation and proliferation of CTLs, but also activation of macrophages for phagocytosis and lysis. Activated macrophages, in turn, secrete multiple pro- and anti-inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-10, IL-12, IL-18 and TNF-α to maintain the inflammatory response with immune cell activation and proliferation for pathogen elimination. When the pathogen has been cleared CTLs will

eliminate antigen-presenting DCs and NK cells, and T regulatory cells will limit CTL activation, altogether to re-establish immune homeostasis[7].

Defects in NK-cell and CTL cytotoxicity, as in HLH, result in an ineffective clearance of the pathogen and prolonged antigen presentation, overstimulating NK cells and CTLs to secrete excessive amounts of IFN-γ, leading to overactivated CTLs and macrophages, producing a cytokine storm with characteristic HLH manifestations; schematically illustrated in Figure 2[5,6,11].

The hypercytokinemia has been linked to delayed detachment of the defective cytotoxic lymphocyte from the target cell with prolonged synapse time causing hypersecretion of cytokines[83]. The role of overactivated CTLs and IFN-γ in the pathogenesis of HLH has been elucidated by several murine models, and HLH manifestations have also been inhibited by anti-$CD8^+$-antibodies and IFN-γ-neutralization[84-86]. Most animal models require a viral trigger to develop HLH and show a wide spectrum of disease severity, which seems related to the degree of residual cytotoxic function[87,88]. Interestingly, in a study on PID, patients with T- and NK-cell deficiency were able to manifest typical symptoms of an HLH syndrome. In these T- and NK-deficient patients with HLH syndrome perhaps activated macrophages and the associated cytokine storm are the main drivers of hyperinflammation.[20]. The extent to which IFN-γ plays a role in the pathogenesis of primary HLH in humans and in the heterogeneity of HLH phenotypes, remains to be elucidated. However, elevated levels of IFN-γ in patients with primary HLH have been reported, and remission of HLH activity in selected cases of primary HLH with anti-IFN-γ therapy (emapalumab), suggests IFN-γ as an important mediator of HLH pathogenesis[5,89,90]. Etoposide, the mainstay of treatment for HLH, has been shown to selectively deplete activated CTLs and efficiently inhibit cytokine production in a murine model of HLH, further supported *in vivo* by resolution of HLH with etoposide-containing therapy[91]. Importantly, NK cell cytotoxicity has been shown to reduce tissue infiltration by activated macrophages and limit CTL responses and activation, thereby, reducing HLH manifestations and fatal outcome in a murine model[92].

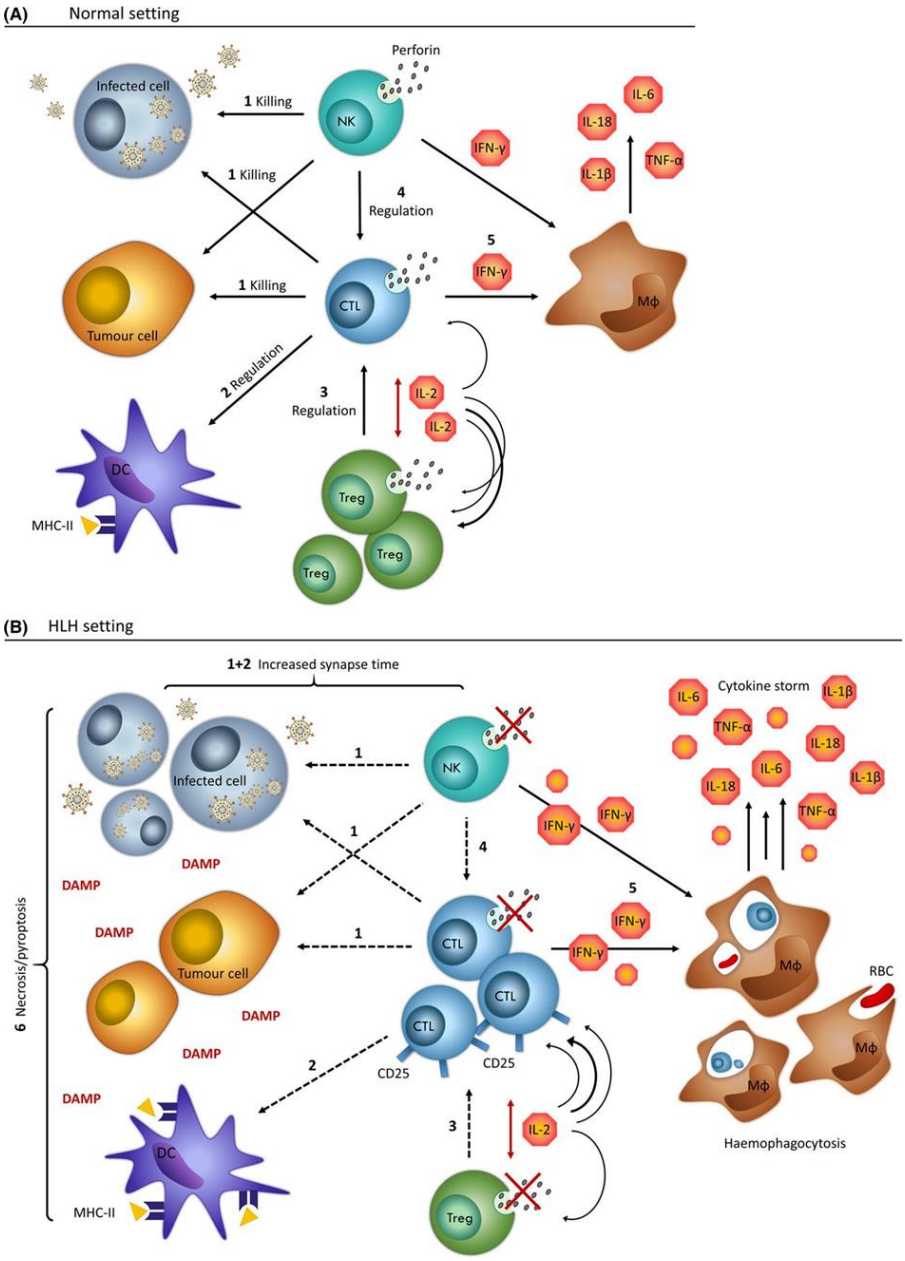


Figure 2. Hypothesized pathogenesis of primary HLH.
Brisse *et al*, 2016, Br J Hematology, with permission from John Wiley and Sons[7].
(A) Normal situation: 1. CTLs and NK cells eliminate tumour cells and/or infected cells via apoptosis. 2. When the immunological stimulus is cleared, the CTLs will inhibit further antigen presentation by removing antigen-presenting DCs. 3. Tregs compete with the activated CTLs for the available IL2 and thus limit the proliferation of CTLs. They may also directly eliminate activated CTLs. 4. NK cells likewise control the size of the activated CTL pool via induction of apoptosis. 5. This limits the amount of CTL-derived IFN-c and consequently the extent of macrophage activation and additional cytokine production. (B) In the setting of primary HLH: 1. CTLs and NK cells fail to eliminate tumour cells and/or infected cells, which continue to replicate, resulting in persistent antigenaemia. 2. CTLs no longer remove the antigen-presenting DCs, leading to prolonged and heightened antigen presentation, stimulating antigen-specific T cells. 1+2. The inability of CTLs and NK cells to eliminate target cells causes an increase in synapse time, stimulating the effector cells to produce more cytokines. 3. Tregs can no longer outcompete the hyperactivated CTLs for the lower amount of available IL2 because the expression of CD25 is more highly upregulated in the latter. Treg numbers drop and CTLs continue to proliferate. 4. Similarly, lacking their cytotoxic capacity, NK cells no longer control the size of the activated CTL pool. 5. The activated CTLs produce massive amounts of IFN-c inducing excessive macrophage activation and directly provoking haemophagocytosis. In turn, the activated macrophages produce enormous amounts of pro-inflammatory cytokines, creating a cytokine storm. 6. As cells can no longer be eliminated via immunologically silent apoptosis, necrosis and/or pyroptosis will occur, resulting in the release of DAMPs that instigate further inflammation. Solid black arrows indicate the effective function of the processes as described by the numbers written above them (A), dotted black arrows indicate impaired function of these processes (B). Red arrows refer to the competition for available IL2 consumption (A+B). HLH, haemophagocytic lymphohistiocytosis; CTL, cytotoxic T lymphocyte; DAMP, danger-associated molecular pattern; DC, dendritic cell; Mφ, macrophage; MHC, major histocompatibility complex; NK, natural killer; RBC, red blood cell; Treg, regulatory T cell.

1.5 SECONDARY HLH

Secondary (acquired) HLH (sHLH) is by far more common than primary (genetic) HLH, and can occur in all age groups, but is more common in adults[93,94]. A quick search on PubMed reveals that prior to 1990 there were barely 200 publications on HLH, which by year 2000 had increased to about 800 publications, but today, we find over 6000 publications on HLH. There has been an explosive rise in awareness of HLH in the last decades, in particular secondary HLH, and even so, it is reported to be underdiagnosed[95]. The incidence of sHLH is unknown and varies greatly between study populations. A nationwide survey on HLH in Japan, with 799 reported HLH cases, revealed an annual incidence of 1 in 800,000, where the absolute majority were sHLH[96]. Even mortality in HLH shows heterogeneity depending on underlying disease and trigger, and length of follow up, but is overall high, reported between 20 - 88% in a scoping review on adult HLH, with the highest mortality in malignancy-associated HLH compared to other secondary forms of HLH[97].

Secondary HLH is most commonly associated with infections, malignancies, autoimmune and autoinflammatory diseases, and less commonly with solid and hematological transplantation, immune activating therapies such as BiTEs (bi-specific T-cell engagers) and CAR-T (chimeric antigen receptor T-cells), and other less frequent associations such as pregnancy and pharmaceutical drugs[6,93]. Immunosuppression in patients with sHLH is not unusual [12,98,99]. Globally, there is geographical asymmetry of the reported predominant trigger factors in sHLH, with an overrepresentation of malignancy-associated HLH (mal-HLH) in Asia, predominance of EBV-associated HLH (EBV-HLH) in USA and Asia, and human immunodeficiency virus (HIV)-associated HLH in Europe, suggesting a possible heterogeneity in population genetic predisposition or variety in triggering factors[93]. However, mal-HLH is proportionately high even in large European adult HLH studies[98,99].

1.5.1 Similar but not the same

Much of today's knowledge, diagnostic tools and management of sHLH originates from primary HLH, a genetic immune deficiency predominantly found in children that requires prolonged therapy with immunosuppressive drugs to survive to allogeneic HSCT. Although genetic and acquired HLH have similar clinical presentation and hyperinflammatory backbone, they have their dissimilarities that require a different diagnostic and therapeutic approach. Hypomorphic mutations with a more indolent clinical course have been identified in adults with HLH, but these are a minority[16-18]. Secondary HLH has a more complex scenario than just a genetic etiology driving the hyperinflammation. In a setting of temporary immune dysregulation, underlying diseases or conditions that predispose to HLH, such as malignancy or immune suppression, and external factors that trigger hyperinflammation, such as infections, play a vital role in the etiopathogenesis of sHLH.

The HLH-2004 diagnostic criteria, based on the first diagnostic guidelines in 1991, are founded on observations from a pediatric population with predominantly primary HLH, but are still valid and widely used for evaluation of sHLH, despite certain limitations[11]. Based on an adult

population, Fardet et al created a criterion-weighted probability score for hemophagocytic syndrome in adults (HScore), in 2011 (Table 1)[12]. The HScore ranges from a score of 90 with <1% probability of HLH to a score >250 with 99% probability of HLH. The best cut-off score was 169 with a sensitivity of 93% and specificity of 86%, and correct classification of 90% of the patients with HLH. Other described common manifestation, not included in the criteria, in secondary and adult HLH (and frequently also seen in primary HLH) are coagulopathy, elevated lactate dehydrogenase (LDH), bilirubin, D-dimers and C-reactive protein (CRP), hyponatremia, hypoalbuminemia, rash and CNS involvement[98,100-103].

1.5.2 Diagnostic evaluation of secondary HLH

Today, it is suggested that the HLH-2004 criteria be used in the evaluation of adult HLH, with HScore also considered a useful diagnostic tool[103,104]. In certain settings, where HLH is highly suspected, diagnosis and treatment may be started without fulfillment of 5 HLH-2004 criteria; such as in patients with an unexpected rapid progress and severity of symptoms and laboratory abnormalities or in patients with CNS involvement. In the author's experience these former patients commonly fulfill 4/8 HLH-2004 criteria and frequently also have a high Hscore. Repeated evaluations are necessary due to the rapid course an evolving HLH may take. A recent study on the reliability of HLH-2004 criteria and HScore in an adult intensive care setting found the best prediction accuracy of HLH diagnosis to be ≥4/8 fulfilled HLH-2004 criteria and HScore ≥168[104]. The particular cytokine pattern of highly elevated IFN-γ and IL-10 with lower IL-6 may also support the diagnosis of HLH[89]. Separate pieces of a puzzle will not give you the diagnosis of HLH, but it's the picture you see when they are all put together; combining HLH criteria, underlying disease and risk factors, and the clinical progression for the evaluation of HLH.

The search for a trigger in sHLH is imperative! Extensive microbial testing, including all types of pathogens, is essential. If possible, a bone marrow examination should be performed, not only to identify hemophagocytosis, but more importantly to exclude any malignancy; preferably prior to administration of corticosteroids or cytotoxic drug, although it should not delay vital treatment. Extensive imaging, including positron emission tomography/computer tomography (PET/CT) imaging, and generous tissue sampling (biopsies) are required to identify even occult malignancies that may be triggering the hyperinflammation[103,105]. Reports on CNS involvement in sHLH are limited but has been reported ranging from 21 to 89% in adult HLH patients, with potentially devastating consequences, why lumbar puncture and MRI should always be considered[64,65,106]. Functional lymphocyte cytotoxicity and genetic testing are not essential in sHLH since HLH-causing mutations are rarely found as the source of hyperinflammation. However, it may be explanatory, and recommended, in cases of relapsing or refractory HLH, young age, family history of consanguinity, severe EBV infection in a young male, other manifestations (e.g. albinism) indicating a possible PID and HLH with an unknown trigger[103].

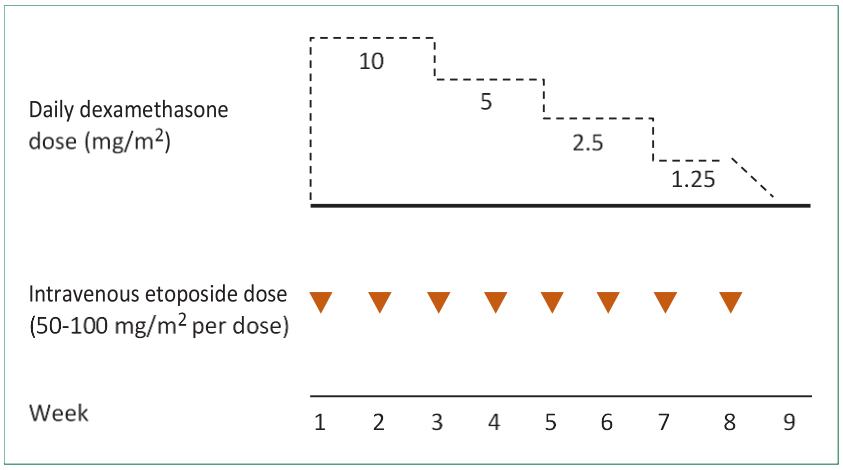
1.5.3 Treatment of secondary HLH

Treatment of sHLH must be tailored with several considerations in mind, such as different etiologies and triggering factors, varying degrees of immune dysregulation and immunosuppression, and comorbidities with lower tolerance to cytotoxic drugs with increasing age[103,107-109]. Decision to start HLH-directed therapy should always be based on the underlying condition, clinical progress and organ function[110]. HLH is a life-threatening and rapidly progressive condition, which requires prompt diagnosis and timely tailored treatment, of the right kind and intensity[103,111]. Not surprisingly, treatment for the different forms of sHLH varies. Treatment recommendations for sHLH have previously mainly been based on experiences from primary HLH, case-series studies, case reports and expert consensus, but more recently there is also data from retrospective and prospective studies, not least from East Asia.

For all forms of sHLH, it is imperative to treat the triggering factor, i.e. to remove a main elicitor of the cytokine storm. In moderately active HLH, that does not respond to trigger-directed therapy or appropriate empirical treatment and supportive care, addition of corticosteroids is recommended, such as dexamethasone or pulsed corticosteroids[45,103,110]. The benefit of adding intravenous immunoglobulins (IVIG) is debated, but has been shown beneficial in some studies on infection-associated HLH and autoimmunity/autoinflammatory-associated HLH (also known as macrophage activation syndrome-HLH, MAS-HLH), with doses such as 0.5g/kg/day for 3 days, while other studies suggest IVIG to be removed in MAS-HLH treatment[102,112-115]. The addition of IL-1 receptor antagonist (anakinra) is proving beneficial in several forms of sHLH, and other cytokine-inhibitors, such as anti-IFN-γ are also being trialed[115-119]. In cases of severe HLH with imminent organ failure, not uncommonly mal-HLH or EBV-HLH, addition of dose-adjusted etoposide is recommended[44,103,120-122]. The HLH-94 protocol prescribes etoposide 150 mg/m2 body surface area (BSA)/dose, initially biweekly. However, reduced age-adjusted etoposide doses of 50-100 mg/m2 BSA/dose, further reduced in the presence of renal insufficiency, on a weekly basis, is frequently sufficient in sHLH, as suggested by Henter *et al* for severe influenza-associated HLH (Figure 3).

Furthermore, aggressive supportive care and broad antimicrobial and antiviral prophylaxis, against opportunistic diseases including aspergillosis in these immunocompromised patients, is of uttermost importance to prevent further organ damage. HLH-directed treatment with weekly etoposide must be re-evaluated after every dose to determine tapering of treatment and cessation, since length of treatment should be guided by remaining disease activity, and not a predetermined protocol. The risk of treatment-related AML lies no higher than 1% with accumulated etoposide dose below 1500 mg/m2 BSA, and hence, the risk of morbidity and mortality from severe HLH is higher[44]. Useful markers to monitor disease activity and therapy response are ferritin and sIL-2R, as well as platelets, fibrinogen and liver transaminases[46,120,123].

Figure 3 Schematic overview of modified etoposide treatment of severe sHLH



Adapted from Henter *et al*, Cytotoxic therapy for severe avian influenza A (H5N1) Infection. Lancet, 2006[124].
The continued administration of weekly etoposide should be reevaluated before each dose. For treatment option details, see text.

