Confirmed efficacy of etoposide and dexamethasone in HLH treatment: Long term results of the cooperative HLH-2004 study

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KEY POINTS

Key point 1:

Early introduction of cyclosporine did not improve HLH outcome in patients treated with the HLH-94 etoposide-dexamethasone backbone (*P*=.06)

Key point 2:

HLH-2004 may possibly be improved by risk group stratification, less therapy reduction weeks 7-8 for verified FHL patients, and earlier HSCT

ABSTRACT

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening hyperinflammatory syndrome comprising familial/genetic HLH (FHL) and secondary HLH. In the HLH-94 study, with an estimated 5-year probability of survival (pSu) of 54% (95% CI, 48-60%), systemic therapy included etoposide, dexamethasone and, from week nine, cyclosporine A (CSA). HSCT was indicated in patients with familial/genetic, relapsing, or severe and persistent disease. In HLH-2004, CSA was instead administered upfront, aiming to reduce pre-HSCT mortality and morbidity.

During 2004-2011, 369 children aged <18-years fulfilled the HLH-2004 inclusion criteria (5/8 diagnostic criteria, affected siblings, and/or molecular diagnosis in FHL-causative genes). At a median follow-up of 5.2 years, 230/369 (62%) patients were alive (5-year pSu 61%, 56-67%). The 5-year pSu in children with (n=168) and without (n=201) family history/genetically verified FHL was 59% (52-67%) and 64% (57-71%), respectively [familial occurrence (n=47): 58% (45-75%)]. Comparing with historical data (HLH-94), using HLH-94 inclusion criteria, pre-HSCT mortality was non-significantly reduced from 27% to 19% (*P*=.064 adjusted for age and gender). Time from start of therapy to HSCT was shorter compared to HLH-94 (*P*=.020 adjusted for age and gender) and reported neurological alterations at HSCT were 22% in HLH-94 and 17% in HLH-2004 (using HLH-94 inclusion criteria). Five-year pSu post-HSCT overall was 66% [verified FHL 70% (63-78%)]. Additional analyses provided specific suggestions on potential pre-HSCT treatment improvements.

HLH-2004 confirms that a majority of patients may be rescued by the etoposide/dexamethasone combination but intensification with CSA upfront, adding corticosteroids to intrathecal therapy, and reduced time to HSCT did not improve outcome significantly.

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is a devastating disturbance of the immune system leading to an uncontrolled accumulation of macrophages and lymphocytes, and hyperinflammation. Without adequate therapy it is often fatal[1]. Traditionally, HLH is divided into primary (inherited) and secondary (acquired) forms[2]. Both can be diagnosed at any age.

The most common form of primary HLH is familial (FHL). FHL is autosomal recessive, and mutations in four genes (*PRF1*, *UNC13D*, *STX11*, and *STXBP2*) are today known to cause disease; all affecting the perforin pathway of lymphocyte cytotoxicity[3-6]. Primary HLH also includes X-linked lymphoproliferative disease (XLP), Griscelli syndrome type 2 (GS2), Chediak-Higashi syndrome (CHS), and Hermansky-Pudlak syndrome (HPS). Secondary HLH (sHLH), more common in adults, is often associated with underlying conditions including severe infections, malignancies, or inflammatory disorders[2,7-9]. Both primary and secondary HLH are often triggered by infections[10].

Clinical manifestations of HLH include prolonged fever, splenomegaly, cytopenias, hypertriglyceridemia, hypofibrinogenemia, and hyperferritinemia. Hemophagocytosis is not required for diagnosis[11]. The CNS is commonly affected at onset, often leading to neurological late effects[1,12-16]. It can be difficult to distinguish between primary and secondary HLH at onset, and both often require chemotherapy and/or immunotherapy. For primary cases, hematopoietic stem cell transplantation (HSCT) is required for cure[17-19], and may also be necessary for some forms of sHLH such as chronic active EBV infection (CAEBV)[20,21].

Three decades ago, long-term survival in HLH was <5%[1]. Fortunately, thanks to collaborations worldwide and new treatment protocols including HSCT, many patients are now long-term survivors[12,22-26]. In 1989, the Histiocyte Society established an HLH registry to which 122 patients were reported with a 5-year survival of 21%[27]. In 1994, the Histiocyte Society launched the first international therapeutic study on HLH, HLH-94 (Figure 1A)[12], with >200 eligible patients recruited worldwide. It resulted in a remarkably improved outcome with a 5-year probability of survival (pSu) of 54%±6%[15,24,26,28]. Moreover, XLP, GS2 and CHS also responded well[29]. However, early mortality and late neurological effects remained problematic: 29% died before HSCT and 19% displayed late neurological sequelae[15,24,26]. A subsequent study, HLH-2004, was therefore initiated (Figure 1B). Here we present a summary of HLH-2004 with focus on survival, mortality, toxicity, and comparisons with the historical control HLH-94.

TREATMENT PROTOCOL, PATIENTS AND METHODS

The HLH-2004 treatment protocol

HLH-2004 was based on HLH-94, with eight weeks initial treatment followed by a continuation phase, both including etoposide and dexamethasone (Figure 1 A-B). The first and main aim was "to provide and evaluate a revised initial and continuation therapy, with the goal to initiate and maintain an acceptable condition in order to perform a curative SCT, for patients with familial, relapsing, or severe and persistent HLH"[11]. As compared with HLH-94, three treatment changes were made: 1) Due to high mortality weeks 1-8, treatment was intensified by administrating cyclosporine A (CSA) upfront, aimed at increasing immunosuppression without inducing additional myelotoxicity. Notably, CSA had been reported to inhibit production of interferon (IFN)-gamma[30], and to be beneficial in initial HLH treatment[31]. 2) To reduce time to HSCT, it was recommended that HSCT be performed as soon as an acceptable donor was available. 3) Given their beneficial systemic effect, corticosteroids were added to the intrathecal (IT) methotrexate (MTX) treatment recommended for a subset of patients. For non-verified FHL patients with complete disease resolution after eight weeks, it was recommended that treatment was stopped.

The choice of HSCT donor, timing, conditioning regimen, and graft-versus-host-disease (GVHD) prophylaxis were determined by the treating physician. A suggested conditioning regimen was however provided, including busulfan, cyclophosphamide, and etoposide, with CSA and methotrexate (or mycophenolate) as GVHD prophylaxis. When using an unrelated donor antithymocyte globulin (ATG) was also recommended. When the study was initiated 2004, limited data was available on reduced intensity conditioning (RIC), hence no firm suggestions on RIC were provided[32].

Study-specific clinical report forms (CRF) were completed by the treating hospitals at start of treatment and after two weeks, four weeks, two months, six months, and thereafter annually. For transplanted patients, data were reported at transplantation, 100 days post-HSCT, and then annually. Serious adverse events (SAEs) and mortality reports were sent to the principal investigator, and subsequently to the external Data Safety Monitoring Board. The study started January 1, 2004, and closed for enrollment on December 31, 2011. Cut-off for data entry was March 31, 2016.

The HLH-2004 study was approved by the Histiocyte Society and the Ethics Committee of the Karolinska Institute, and registered at clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT00426101).

Patients

In HLH-94, 5/5 criteria [fever, splenomegaly, bicytopenia (Hb <90 g/L, platelets $<100x10^9$/L, ANC $<1.0x10^9$/L), hypertriglyceridemia (fasting triglycerides ≥2.0 mmol/L) and/or

hypofibrinogenemia (fibrinogen ≤1.5 g/L), and hemophagocytosis], and/or an affected sibling (i.e. "familial patients") were required[12]. In HLH-2004 three new diagnostic criteria were implemented: ferritin ≥500 µg/L, low/absent (<10 Lytic Units) NK-cell activity, and soluble CD25 (sIL-2r) (≥2400 U/ml)[11]. HLH-2004 enrollment required: 5/8 diagnostic criteria, an affected sibling, and/or a molecular diagnosis in FHL-causative genes (*PRF1, UNC13D, STX11, STXBP2*). Inclusion age was increased from <16 to <18 years[11,12]. Inclusion criteria were: no prior cytotoxic or CSA treatment, no other underlying disease, an intention to treat according to HLH-2004, and reported follow-up. Modifications of dosages were allowed, as long as all medications were administered. Supportive therapy or anti-infectious agents, including rituximab for EBV-infection (n=14), was accepted.