Remaining HLH activity after 8-12 weeks of treatment should prompt further search for other underlying triggers or possible genetic predisposition, and may respond to maintenance therapy such as that in the HLH-94 protocol. Salvage therapy for refractory or relapsing HLH includes treatment intensification with chemotherapy, such as intensification of the HLH-94/2004 protocol, other chemotherapy, such as the DEP (liposomal doxorubicin, etoposide, methylprednisolone) regimen, alemtuzumab, and possibly allogeneic HSCT[125-127]. JAK1/2 inhibitor ruxolitinib has been successfully used upfront and for refractory disease in HLH[128-130]. Prognostic markers of poor survival in sHLH reported in several larger studies are lymphoma, high age, highly elevated ferritin, thrombocytopenia, multiple infection triggers and lack of etoposide treatment[99,100,122,131,132].

Immunotherapies, such as CAR-T, BiTEs and checkpoint inhibitors, may induce a cytokine release syndrome similar to HLH. Successful inhibition of the cytokine release storm is seen with treatment interruption or corticosteroids and inhibitors of IL-6 (tocilizumab)[133-135].

1.5.4 Infection-associated HLH

The first report on infection-associated HLH (IA-HLH) was in 1979 in a case series of 19 children. In fourteen of nineteen patients there was immunosuppression and infection by herpes virus documented. Therapy consisted of supportive care and withdrawal of immunosuppressive treatment; thirteen children survived[136]. However, mortality of IA-HLH, reported at 17-67%, varies greatly with etiology and age of the patient[93,96,98,99]. It is important to stress that infections also are a common trigger of primary HLH, and hence, cannot distinguish between the primary and secondary form of HLH [43,137].

Viruses are the most common cause of IA-HLH, predominantly EBV, but also HIV, cytomegalovirus (CMV) and influenza virus. However, HLH has also been associated with bacteria, fungi and protozoa infections[93,138]. Diagnostics should include viral polymerase chain reaction (PCR) wherever possible to identify disseminated infections[139]. A large study of 151

adults with sHLH identified two or more infectious triggers as the only independent prognostic factor associated with death[99]. Aggressive disease-specific therapy to eliminate the infectious trigger is vital, with addition of corticosteroids and IVIG when necessary. Occasionally, it may suffice with specific trigger-directed treatment to resolve the HLH if it is mild[136]. HLH associated with intracellular pathogens, such as leishmaniasis, rickettsia, and tuberculosis, also generally respond well to solely disease-specific treatment[140]. Prognosis of HIV-associated HLH, mostly caused by opportunistic infections and lymphoma, has improved with antiretroviral treatment, but still has a mortality of about 30%, despite treatment with corticosteroids, IVIG and in more severe cases etoposide[141].

Epstein-Barr virus-associated HLH

A nationwide survey on HLH in Japan, with 567 patients included for further analysis, revealed EBV-associated HLH (EBV-HLH) in 29% of cases, a majority under 15 years old, and 24% with other forms of IA-HLH[96]. Furthermore, 5-year overall survival exceeded 80% in EBV-HLH, but older patients and those with severe EBV-HLH did worse. EBV-HLH primarily occurs in primary EBV infections, determined by serology, but it also occurs in EBV reactivations, with a worse prognosis[96,142]. Besides fulfillment of HLH-criteria, diagnosis of EBV-HLH requires an active EBV infection, determined by EBV-DNA copies and EBER-ISH (EBV-encoded RNA in situ hybridization), or demonstration of clonality[143,144]. Importantly, the presence of an EBV infection does not rule out mal-HLH, which must be excluded for correct therapeutic approach. EBV is commonly known to infect B-cells in infectious mononucleosis, but in EBV-HLH infection of T cells, as well as NK cells, is observed, and frequently EBV/T-cell receptor clonality[144-147]. Higher EBV genome copies are found in EBV-HLH compared to infectious mononucleosis, and a viral load $>10^3$ copies/mL is commonly found in EBV-HLH[96,148]. A higher viral load has been correlated with increased mortality[149].

An early report from 1998 on virus-associated HLH (VA-HLH) reported the survival of only 27 of 99 patients with EBV-HLH[150]. Treatment of EBV-HLH with the HLH-94 protocol, containing corticosteroids and etoposide, greatly improved survival to >80%, without HSCT[151]. Importantly, early administration of etoposide, within 4 weeks of diagnosis, significantly improves survival in severe EBV-HLH[121]. Addition of rituximab (anti-CD20 monoclonal antibody) may further reduce EBV load and improve therapeutic efficacy[152]. This treatment approach has shown a sustained long-term survival of patients with EBV-HLH close to 80%, including 19% HLH reactivations[153]. On the other hand, a step-wise approach starting with corticosteroids and CSA, with treatment intensification with the addition of chemotherapy if there is an insufficient response has also been shown successful, with 54% remitting without chemotherapy[147]. A 3-year survival of above 90% in pediatric EBV-HLH has been reported, with most children (60%) treated with corticosteroids, etoposide and CSA[154]. A recent report on ruxolitinib, a Janus kinase (JAK)1/2 inhibitor, as upfront treatment of EBV-HLH gave 75% complete remission at 6 months follow-up[155]. Fever, plasma levels of ferritin, sIL-2R, LDH and EBV-DNA copies are useful markers to monitor therapy response and course of disease[143,147]. Highly elevated sIL-2R at diagnosis is associated with worse outcome[156].

Reactivations of EBV-HLH may respond to treatment intensification, but a minority of patients with refractory EBV-HLH may be candidates for HSCT, with an overall good outcome in EBV-HLH, somewhat better in children than adults[126,153,157]. Patients with refractory EBV-HLH and sustained high (>10^3-10^4 copies) or increasing EBV-DNA copies should be evaluated for chronic active EBV (CAEBV), a EBV-positive T/NK lymphoproliferative disease, since these patients have a dismal prognosis with only chemotherapy and require allogeneic HSCT for better outcome[158-161]. Salvage therapy for EBV-HLH with DEP-regimen showed 73% overall response, but a 50% mortality due to HLH recurrence or infections[162]. Recently, escalating doses of ruxolitinib as a bridge to allogeneic HSCT in an EBV-HLH patient was reported successful[163].

Influenza AH1N1-associated HLH

The "Spanish flu" of 1918-1919, caused by influenza AH1N1 virus, was the worst pandemic recorded in recent history with 25-50 million estimated deaths[164]. A distinct feature of the 1918 pandemic was the "W-shaped" age-mortality curve, with almost half of the influenza deaths occurring in the age group 20-40 years[165]. A review on the more recent 'avian' influenza AH5N1 found 907 (94.6% confirmed) human cases reported between 1997 and 2015, more than half (53%) fatal with a mortality peak in the age group 24-35 years[166]. Although the 2009 pandemic 'swine' influenza AH1N1 was milder with an estimated infection fatality risk of 1-10 per 100,000 infections, young adults were still at 2-4 times higher risk of fatal outcome compared to seasonal influenza[167,168]. Hospitalized influenza AH1N1 patients had an overrepresentation of previously healthy, young adults, and pregnant women, with median age 30-50 years, that were likely to be admitted to the intensive care unit (ICU) with complications of hypoxemia, septic shock and organ failure, with frequent use of rescue therapies and a high mortality[169-171]. A significant proportion (20-30%) of critically ill 2009 influenza AH1N1-infected patients required further support with extracorporeal membrane oxygenation (ECMO) mainly due to acute respiratory distress syndrome (ARDS), with mortality reports varying between 8-65%[172,173].

Fatal pandemic influenza A may share many characteristics with HLH, such as high levels of proinflammatory cytokines and hemophagocytosis, and a similar sepsis-like inflammatory condition with multiple organ failure[171,174,175]. There are several reports on influenza AH1N1-associated HLH (AH1N1-HLH), describing similar characteristics to other well-recognized virus-associated HLH types, such as EBV-HLH[150,176-181]. These reports describe both successful and unsuccessful treatment of AH1N1-HLH with steroids, IVIG and/or cytotoxic drugs.

Why so many previously healthy young adults succumb to severe illness in influenza AH1N1, with a high mortality rate, remains incompletely understood. It has been postulated that the highly virulent influenza A virus elicits an excessive inflammatory immune response in the young, robust, AH1N1-naïve immune system of these young adults[182,183].

Dengue-associated HLH

Dengue is a mosquito-borne viral infection with a wide disease spectrum, from asymptomatic infection to severe life-threatening illness. Globally, the incidence is estimated at 390 million dengue virus infections per year of which about 96 million manifest clinically[184]. According to the World Health Organization (WHO) about 500,000 cases with severe dengue require hospitalization yearly. There are four serotypes of dengue virus (DENV 1-4), and whilst infection by one serotype is believed to cause life-long immunity, subsequent infections by other serotypes increase the risk of severe dengue. The confirmation of dengue virus infection is by viral isolation, detection of viral RNA by PCR, antigen (namely NS1) or antibodies[185].

Symptomatic dengue causes flu-like symptoms that typically last for 2-7 days. WHO has classified dengue in to two major types: dengue, with and without warning signs, and severe dengue (SD). Dengue should be suspected in patients with high fever (40°C) and at least 2 other flu-like symptoms, such as headache, myalgia and arthralgia, vomiting, lymphadenopathy and rash. About 3-7 days after illness onset, in the so-called critical phase, manifestation of warning signs associated with SD, such as severe abdominal pain and vomiting, tachypnea, fatigue and bleeding, should lead to close observation and intervention. WHO 2009 classification for dengue has further defined criteria for SD as: 1) severe plasma leakage, leading to shock and/or fluid accumulation with ARDS, 2) severe bleeding, as evaluated by clinician, and 3) severe organ impairment of liver (with elevated liver transaminases ≥1000 U/L) and/or CNS (with impaired unconsciousness), and/or heart and other organs[185]. Treatment of dengue mainly consists of antipyretics and fluid balance control, where increasing severity of dengue requires more extensive fluid management. Patients with SD may also require additional supportive care and adjuvant therapy, as recommended by WHO guidelines. Dengue is one of the most common reasons for ICU admission in endemic countries. Deaths in dengue have been estimated to between ~10,000-20,000 per year with a rising trend in both incidence, severity and mortality in the last decade [186,187].

Patients with SD, irrespective of serotype, are at increased risk of developing HLH (dengue-HLH). Dengue-HLH affects children and adults, with most reports originating from India, Puerto Rico, Thailand and Malaysia, with a case fatality report of 14.6%[188]. Persistent fever, splenomegaly, hepatomegaly, cytopenias, hyperferritinemia, elevated liver enzymes and multiple organ dysfunction in a deteriorating severe dengue patient should raise suspicion of HLH[188,189]. Ferritin has been proposed as a biomarker for disease severity in dengue, and hyperferritinemia should prompt physicians to evaluate for SD and HLH. One study showed good prediction sensitivity and specificity of ferritin for disease severity particularly at the time of defervesence, when manifestations of SD are commonly observed[190]. Hyperferritinemia in dengue has also been associated with viremia, thrombocytopenia, elevated liver enzymes, coagulopathy and sIL-2R, all common in states of hyperinflamation[191]. Current treatment recommendations for SD with fluid management and supportive care may be successful to treat patients with milder forms of dengue-HLH, but several reports demonstrate the need for additional HLH-directed therapy, predominantly corticosteroids and IVIG, but in selected cases also cytotoxic drugs, preferentially etoposide[189,192-194].

1.5.5 Malignancy-associated HLH

Malignancy-associated HLH (Mal-HLH) occurs in both children and adults, but the likelihood of an underlying malignancy increases with age. In several large cohorts mal-HLH account for around half of the cases of HLH in adults[93,98,100,132]. A Swedish population-based study reported the incidence of HLH in adults with hematological malignancies to be 1%[195]. Mal-HLH occurs in the setting of a neoplasms in two different situations: as a presenting feature at diagnosis or relapse of a malignancy ("malignancy-triggered HLH", MT-HLH), or as a manifestation during or after chemotherapy ("HLH during chemotherapy", Chemo-HLH)[108].

The pathobiology of MT-HLH is still unclear, but besides the persistent antigen stimulation by the malignant cells, they possess other characteristics that can co-trigger hyperinflammation in MT-HLH. Non-Hodgkin lymphomas (NHL), in particular, secrete numerous inflammatory mediators and growth factors, such as IL-6, IFN-γ and TNF-R1[196,197]. EBV is a well-known trigger of HLH and of EBV-associated lymphomas, especially Hodgkin lymphomas[198]. In a Japanese study on lymphoma-associated HLH (LA-HLH), EBV genome was detected in 83% of T/NK-cell LA-HLH and only in 13% of B-cell LA-HLH[199]. MT-HLH predominantly occurs with hematological malignancies, and most frequently T- and NK-cell lymphomas and leukemias, as well as B-cell lymphomas, particularly diffuse large B-cell lymphoma (DLBCL) and Hodgkin lymphoma[93,94,108]. T-cell malignancies are the most common mal-HLH trigger in children[200]. Importantly, mal-HLH can occur even at young age when primary HLH could be suspected, and with a concurrent infection a true mal-HLH could be masked[201].

Chemo-HLH occurs most frequently during or after chemotherapy of leukemias and lymphomas, with the highest risk in malignancies treated with an aggressive chemotherapy regimen, such as acute leukemias, and commonly but not exclusively, during the more chemo-intensive induction and consolidation phases. The cytotoxic drugs cause or enhance immune dysregulation, including T and NK cells, and predispose the immunocompromised patient to infections and HLH[109]. Chemo-HLH is almost always associated with infectious triggers, and due to the immune compromised milieu, infections by bacteria, viruses (frequently herpesviridae and adenovirus) and fungi are all common[200,202].

The age-dependency of mal-HLH demands a more thorough search for neoplasms with increasing age. Not uncommon, the perseverant search for the trigger of an HLH of unknown etiology finally detects an occult lymphoma[108]. Splenectomy in a study of 22 patients with refractory HLH of unknown etiology revealed 7 patients with occult lymphoma in the spleen, why splenectomy may be appropriate in selected patients[203]. There are no specific criteria for the definition of MT-HLH and chemo-HLH, and currently, despite their limitations, HLH-2004 criteria are recommended, frequently used with support of the HScore[11,12,108]. In an attempt to improve diagnostic criteria for mal-HLH, variations of the current criteria have been suggested; one such being an extended 18-point HLH criteria set, including additional parameters associated with mal-HLH and readily available in clinical practice, which detected a higher number of mal-HLH patients with poor survival, than with HLH-2004 criteria alone, that could have benefited from timely HLH-directed therapy[199,204]. Soluble IL-2R is a sensitive marker of

HLH, as well as a marker associated with tumor burden in NHL[205,206]. An elevated sIL-2R/ferritin ratio may further support diagnosis of LA-HLH compared to non-malignancy-associated sHLH[207,208].

It is important to distinguish between MT-HLH and chemo-HLH, since they have different therapeutic approaches. As with other forms of sHLH, treatment must be adjusted to the underlying disease, clinical progress and organ function in the patient. Timely and adequate neoplasm-directed therapy is of uttermost importance in MT-HLH, but whether HLH-directed therapy or malignancy-directed therapy should be given first or in combination is still uncertain. Daver *et al* suggest a 2-step approach (that the author personally finds in favor) with first, HLH-directed therapy to reduce and halt the frequently aggressive cytokine- and hyperinflammation-induced organ dysfunction, which may not allow for adequate neoplasm-specific therapy, and second, malignancy-directed therapy when organ function and condition of the patient is acceptable for appropriate neoplastic treatment[109]. If HLH-directed therapy with a modified age-adjusted HLH-94 protocol, including etoposide and corticosteroids as previously suggested is insufficient, chemotherapy intensification or salvage therapy may be successful[103,108,109,162]. Since many lymphoma protocols, such as the CHO(E)P-protocol commonly used for LA-HLH, include corticosteroids and etoposide (or allow for its addition), they can target both the malignancy and to some extent the T-cell promoted hyperinflammation, however, it is not seldom insufficient in more severe mal-HLH[100,102,110,209]. Addition of rituximab in malignancies with high EBV genome copies should be considered. Furthermore, treatment of any concurrent infections, which may contribute to the hyperinflammation, and wide antimicrobial prophylaxis and screening surveillance for secondary infections is strongly recommended[108]. Despite aggressive HLH- and malignancy-directed therapies, mortality in mal-HLH still remains high, many with active HLH at death, and LA-HLH is a persistent prognostic factor of poor outcome in adult HLH[100,122,131,132,200,210,211]. T/NK-cell lymphomas show a particularly poor survival[199,210,212]. There are few reports on allogeneic HSCT in sHLH, but it may be indicated in selected malignancies[97,103].

The immunocompromised state of the patient with chemo-HLH and concurrent infection demands immediate withdrawal of immunosuppressive therapy and aggressive infection-specific treatment, along with adequate supportive care. Addition of corticosteroids, with or without IVIG, is frequently required, whilst etoposide should be used sparingly to avoid further immune dysregulation[108]. In the author's experience, anakinra may also be beneficial, in particular as an alternative to etoposide in case of severe neutropenia. Clinical severity should guide the extent of HLH-directed therapy necessary. Although rare, relapse of malignancy as a contributing trigger to chemo-HLH should be excluded. Despite succeeding to eliminate the HLH, mortality in chemo-HLH remains high, and in one study 6-month survival was reported at 63%[200,202]. Therefore, to decrease risk of chemo-HLH broad anti-microbial prophylaxis and regular surveillance is strongly recommended[108].

1.5.6 Macrophage activation syndrome-HLH (MAS-HLH)

Macrophage activation syndrome is a form of sHLH in the context of rheumatologic disorders, i.e. systemic autoimmune or autoinflammatory diseases, such as systemic juvenile idiopathic arthritis (sJIA) and systemic lupus erythematosus (SLE) in children, and adult-onset Still's disease (AOSD) and SLE in adults[213-215]. In a 2016 revised classification of histiocytic disorders, the term MAS-HLH was used to classify HLH associated with systemic juvenile idiopathic arthritis (sJIA), adult onset Still's disease (AOSD), systemic lupus erythematosus (SLE), vasculitis, and other defined autoimmune conditions[14]. Similar to other forms of sHLH, MAS-HLH is a product of immune dysregulation and cytokine storm[216]. The frequency of overt MAS-HLH complicating sJIA is ~10%[213,217]. However, up to 30-40% may present with occult MAS-HLH[218,219]. MAS-HLH is reported to occur in ~10-15% of adults with AOSD and ~4% in SLE. Infections and onset of disease are common triggers of MAS-HLH[214,215,220,221]. The mortality in MAS-HLH remains high, reported at ~4-15%[222].