Altogether 369 patients (all with active HLH) from 27 countries were eligible (Argentina, Austria, Bahrain, Belarus, Belgium, Brazil, Canada, China, Czech Republic, Denmark, Germany, Greece, Italy, Japan, Korea, Malaysia, Netherlands, Norway, Oman, Portugal, Serbia, Spain, Sweden, Switzerland, Turkey, United Kingdom, and United States of America). For the HLH-94 criteria and ferritin, information was available in 347 to 351/369 (94-95%), while information on NK-cell activity and sIL-2r was available for 240 (65%) and 223 (60%), respectively (Table 1). Hemophagocytosis was found in 287/349 (82%) with information available, and in other organs than bone marrow in only 15/287 (most commonly the spleen, n=8). In addition, information on AST and ALT values were available in 319/369 (86%) and 333/369 (90%), respectively, of which 243/319 (76%) and 186/333 (56%) were >120 U/L.

The patients (n=369) were evaluated as one single cohort but also as subgroups: patients with verified FHL-causative mutations or an affected sibling (n=168), and patients without any of these two characteristics (n=201). Separately, to compare the HLH-2004 and HLH-94 protocols, HLH-2004 patients were also divided into two "criteria subgroups", one where HLH-94 criteria were fulfilled (n=240), and one where HLH-2004 criteria were fulfilled but not HLH-94 criteria (n=129).

Furthermore, as stated in the protocol, patients with XLP, GS2, CHS, and HPS (n=29) were analyzed separately[11]. Patients with other known underlying defined diseases or immunodeficiencies, i.e. malignancies, systemic rheumatic diseases, Langerhans cell histiocytosis, Kawasaki disease, or Leishmaniasis, were excluded from the present analyses.

Statistical analyses

The Mann-Whitney U-test was used for continuous variables, and Fisher's exact test and chi-square tests for categorical variables. Probability of survival (pSu) was estimated using the Kaplan-Meier method for univariate tests, and Cox Proportional Hazards Model for multi-variate tests. Competing risk methodology was used when analyzing time to HSCT and death prior to HSCT[33]. Confounders (age and gender) were adjusted for. Two-sided 95% confidence intervals (CI) were calculated and *p*-values <0.05 were considered significant. Analyses were performed

using SPSS (version 23, IBM SPSS for Windows, IMB Corp, Chicago, IL) or R (version 3.2.3, The R foundation for statistical computing, Vienna, Austria).

RESULTS

General characteristics and outcome

Altogether 369 patients were eligible. This makes HLH-2004 the largest prospective study on HLH to date. From the first HLH registry in 1989 to the two therapeutic studies HLH-94 and HLH-2004, >700 patients have been evaluated[26,27], a remarkable amount for such a rare disorder.

In HLH-2004, 230/369 (62%) patients (median follow-up 5.2 years) were alive at last follow-up with an estimated 5-year pSu of 61% (56-67%) (Figure 2A). One aim with HLH-2004 was to induce resolution so that HSCT could be performed, and 187 patients (51%) were reported to have achieved resolution at two months (20 of whom had had a reactivation). An overview of the outcome in HLH-2004 and HLH-94 is presented in Table 2.

Primary and secondary HLH

Altogether 295 patients (80%) were evaluated for FHL-causative genes, 67 were not studied (missing data =7). Many analyses were made retrospectively, sometimes post-mortem. Of these 295, 158 displayed biallelic FHL-causative mutations while 137 did not (90 were screened for ≥3 FHL genes, 43 for 1-2 FHL genes, and four had monoallelic mutations reported).