Several guidelines and criteria for the diagnosis of MAS-HLH have been suggested throughout the years. The initial HLH-2004 criteria and preliminary diagnostic guidelines for MAS-HLH had limitations for the diagnosis of MAS-HLH[11,223-225]. Through international collaboration a set of diagnostic criteria complicating sJIA was developed, but primarily for use in clinical trials and research[226]. Thereafter, a weighted MAS-HLH score (MS score) for identification of MAS in sJIA was developed, as well as an MH score to help clinicians distinguish MAS-HLH from primary HLH[227,228]. While MAS-HLH has many similarities with other forms of sHLH, there are differences in clinical presentation that must be considered, such as frequently normal but decreasing levels of platelets, neutrophils and fibrinogen at presentation, with rising ferritin and aspartate aminotransferase (AST). Consequently, the importance of the dynamics of laboratory tests at diagnosis and during development of MAS-HLH has been highlighted[229,230]. Typical manifestations of MAS-HLH include prolonged fever, hepatosplenomegaly, lymphadenopathy, CNS involvement, hemorrhagic manifestations, falling blood cell line values and fibrinogen, and increasing ferritin, triglycerides and LDH[217]. Diagnostic inflammation markers, such as ferritin and s-IL2R, have been associated with disease activity in systemic rheumatologic diseases and MAS-HLH[219,231]. In MAS-HLH, CNS involvement has been reported in ~30-40% of the patients and there are also increasing reports of severe pulmonary involvement, causing high morbidity and mortality[222,232,233].

The treatment of MAS-HLH is targeted at reducing inflammation, preferably without too much immunosuppression to fight infections, primarily including therapies such as corticosteroids, IVIG, IL-1 receptor antagonist (anakinra), CSA and plasma exchange, making the approach somewhat different to other forms of sHLH[103,113,217]. It has also been observed that therapeutic choice may differ with the specialty of the treating physician[222]. Addition of cyclophosphamide and tacrolimus in corticosteroid-resistant MAS-HLH may be efficient[215]. However, both children and adults with MAS-HLH have shown excellent response to the addition of anakinra, with few side-effects and with long-lasting remission[115,116]. Severe forms of MAS-HLH, particularly with CNS and pulmonary involvement, require a prompt and likely more

aggressive therapeutic management, but further studies are required for improved management of these patients[44,103,234].

1.5.7 The pathogenesis of secondary HLH

The pathogenesis of secondary HLH remains incompletely understood, but growing evidence suggests it is multifactorial, and consequently, sHLH has been described as a "threshold" syndrome[7]. Secondary HLH is "classically" characterized by the absence of underlying genetic mutations. However, some cases of adult HLH, may be explained by hypomorphic mutations and sequence variants, in homozygous and heterozygous state, identified in HLH-causing genes, some associated with decreased NK cell function[16-18]. Some variants are also common in the healthy population, such as A91V-*PRF1* found in up to 10% of the healthy caucasian population, implying they may contribute with a risk but not a certainty of HLH[235,236]. Similar findings have been made in known HLH-associated genes in MAS-HLH, as well as sequence variants in other genes potentially involved in the cytolytic pathway[237-239]. However, polymorphisms in genes involved in other pathways than the cytotoxicity pathway, such as genes in cytokine production and signaling or inflammasome activation (e.g. *IRF5*, *NLRC4*, *IFNGR1/2*) have been associated to an increased susceptibility to HLH[240-242]. Interestingly, heterozygous mutations in known HLH-causing genes have also been identified in cases of fatal influenza AH1N1[243]. Combination of heterozygous mutations in two genes involved in the degranulation pathway has shown synergistic effect on cytotoxicity in patients with sHLH, and a mouse model could further show that accumulation of monoallelic mutations impaired lymphocyte cytotoxicity and increased the risk of developing HLH[244,245]. Repeated TLR9 stimulation with CpG (oligodeoxynucleotides that stimulate immune response) in a wildtype murine model gave rise to a MAS-like manifestation, independent of IFN-γ, illustrating development of HLH without genetic mutations[246,247].

Defects in cytolytic activity of cytotoxic lymphocytes and decreased numbers of NK cells have been observed in patients with sJIA and MAS-HLH[248,249]. In AOSD, NK cell deficiency and associated decreased cytotoxic activity have been correlated with elevated levels of IL-18 and IL-18BP (binding protein), markers of disease activity. Furthermore, NK cell proportions and cytotoxic dysfunction were improved on treatment[250]. High levels of free-circulating IL-18, due to disproportionate increase in IL-18 and IL-18BP, has also been reported in other forms of sHLH with associated impaired NK cell cytotoxicity and decreased NK cell numbers[251]. Decreased NK cell cytotoxicity associated with decreased frequency of NK cells has also been observed in children with sepsis, and low frequency of NK cells and CTLs observed in critically ill adults, associated with increased mortality[252,253]. On the other hand, reduced NK cell cytotoxicity has been observed in septic patients despite the presence of $CD56^{+}CD16^{+}$ NK cells[254]. The excessive cytokines in sHLH, alike the cytokine storm in primary HLH, may also directly impact NK cell and CTL function. The MAS-HLH cytokine storm includes cytokines such as IL-1, IL-6, IL-18 and IL-33[10]. Overstimulation of NK cells and CTLs by cytokines, such as IL-12 and IL-18, can induce exhaustion and apoptosis[255]. Overexposure of IL-6 has shown reduced expression of cytotoxic proteins with defective NK cell cytotoxicity, reversed

with anti-IL6 antibody (tocilizumab)[256]. Elevated levels of anti-inflammatory IL-10 are also found in HLH, and have been shown to play a protective role in a murine model of sHLH[89,246]. The observed defects in lymphocyte cytotoxicity and reduced frequency of cytotoxic lymphocytes observed in sHLH, and in critically ill, is likely to be transient, but nevertheless can cause hyperinflammation, akin that seen in primary HLH, and hence, result in sHLH.

In addition to continuous cytokine overstimulation, infection pathogens themselves may affect NK and CTL function. Latent-membrane-protein-1 in EBV can reduce CTL cytotoxicity by inhibition of SAP gene expression[257]. Influenza AH1N1 has been shown to infect NK cells and induce apoptosis, reducing NK cell numbers[258]. Lipopolysaccharide (LPS), a component of the bacterial cell membrane and potent inducer of immune responses, can directly induce production of IFN-γ in NK cells but simultaneously decreases NK cell degranulation[259]. Tumor cells often have increased resistance to apoptosis, prolonging exposure and synapse time with cytotoxic lymphocytes, and hence, hypersecretion of cytokines[83].

There are several factors that may contribute to the pathogenesis of sHLH, unlike familial HLH, where the genetic defect alone is enough for the development of HLH. The accumulation of factors and defects amplify the immune dysfunction, which at some point becomes an uncontrolled hypercytokinemia and hyperinflammation, leading to development of HLH, and to the theory of HLH as a 'threshold' syndrome (Figure 4)[7,260].

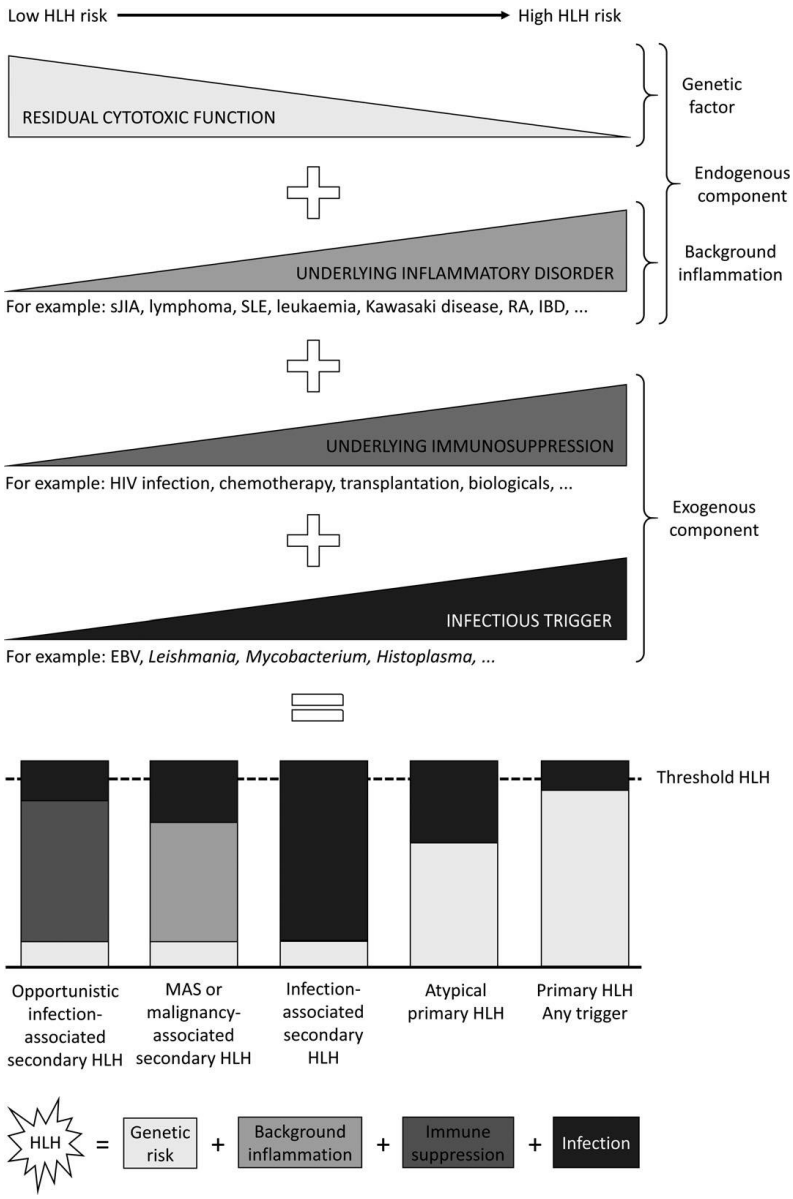


Figure 4. Threshold model of the HLH spectrum.
Brisse *et al*, 2016, Br J Hematology, with permission from John Wiley and Sons[7].
Genetic factors, underlying inflammatory condition, underlying immunosuppression and different strengths of infectious triggers are superimposed until a certain threshold point is reached, beyond which inflammation is no longer controlled and fulminant HLH develops. Diverse pathways and etiologies can thus result in the same common end stage of HLH. EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; HLH, haemophagocytic lymphohistiocytosis; IBD, inflammatory bowel disease; MAS, macrophage activation syndrome; RA, rheumatoid arthritis; sJIA, systemic juvenile idiopathic arthritis; SLE, systemic lupus erythematosus.

1.6 MARKERS OF INFLAMMATION IN HLH

1.6.1 Ferritin

Ferritin is a ubiquitous, highly preserved protein, its main function being to maintain iron homeostasis. However, time and evolving research has also shown ferritin to be involved in many inflammatory processes. Ferritin is a predominantly intracellular iron-binding protein providing iron for incorporation into heme in red blood cells and other essential cellular processes, at the same time preventing potential toxic effects of free-iron, such as generation of tissue-damaging reactive oxygen species. Besides free iron, ferritin expression is also regulated by hormones, oxidative stress and inflammation[261,262]. Ferritin is described as having

both pro- and anti-inflammatory properties, interacting with immune cells and immune responses, and stimulating pro- and anti-inflammatory cytokine production[263,264]. On the other hand, proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, induce ferritin transcription and secretion in macrophages and mesenchymal cells, enhancing further production of proinflammatory cytokines, as illustrated by the hyperferritinemic cytokine-storm in HLH[261,265].

The normal range of serum ferritin is gender and age specific with a maximum level of approximately 300 μg/L for adults. Elevated ferritin levels have been described in many conditions, such as infections, shock, iron overload, hemolytic anemia, liver disease, renal failure, malignancies, autoimmune diseases and stem cell transplantation, as well as in HLH[266-269]. Ferritin was described as a marker of hyperinflammation in HLH in 1988, and is used as a diagnostic criterion of HLH[11,270]. In children, ferritin levels >10,000 μg/L are reported specific and sensitive for primary HLH in children, whereas its specificity in sHLH and adults is debated[266,271]. The highest levels of ferritin, i.e. extreme hyperferritinemia (>10,000 μg/L), are observed in HLH, liver failure, renal failure, hematological malignancies, infections, iron overload (chronic transfusion and hemochromatosis), MAS-HLH and rheumatological diseases[271-273]. Some studies report the highest median and peak ferritin values in patients with HLH[268,269,273], while others report higher ferritin values in other conditions[271,272]. The current HLH-2004 criteria require a ferritin level >500 μg/L for HLH diagnosis, a level determined from a pediatric FHL cohort (in the HLH-94 study) where 26/31 patients had ferritin >500 μg/L, which by many is considered too low a level[11]. Several new ferritin cut-off levels for HLH diagnosis have been suggested, ranging from 2,000-10,000 μg/L, while a larger adult study did not even find ferritin >50,000 μg/L to be predictive for HLH[271,273-276]. Low glycosylated ferritin has been proposed as a good and possibly more sensitive marker for HLH, but has not been widely recognized, most likely due to restricted availability[277,278]. Hyperferritinemia has also been associated with increased risk of ICU admission and mortality in children and adults[276,279-281].

Despite the controversies, there seems to be a general agreement that extremely elevated levels of ferritin should raise suspicion of HLH, and hence, ferritin may be considered a good marker for HLH, even if not fully predictive, and is readily available at an affordable cost[282]. This is particularly important since an undetected evolving HLH can progress rapidly with deadly consequences. Ferritin may also readily be used to monitor disease course and therapy response[45,46].

1.6.2 Soluble IL-2 receptor

Soluble IL-2R (sIL-2R, also known as sCD25) is a marker of T-cell activation and of disease activity in inflammatory conditions, such as HLH, and where T-cell activation is prominent. Soluble IL-2R ≥2,400 U/mL is a criterion for HLH diagnosis in the current HLH-2004 criteria[11], a level defined from previous observation of elevated sIL-2R in children with HLH, were a majority of children in a German FHL cohort of 65 patients had sIL-2R >2,400 U/mL[156,283,284]. In HLH reports, sIL-2R levels may exceed 200,000 U/ml, but medians fall

around 3,000-21,500 U/ml[285]. A review on the clinical utility of sIL-2R in HLH reported two studies showing good sensitivity for sIL-2R ≥2,400 U/mL in the diagnosis of HLH and MAS-HLH[123]. Several studies have reported sIL-2R to be a good marker of disease activity and for monitoring response to therapy in HLH and in sJRA and MAS-HLH[156,219,284,286,287]. Initial levels of sIL-2R >10,000 U/mL have been associated with a worse prognosis, and higher levels observed in EBV-HLH and mal-HLH[156,287]. Few comparative studies have been done in adults, but two recent studies showed varying results on the sensitivity of sIL-2R for HLH diagnosis. Hayden *et al* reported a good sensitivity, whilst Naymagon *et al* reported poor discrimination between HLH and other diagnoses (hematological malignancies, sepsis and rheumatologic disease). However, both studies reported a high prevalence of HLH (93% and 67%, respectively) for sIL-2R levels >10,000 U/mL[205,288].

Highly elevated levels of sIL-2R can also be found in T-cell malignancies, such as hairy cell leukemia, T-cell leukemia and anaplastic large cell lymphoma, for which sIL-2R is a marker of tumor burden and for monitoring response to therapy[206,289]. Immune activation in HIV patients can be monitored with sIL-2R in combination with other parameters[290]. Acute and chronic liver failure, associated with T-cell activation, also display elevated levels of sIL-2R, making it a less specific marker for HLH activity when combined with liver failure[291,292].

1.7 CRITICALLY ILL IN THE INTENSIVE CARE UNIT (ICU)

1.7.1 Inflammatory responses in critically ill in the ICU

Every year in Sweden there are approximately 45,000 admissions to intensive care, and the 30-day mortality rate lies around 6-8%, with a decreasing trend in the last few years, according to the Swedish Intensive Care registry (SIR). The standardized mortality ratio (SMR), the ratio of expected and observed 30-day mortality after admission to ICU, in Sweden is slightly below 1.0, and stable over the years, meaning survival is better than that expected from ICU scoring systems, such as SAPSII (Simplified Acute Physiology Score), measuring disease severity and predicted mortality.

Increasing incidence of hospitalization and ICU admission due to sepsis is reported in the last two decades[293-295]. Despite a decreasing trend in mortality rate for sepsis, it still remains high and the is main cause of morbidity and mortality in patients treated in ICU[296,297]. The initial definition of sepsis in 1991 was based on the view that sepsis resulted from a host's systemic inflammatory response syndrome (SIRS) to infection (Table 3)[298].

Table 3. SIRS (Systemic Inflammatory Response Syndrome)

Two or more criteria fulfilled:
Temperature >38°C or <36°C
Heart rate >90/min
Respiratory rate >20/min or $PaCO_2$ <32 mm Hg (4.3 kPa)
White blood cell count >12,000 x10^9/L or <4,000 x10^9/L or >10% immature bands

Criteria from Bone et al.[298]

A review of the definitions in 2001 resulted in mostly unchanged definitions for sepsis, severe sepsis and septic shock[299]. In 2016, a new (third) international consensus definition for sepsis and septic shock was published. Sepsis was then defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection, in practice represented by an increase in sequential organ failure assessment (SOFA) score of 2 points or more, which is associated with an in-hospital mortality greater than 10%. Septic shock is defined as a subset of sepsis in which profound circulatory, cellular and metabolic abnormalities are associated with a greater risk of mortality[300]. Acute, refractory multiple organ dysfunction syndrome (MODS) is a main cause of death at tertiary intensive care units with a reported mortality rate of 44-76%, similar to that reported for septic shock[300,301]. MODS can be defined as an acute and potentially reversible dysfunction of 2 or more organ systems caused by multiple factors, and a consequence of a dysregulated inflammatory and immune response[301]. Besides SIRS, sepsis and MODS, critically ill patients may have other underlying inflammatory conditions, such as autoimmune diseases and malignancies.

Thrombocytopenia, clinically defined as platelets <150 x10^9/L, is a common occurrence at ICU and is associated with an increased mortality, in particular in patients with sepsis. Thrombocytopenia is frequent in sepsis, up to 47% in a study in ICU patients with sepsis, and is associated with a higher incidence of organ dysfunction, including acute kidney injury with elevated creatinine, use of vasopressors, hyperbilirubinemia, as well as hypoalbuminemia and elevated LDH[302,303]. An absent or blunted resolution of thrombocytopenia in critically ill and septic patients may indicate continuing critical illness and is associated with a worse outcome[303,304]. Hypoalbuminemia is also a well-known independent predictor of poor outcome associated with increased mortality, morbidity and length of ICU care, in particular in patients with sepsis, who benefit from albumin replacement[305-307]. Hemophagocytosis is neither mandatory nor unique to HLH, and found in critically ill patients with for example sepsis. However, the presence of hemophagocytosis in the bone marrow of thrombocytopenic patients has been noted to be particularly high in critically ill patients with sepsis and septic shock, frequently in association with other signs of critical illness and MODS[281,308,309]. Although hemophagocytosis should trigger suspicion and evaluation of HLH, it is a common occurrence in other conditions such as infections and malignancies without HLH diagnosis[309-313].