168 (46%) patients were either familial cases or displayed biallelic gene defects in *PRF1*, *UNC13D*, *STX11*, or *STXBP2*, i.e. had verified FHL, with an overall 5-year pSu of 59% (52-67%), as compared to 64% (57-71%) in patients without verified FHL (n=201) (Figure 2B). One hundred fifty-eight of 168 patients were identified with biallelic FHL-causative mutations (5-year pSu 61%, 53-69%). 59/158 displayed *PRF1* mutations (5-year pSu 55%, 43-69%); 73/158 *UNC13D* mutations (5-year pSu 64%, 53-76%); 6/158 *STX11* mutations (5-year pSu 63%, 21-105%), and 20/158 *STXBP2* mutations (5-year pSu 64%, 45-89%). 47 patients had an affected sibling (5-year pSu 58%, 45-75%) (Figure 2D), of whom 37 (79%) had biallelic FHL-causative mutations identified.

Additionally, patients with mutations in other HLH-associated genes were studied separately. These 29 patients [XLP (n=16), GS2 (n=11), CHS (n=1), HPS (n=1)] had a 5-year pSu of 59% (43-81%).

Infections at diagnosis

137 patients (37 %) had a reported infection at diagnosis, 32 of these (23%) were patients with verified FHL. Of these, 93 either had EBV (n=74), CMV (n=14) or combined EBV/CMV (n=5) infection at onset (DNA copies were often not reported). 16/93 EBV/CMV-infected patients had verified FHL (EBV=6, CMV=8, combined EBV/CMV =2). 17/74 (23%) EBV-infected patients (five with verified FHL) underwent HSCT with nine HSCT-survivors, while 11/74 died without a HSCT (none with verified FHL) and 46/74 (62%) were alive without a HSCT.

Mortality in patients not receiving HSCT

In order to identify therapeutically meaningful information, pre-HSCT fatalities during different treatment-related time periods were thoroughly analyzed. 75/369 patients (20%) died without HSCT; 50 within the first two months of treatment (17 during the first ten days, 17 during days 11-28, and 16 during days 29-59; median age at onset four, 30, and 13 months, respectively). Another nine died days 60-119 (median age 17 month) and 16 thereafter (eight >365 days after onset) (median age at onset 17 month). Notably, only 28/75 (37%) who died pre-HSCT had verified FHL (verified biallelic mutations=23, affected sibling=5).

The 17 patients who died days 11-28 had remarkably high median age at onset and, notably, 10/17 (59%) were aged ≥1-year and without verified FHL as opposed to 3/17 (18%) who died during the first ten days (P=.032) (Figure 3). Among these ten patients, septicemia, infections, and/or bleedings (associated with thrombocytopenia) were diagnoses reported by the treating physicians in seven patients; possibly associated with treatment toxicity. Analyzing the treatment period up to six weeks, this pattern was further strengthened since 17/25 (68%) who died days 11-42 were aged ≥1-year and without verified FHL as compared to 3/17 who died during the first ten days (P=.018) (Figure 3).

In contrast, among the eight (four with verified FHL) who died days 43-59, six (75%) were reported, by the treating physicians, to suffer “refractory HLH”, “fulminant HLH”, “active HLH”, “multiorgan failure”, “progressive disease”, and “HLH reactivation”.

The information above suggest that treatment possibly should be (1) risk group stratified and (2) less aggressive days 11-42 for some patients, and (3) with less reduction of initial treatment days 43-59 for verified FHL patients.

Finally, there was an overrepresentation of deaths after the first 100 days in patients that had had resolution and reactivation, and 8/16 children who died after day 120 had verified FHL, stressing the importance of acute/subacute HSCT for these patients (Figure 3).

Serious adverse events (SAEs)

No suspected unexpected serious adverse reaction (SUSAR) was reported. In total, 89 SAE grade III or IV were reported, of which 48 reported one or more suspected causative drugs. With regard to one single suspected drug, 13 reported CSA (the most common complication being CNS affection, n=6), six reported etoposide (the hepatobiliary system being most frequently affected, n=3), and six reported dexamethasone (five with cardiac hypertension). In fifteen reports, two drugs were suspected (CSA=14, dexamethasone=14, etoposide=2), with the most common SAE being cardiac hypertension associated with dexamethasone and CSA (n=11). Eight reports suspected all three drugs to be involved, with infections (n=5) being most frequent. Posterior reversible encephalopathy syndrome (PRES) was altogether mentioned in three SAE reports (two were drug-associated, both with CSA).

In addition, as in HLH-94[26], one patient developed acute myeloid leukemia (AML). This patient received no etoposide after three weeks of therapy due to infections, and was in complete resolution at two months. Two years later the patient was diagnosed with AML, underwent HSCT but died.

HSCT outcome

188/369 patients (51%) in HLH-2004 underwent HSCT. Of these, data was available in 185 of whom 121 (65%) were alive at last follow-up (5-year pSu post-HSCT 66%, 59-73%) (Figure 4A). 135/188 (72%) had verified FHL with 5-year pSu of 70% (63-78%) (no data=2) as compared to 54% (63-78%) for patients without verified FHL (n=52) (P=0.06, log rank) (Figure 4B).

Fifteen (8%) were transplanted before two months of therapy, 60 (32%) between two and four months. Six months after starting treatment, 118 (64%) had received HSCT. The median time to HSCT was 148 days (range 25–2105).

Sixty-four patients died post-HSCT, 36 (56%) prior to day +100, 57 (89%) within the first year, and seven thereafter. According to reporting clinicians, 31 (48%) deaths were reported as transplant-related mortality (TRM), 16 (25%) as dead of disease/relapse/graft failure, in 11 (17%) the cause of death could not be specified as TRM or death of disease, one (2%) died of a surgical complication. Mortality data was missing in five cases.

Patients alive without HSCT

Both HLH-2004 and HLH-94 were designed so that patients without familial/genetic, relapsing, or severe and persistent HLH should stop treatment after two months. In HLH-2004, 88/369 (24%) patients (median age at onset 2.3 years, range 33-5675 days) stopped therapy and were off HLH treatment for >1-year and alive without HSCT, i.e. presumed sHLH. Interestingly, two of these 88 patients, both alive for ≥5-years, displayed biallelic *PRF1* p.Ala91Val mutations; a variant debated but sometimes considered a polymorphism[34]. Notably, six patients had reactivated after stopping initial therapy and restarted initial therapy before they finally became off treatment, but despite their reactivations these six were all alive without HSCT. Another patient was off HLH treatment ≥1-year but died due to other reasons (congenital heart failure). In addition, 18 were alive without HSCT with <1-year follow-up.

Comparisons between HLH-2004 and HLH-94

When comparing HLH-2004 with the historical control HLH-94 (n=232), HLH-2004 patients were divided into two groups. In one group patients fulfilled all five HLH-94 criteria (n=240) ("HLH-2004, 1994-criteria") and in the other five of eight HLH-2004 criteria but not the HLH-94 criteria (n=129) ("HLH-2004, 2004-criteria"). Patients who fulfilled the HLH-94 criteria in both studies were compared[12]. Baseline characteristics were comparable between the two studies (Table 2). Patients with an affected sibling (HLH-2004, n=47, and HLH-94, n=52) also underwent special evaluation[11,26].

Treatment intensification

For the "HLH-2004, 1994-criteria" subgroup, 151 (63%) were alive at last follow-up. This subgroup had a non-significant tendency to better survival (5-year pSu 62%, 56-68%) compared to HLH-94 (5-year pSu 56%, 50-63%) with an HLH-94 vs HLH-2004 Hazard Ratio (HR) of death of 1.23 (95% CI, 0.93-1.64) (*P*=.15) (Figure 2C). Patients included in the "HLH-2004, 2004-criteria" subgroup displayed a 61% 5-year pSu (53-70%). 5-year pSu for familial patients in HLH-2004 was 58% (45-75%) and 56% (44-71%) in HLH-94 (Figure 2D).

In the "HLH-2004, 1994-criteria" subgroup, 46 (19%) died pre-HSCT compared to 63 (27%) in HLH-94. Pre-HSCT mortality was further compared using the competing risk methodology, with death and HSCT considered competing events[33]. The HLH-94 vs. the "HLH-2004, 1994-criteria" subgroup displayed a HR of 1.45 (0.99-2.12; *P*=.055), approaching borderline statistical significance to enhanced pre-HSCT survival in HLH-2004. Adjusted for age and gender, the HR was 1.44 (0.98-2.11; *P*=.064). Among familial patients in HLH-2004, 12/47 (26%) died before HSCT, as compared to 12/52 (23%) in HLH-94.