Ferritin levels >3,000 µg/L in children have been associated with an increased risk of ICU admission and death. Furthermore, a stepwise increasing ferritin level was positively correlated with hazard ratio of death[279]. Children in ICU with sepsis and septic shock displayed higher ferritin levels, >500 µg/L (overall range 21-2210 ng/ml), and an increased risk of death than other critically ill children. Interestingly, the children with a moderate rise in ferritin (200-500 ng/ml) showed the lowest mortality (9%), suggesting that a limited hyperferritinemic response in sepsis may be protective[280]. Similarly, in adults in surgical ICU, higher ferritin levels were observed in patients who developed sepsis (mean ferritin 1,585 µg/L), and consequently, associated with more severe illness, with the highest ferritin levels and fatal outcome in patients who developed MODS[314]. These earlier studies reported lower levels of ferritin in critically ill patients than the ferritin levels observed in a majority of patients with HLH. A recent large

retrospective ICU study of 2,623 critically ill adults with ferritin >500 µg/L reported maximum ferritin levels of 1,545 µg/L (range 964-4,175), 1,448 µg/L (range 836-2,803) and 974 µg/L (range 696-1,795) for patients with septic shock, sepsis and other conditions (e.g. liver disease, renal disease, autoimmune disease, infections and malignancies), respectively. Ferritin levels in septic shock and sepsis patients were significantly higher than for other diagnoses. Nevertheless, the highest ferritin levels were observed in patients with HLH (31,674 µg/L, range 15,121-87,975). Even in this study, maximum ferritin levels were associated with increased in-hospital mortality[276]. Notably, ferritin studies in critically ill in ICU generally report lower ferritin levels for other conditions than HLH compared with ferritin levels observed in patients with hyperinflammation and HLH.

Soluble sIL-2R is also a good biomarker of sepsis, irrespective of severity of illness, in patients admitted to intensive care[315]. Non-survivors at ICU display higher levels of sIL-2R, IL-10 and SOFA score compared to survivors[316]. Adults with sepsis in HLH studies have shown sIL-2R values generally up to about ~4,000-8,000 U/mL, with only a few higher values up to ~30,000 U/mL[205,288]. Elevated plasma levels of soluble IL-2R in sepsis are associated with increased percentages of $CD4^+CD25^+$ regulatory T cells, which together with elevated IL-10 characterize the compensatory anti-inflammatory response syndrome after the initial proinflammatory response in sepsis, and are thought to play a role in sepsis-induced organ injury, such as acute kidney injury (AKI)[317-319]. Accordingly, elevated levels of sIL-2R and IL-10 are associated with septic AKI, with reported sIL-2R levels up to 50ng/mL[320]. Soluble IL-2R is excreted and catabolized in the kidneys, and therefore, decreased renal clearance may augment elevated plasma sIL-2R levels, but apparently less than the increase observed in sepsis[320-322].

1.7.2 Hyperinflammation and HLH in the ICU

Even though the last decade has shown heightened awareness of HLH in critically ill in intensive care, its diagnosis still remains challenging due to the substantial clinical similarities with commonly encountered hyperinflammatory responses encountered in ICU, such as sepsis, SIRS, MODS and autoimmune disorders, rendering HLH still likely underdiagnosed at ICU; incidence recently reported at 1.5%[95,285,323,324]. However, critically ill patients with HLH generally have an unexpected and disproportionate inflammatory response with imminent or rapidly evolving MODS despite appropriate empirical treatment and aggressive supportive care. Early recognition and prompt HLH-directed therapy is crucial for improved survival[107,111]. A recent systemic review on HLH in critically ill reported a mortality of 57.8%, with infections being the most common trigger, but the highest mortality seen in HLH of unknown etiology and mal-HLH[325].

The reliability of the HLH-2004 criteria and HScore in ICU have been questioned, but in a large ICU cohort a cut-off of 4 fulfilled HLH-2004 criteria and HScore of 168 predicted HLH with 95% and 100% sensitivity, respectively, and 94% specificity. Furthermore, sensitivity and specificity of the HLH-2004 criteria were reported improved by adjusting ferritin level to >3,000 µg/L. Not surprisingly both higher number of HLH-2004 criteria and HScore were

associated with increased mortality in this study[104]. Based on these findings, 4 or more HLH-2004 criteria may be enough to consider initiation of HLH-directed therapy. Although none of the criteria used for HLH diagnosis are specific for HLH, and each single criterion can be observed in other inflammatory conditions, it is the combination of criteria in the unexpected galloping scenario of hyperinflammation that should make us suspicious of HLH. Typically, critically ill with HLH have more marked hyperferritinemia and severe cytopenias, in particular thrombocytopenia, and higher SOFA score requiring more life-supporting treatments, than other inflammatory conditions in critically ill. Splenomegaly is rare in critically ill outside of HLH diagnosis[131,285,324,326]. Older age, higher SOFA score, shock on admission and lymphoma have been associated with increased risk of mortality in critically ill with HLH[131,324,326]. A recent systemic review of HLH in critically ill reported IVIG treatment in IA-HLH to be associated with improved survival[325].

Many critically ill patients with MODS and signs of extreme inflammation do not, at least initially, fulfill 5/8 HLH-2004 criteria, but may still require a different treatment approach to other patients with MODS in ICU. Carcillo *et al* have suggested 3 inflammation phenotypes in sepsis-induced multiple organ failure, including multiple organ failure associated with 1) immunoparalysis, 2) thrombotic microangiopathy-driven thrombocytopenia, and 3) sequential liver failure[327]. They observed a higher mortality, and increased presence of sHLH, in pediatric ICU patients with an inflammation phenotype associated multiple organ failure than in the children with multiple organ failure without inflammation, suggesting they are a subset of patients with a different pathophysiology and underlying immune dysregulation, including a deficiency of NK cells and CTLs[328-330]. The presence of MAS-HLH or hepatobiliary dysfunction and coagulopathy syndrome (HDB/DIC), a MAS-like syndrome, is reported as an independent risk factor of 10-day mortality in patients with infection and SIRS, with ferritin levels >4,420 µg/L associated with increased levels of IL-6, IL-18, IFN-γ and soluble CD163 and a 66% mortality. The patients with MAS-HLH and HBD/DIC had significantly more ARDS, AKI, shock and higher levels of ferritin and triglycerides. Furthermore, a poor decline in ferritin was predictive of adverse outcome, as seen in patients with primary HLH[46,331].

In 1994, a study on the effect of recombinant human IL-1 receptor antagonist in adults with sepsis showed no increased survival, however, secondary analyses found an increased survival in septic patients with organ dysfunction and with a predicted mortality >24%[332]. A post-hoc analysis showed significant improvement in 28-day survival with IL-1 receptor antagonist treatment (anakinra) (65.4% anakinra vs 35.3% placebo) in the patients with sepsis and concurrent HBD/DIC, who also displayed higher predicted mortality scores, shock and AKI. The high and similar survival seen in non-HBD/DIC patients treated with anakinra or with placebo, around 71% for both groups, suggests the HBD/DIC patients to be a subset of critically ill patients with more inflammatory pathophysiology and severity of illness, that benefit from additional anti-inflammatory therapy[117]. There are now several successful reports on the efficacy of anakinra in infection/sepsis and MODS associated hyperinflammation/sHLH, as an addition to the recommended anti-inflammatory therapy with corticosteroids and IVIG, besides

the crucial aggressive supportive care[113,118,119,329,333,334]. Selected patients may also benefit from the addition of plasmapheresis[113,335,336].

In the last few years, many studies including some larger ones have enriched our understanding of HLH in critically ill in intensive care. HLH in critically ill patients at the extreme of the severity scale of the ladder of hyperinflammation observed in the ICU scenario. Challenges remain to identify the subset of patients who would benefit from hyperinflammation-specific therapy, and the question is: are we still missing some and, if so, how do we identify them for early appropriate intervention?

2 RESEARCH AIMS

The overall aim of the thesis was to, with our previous knowledge in primary HLH, study hyperinflammation and the development of HLH in critically ill in different settings, to distinguish these patients for future early identification and appropriate therapeutic intervention. A second aim was to investigate if we, by our knowledge gained in the biology of primary HLH, could contribute to a better understanding of the biology of secondary HLH.

The specific aims were:

Paper I:

- to study the value of ferritin and sCD25 (sIL-2R) as parameters of hyperinflammation in critically and their association with other parameters of critical illness, as potential parameters to identify hyperinflammatory patients on admission to ICU.
- to study the association of ferritin and sCD25 (sIL-2R) with NK cell proportions and NK cell cytotoxicity/degranulation in critically ill with signs of hyperinflammation.
- to identify any HLH-associated genetic mutations/variants in critically ill.

Paper II:

- to study the frequency, characteristics and outcome of HLH associated with pandemic influenza AH1N1 infection in a selected cohort of critically ill requiring ECMO, to assess if these patients can be distinguished for early appropriate intervention.
- to study NK cell proportions and NK cell cytotoxicity/degranulation in critically ill with pandemic influenza AH1N1 infection on ECMO support, and possible associations with HLH and mutations/variants in HLH-associated genes.

Paper III:

- to evaluate possible benefits of additional HLH-directed therapy with moderately dosed etoposide in patients with severe MAS-HLH.

Paper IV:

- to study the frequency, clinical features, risk factors and mortality of dengue-associated HLH in a large population of critically ill with severe dengue, for future intervention studies.

Paper V:

- to identify and characterize patients with HLH in a selected cohort of critically ill in ICU with higher risk of hyperinflammation and adverse outcome, and furthermore, compare them with critically ill with fatal outcome who did not fulfil the diagnosis of HLH; a future aim being earlier diagnosis and appropriate intervention.
- to study lymphocyte proportions and lymphocyte cytotoxicity/degranulation in critically ill and those who develop HLH, for improved understanding of the pathogenesis of hyperinflammation in critically ill.

3 PATIENTS AND METHODS

Below is a summary of Patients and Methods. Details of the patient cohorts and methods used in each study are presented in the methods section of each individual papers.

3.1 STUDY POPULATION

The study populations in **Paper I-V** are all different, although study **I** and **II** were carried out under the same ethics approval.

Paper I was a prospective observational study in adult patients (≥18 years) admitted to the Intensive Care Unit at Karolinska Huddinge Hospital, Stockholm, Sweden during a period of 11 months. Patients observed by the intensivist to have a serum ferritin >500 µg/L in routine blood tests on admission to ICU were included in the study on patient consent. Thirty-nine patients were included in the study. In two patients with multiple ICU admissions, only the admission with the highest ferritin value was included in the study. Patients admitted for post-operational care after liver transplantation (n=7), with expected hyperferritinemia from surgical hepatocellular injury, and patients on ECMO support (n=13) were excluded. Thirty-two eligible patients were further analyzed in the study.

In **Paper II** we included the patients on ECMO support, excluded in **Paper I**, with PCR-confirmed 2009 pandemic influenza AH1N1 infection. Of the 13 enrolled patients, informed consent was missing for two patients, who were excluded. The remaining 11 patients in the study were all treated at the ECMO Centre at Karolinska University Hospital, Stockholm, Sweden, during the period July 2009 to February 2010, when the influenza AH1N1 pandemic was current. During the study period a total of 16 patients were treated at the ECMO Center at Karolinska University Hospital for pandemic influenza AH1N1 infection, whereof 11 were included in our study (69%).

Paper III is a case-series report of seven children with MAS-HLH treated with etoposide. The underlying systemic autoimmune disease of the children were: 3 with systemic juvenile idiopathic arthritis (sJIA), 2 with atypical sJIA and 2 with systemic lupus erythematosus (SLE). The children were in the ages 0.4 to 16 years (median 9 years) at the onset of MAS-HLH and were treated with etoposide during 2010-2017. Four children were cared for at Karolinska University Hospital, Stockholm, two children at Sahlgrenska University Hospital, Gothenburg, and one patient at Umeå University Hospital, all in Sweden. The last three patients were treated in close collaboration with Karolinska University Hospital.

In **Paper IV**, all patients ≥18 years with severe dengue (SD) and a positive NS1 antigen test, and/or positive IgM or IgG by enzyme linked immunosorbent assay (ELISA), admitted to the multidisciplinary ICU at Hospital Sultanah Aminah in Johor Bahru, Malaysia, during 2010-2014, were included in the study. 180 patients with severe dengue were studied in **Paper IV**.

Paper V is a prospective observational study at four intensive care units (ICU) in Stockholm and Norrtälje, in Sweden, including the ICU at Karolinska University Hospital Solna and

Huddinge, Danderyd Hospital and Norrtälje Hospital. Adults (≥18 years) admitted to the ICU, between January 9, 2012, and December 31, 2014, were included in the study if: 1) informed consent from patient or next of kin was obtained, 2) the patient fulfilled criteria for SIRS, and 3) had persistent inappropriate thrombocytopenia with platelets <70 $x10^9$/L, or platelets <100 $x10^9$/L with serum ferritin >2,000 µg/L, or simply serum ferritin >5,000 µg/L. Patients could be re-evaluated for study inclusion at any time during ICU care if inclusion criteria were not fulfilled. Inclusion criteria with hyperferritinemia, a marker of inflammation, and thrombocytopenia, associated with poorer outcome in critically ill, were used to select a study population of critically ill with more severe illness and more likely to develop hyperinflammation. Finally, 50 eligible patients were studied in **Paper V**. Patients in ECMO (n=12) were excluded and will be studied separately due to the complexity of ECMO treatment.

3.2 ETHICS

The Ethics Committee at Karolinska Institutet, Stockholm, Sweden, approved the studies in **Paper I and II** (protocol nr 2009/248-31/4, March 4, 2009), as well as **Paper III** (2006/228-31/3, March 22, 2006; 2010/1596-31/4, November 3, 2010; and 2013/1723-31/4, November 27, 2013) and **Paper V** (protocol nr 2011/802-31/3, June 15, 2011; and 2012/861-32, May 11, 2012). The study for **Paper IV** was approved by the Medical Research and Ethics Committee in Malaysia (NMRR-15-1476-26640, October 20, 2015). Informed consent was obtained for all patients in **Papers I-III and V**.

3.3 METHODS

3.3.1 Clinical definitions

The current HLH-2004 and HScore diagnostic criteria for HLH have been described elsewhere (please refer to Table 1in section 1.2)[11,12].

Retrospective evaluation of HLH, using adapted HLH-2004 criteria, was done for all the patients included in **Paper II**, even if they had received a diagnosis of HLH during the time on ECMO support (n=2). Due to ECMO treatment the diagnostic criteria of fever and hemoglobin <90 g/L could not be used, since ECMO regulates body temperature and hemoglobin is maintained >100 g/L by regular blood transfusions for optimal oxygenation. Consequently, patients were classified as having HLH if they fulfilled 4/7 HLH-2004 criteria (fever excluded and cytopenia evaluated only on platelet and neutrophil values). Additionally, patients were subsequently evaluated with the HScore, an HLH probability score validated in adults with best cut-off score for HLH diagnosis at 169, accurately classifying 90% of patients[12]. Number of fulfilled HLH-2004 criteria and score of the HScore were determined from available parameters for every day during ECMO care.

Paper III included children with sJIA, atypical sJIA and SLE. The diagnosis of sJIA was made according to the International League of Associations for Rheumatology criteria[337]. The two patients with 'atypical' sJIA did not present with arthritis at diagnosis. The diagnosis of SLE was made according to the 1997 American College of Rheumatology revised SLE

criteria[338,339]. All patients fulfilled the criteria for macrophage activation syndrome complicating sJIA or SLE, respectively, that have been previously described (please refer to the section on MAS-HLH in Introduction).

In **Paper IV**, HLH diagnosis was retrospectively evaluated and defined by two different approaches, using 1) HLH-2004 criteria and 2) the HScore. An HLH diagnosis with HLH-2004 criteria was given if ≥4/8 HLH-2004 criteria were fulfilled (information on HLH genetics, NK cell cytotoxic activity and sIL-2R were not available), and with the HScore if the patient had a probability of HLH ≥70% (score ≥180). Severe Dengue (SD) was defined according to the WHO 2009 classification previously described (please refer to section 1.5.4, Dengue-associated HLH).

The definition of systemic inflammatory response syndrome (SIRS) used for inclusion of patients in **Paper V** has been defined elsewhere (please refer to Table 3 in section 1.7.1)[298]. All patients were retrospectively evaluated for HLH using both HLH-2004 criteria and the HScore. A patient was classified as having HLH if 1) a diagnosis of HLH had been established during ICU care, or 2) ≥5/8 HLH-2004 criteria were fulfilled AND HScore was ≥169. For each patient an HLH evaluation was performed for every day during ICU care.

Patients in **Paper I and V** were classified as having sepsis, sever sepsis or septic shock as recorded in the medical files by the treating physician. At the time of the studies, sepsis was defined according to the 2001 International Sepsis Definitions Conference, which were very similar to the original definitions 1991[298,299].

3.3.2 Collection of clinical data

For **all papers** clinical and laboratory data was retrieved from the patients' medical files, except for lymphocyte cytotoxicity, cell counts and genetic sequencing, that were performed separately as research assays at Karolinska Intitutet (see below). Assay of sIL-2R (sCD25) was performed by the regular hospital laboratory by a chemiluminescence assay (Immulite 1000, Siemens, Germany) with a detection range of 5-7,500 U/mL, with all values above 7,500 reported as >7,500 U7mL. In **Paper I**, routine blood tests and additional study tests taken on admission to ICU only were analyzed in the study, as well as survival at time of ICU discharge. In **Paper II**, all patients were followed until ICU discharge, but blood samples for lymphocyte cytotoxicity function were obtained from 2 patients after recovery (~3 years after discharge), and included in the study. In Paper III, data was retrieved by treating physician in each respective hospital where the child was treated. The follow-up time of the children was 2 to 9 years (median 6 years) after MAS-HLH onset. Data for **Paper IV** was retrieved from the ICU Registry, case report forms and medical files at the hospital Sultanah Aminah in Johor Bahru, Malaysia. Patients were followed until ICU discharge or death, whichever occurred first. In **Paper V**, additional relevant data was retrieved from the Swedish ICU registry. Follow-up time was until hospital discharge or death, whichever occurred first.

For details on data collected for each study please refer to the individual papers. Since all studies were observational, patient care, clinical examinations and treatments were all done according to local routines and at the discretion of the treating physicians.

3.4 LYMPHOCYTE CYTOTOXICITY ASSAYS AND CELL COUNTS

Assessment of cell percentages, NK cell cytotoxicity and degranulation was performed in the same manner for **Papers I, II, III and V**. For **Paper V** absolute cell counts were also assessed. Collected whole blood samples, in EDTA-containing vials, were processed within 24 hours from venous puncture. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation and resuspended in complete medium. Percentages and absolute counts of white blood cells (total leukocytes, granulocytes, lymphocytes and monocytes) and lymphocyte subpopulations (B-lymphocytes, T-lymphocytes and NK cells) were determined by flow cytometry before and after Ficoll separation. Furthermore, in **Paper V**, subgroups of T- and NK cells were also counted, including $CD56^{dim}$ and $CD56^{bright}$ NK cells, CTLs ($CD3^+CD8^+$), activated CTLs ($CD8^+HLA\text{-}DR^+$), exhausted CTLs ($CD8^+\text{-}PD1^+$), CD4 T cells ($CD3^+CD4^+$), activated $CD4^+$ ($CD4^+HLA\text{-}DR^+$), exhausted $CD4^+$ ($CD4^+\text{-}PD1^+$). For cytotoxicity assays, $CD3^-CD56^{dim}$ NK cells, and $CD3^+CD8^+$ T cell subsets for **Paper V**, were isolated from PBMCs by negative selection using magnetic beads, and resuspended in complete medium for cytotoxicity and degranulation assays.