In the "HLH-2004, 1994-criteria" subgroup, 48/240 (20%) were off treatment for >1-year and alive without HSCT (median age at onset 2.5 years), results similar to HLH-94 (48/232, 21%) (median age 2.4 years). Median age at start of therapy, as well as the proportion of patients alive without HSCT, i.e. presumed sHLH, were therefore similar in the two studies.

During the first two months, when CSA was administered in HLH-2004, using the same inclusion criteria, reactivations (15/240, 6%) and mortality 27/240 (11%) in HLH-2004 were comparable to HLH-94; 18/232 (8%) and 33/232 (14%), respectively (Table 3).

Results related to HSCT

For the "HLH-2004, 1994-criteria" subgroup, median time to HSCT was 154 days (range 25-2105 days) whereas in HLH-94 it was 180 days (range 42-1189); HR=1.25 (0.99-1.6, *P*=0.074). Adjusted for age and gender, HR was 1.34 (1.047-1.71, *P*=.020), i.e. time to HSCT was significantly reduced in HLH-2004. Among familial patients, median time to HSCT was 126 days (range 27–2105) in HLH-2004 and 165 days (range 50-1189) in HLH-94 (HR=1.09, 0.69-1.73, *P*=0.70).

In the "HLH-2004, 1994-criteria" subgroup, 135 (56%) were transplanted with a 5-year pSu post-HSCT of 67% (60-76%) (no data=2). In HLH-94, 5-year pSu post-HSCT was 66% (58-75%) (Figure 4C). For familial patients in HLH-2004, 5-year pSu post-HSCT was 79% (66–94%) as compared to 70% (57-86%) in HLH-94 (Figure 4D).

Neurological alterations

In HLH-2004, 32% displayed CNS involvement at diagnosis, and 17% had CNS complications after two months of therapy. For the "HLH-2004, 1994-criteria" subgroup, 81/240 (34%) had neurological alterations at start of therapy, 37/205 (18%) displayed CNS complications at two

months, and 22/133 (17%) at start of conditioning. In HLH-94, the corresponding numbers were 32% at onset, 13% at 2 months, and 22% at HSCT (*P*=.62, *P*=.22, and *P*=.26, respectively), i.e. the outcome of neurological alterations was not significantly different between the studies.

DISCUSSION

HLH-2004, the second international HLH-study launched by the Histiocyte Society, recruited 369 eligible patients and is, to our knowledge, the largest prospective therapeutic study on HLH to date. Combined with HLH-94 and the first international registry from the Histiocyte Society, over 700 patients have been evaluated of whom over 600 were prospectively recruited[11,26,27]. This is outstanding for such a rare disorder, making the Histiocyte Society an example of what researchers and physicians can accomplish for patients through worldwide collaboration. HLH-2004 itself displays the same international collaborative success with 27 participating countries.

Long-term survival in HLH has improved dramatically over the last decades, from a few percent in the early 1980s, with one long-term survivor with verified familial HLH first treated in 1981 still alive[1,27,35,36], to an overall 5-year pSu of 54% in HLH-94, and a 5-year pSu of 61% in HLH-2004[24,26]. The reduced pre-HSCT mortality from 27% in HLH-94 to 19% in HLH-2004 was rewarding. Furthermore, both FHL patients (with biallelic mutations in *PRF1*, *UNC13D*, *STX11*, *STXBP2*, or an affected sibling) and patients with related disorders (XLP, GS2, CHS, and HPS) displayed an overall 5-year pSu of 59%, confirming that the treatment is valuable for patients with all these genetic defects[29].

During the first two months of treatment, there was no significant reduction in mortality and reactivations in HLH-2004 as compared to HLH-94. However, there was a trend to reduced pre-HSCT mortality (P=.064 adjusted for age and gender), statistically analyzed using competing risk methodology[33]. However, a weakness of HLH-2004 was the non-randomized design, hence the historical control HLH-94 was used which obviously makes conclusions less reliable, in particular since supportive care, including anti-infectious treatment, has in all likelihood improved over time.