NK cell cytotoxicity was assessed by a standard 4 h ^{51}Cr-release assay, as previously described[283,340]. Briefly, isolated effector NK cells, either fresh or after IL-2 stimulation, were co-incubated for 4 h with ^{51}Cr-labeled K562 (human erythroleukemia cell line) target cells at different effector-to-target cell ratios. Lymphocyte cytotoxicity was calculated as lytic units (LU^{25}) at 25% target cell lysis. LU <10 was considered pathologically low in all papers mentioned.

CD107a (lysosomal-associated membrane protein 1, LAMP-1) is a transmembrane protein in secretory lysosomes. Cell surface expression of CD107a increases upon exocytosis of secretory vesicles in NK cells and CTLs, and can be measured to assess degranulation capacity of the lymphocytes. Natural cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC) were assessed by stimulation of PBMC with K562 cells or with P815 cells supplemented with either anti-CD16 (for NK cells) or anti-CD3 (for CTLs), respectively. After incubation, centrifugation and resuspension, cells were labeled with antibodies and analyzed by flow cytometry, with quantification of CD107a surface expression on gated NK cell and CTLs. Lymphocyte degranulation assays were considered pathological if K562 NK cell degranulation was <5% (absent) or 5-10% (defective), anti-CD16 NK cell degranulation was <13%[341], and anti-CD3 CTL degranulation was <23%[340]. Assessment of secretory vesicle proteases, such as perforin and granzyme A and B, was achieved be intracellular staining. All lymphocyte cytotoxicity assays were performed at the Center for Infectious Medicine, Karolinska Institutet, Huddinge, Sweden.

3.5 GENETICS

In **Paper I**, genetic sequencing was performed in patients with abnormal or defective NK cell degranulation, low NK cell "killing" and/or low percentage of NK cells as defined above; n=11. In **Paper II and III**, genetic sequencing was performed in the patients with available DNA sample, n=5 and n=6, respectively. Similar for all studies, genomic DNA was isolated from peripheral blood according to standard procedures and sequenced for mutations in known HLH-causing genes. Specific primers, available upon request, were used to amplify exons and exon/intron boundaries of *PRF1, STX11, UNC13D* and *STXBP2* by PCR. Subsequently, PCR products were sequenced by direct sequencing and data was analyzed using SeqScape software, and in silico predictions performed using SIFT and PolyPhen-2. Rare variants (minor allele frequency, MAF, <1%) were reported. All sequencing was performed at the Center of Molecular Medicine, Karolinska Institutet, Stockholm, Sweden.

3.6 STATISTICS

Please refer to the individual papers for details on the statistical analyses used in the study. **Paper III** was a case-series report without statistical analysis. Therefore, this section refers to remaining papers. For descriptive analysis, frequencies, percentages, medians, interquartile range and minimum-maximum range was used. Comparison of categorical variables was analyzed with chi(X^2)-squared test, or Fisher's exact test for smaller-sized samples, whilst Mann-Whitney *U* test was used for continuous variables. Spearman's rank correlation test and Fisher's exact test were used to analyze associations between variables. The distribution of continuous variables was assessed by histograms, and where appropriate, continuous variables were log-transformed using the natural logarithm. In **paper IV and V**, univariable and multivariable logistic regression were used to evaluate risk factors of death. Subsequently, in **Paper V**, the selected multivariable model was evaluated using a receiver operating curve (ROC) and assessing area under the curve (AUC). Furthermore, survival of patients in ICU with severe dengue was assessed with a Kaplan-Meier survival curve. Soluble IL-2R (also sCD25) has a detection range of 5 to >7,500 U/mL in the laboratory. Therefore, values reported as >7,500 U/mL were assigned the exact value of 7,500 in relevant statistical analysis in **papers I, II and V**. Throughout the papers, *p*-values <0.05 were considered statistically significant.

4 RESULTS AND DISCUSSION

Secondary HLH (sHLH) is a condition of hyperinflammation associated with immune dysregulation and an inappropriate response to triggers, such as infections, malignancies and autoimmune or autoinflammatory diseases, resulting in a highly fatal cytokine storm with multiple organ dysfunction[7,19,93]. It is, nevertheless, a treatable condition if promptly recognized for timely and appropriate HLH-directed therapy. However, the diagnosis in critically ill is challenging due to the clinical overlap with other common systemic inflammatory responses, and consequently, underdiagnosed[95,103,107]. There are no clinical manifestations or laboratory tests that are specific to HLH, yet some markers of inflammation, such as elevated ferritin and sIL-2R, and routine laboratory tests, such as low platelets and elevated AST, may be indicative of HLH and useful to identify patients with hyperinflammation, in particular if concurrent with other typical manifestations of HLH and in the setting of progressive uncontrolled inflammation and organ dysfunction[107,110,111,120]. The pathogenesis of this eruptive phenomenon is not fully understood, but is generally viewed as a multifactorial 'threshold' syndrome with additive factors increasing the hyperinflammation and susceptibility to HLH, until a break point of uncontrolled inflammation is reached and HLH develops. Cytotoxic lymphocytes and defect cytotoxicity, causing primary HLH, are suggested contributors in the pathogenesis of sHLH[7,260].

The main purpose of this thesis was to study if we could use our knowledge gained in primary HLH to better characterize and understand hyperinflammation and HLH in critically ill, for earlier diagnosis and intervention, and improved treatment of these patients. Additionally, with our knowledge of the biology of primary HLH, we wanted to investigate the biology in critically ill with hyperinflammation for a better understanding of the pathogenesis of secondary HLH. This was achieved by first studying the inflammatory markers ferritin and sIL-2R in critically ill patients in ICU, and then, moving on to hyperinflammation and HLH in critically ill in different scenarios, and all along, studying cytotoxic lymphocytes and possible associated genetic variations when possible.

4.1 AGE, GENDER AND COMORBIDITIES

We studied 32 critically ill patients in ICU in **Paper I**, 11 patients with influenza AH1N1 in ECMO support in **Paper II**, seven children with MAS-HLH in **Paper III**, 180 patients with severe dengue in **Paper IV**, and 50 critically ill patients in ICU in **Paper V**. The study populations in Paper I, II, IV and V were adults >18 years, whilst the patients in **Paper III** were children <18 years. There was a male predominance throughout the studies and the median overall age in **Paper I and V** was 63 and 62 years, respectively, in line with previous findings in studies on critically ill patients with sepsis, a largely represented patient category in our studies[296,297]. The median ages of our study populations in ECMO with severe pandemic AH1N1 infection (**Paper II**) and with severe dengue (**Paper IV**) were lower, 31 and 35 years, respectively. The increased risk of hospitalization with complications and fatal outcome in young adults, and pregnant women, during the influenza AH1N1 pandemic likely explains the observed younger, largely previously healthy, study population in **Paper II**[168-171]. Although,

children are at increased risk of developing severe dengue (SD), the incidence in adults shows geographical heterogeneity. However, the younger study population observed in **Paper IV**, is similar to the incidence peak of SD observed in young adults aged ~20-40 years in Southeast Asia and Saudi Arabia; an age group with more secondary and tertiary dengue infections that tend to be more severe[185,342-344].

Overall in our ICU studies (**Paper I**, **II and V**), 54-68% of critically ill had one or more comorbidities at ICU admission, with similar proportions in patients with sepsis as in a previous Swedish nationwide study[297]. In our studies comparing patients with HLH ("HLH") and without HLH ("non-HLH"), we observed patients with HLH to be younger, predominantly male and with fewer comorbidities. HLH studies in literature show a generally young age in patients with HLH, with a mean of 49 years in a review with 775 adult HLH cases[93]. Most HLH studies compare HLH survivors and HLH non-survivors, where non survivors are predominantly older, and increasing age is an independent risk factor of mortality in a few reports[122,131,181]. However, few studies compare HLH and non-HLH patients, but in those studies, similar to our study, HLH patients have been found to be younger than non-HLH patients[98,276]. In line with our findings, reports in the literature show HLH patients to be predominantly male, and male gender has also been identified as a risk factor of mortality in HLH[93,102]. Three of four HLH patients with influenza AH1N1 infection, in **Paper II**, were previously healthy without comorbidities. In our larger ICU study (**Paper V**), 44% of HLH patients had comorbidities as opposed to 81% of non-HLH patients. Why are healthy young adults succumbing to severe illness and hyperinflammation or HLH? The answer to this question is not completely understood and likely multifactorial, and will be discussed further in later sections of the thesis.

4.2 REASONS FOR ICU ADMISSION, ASSOCIATED DISEASES AND SEPSIS

Secondary HLH is typically associated with malignancy, systemic autoimmune and autoinflammatory diseases and infections, with a predominance of viral infections, especially herpes viruses, but also others, such as pandemic influenza[93]. Among our 11 patients with pandemic influenza AH1N1 infection on ECMO support, one patient had stable inflammatory bowel disease at the time of AH1N1 infection and HLH, suggesting the AH1N1-infection as the main trigger. Another patient with stable chronic lymphatic leukemia did not develop HLH, but showed manifestations of hyperinflammation, and a HScore of 136, most likely triggered by his severe AH1N1 infection. Clinically significant co-infections were few in the AH1N1-infected ECMO patients and mostly found in bronchoalveolar lavage, a finding also reported in previous ECMO studies on AH1N1 infection, and with opportunistic pathogens (coagulase-negative staphylococci and *Candida* species) in our study patients[172,345]. All 11 patients were on empiric antimicrobial therapy including broad-spectrum antibiotics since or prior to ECMO admission. Beutel *et al* describe a similar scenario without uncontrolled bacterial co-infections in their HLH patients with AH1N1 infection on ECMO support, indicating the influenza AH1N1 infection to be the main trigger of HLH in these cases[181]. However, it cannot be

excluded that low-virulence co-infections contributed to the development of HLH in our two patients with co-infection.

Sepsis is an increasing and leading reason for admission to ICU worldwide[293,296]. Sepsis or septic shock were predominant diagnoses at ICU admission in our studies as well. In **Paper I**, 75% had sepsis, according to the international consensus definition of sepsis and septic shock at the time, where reason for ICU admission was defined as septic shock (n=11), respiratory failure (n=9), coma (n=3) or acute renal failure (n=1). In **Paper V**, 66% of patients were admitted to the ICU due to sepsis (30%) or septic shock (36%) and 24% with respiratory failure (whereof 7/12 had bacteremia), similar to another large ICU study on HLH where these diagnoses were evaluated[324]. Furthermore, 37 (74%) patients had a clinically significant infection (defined as positive blood culture or significant deep infection and /or significant positive virus PCR in blood, tracheal secretion or CSF): bacterial infection n=32 (64%), EBV n=3 (6%), HIV n=1 (2%), other viruses n=7 (14%, such as adenovirus, HHV6, CMV and influenza AH1N1) and aspergillosis n=4 (8%), with a statistically similar proportion in HLH and non-HLH patients. Viral infections were more prominent in HLH patients than non-HLH patients in **Paper V**, but bacterial infections remained predominant. Furthermore, simultaneous infection with bacteria and virus was encountered in 7/50 patients, whereof 5 were in HLH patients, both pathogens likely contributing to development of HLH. In other ICU-HLH studies viral infections are commonly reported, but rarely bacterial, making a comparison difficult, but in a systematic HLH review bacterial and viral triggers were almost equal[131,324-326]. However, overall in adult HLH studies, viruses are a more commonly reported infectious trigger, contrary to our findings in critically ill in ICU. The high number of patients with sepsis or septic shock and bacterial infections in **Paper V** may be due to a selected cohort of more severely ill ICU patients, or different ethnical and geographical background compared to some other studies. **I**nfection rates were similar in **Paper I** with a less selected cohort. In line with other studies, we observed a similar high proportion of previous immunosuppression (42-44% overall in **Paper I and V**), more marked in patients with HLH (66.7%)[98-100,102,132]. Two patients in **Paper III** had a viral infection (EBV and varicella-zoster virus) and one an *E.coli* urinary tract infection as possible co-triggers of MAS-HLH. Infections are common triggers of MAS-HLH, along with onset of disease[214,215,217].

In line with previous studies we observed a high number of malignancies, and in **Paper V** almost all were hematological malignancies (n=12, 24%), with a significant predominance in patients with HLH (50% vs 9.4% in non- HLH, p=0.004). Associated diseases in HLH were not considered mutually exclusive. Whether infections or malignancies predominate in previously studied HLH cohort varies[98,100,102,131,132,324,326]. There was a surprisingly large number of leukemia-associated HLH (n=7) in **Paper V**, whereof many had HLH during chemotherapy with severe immunosuppression and concurrent infection. Systemic autoimmune diseases were prevalent in <19% of patients in **Paper I and V**. MAS-HLH only comprised 5.6% (n=1) of our HLH cases in **Paper V**, but one other patient with systemic autoimmune disease, who did not fulfill the study definition for HLH, was clinically a MAS-

HLH on examination of the medical files. Other HLH studies also report low proportions of MAS-HLH, generally below 20%[98,100,102,131,132,324,326].

4.3 MARKERS OF HYPERINFLAMMATION IN CRITICALLY ILL

Hyperferritinemia has been associated with many conditions and defined as a marker of inflammation. Markedly elevated serum ferritin levels were associated with HLH already in 1988[270]. Ferritin >500 µg/L is a diagnostic criterion for HLH in the HLH-2004 diagnostic criteria[11]. However, several studies thereafter have suggested a higher cut-off level for better specificity, and the weighted HScore for probability of HLH in adults has the lowest scoring-level for ferritin at >2,000 µg/L[273-276]. Although ferritin >10,000 µg/L is reported as considerably specific for pediatric and primary HLH, the scenario in adults appears substantially different, with heterogeneity in different study populations and reported marked hyperferritinemia in several other conditions than sHLH[266,271,276]. Despite the controversy, ferritin is nevertheless considered a good marker to suspect HLH, when highly elevated, and to follow therapy response and disease activity[46,103,268,273,276]. Our knowledge of hyperferritinemia in critically ill in intensive care has expanded greatly only in the last few years, and generally lower ferritin levels are reported in other conditions than HLH. A recent large ICU study of 2,623 adults with ferritin measurements observed a maximum ferritin median of 1,314 µg/L (interquartile range, IQR, 788-2,576), all patients included, whereof 40 identified HLH patients had a median of 31,674 µg/L (IQR 15,121-87,975)[276].

In **Paper I**, our earliest study, the low ferritin level >500 µg/L was chosen as an inclusion criterion following the HLH-2004 diagnostic criterion for ferritin, and when the ferritin scenario in ICU was still fairly unexplored. Thirty-two patients were included in the study, 75% with sepsis, and their admission parameters were studied. The overall median serum ferritin level was 2,095 µg/L (range 545-49,078), with sepsis patients having a higher median ferritin than non-sepsis patients, 2,394 µg/L (range 545-49,078) and 1,419 µg/L (range 624-5110), respectively, at ICU admission. Ferritin levels >5,000 µg/L (n=10) were observed in nine patients with sepsis, whereof eight had ferritin levels >10,000 µg/L, and the remaining ferritin value >5,000 µg/L was found in a patient with an undiagnosed lymphoma, a disease associated with hyperinflammation[93,108]. Hyperferritinemia has been associated with an increased risk of admission to ICU, development of MODS and mortality, particularly in patients with sepsis[276,279,280,314]. Ferritin levels >10,000 may not be specific for HLH in adults, but on the other hand, a majority of patients in ICU have ferritin levels <3,000 µg/L[269,276,314]. Forty-two percent of our patients had ferritin levels <2,000 µg/L. Therefore, ferritin levels >5,000 µg/L, and even more so >10,000 µg/L, should prompt physicians to evaluate for hyperinflammation, clearly associated with hyperferritinemia.

In **Paper V**, HLH patients had a significantly higher ferritin level on admission to ICU (median 20, 682 and 5,492 µg/L, p=0.032, respectively) and developed higher maximum ferritin levels (median 41,564 and 10,924 µg/L, p=0.016, respectively) compared with patients without HLH, supporting previous findings of higher ferritin levels in patients with hyperinflammation, including critically ill in ICU[98,276]. Similar to Lachmann *et al*, even sepsis and septic shock

patients in **Paper V** had significantly lower maximum ferritin levels than HLH patients, with 5,492 µg/L, 12,487 µg/L and 41,564 µg/L, respectively (p=0.014)[276]. Furthermore, maximum ferritin levels were positively correlated to HScore (r_s=0.504, p<0.001), an HLH scoring system with increasing probability of HLH with increasing score, and therefore, indirectly increasing probability of hyperinflammation. A study on sHLH among SIRS patients, also showed ferritin to be the biomarker strongest correlated to both HLH-2004 and the Hscore[346]. Influenza AH1N1-infected patients in ECMO similarly showed higher maximum ferritin levels in the patients who developed HLH (10,735 µg/L vs non-HLH 1,204 µg/L, p=0.024). Patients with MAS-HLH are characterized by hypercytokinemia and hyperinflammation, and all seven MAS-HLH patients in **Paper III** had ferritin >5,000 µg/L (median of 20,778 µg/L, range 5,025-121,937) prior to starting etoposide treatment, when their MAS-HLH was still active[216]. Ferritin has been positively associated with disease activity in sJIA and MAS-HLH, and ferritin levels in our patients decreased with resolution of MAS-HLH[230,231,347,348]. Also in dengue, ferritin has been associated to disease severity[190]. The median peak ferritin level of the 180 patients with severe dengue in **Paper IV** was 22,236 µg/L (range 565 to >100,000), and even higher in those who developed dengue-HLH (26,603 µg/L with HLH-2004 criteria and 29,531 µg/L with HScore criteria), and lower in those who did not. Furthermore, hyperferritinemia was associated with the development of HLH in SD (OR 1.7, p=0.048). Altogether, our findings support the presence of hyperferritinemia in critically ill patients, it's correlation with inflammation, starting at sepsis and increasing to the hyperinflammatory condition HLH, as well as the importance of ferritin as a marker of inflammation and a diagnostic tool to identify patients with potentially life-threatening hyperinflammation.

Compared to ferritin, sIL-2R has been less extensively studied. As a marker of T-cell activation, highly elevated levels of sIL-2R can be observed in all forms of HLH, and associated with disease activity, therapy response and prognosis[156,205,219,284,286,287]. Soluble IL-2R ≥2400 U/mL is a criterion in the HLH-2004 diagnostic criteria for HLH[11]. The sensitivity and specificity of sIL-2R appears as controversial as for ferritin, but commonly sIL-2R levels >10,000 U/mL are reported to have a high specificity for HLH[205,288]. Soluble IL-2R levels in patients with sepsis in other studies are rarely higher than ~4,000 and ~8,000 U/mL. Nonetheless, sIL-2R is suggested as a marker for diagnosis of sepsis, and reported higher in non-survivors than survivors[315,316]. Elevated sIL-2R is also associated with acute kidney injury, frequently present in patients with sepsis, potentially further elevating serum sIL-2R levels[320]. A limitation of our studies (**Paper I**, **II, III and V**) was the capped detection range of the sIL-2R assay with all levels above 7,500 U/mL reported as >7,500 U/mL rather than an exact value.

In **Paper I**, sIL-2R showed a similar distribution pattern to ferritin, with the highest sIL-2R levels observed in patients with sepsis, having a median of 3,366 U/mL (range 1,085 to >7,500) compared to 1,439 U/mL (range 833-3,287) in non-sepsis patients. Our findings are in line with previous studies indicating elevated levels of sIL-2R in sepsis and inflammation[315,316,320]. Using the HLH criterion level sIL-2R ≥2,400 U/mL, we could observe in **Paper I** that overall, half of the patients had sIL-2R levels over 2,400 U/mL, whereof all but two had sepsis. The two non-sepsis patients with sIL-2R >2,400 U/mL were a patient with newly relapsed diffuse large

cell B-lymphoma, and a patient newly treated for a mantle cell lymphoma with high-dose chemotherapy and autologous SCT, both conditions that can be associated with T-cell activation and elevated levels of sIL-2R[108,349]. The positive correlation we observed with sIL-2R and creatinine is likely related to the reported sepsis-induced AKI, associated with elevated CD4+CD25+ T-regulatory cells and accompanying sIL-2R increase, and decreased sIL-2R excretion due to renal dysfunction[319,320,322].

In **Paper V**, patients with HLH, characterized by T-cell activation and hyperinflammation, showed significantly higher maximum sIL-2R levels (median 7,500 U/mL, IQR 2,796-7,500) than non-HLH patients (median 2,992 U/mL, IQR 1,832-4,125) (p=0.005), despite the capped sIL-2R levels giving false low levels in HLH patients. Of the 10 patients with sIL-2R >7,500 U/mL, nine were HLH patients (7/9 with sepsis or septic shock), and the remaining patient without HLH was a man with rheumatic disease admitted to ICU with an *E.coli* septic shock and a ferritin of 4,587 µg/L with fatal outcome. On the other hand, patients with sepsis and septic shock who did not develop HLH had a maximum sIL-2R median of 2,250 U/mL (IQR 985-5,865) and 3,358 U/mL (IQR 2,920-4,683), compared with a median of 7,500 U/mL in the HLH patients (p=0.030). These findings are in line with previous studies where sIL-2R levels >10,000 U/mL show high specificity for HLH diagnosis, and lower levels are generally reported in sepsis, rarely >7,500 U/mL, as we observed in our study[205,288]. No correlation between sIL-2R and creatinine was found in **Paper V**, but kidney dysfunction was as expected common, with an overall median maximum creatinine of 228 µmol/L (IQR 128-370), similar for HLH and non-HLH patients, possibly affecting sIL-2R levels in general. Two of 3 assayed HLH patients with AH1N1 infection in ECMO (**Paper II**) had sIL2R >7,500 U/mL compared with none in the non-HLH group. In **Paper III**, sIL-2R was assayed in 5/7 MAS-HLH patients with a median of 3,460 U/mL (IQR 2,741 to >7,500) and 2/5 showing levels >7,500 U/mL. Altogether, our findings show an association of elevated sIL-2R with sepsis and further with hyperinflammation in critically ill, with significantly higher levels in HLH.

In our first study, **Paper I**, we looked at correlations between ICU admission parameters of inflammation and critical illness. Perhaps not surprisingly, ferritin and sIL-2R, both markers of inflammation, were positively associated with each other and with CRP, another inflammation marker, in the entire cohort and in the sepsis subgroup. Furthermore, hyperferritinemia and elevated sIL-2R were associated with thrombocytopenia (Table III, **Paper I**). Thrombocytopenia (platelets <150 $x10^9$/L) is a common occurrence in critically ill, comprising about half of the patients with sepsis, and is associated with a higher risk of adverse outcome, including organ dysfunction, hypoalbuminemia and increased mortality, not the least if resolution of the thrombocytopenia is absent[302-304]. Thrombocytopenia is also a prominent feature of hyperinflammation and HLH, and frequently an early manifestation in the author's experience[93,99,102,122,131,324,326]. In **Paper V**, with more severe critical illness, the entire cohort displayed a marked thrombocytopenia at nadir (median 30 $x10^9$/L, IQR 12-63), significantly lower in HLH patients already on admission to ICU compared with non-HLH (30 vs 85 $x10^9$/L, respectively, p=0.001), with the caveat that platelets <70 $x10^9$/L was one of the inclusion criteria of the study. Additionally, hypoalbuminemia is an independent predictor of adverse

outcome in critically ill, and in our study (**Paper I**) it was associated with elevated sIL-2R and with a similar trend for hyperferritinemia (p=0.059) (Table III)[305,307]. Hypoalbuminemia is also commonly found in HLH but not included in the diagnostic criteria[100,285]. **Paper V** showed an overall marked hypoalbuminemia at nadir, median 18 g/L (IQR 15-20), already marked on admission (median 21 g/L), with no apparent difference between HLH and non-HLH patients, indicating a generally critically ill study population. Overall, we show a correlation between markers of inflammation, hyperferritinemia and elevated sIL-2R, and other indicators of adverse outcome in critically ill, such as thrombocytopenia and hypoalbuminemia; all factors commonly observed in HLH.

4.4 HLH IN CRITICALLY ILL WITH INFECTIONS

4.4.1 HLH associated with pandemic influenza

Severe pandemic influenza A infection may develop many features similar to those found in HLH, such as hypercytokinemia, multiple organ failure, infiltration of organs with reactive histiocytes and lymphocytes with hemophagocytosis[171,174,175,350]. During the study for **Paper I**, a large proportion of patients with severe influenza AH1N1 and ARDS were noted to require ECMO support at the ECMO center in Karolinska University Hospital in Stockholm, Sweden. This observation was later confirmed by reports in literature, such as a systemic review, revealing that 20% of patients admitted to ICU with severe influenza AH1N1 required ECMO support[172,173]. Following the 2009 pandemic, manifest HLH in association with severe influenza AH1N1 infection was described in case reports[176-178,180]. In 2011, Beutel *et al* reported HLH in nine of 25 critically ill patients with influenza AH1N1, several on ECMO support[181].

In **Paper II** we describe a well-defined cohort of 11 critically ill patients with severe pandemic influenza AH1N1 infection on ECMO support, with the aim to characterize and distinguish the patients that developed HLH. Four of the eleven ECMO-treated patients with severe AHN1 infection developed HLH, and they could be distinguished from the patients who did not, despite an existing grey zone of inflammatory states in between. CRP was increasingly high in all patients, indicating inflammation in general. All patients required vasopressor support and continuous renal replacement therapy (CRRT), and the overall median duration on ECMO was 16 days, without a significant difference between HLH and non-HLH patients (31 and 13 days, respectively, P=0.23). Three of 4 HLH patients, compared to 1/7 non-HLH, required veno-arterial ECMO due to additional circulatory failure, suggesting these patients to be more severely ill with more organ failure.

The patients who developed HLH showed characteristic features such as fever prior to ECMO start, organomegaly, bicytopenia, hyperferritinemia, hemophagocytosis in bone marrow, elevated sIL-2R, elevated liver tests, hypoalbuminemia and abnormal lymphocyte cytotoxicity. HLH patients fulfilled a higher number of HLH-2004 criteria and had significantly higher maximum HScore of 226 (range 213-240) compared to non-HLH patients, maximum HScore 86 (range 19-136) ($p<0.01$). The lowest HScore among HLH patients was 213, corresponding

to >93% probability of HLH, as opposed to the highest HScore of 136 in the non-HLH group, corresponding to a probability of HLH of almost 16%[12]. There was an overall marked thrombocytopenia in the study cohort. Five of 11 patients, whereof 3 with HLH, had bicytopenia (hemoglobin excluded as explained in the *Methods* section) according to HScore criteria, but not by HLH-2004 diagnostic criteria. As previously mentioned, HLH patients showed higher peak ferritin levels than non-HLH patients (10,735 and 1,204 µg/L, respectively, p=0.024), a difference that was not significant for ferritin values at admission to ECMO, suggesting HLH patients may have had a larger ferritin increase due to more hyperinflammation. Only one non-HLH patient had a peak ferritin >2,000 µg/L (12,933 µg/L), which was a patient with chronic lymphocytic leukemia and invasive candidiasis, who had the highest HScore of all the non-HLH patients. Higher sIL-2R levels and more marked hypoalbuminemia in HLH patients has also been previously mentioned.

On abdominal radiology, all four HLH patients had organomegaly, whereof 3 had hepatosplenomegaly (p=0.048). Organomegaly, in particular splenomegaly, is supportive of the diagnosis of HLH and present in ~70%, commonly in association with malignancy and infection[93,102,208,285]. The HLH patients in our study also developed higher peak levels of alkaline phosphatase (ALP) (p=0.012) and gamma-glutamyl transferase (GGT) (p=0.024), signs of obstructive hepatitis, which may be suggestive of characteristic periportal lymphocytic infiltration in HLH[6]. Congestive hepatopathy from right ventricular failure due to ARDS, particularly in patients on veno-arterial ECMO, could also explain the signs of obstructive hepatitis and hepatomegaly, but splenomegaly is generally absent, and was found in three of our HLH patients[351]. Our patients show characteristics similar to those described by Beutel *et al* in their patients with severe influenza AH1N1 that developed HLH, and in case reports in the literature[177-179,181,352]. Whether ECMO-related SIRS, an inflammatory response observed at initiation of ECMO, could contribute to triggering HLH remains uncertain. However, similar to Beutel *et al*, we found HLH to develop later in the course of ECMO treatment (median 15 days), suggesting them to be separate entities[181,353].

All eleven patients in our study (**Paper II**) survived compared to other reported fatality rates of 8-65% for patients with severe AH1N1 infection on ECMO support and in sHLH[97,173]. Beutel *et al* reported the very high mortality of 84% in patients with AH1N1-associated HLH (n=9, all on ECMO support), despite HLH-directed treatment in six patients (all corticosteroids and 4 with additional etoposide), compared to 25% in the group without HLH[181]. Two of our 4 HLH patients in **Paper II** received anti-inflammatory treatment: one patient diagnosed with HLH at ECMO (day 32) with unremitting inflammation and multiple organ failure was administered high dose dexamethasone (10 mg/m^2 BSA/day), a single dose etoposide (75 mg/m^2 BSA) and 5 days IVIG, and rapidly improved[180], and the other patient, improved after 5 days IVIG. Another patient with HLH improved with a slow recovery after optimized antimicrobial treatment. Case reports of AH1N1-HLH in the literature report successful and unsuccessful treatment with corticosteroids ± IVIG[176-179]. In 2006, Henter *et al* suggested cytotoxic therapy for patients with influenza AH5N1-associated hyperinflammation, and subsequently, in 2010 our group described the first case of AH1N1-HLH successfully treated

with etoposide (the above mentioned patient)[124,180]. The value of corticosteroids in the treatment of virus-associated hyperinflammation has been debated. However, a large randomized trial on dexamethasone in the treatment of Covid-19 resulted in lower 28-day mortality for patients with severe Covid-19 on respiratory support[354].

Overall, our findings support that young, previously healthy patients can develop influenza AH1N1-HLH. Even on ECMO support patients with HLH can be distinguished from other critically ill with varying degrees of hyperinflammation, using currently available HLH diagnostic tools (HLH-2004 criteria and HScore) combined with the clinical picture of persistence and progress of an inappropriate inflammatory response. Consequently, monitoring for signs of hyperinflammation and progressive organ dysfunction is recommended, since prompt HLH diagnosis and timely HLH-directed treatment, of adequate intensity, is of the essence. Cytotoxic therapy, such as etoposide, may be beneficial in a few selected cases of severe AH1N1-HLH.

4.4.2 HLH associated with severe dengue

Dengue is a global disease with a high incidence and high mortality in its severe form, severe dengue (SD)[184,187]. HLH associated with SD has been reported in case reports or smaller cohorts in adults and children[188,189,192-194]. In **Paper IV**, we report, to our knowledge, the largest study, except a recent meta-analysis, on dengue-associated HLH (dengue-HLH) in adults, with a comprehensive report on characteristics, clinical symptoms and laboratory findings, treatment and outcome[188]. Of 8,802 ICU admissions to Hospital Sultanah Aminah during the defined 5-year period, 287 had dengue infection, 9 with missing medical records. Of these 278 dengue patients 71% (197) had SD, whereof 17 aged <18 years were excluded, and remaining 180 patients (57% males) with SD were studied in **Paper IV**. As per WHO criteria for SD, our study population suffered from severe plasma leakage (n=108), and/or severe organ involvement (n=99) with predominantly liver involvement, and/or severe bleeding (n=99)[185]. Of the 180 SD patients 75 patients had two or more of these SD criteria. Eighty-seven of 180 patients (48%) had severe involvement of liver and/or CNS, a clinical presentation with characteristics similar to HLH[19]. Not surprisingly, altogether liver tests were elevated: alanine aminotransferase (ALT) and LDH about 5 times the upper limit of normal (ULN) and AST about 10 times the ULN. Overall there was a marked thrombocytopenia with median platelet nadir 15.5 $x10^9$/L, as previously mentioned, a finding frequently observed in critically ill patients. Life support treatments (LST), such as mechanical ventilation, inotropic support and CRRT were required in 39%, 31% and 18%, respectively.

Seventy-seven of the 180 SD patients had 4 or more HLH-2004 diagnostic parameters available for evaluation of HLH, whereof 31 (40%) were evaluated as having HLH, fulfilling ≥4/8 HLH-2004 criteria (group named "HLH-2004 ≥4"). For evaluation by HScore, 89 of 180 SD patients had enough parameters available to evaluate for a probability of HLH of 70% or greater (≥180 points), whereof 21 (24%) were evaluated as having HLH with HScore probability of ≥70% (HLH group named "HSprobability ≥70"). Seventeen of the aforementioned patients had both ≥4/8 HLH-2004 criteria and HScore probability of ≥70%. Only four SD patients had previously

known immunosuppression, whereof one was evaluated as an HLH patient. This is a very low proportion of previous immunosuppression in critically ill and HLH compared with **Papers I and V**. However, similar to **Paper II**, the study population is comprised of a younger (median age 34.9 years), healthy population, like other dengue studies, which may explain the difference[131,324,342-344]. In **Paper IV**, clinical and laboratory findings in SD that were associated with developing HLH, by either definition of HLH, included hepatomegaly, peak AST and ALT, peak LDH, peak ferritin, lowest fibrinogen, and negatively associated was increasing age at ICU admission. These findings are similar to the another study on risk factors associated with dengue-HLH reported by Ellis *et al*[194]. In **Paper IV**, patients with HLH had AST levels 50-60 times the ULN and ALT and LDH 10-17 times the ULN, which is about 3 (or more) times higher than the SD patients without HLH. Liver involvement is a common feature in SD, and was also predominant in our study population, with elevated AST more prevalent than ALT, and 10-fold increase or more observed in a smaller percentage of more severe cases[355-357]. Liver involvement is also a common feature of HLH with elevated liver transaminases and LDH[93,99,102]. In both severe dengue and HLH, AST is commonly more prominent than ALT, compared with other viral infections, and hypercytokinemia and activated infiltrative lymphocytes are involved in the pathogenesis[6,355]. Patients with dengue-HLH were also observed to have higher peak ferritin levels, in line with other patients with HLH, as previously discussed.

Altogether 39/180 (22%) patients with SD died, including 12/31 (39%) "HLH-2004 ≥4" HLH patients and 9/21 (43%) in the "HSprobability ≥70" group. Case fatality rates reported in severe dengue, for adults and children, vary in the range of 1-29%, the highest case fatality rate from Malaysia, the origin of our study[185,187,188,344]. Case fatality rates reported for dengue-HLH in children and adults vary around 4.5-14.6%, numbers much lower than those observed in our adult study[188,194]. Our study population was much larger than other studies, but with the limitation that only 77/180 severe dengue patients could be evaluated for HLH, leaving a large margin for the possibility of higher incidence and both higher or lower mortality in dengue-HLH. Our study showed a 47% mortality in patients with severe liver involvement, and increasing levels of ferritin and AST were associated with an incremental risk of death. Severe organ involvement, hepatomegaly, peak AST, ALT, LDH, ferritin and creatinine, thrombocytopenia and higher ICU mortality scores were all mortality-associated risk factors, but in multivariable regression, peak AST (log, OR 2.8, p=0.0019), peak creatinine (OR 7.3, p=0.0065) and SOFA score (OR 1.4, p=0.0051) were the only independent risk factors of death. Median duration from ICU admission to death was only 2 days (IQR 1-5), with 64% of deaths occurring during the first 3 days of ICU admission, indicating the need for fast and effective therapies.

The basis of SD treatment is antipyretics and fluid replacement therapy, and appropriate additional supportive therapy. Several case series studies have reported successful treatment of dengue-HLH with addition of corticosteroids, such as methylprednisolone pulses or dexamethasone, a few with addition of IVIG as well, and one pediatric study had good survival with IVIG only[189,192-194,358,359]. In our study cohort, 25 patients, all with at least severe organ

involvement (24 with liver involvement) and highly elevated AST, ALT and ferritin, were administered corticosteroids, whereof 13 (52%) survived, of which 8 had been administered dexamethasone. Of HLH patients, 15 and 14 patients in "HLH-2004 ≥4" and "HSprobability ≥70", respectively, received corticosteroids, of which 7 and 6, respectively survived. Clearly, a portion of patients die in dengue-HLH despite corticosteroids therapy, suggesting the need for additional therapy. Ellis *et al* report on 8 of 22 pediatric dengue-HLH cases treated with additional etoposide, and only one dengue-HLH related death in the study[194]. There are no other studies, to the author's knowledge, reporting the use and outcome of etoposide for severe dengue-HLH. Etoposide is an important component of HLH-directed therapy in selected cases of sHLH, such as in severe EBV-HLH, where early administration of etoposide shows a drastic improvement in survival[121]. Etoposide has been shown to selectively ablate activated T cells, and together with corticosteroids, achieve a reduction of lymphocytes and cytokine secretion[91]. T cells are infected in both EBV-HLH and in acute dengue, where they support viral replication[145,360]. Altogether, this could provide a rationale for the use of etoposide in selected cases of severe dengue-HLH where corticosteroids are not sufficient.

Our study (**Paper IV**), and others, support that dengue-HLH should be considered in deteriorating severe dengue patients with increasing organ dysfunction and inflammation, with markedly elevated liver enzymes (particularly AST) and ferritin. Furthermore, there is a need for future studies on dengue-HLH and evaluation of HLH-directed therapy with corticosteroids and IVIG, and the addition of cytotoxic therapy, such as etoposide, in selected cases of severe dengue-HLH.