Altogether 121/185 (65%) patients who underwent HSCT were alive at last follow-up and the overall 5-year pSu post-HSCT was 66%, unfortunately not better than in HLH-94 (Figure 4C). However, time to HSCT (median 154 days) was significantly reduced in HLH-2004 compared to HLH-94 (P=.020, adjusted for age and gender).

CNS involvement including encephalopathy is frequent in HLH and may result in potentially severe late neurological effects[15,16]. In HLH-2004, IT corticosteroids were added to the IT MTX suggested in HLH-94 and, in addition, CSA was initiated on day 1; both were alterations that hopefully would reduce late neurological effects. In HLH-2004, 32% displayed neurological alterations at diagnosis, 17% after two months, and 19% at start of HSCT conditioning; proportions not significantly different from those in HLH-94. Notably, despite the increased focus on CNS symptoms in HLH-2004, the proportion of children with reported neurological alterations at HSCT was 22% in HLH-94 and 17% in HLH-2004 (using the HLH-94 inclusion criteria). It has been suggested that CSA may contribute to PRES in HLH[37,38], but PRES has also been associated with other drugs, including corticosteroids[39,40]. Notably, encephalopathy is

common in the natural course of HLH and CSA has been reported to be beneficial in rheuma-associated HLH/MAS[41]. In our data, we found no evidence of a marked increase of CSA-induced PRES.

Corticosteroids are important anti-inflammatory drugs for HLH, with dexamethasone having higher concentrations in the CSF and a longer half-life in the CNS than prednisone[42]. Importantly, etoposide can compensate for the cytotoxic defect in FHL and, more specifically, if lymphocytes isolated from FHL patients were subjected to etoposide *in vitro* this normalized the otherwise deficient induction of apoptosis[43]. Moreover, etoposide can substantially alleviate all symptoms of murine HLH, and the therapeutic mechanism of etoposide involves potent selective deletion of activated T-cells and efficient suppression of inflammatory cytokine production[44]. Etoposide may induce secondary AML[45], but only two of over 600 eligible patients included in the HLH-94/HLH-2004 studies were reported to develop malignancies, hence we feel secure to recommend treatment with etoposide for future HLH studies.

Importantly, based on reports by treating physicians, treatment with HLH-94/HLH-2004 may, possibly, be further improved (Figure 3). 1) Only 28/75 (37%) of the patients who died pre-HSCT had verified FHL whereas in 47/75 a verified genetic defect was not identified or not analyzed and, notably, 32 of these 47 (68%) were ≥1-year at onset of disease. Deaths in these somewhat older patients without verified FHL were significantly overrepresented during days 11-28, and many of these deaths may be associated with toxicity suggesting that reduced early cytotoxic therapy might be beneficial for these patients (such as etoposide once weekly instead of twice)[55]. 2) In contrast, many who died days 43-59 suffered active HLH, possibly suggesting a value of intensified therapy during this phase for verified FHL patients compared to the reduced treatment intensity in HLH-2004. 3) Finally, 8/16 (50%) who died >120 days after start of initial treatment had verified FHL. Many of these died from reactivation of disease, stressing the importance of an early HSCT.

The results of HLH-2004 compare well with the largest study reported on ATG-based therapy with an overall survival of 21/38 (55%) obtained in a highly experienced center[25]. Nevertheless, despite the reduced pre-HSCT mortality in HLH-2004 from 27% to 19%, there is still a need for improved pre-HSCT survival and interesting trials with alternative therapeutic approaches for HLH have been initiated including ATG in combination with etoposide (ClinicalTrials.gov Identifier: NCT01104025), alemtuzumab (NCT02472054)[46], tocilizumab (NCT02007239)[47,48], ruxolitinib (NCT02400463)[49,50], and a targeted anti-interferon gamma monoclonal antibody (NCT01818492)[51-54]. Whether these drugs can be sufficient to halt HLH alone or require the addition of etoposide remains to be elucidated, as well as how they are best used (doses, drug combinations, in adults/children, etc).