4.5 HLH IN CRITICALLY ILL IN THE ICU

A diversity of inflammatory responses is frequently encountered in critically ill in intensive care, where a subset of these patients are likely to develop hyperinflammation and sHLH; a condition likely underdiagnosed in critically ill at ICU but crucial to identify for appropriate antiinflammatory therapy and improved survival[107,285,323,361]. **Paper V** studied 50 critically ill patients in intensive care, included on criteria of SIRS, hyperferritinemia and cytopenia (thrombocytopenia), indicators of inflammation and critical illness that could be part of a developing HLH or some other inflammatory response in critically ill. Overall, there was a high degree of inflammation observed in the study population in **Paper V**, with 66% admitted for sepsis and septic shock and an overall median CRP of 335 mg/L (IQR 166-424). They exhibited extensive organ failure (MODS) and high risk of mortality, with a median SOFA score on admission of 11 (IQR 8.0-13.5) and highest SOFA score 13 (IQR 10.0-16.0). Initial and highest SOFA scores >11 have been shown to correspond to a mortality >80%, with highest SOFA score having the strongest correlation with mortality[362]. The need for LST in **Paper V** was high, with 70% requiring mechanical ventilation, 90% vasopressor/inotropic support and 52% CRRT. 28-day mortality (from ICU admission) and hospital mortality was 46% and 56%, respectively. HLH and similar hyperinflammatory conditions, such as MAS-like MODS, should be suspected in the critically ill patient with an unexplained or disproportionate inflammatory response (e.g. fever, cytopenias, hyperferritinemia, organomegaly,

hemophagocytosis), in particular with concomitant progressive MODS and inadequate response to appropriate empiric therapy and supportive care[107]. The high proportion of inflammatory responses and MODS in our study population was a breeding ground for hyperinflammatory conditions.

Evaluation for HLH in the 50 critically ill patients in **Paper V** resulted in 18 patients categorized as HLH (termed "HLH") and 32 without HLH (termed "non-HLH"). HLH-2004 criteria and HScore have demonstrated a high diagnostic accuracy for HLH in critically ill ICU patients, with the best prediction accuracy at a cutoff of 168 for HScore and 4 fulfilled criteria of eight for the HLH-2004 criteria in a large ICU study, and cutoff at 144 for HScore in another smaller ICU study[104,363]. Improved accuracy of HLH-2004 criteria could be achieved by adjusting ferritin cutoff to 3000 µg/L (instead of 500 µg/L)[104]. To reduce the risk of overdiagnosing HLH, the patients in **Paper V** defined as HLH had to have ≥5/8 HLH-2004 criteria and HScore ≥169 (n=14) and/or have been diagnosed with HLH during ICU care (n=12). In the latter group, four patients didn't fulfill ≥5/8 HLH-2004 criteria, but fulfilled 4/8 criteria, whereof two had HScore ≥190 and the other two had HScore of 144 and 166, respectively. On review of medical files of the latter two patients, one patient developed HLH during chemotherapy and another had AH1N1-associated HLH. There is no test or finding that is specific for HLH, but it is the magnitude and combination of findings together with the overall clinical presentation in a setting of hyperinflammation that allows for diagnosis of HLH. The HLH patients presented with a median of 5/8 fulfilled HLH-2004 criteria (range 4-7, IQR 5-5.75) and a median HScore of 207 (range 144-289, IQR 189-221), compared with a median of 3/8 fulfilled HLH-2004 criteria (range 2-5, IQR 2.75-4) and a median HScore of 138 (range 35-238, IQR 112-154) for non-HLH patients. Seventy-eight percent of the HLH patients fulfilled ≥5 HLH-2004 criteria and 89% had HScore >169, compared to 9% and 22%, respectively, in non-HLH. Six patients fulfilled 4/8 HLH-2004 criteria and had a HScore >169, which according to Knaak *et al* would be highly suggestive of HLH[104]. Our study (**Paper V**) shows a high incidence of sHLH (36%) compared with 0.63-2.2% in other larger ICU studies, which include all critically ill patients in ICU with a ferritin measurement >500 µg/L or critically ill with ≥2 SIRS criteria[324,346,363]. Compared with the latter studies, we have a selected cohort of more critically ill and hyperinflammatory patients as reflected by our many high HScores and ferritin levels, which in the aforementioned studies were parameters associated with increased mortality, and hence, with disease severity.

Eighty-one percent of the HLH patients fulfilled the criteria of fever >38.4°C, compared to 58% in non-HLH. Of note, infections rates were generally high, with 83% in HLH and 69% in non-HLH. Cytopenias were significantly more severe (p=<0.005) and organomegaly more prominent in HLH patients, with splenomegaly found in 50% and 5% of HLH and non-HLH patients, respectively (p=0.008). Peak triglyceride levels were higher in HLH than in non-HLH (p=0.005), but there was no significant difference for fibrinogen, which was rarely below the HLH criterion of 1.5g/L, possibly due to use of plasma in the supportive care of critically ill patients. As previously discussed (please refer to section 4.3), HLH patients exhibited extreme hyperferritinemia at admission and at the maximum level (41,564 µg/L) compared with non-

HLH patients, even when comparing with maximum ferritin levels in non-HLH patients with sepsis (5,492 µg/L) and septic shock (12,487 µg/L) (p=0.014). These findings are in line with a recent large study on hyperferritinemia in critically ill at ICU (peak ferritin sepsis 1,448 µg/L, septic shock 1,545 µg/L and HLH 31,374 µg/L), but where our patients with sepsis and septic shock display higher ferritin levels, likely affected by our smaller cohort selected for elevated ferritin and some variation in the definition of HLH[276]. Maximum sIL-2R, also previously discussed (please refer to section 4.3), was higher and more frequently >7,500 U/mL in HLH patients, with a lower sIL-2R median even in septic non-HLH patients (3,306 U/mL). Hemophagocytosis, not mandatory for the diagnosis of HLH and prevalent in critically ill, was found in more HLH than non-HLH patients, but the data is inconclusive due to many missing values, especially in the non-HLH subset.

Altogether, these findings of fever, marked cytopenias, splenomegaly, elevated triglycerides, highly elevated sIL-2R and extreme hyperferritinemia, in the setting of disproportionate inflammation in critically ill in ICU, are supportive of the diagnosis of HLH and should trigger prompt further evaluation and appropriate therapy[103,107,111,285]. It is imperative to identify and treat the underlying trigger of HLH for better management and survival[103]. In adults, infections and malignancies are predominantly associated with HLH, and the likelihood of an underlying malignancy increases with age, why sHLH of unknown etiology should include an exhaustive search for an occult neoplasm[93,103,108]. As previously detailed (please refer to section 4.2), infections (bacterial and viral) were the most frequent underlying trigger, found in 84% of the HLH patients, followed by hematological malignancies found in 50%, causes that were not considered mutually exclusive. HLH may occur in the context of immune suppression, and even if the pathobiology is not fully understood, immunosuppression, whether acquired or inherent, contributes to immune dysregulation and lowering the threshold for development of HLH[7,361]. A high prevalence of immunosuppression can be observed in critically ill with HLH, and in our study population 67% of the HLH patients were previously immunosuppressed compared with 28% in non-HLH patients (p=0.019).

Mortality in sHLH is high and in critically ill it is reported to be around 58% overall, with variations by underlying trigger, in a recent review[325]. We observed a similar 28-day mortality for HLH (50%) and non-HLH (44%) patients, but HLH patients tended to have a higher hospital mortality (73% and 47%, p=0.159), likely due to patients with malignancy-HLH who finally succumbed to their disease at the ward after ICU. Altogether, 23 patients died within 28 days of ICU admission ("non-survivors (NS)"), whereof 9 had HLH ("HLH-NS") and 14 did not ("non-HLH-NS"). Twenty-seven patients survived past 28 days from ICU admission ("survivors (S)"), of which 9 were HLH patients ("HLH-S") and 18 were non-HLH patients ("non-HLH-S"). The minimal difference in SOFA scores and similar requirement of LST between HLH and non HLH patients, suggest a somewhat homogenous severity of illness, albeit overall high, in the study population. However, further analyses of SOFA score and LST between S and NS within the HLH and non-HLH subsets identified a significant difference between survivors and non-survivors in the non-HLH cohort, not observed within the HLH subset. Non-HLH-NS demonstrated more severity of illness with higher SOFA score on

admission (13.5 and 9.0, respectively, p=0.003) and at maximum (16.5 and 10.0, respectively, p<0.001) and a trend of needing more LST compared to non-HLH-S. Non-HLH-NS had a similar severity of illness on admission to HLH patients, but surprisingly developed more severe organ failure than HLH patients (maximum SOFA 16.5 and 13.0, respectively, p=0.029), but were also notably older (median 72 and 58 years, p=0.001).

Overall, non-survivors had significantly higher maximum levels of ferritin than survivors (43,943 and 8,148 µg/L, p=0.001), and 5-fold higher ALT (658 vs 150 U/mL) and LDH (1,956 vs 534 U/mL), and a magnificent 12-fold higher AST (2,102 vs 169 U/mL) (p≤0.001). Interestingly, non-survivors also demonstrated an 8 to 9-fold faster daily rate of increase of ferritin (10,418 vs 1,382 µg/L, p=0.003) and liver transaminases (p<0.01) compared to survivors. This same pattern was evident between non-survivors and survivors in the non-HLH cohort, but with 1,5-3 times larger disparity, indicating a more severe liver inflammation or hepatocellular damage in the non-HLH-NS. HLH-NS also demonstrated significantly higher maximum ferritin levels (44,240 and 14,308 µg/L, p=0.047) and faster rate of increase than HLH-S (18,730 and 2,808 µg/L/day, p=0.049). Overall, each successively higher rate of increase of ferritin was associated with a higher risk of death. Higher ferritin levels in non-survivors have been previously described in critically ill in ICU, and hyperferritinemia also associated with increased mortality[276,279,280,314,346]. It has also been observed in survivors and non-survivors of HLH in other studies, but not always statistically significant, although with an evident overall hyperferritinemia[131,324]. Even though variables including peak ferritin, AST, ALT, LDH and SOFA score, and number of HLH-2004 criteria and HScore, were all univariably associated with mortality, it was only peak AST in the whole cohort and SOFA score for non-HLH that were independently associated with mortality in **Paper V**. The HLH subset was too small for a meaningful multivariable analysis. Fastest rate of increase of ferritin has not, to our knowledge, been previously studied in HLH in the comparison of survivors and non-survivors, and we suggest it may be used to help identify the patients at higher risk of adverse outcome for earlier appropriate intervention and hopefully improved survival.

Maximum ferritin and fastest rate of increase of ferritin did however not distinguish our HLH and our non-HLH-NS, the latter with maximum ferritin levels similar to those observed in the HLH patients (32,781 and 41,564 µg/L, respectively). However, neither splenomegaly nor malignancies were identified in non-HLH-NS, who also had significantly lower number of fulfilled HLH-2004 criteria and HScore than HLH patients (median HScore 138 and 207, respectively, p=0.001), similar to that seen in non-HLH-S (HScore 132), suggesting an overall milder inflammatory response in non-HLH patients. This is in contrast to the HLH patients who showed all the previously described manifestations of HLH and hyperinflammation. In HLH, infiltration of activated lymphocytes and histiocytes cause organomegaly and hepatitis, while ferritin is released from activated macrophages[6]. Hyperferritinemia may also arise from ferritin released by hepatocytes in acute hepatocellular damage, with concurrent ALT elevation[364,365]. Non-HLH-NS exhibited severe and rapidly progressive liver damage, with high and rapidly increasing ferritin levels, but also a high proportion of elevated ALT. A high ferritin/ALT ratio was found to distinguish hyperferritinemic HLH patients from

hyperferritinemic non-HLH-NS (ratio 334 and 29, respectively, p=0.004), suggesting that the hyperferritinemia in HLH patients is predominantly inflammatory, released from macrophages, compared to a larger contribution from hepatocellular damage in the hyperferritinemic non-HLH-NS patients. Elevated ferritin/ALT ratio has been found in patients with fulminant hepatic failure, generated by activated macrophages, compared with acute viral and drug-induced hepatitis[366]. With the best ferritin/ALT ratio cutoff of 127 (sensitivity and specificity 72%, AUC 0.78, 95% CI 0.65-0.91), three non-HLH-NS above the cutoff also had HScore >169, whereof two showed typical clinical presentation of HLH but only fulfilled 4 HLH-2004 criteria (of 5 or 6 available parameters for evaluation), recently determined as enough criteria for good accuracy of HLH diagnosis in critically ill[104]. To our knowledge, this is the first HLH study to analyze ferritin/ALT ratio, and we suggest it as a useful additional tool to identify hyperinflammation-driven hyperferritinemia, that could benefit from anti-inflammatory therapy.

Altogether, our study was able to support hyperferritinemia as a good marker of hyperinflammation and HLH in critically ill. However, rapidly rising hyperferritinemia was also observed in a subset of patients who manifested fewer signs of hyperinflammation but were still at risk of adverse outcome. Fastest rate of increase of ferritin and ferritin/ALT ratio may be useful parameters to help identify and distinguish these risk patients, but this requires further studies in a larger and more diverse study population of critically ill.

4.6 MODERATELY DOSED ETOPOSIDE FOR SEVERE MAS-HLH?

As for other forms of HLH, the last decades have provided an advancement in the pathogenesis, diagnosis and treatment of MAS-HLH. Treatment of MAS-HLH has improved significantly with the addition of anakinra to the conventional treatment of MAS-HLH including, high dose corticosteroids, IVIG and CSA[115,116,217]. However, mortality and morbidity are still high, including CNS complications and increasing reports of severe pulmonary involvement[222,232].

In **Paper III** we report seven children with sJIA, atypical JIA or SLE, who developed severe MAS-HLH with CNS involvement and/or without sufficient response to conventional MAS-HLH therapy, and they were therefore treated with additional etoposide, administered in moderate doses with the aim to provide highly efficient anti-inflammatory therapy with limited side-effects. All children, aged 0.4-16 years (median 9) at onset of MAS-HLH, were severely ill and fulfilled MAS criteria for sJIA or MAS criteria for SLE, accordingly[224,226]. The children presented with typical MAS-HLH manifestations including persistent fever, splenomegaly, falling and low blood counts, and elevated ferritin, sIL-2R and liver transaminases. All but one patient fulfilled ≥4/8 HLH-2004 criteria, that may be less appropriate for diagnosis of MAS-HLH initially, but criteria are successively fulfilled as the MAS-HLH aggravates[11]. Consequently, 6 of 7 patients required care in the ICU. Five children had CNS involvement, 2 moderate/severe and 3 very severe, where the latter presented with initial disorientation followed by rapid deterioration of cerebral function, seizures and finally unconsciousness (one patient with progressive brain edema), with gravely pathological EEG and CNS MRI showing encephalitis and extensive encephalopathy. Two patients also developed posterior reversible

encephalopathy syndrome. Five children had pulmonary involvement, whereof 3 were severe with acute respiratory distress requiring mechanical ventilation due to lung disease, including pulmonary alveolar proteinosis and pulmonary arterial hypertension, as described in previous studies on sJIA-associated lung disease[232,233].

All patients, except for one sJIA patient, were on oral steroids at MAS-HLH onset. Furthermore, all sJIA patients were on inhibitor treatment (IL-6 or IL-1 inhibitor), and all SLE patients on oral steroids and hydrocychloroquine, at MAS-HLH onset. Previous reports demonstrate that IL-6 and IL-1 inhibitors do not offer full protection against MAS-HLH development, as observed in our study[216]. CNS and pulmonary involvement are both reported as independent risk factors of a more severe course of MAS-HLH[222]. Younger age at diagnosis, exposure to inhibitors (IL-6 and IL-1) and more adverse reactions, high serum IL-18 levels and in particular prior episodes and ongoing MAS-HLH at diagnosis of pulmonary involvement have been associated with sJIA-associated lung disease[232,233,367]. Furthermore, it has been hypothesized that exposure to inhibitor therapy may promote lung disease in a few treated patients[232]. All children in our study with pulmonary involvement had exposure to inhibitor treatment prior to MAS-HLH onset, and 2 of the patients with severe lung disease were of young age (5 months and 5 years). Of the three children with severe pulmonary involvement, two had suffered prior MAS-HLH episodes and one patient developed pulmonary involvement concurrent with the first MAS-HLH episode.

In 4 of 7 MAS-HLH patients in our study, etoposide treatment was mainly initiated due to rapid progression of severe CNS symptoms and HLH (n=3) or due to absent improvement of CNS symptoms (n=1) despite conventional MAS-HLH therapy with high dose methylprednisolone pulses. Two of these patients experienced regression of CNS symptoms within 6 days from initiation of the moderately dosed etoposide therapy, whilst the other two patients had a slower improvement within 2-4 weeks, of which one ended up with severe neurological sequelae. The latter patient, already with severe CNS symptoms at admission, was the only patient in our case series who did not fully recover. In the remaining 3 of 7 MAS-HLH patients, etoposide treatment was primarily initiated due to progression of HLH and/or pulmonary disease despite conventional MAS-HLH therapy (high dose methylprednisolone pulses or corticosteroids and CSA), and all three patients showed improvement of HLH and/or pulmonary symptoms after initiation of etoposide. Two of these patients had reactivation of their MAS-HLH, which also responded well to re-introduced moderately dosed etoposide treatment. One patient with several recurring MAS-HLH episodes finally underwent allogeneic HSCT and is well without MAS-HLH reactivations. In some patients there was a trend of improvement of laboratory findings as a response to high dose corticosteroid pulses, but the clinical severity and lack of improvement of the patients' CNS and pulmonary involvement prompted the addition of etoposide. Besides one patient with neutropenic septicemia after the first etoposide dose, likely etoposide-toxicity related, no serious adverse events were encountered in our case series. Limited case reports in the literature on additional etoposide treatment for MAS-HLH have also shown rapid recovery without serious adverse events[368]. The risk of secondary leukemia associated with etoposide has in particular been with cumulative etoposide doses >1500 mg/m^2,

which is far beyond the cumulative dose reached with a limited number of moderately dosed etoposide doses[43,369].

CNS involvement is frequent in HLH and MAS-HLH, reported at ~35%, and may lead to neurological late-effects[41,222]. In our study, 5/7 (71%) MAS-HLH children had CNS involvement, likely a higher proportion due to more severely ill MAS-HLH patients being referred to Karolinska University Hospital as a tertiary care reference center. Even so, only one patient suffered neurological sequelae, and all of the patients showed improvement after etoposide initiation, suggesting etoposide in a moderate dose may be beneficial in the treatment of severe MAS-HLH with CNS involvement to reduce long-term CNS damage. Systemic etoposide is included in treatment recommendations by experts of CNS involvement in HLH[64]. Mortality in MAS-HLH with pulmonary involvement is high, recently reported at 42% for 5-year survival. In our study, all patients with MAS-HLH and pulmonary involvement survived without sequelae at a follow-up of 2-8 years. Histology of lung tissue from patients with sJIA-associated lung disease (including MAS-HLH) have shown predominant lymphocyte inflammation with lymphoplasmacytic infiltrates, and transcriptional profiling has shown an upregulation of T-cell activation networks[367]. The selectively ablative effect of etoposide on activated T lymphocytes could support the hypothesis of efficacy of etoposide on the pulmonary disease[91]. Our positive experience of additional moderately dosed etoposide for the treatment of selected cases of severe MAS-HLH including CNS or pulmonary involvement, is shared with others. A report of adult MAS-HLH where etoposide was used upfront, due to initial severity of MAS-HLH, or as second or third line therapy in combination with cyclophosphamide in a subset of patients, showed overall efficacy of 100% and 73%, respectively[234].

4.7 BIOLOGY OF SECONDARY HLH

Familial HLH is caused by mutations in genes involved in the perforin-dependent cytolytic pathway of cytotoxic lymphocytes, resulting in a cytokine storm[5,8,9]. While hypercytokinemia is involved in the pathobiology of all forms of HLH, the precise pathogenesis of secondary (acquired) HLH is incompletely understood[7].

4.7.1 Lymphocytes and cytotoxic function

Lymphocyte cytotoxicity function assays were performed on available samples in **Papers I-III and V**. In **Paper I**, 19 of 32 (52%) ICU patients were assayed for NK cell cytotoxicity and NK-cell frequency (%). Overall, 11 of 19 critically ill patients had NK cells <5% in PBMCs, whereof seven had sepsis. On the other hand, sepsis was also common in patients with normal NK cell percentages. Generally, there was a trend that patients with higher ferritin levels had low percentages of NK cells (p=0.196). Unfortunately, only four of 10 patients with ferritin levels >10,000 µg/L had samples available for lymphocyte assay evaluation. However, in **Paper V**, we had 47 samples from critically ill ICU patients available for cell counts and lymphocyte cytotoxicity assays to further investigate our findings in **Paper I**. Interestingly, we encountered a study cohort with a general lymphopenia, with low median absolute counts

(cells/µL whole blood) of lymphocytes and subpopulations, including NK cells ($CD3^-56^+$), CTLs ($CD3^+CD8^+$) and CD4 T cells ($CD3^+CD4^+$), compared with 200 healthy blood donors (all X^2-tests $p<0.001$). Fifty-seven percent of critically ill had NK cells <5%, but when analyzing absolute counts 39/47 (83%) had NK cell numbers below the normal 5th percentile in our healthy control population (<80 cells/µL whole blood). The result was similar if patients with previous chemotherapy were excluded (n=30/37, 81%). Furthermore, 42/46 (91%) had CTL counts below the normal 5th percentile (<168 cells/µL whole blood), and similar (89%) with previous chemotherapy excluded. Prolonged lymphocyte depletion, including B and CD4 lymphocytes, in lymphoid tissue has been described in septic patients and associated with MODS and sepsis-related death[370,371]. Additionally, in line with our findings, generalized peripheral lymphopenia in critically ill ICU patients has also been reported, with a relative reduction of CTLs, and failed restoration of T-cell depletion and lymphopenia observed in non-survivors, whereas survivors showed higher absolute T lymphocyte counts at ICU admission[253].

Notably, HLH patients had significantly lower absolute counts of NK cells, CTLs and CD4 T cells compared to non-HLH patients (p=0.016, p=0.036 and p=0.002, respectively). In line with Halstead *et al*, who reported decreased $CD56^{dim}CD16^+$ cytotoxic NK-cells, we also observed lower $CD56^{dim}$ NK cells ($CD3^-CD56^{dim}$ $CD16^+$) in HLH patients, however, we could not find a statistically significant correlation to reduced NK cytotoxicity, but more in-depth analyses may be justified[252]. Non-survivors in our study cohort demonstrated a trend towards higher percentage of exhausted CTLs ($CD3^+CD8^+PD1^-$) compared with survivors (p=0.064), which was also significant when comparing non-HLH non-survivors and survivors (42.5% and 9.0%, respectively, p=0.043), but not in the HLH cohort. Lymphocytes in critically ill with severe sepsis have been reported to upregulate expression of receptors associated with cell activation and exhaustion, alongside increased frequency of T regulatory cells, contributing to the immune suppressed state described in critically ill with sepsis with increased mortality[319,372]. Cell exhaustion and reduced frequency of lymphocytes, importantly NK cells and CTLs, and related cytotoxic dysfunction, have been implicated in the development of immunoparalysis and other inflammation phenotypes with immune dysregulation and multi organ failure in sepsis, and further with the development of hyperinflammation and HLH-like syndromes[327,330,373].

In **Paper I**, six of 19 patients had pathologically low NK cell cytotoxicity (LU<10) and/or abnormal NK cell degranulation (<10%), whereof four also had low NK cells <5% (p=0.016). Furthermore, 5/6 patients with abnormal NK cell cytotoxicity and degranulation had ferritin levels >2,000 µg/L, whereof three had ferritin >10,000 µg/L, and sepsis. Hyperferritinemia was associated with abnormal NK cell cytotoxicity (r_s=-0.462, p=0.047) and degranulation (r_s=-0.504, p=0.030), as was sIL-2R with NK cytotoxicity (r_s=-0.483, p=0.042). Our findings in **Paper I** show hyperferritinemia and sIL-2R to be associated with abnormal NK cell lymphocyte function, which is associated with a low percentage of circulating NK cells. Although a small study population, a statistical correlation between clinical and pathobiological markers of (hyper)inflammation had, to our knowledge, not been previously reported. In **Paper**

V, the lymphopenic setting precluded lymphocyte cytotoxicity assays from being performed or made results unreliable in cases with severe lymphopenia. Consequently, degranulation assays were performed in 40 patients and NK-cell cytotoxicity assays could also be performed in 33 of these patients. Overall, 59% of the assays showed abnormal lymphocyte cytotoxic function (NK cytotoxicity, NK and CTL degranulation). Interestingly, all assayed HLH-NS (n=7) had either abnormal cytotoxicity (LU<10) or defective NK/CTL degranulation, compared to about half of the assayed HLH-S (n=2/5). Furthermore, even non-HLH patients showed a high proportion of defective lymphocyte cytotoxic function (non-HLH-NS, n=6/10 and non-HLH-S, n=7/15). Additionally, of unclear clinical significance, we observed overall significantly lower, but perhaps not abnormal, levels of lymphocyte cytotoxic function compared with the healthy blood donor cohort. Surprisingly, we did not find any significant associations with Fisher's or Spearman's tests for NK cell or CTL counts with LU or degranulation, nor the latter with ferritin. However, the cohort exhibited extensive hyperferritinemia with ferritin >5,000 µg/L in 37/50 patients and >10,000 µg/L in 30/50 patients, making it likely for some association between hyperferritinemia and lymphocyte function, and thus, suggests further study.

Reduced NK cytotoxicity in hyperinflammatory conditions has been previously reported, but studies on lymphocyte cytotoxicity in critically ill, and in adults, are few, making the **Paper V** study one of the larger, to our knowledge. As mentioned earlier, reduced NK cytotoxicity, due to reduced NK frequency, has been described in sepsis[252]. On the other hand, reduced NK cytotoxicity in sepsis has also been reported in the presence of $CD56^{dim}$ NK cells, suggesting there may be other mechanisms than just reduced NK cell numbers[254]. Compared to healthy controls, reduced NK-cell activity can be observed in patients suspected of HLH but not fulfilling the criteria[374]. Defects in NK cytolytic activity and reduced frequency of NK cells have been found in sJIA, MAS-HLH, AOSD and other forms of sHLH, further supporting the hypothesis of reduced cytotoxic lymphocyte numbers and cytotoxic function contributing to the pathogenesis of hyperinflammation[238,248-251]. In line with these findings, we report, in **Paper III**, NK-cells <5% in 5/7 children with MAS-HLH, whereof three also had low absolute NK-cell counts, of which two had both low NK cytolytic activity (LU<10) and defective degranulation.

In **Paper II,** an association between hyperferritinemia and low NK-cell and CTL percentages could be demonstrated, however, even here in a very small sample population. Six of 11 patients with AH1N1 infection in ECMO had samples available for lymphocyte cytotoxicity assays, four patients with AH1N1-HLH and two without HLH. Four patients showed abnormal NK cell cytotoxicity, of which three had HLH with low NK cell and CTL percentages. Lymphopenia with reduced numbers of NK cells, $CD4^+$ and $CD8^+$ T lymphocytes with an excessive CTL immune response has been described in severe influenza AH1N1 infections[375]. AH1N1 virus may directly infect NK cells, inducing apoptosis and contributing to reduced NK cell numbers and the immune dysregulation leading to hyperinflammation[258]. Lymphopenia has been associated with delayed viral clearance, in severe influenza AH1N1 and AH5N1, and further correlated to hypercytokinemia, disease severity and extensive tissue damage[350,375-377].

Beutel *et al* also described prolonged viral shedding in patients with AH1N1-HLH compared to those who didn't develop HLH, and hypothesized it contributes to the development of hyperinflammation, first in the lungs and then systemic[181]. We did not have assays of viral shedding at HLH diagnosis available, but at least 2/4 HLH patients had negative AH1N1-PCR prior to developing HLH, albeit persistent elevated hyperinflammation parameters and low NK cells and CTLs, suggesting more than prolonged viral shedding is in play. Notably, lymphocyte cytotoxicity assays after recovery in one of the HLH patients and in one of the patients without HLH in **Paper II** showed normalized cytotoxicity and cell percentages, supporting the described transient reduction of cytotoxic lymphocytes and defective cytotoxicity in acquired hyperinflammation conditions/HLH[330].

4.7.2 Cytokines

Hypercytokinemia is a cardinal feature of HLH, both primary and secondary, but also dominant in other inflammatory responses, such as sepsis, with many of the same cytokines involved[5,7,378]. In **Paper V**, we found higher levels of IL-18 and IL-10 in HLH patients compared with non-HLH patients. Non-survivors overall demonstrated higher levels of sCD163 and IL-10, the latter even so in HLH-NS and non-HLH-NS compared with their corresponding survivors. In a recent report on biomarkers for HLH diagnosis, elevated IL-18 was determined one of the most efficient biomarkers for early HLH diagnosis, particularly in combination with HScore. Furthermore, sCD163 and IL-10 were also observed elevated in HLH, with higher values in malignancy-HLH than infection-associated HLH[379]. Elevated levels of IL-18 and IL-18BP have typically been found elevated in MAS-HLH, besides other forms of sHLH, and associated with cell exhaustion, decreased NK cells and defective cytotoxicity[250,251,255]. The anti-inflammatory cytokine IL-10, on the other hand, has been shown to play a protective role in a murine model of HLH, and higher levels have been observed in HLH than in the less inflammatory state of sepsis, where it however, contributes to sepsis-related immunoparalysis and further inflammation[89,246].

4.7.3 Genetics

Along with progress of our understanding of primary HLH, hypomorphic mutations and sequence variants in known HLH-causing genes or in genes involved in the cytolytic or related pathways, have emerged in patients with apparent secondary HLH. Whilst some of these mutations or variants may be associated with decreased lymphocyte cytotoxic function and development of adult HLH, others may contribute with a risk but not a certainty of HLH[16-18,235,236]. In **Paper I**, 11 patients with NK cells <5% and/or abnormal lymphocyte cytotoxic function were sequenced for mutations in the previously described known HLH-causing genes. A rare heterozygous variant in *STXBP2* (minor allele frequency, MAF, 0.0001187) was identified in a patient with autoimmune disease and reduced NK cell percentage and NK cytotoxic activity. Mutations in HLH-related genes, including *STXBP2*, *UNC13D* and *PRF1*, associated with defect cytolytic function, have been identified in MAS-HLH patients[237-239,348]. Surprisingly, none of our seven children with severe MAS-HLH in **Paper III** showed any

mutations in HLH-related genes, despite whole genome sequencing being performed for two patients.

We identified two rare heterozygous variants in HLH-related genes in **Paper II**. One patient with AH1N1-HLH with abnormal NK cytotoxicity and reduced NK cell and CTL percentages had a rare heterozygous variant in *UNC13D*. Interestingly, his lymphocyte cytotoxicity assay was normalized after recovery. Another rare heterozygous variant was found in *STX11* in an AH1N1-infected patient who did not develop HLH; no cytotoxicity data was available. The difference in the clinical course of the two patients with heterozygous variants and normalization of NK cell function after recovery in the patient with HLH, supports the theory of mutations and variants having varying penetrance and contribution to the susceptibility of HLH. Heterozygous mutations and rare variants in other HLH-causing genes, some with associated reduced NK cytolytic function, have been described in fatal AH1N1 infections[243].

Overall, our findings reflect the variety of factors that may contribute to the pathogenesis of sHLH, where an accumulation of factors successively lower the threshold to the switch to uncontrolled hyperinflammation and development of sHLH.

5 GENERAL CONCLUSIONS

The overall aim of this thesis was to broaden our knowledge of hyperinflammation and HLH in critically ill, with a focus on intensive care, to better identify the critically ill patients with hyperinflammation that could benefit from anti-inflammatory therapy, to improve morbidity and survival of patients with sHLH. We conclude the following:

- Ferritin and soluble IL-2R, with the highest levels observed in patients with sHLH, are good markers of inflammation that are associated with other parameters of inflammation and adverse outcome, and should raise suspicion of hyperinflammation and prompt evaluation of sHLH diagnosis.

- Patients who developed sHLH were predominantly male, younger and with fewer comorbiditites than criticlly ill who didn't develop sHLH.

- Patients with severe dengue, in particular with severe organ involvement and predominantly liver involvement, are at high risk of developing dengue-HLH associated with a high mortality, which may be reduced by early diagnosis and appropriate treatment.

- Patients with severe influenza AH1N1-infection can develop sHLH, that may benefit from anti-inflammatory treatment, and they manifest characteristic features of HLH despite ECMO treatment.

- Current diagnostic tools for HLH (HLH-2004 criteria and HScore), in the setting of an inappropriate uncontrolled inflammatory response, can be used to identify patients with HLH in critically ill in intensive care.

- Hyperferritinemia and elevated soluble IL-2R are observed in sepsis, septic shock and sHLH, but levels are significantly higher in patients with sHLH.

- Malignancies, infections and immunosuppression are predominant trigger factors in sHLH in critically ill patients.

- Ferritin/ALT ratio may be a useful aid in the diagnosis of sHLH to help distinguish hyperinflammation-dominated hyperferritinemia from hyperferritinemia dominated by hepatocellular injury.

- Ferritin fastest rate of increase may help identify the patients at higher risk of adverse outcome.

- Elevated AST and SOFA score are independent risk factors associated with mortality in critically ill patients in the ICU and with severe dengue.

- Hyperinflammation in critically ill, especially in intensive care, has a wide spectrum with a gradient of inflammatory responses, with HLH at the fatal extremity.

- Secondary HLH in critically ill patients in intensive care is associated with a high mortality.

- Overall, critically ill patients should be monitored for signs of hyperinflammation and rapidly evolving organ failure for prompt HLH evaluation, using currently available diagnostic tools, since appropriate HLH-directed therapy may be beneficial to improve survival.

- Moderately dosed etoposide in patients with severe or refractory MAS-HLH may improve outcome, with limited adverse events.

- Patients with severe critical illness demonstrate reduced numbers of NK cells and CTLs, and a high proportion of defective lymphocyte cytotoxic function.

- Reduced counts of NK cells and CTLs are more prominent in sHLH than in patients without HLH, which can be hypothesized to play a role in the pathogenesis of sHLH.

- Hyperferritinemia can be associated with low NK cell and CTL numbers and reduced lymphocyte cytotoxicity

- Rare variants in HLH-causing genes can be found in critically ill patients with sHLH, and may contribute to an increased susceptibility to develop HLH together with other trigger factors of HLH.

6 FUTURE PERSPECTIVES

Potentially fatal hyperinflammation, as in secondary HLH, has been associated with a number of different conditions with an inflammatory background, where an excessive immune response induces a cytokine storm and hyperinflammation. HLH-directed therapy has been shown beneficial in many such conditions. It is therefore crucial to continue increasing the awareness and recognition of hyperinflammation and sHLH in critically ill patients for appropriate and timely hyperinflammation-directed treatment to improve survival. A current example is improved survival in severe Covid-19 with hyperinflammation, not least pulmonary, with treatments including corticosteroids and cytokine inhibitors.

Importantly, not only increased awareness is necessary, but also improved tools to identify and, importantly, distinguish the patients with hyperinflammation/HLH that may benefit from anti-inflammatory therapy from those patients with similar characteristics but with a different etiopathology that require another therapeutic approach. Although some recent retrospective studies show good diagnostic performance of HLH-2004 criteria and HScore for the diagnosis of HLH in adult critically ill patients, these results should preferably be validated in a large prospective cohort, together with additional new parameters suggested in previous studies including ferritin/ALT ratio and sIL-2R[104,363]. Such studies will further improve diagnostic accuracy in the ocean of overlapping inflammatory and hyperferritinemic responses in critically ill populations, since no criterion is pathognomonic of HLH.

Inflammatory biomarkers such as IFN-γ, IL-10, IL-18, sCD163 and sIL-2R, have been implicated in the pathogenesis of sHLH and suggested as biomarkers of HLH diagnosis, also in combination with each other in specific patterns[7,379,380]. However, further studies in adults and critically ill are warranted to validate previously suggested biomarkers, but also to identify new ones, both for improved diagnostic accuracy and for better understanding of the pathobiology of sHLH. For this purpose, there is an ongoing ambitious prospective study on "Diagnostic biomarkers for adult hemophagocytic lymphohistiocytosis in critically ill patients (HEMICU)" (NCT03510650) that will hopefully bring some more answers and give a strong base for further studies. Furthermore, we need additional and more sensitive biomarkers for monitoring of HLH disease course and therapy response, as well as identifying reactivations in HLH and distinguishing it from secondary infections or other immune responses. Further studies on cytotoxic lymphocytes and their cytotoxic function in critically ill with hyperinflammation compared with other critically ill patients would also help expand our understanding of the pathogenesis of sHLH.

The introduction of cytokine-specific drugs in HLH has added effective therapeutic possibilities to the treatment scenario of sHLH in different settings, including critically ill in intensive care. Future studies on different treatment intensities and combinations in critically ill are warranted. Optimal would be a randomized study for different treatments and forms of hyperinflammation/sHLH, but due to the rarity of sHLH this would require extensive international collaboration. A prospective treatment study in severe dengue with HLH-directed

therapy, where selected patients are treated with corticosteroids and/or etoposide is currently on the way. Our positive experience in moderately dosed etoposide for the treatment of severe or refractory MAS-HLH warrants further studies in a larger cohort for validation. Several studies evaluating different treatments in sHLH, such as anti-interferon-gamma, Janus kinase inhibitors, IL-1 inhibitors and IL-6 inhibitors, are already ongoing.

There are many aspects of the vast field of hyperinflammation left to explore, and hopefully we can continue to be a part of this exciting journey.

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