We conclude that the improvement in survival in HLH/FHL has been extraordinary over the past decades but that the reduced pre-HSCT mortality in HLH-2004 by around one third, from 27% to 19% compared to HLH-94, was not sufficient to reach statistical significance (P=.064).

Positively, time to HSCT was significantly reduced (P=.020), but not neurological alterations. There is thus no strong statistical evidence to suggest HLH-2004 instead of HLH-94. HLH-94 therefore remains the recommended standard of care. Independently, we still recommend the HLH-2004 diagnostic criteria. With regard to improved outcome of HSCT, there are already now promising results reported[32,56,57]. Finally, since there is a need for improved pre-HSCT therapy, it would be valuable to perform a randomized study comparing one of the novel therapeutic approaches with the HLH-94/HLH-2004 concept updated with the knowledge reported here.

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AUTHORSHIP CONTRIBUTION

JIH, ACH, MA, RME, AHF, GJ, SL, and KMC planned the study and recruited patients, with ACH as study coordinator and JIH as principal investigator. IA, EI, KL, MM, VN, and DR served as regional coordinators and recruited patients. SM provided epidemiologic advice. EB performed data entry and compiled data. Results were analyzed by EB, ACH, and JIH, who also wrote the manuscript which was drafted by EB, and reviewed and approved by all authors.

CONFLICT OF INTEREST DISCLOSURES

MA and JIH: Unpaid NovImmune Consultancy.

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FIGURE LEGENDS

FIGURE 1: Overview of the HLH-94 (A) and HLH-2004 (B) treatment protocols.

A. Both HLH-94 and HLH-2004 consist of an initial therapy of 8 weeks, with immunosuppressive and cytotoxic agents, and a continuation therapy thereafter, for patients with familial, relapsing, or severe and persistent, aiming at a hematopoietic stem cell transplantation (HSCT) as soon as an acceptable donor is available. In both HLH-94 and HLH-2004, daily dexamethasone (Dexa) (10 mg/m^2/day weeks 1-2, 5 mg/m^2/day weeks 3-4, 2.5 mg/m^2/day weeks 5-6, 1.25 mg/m^2/day week 7, and tapering during week 8), and etoposide (VP-16) (150 mg/m^2, twice weekly weeks 1-2, then once weekly) is administrated during the initial therapy. The continuation therapy for both HLH-2004 and HLH-94 consists of Dexa every second week (10 mg/m^2/day for 3 days), VP-16 (150 mg/m^2) every second week, and CSA (aiming at 200 µg/L trough value). For patients with progressive neurological symptoms during the first 2 weeks, or if an abnormal cerebrospinal fluid value at onset has not improved after 2 weeks, intrathecal (I.T.) treatment is recommended (up to 4 doses, weeks 3, 4, 5, 6). In the HLH-94 protocol, I.T. methotrexate (doses by age, < 1 year 6 mg, 1-2 years 8 mg, 2-3 years 10 mg, > 3 years 12 mg each dose) is recommended.

B) In HLH-2004, cyclosporine A (CSA) (aiming at 200 µg/L trough value) is administered already upfront during the initial therapy, a modification from HLH-94 where CSA is not administered until the continuation therapy. It is recommended to start CSA with 6 mg/kg daily orally (divide in 2 daily doses), if normal kidney function. Moreover, in the HLH-2004 protocol, in addition to I.T. methotrexate, I.T. prednisolone (doses by age, < 1 year 4 mg, 1-2 years 6 mg, 2-3 years 8 mg, > 3 years 10 mg each dose) is recommended. In HLH-2004, the total treatment period is reduced to 40 weeks as compared to 52 weeks in HLH-94. Figure (A) from Henter *et al*, Med Pediatr Oncol 1997[12], and (B) from Henter *et al*, Pediatric Blood Cancer 2007[11].

FIGURE 2: Overall survival in HLH-2004.

Probability of survival (pSu) for all patients and for different subgroups in the HLH-2004 study. The 5-year pSu is displayed with a 95 % confidence interval.

A. 5-year-pSu for the entire HLH-2004 cohort (n=369).
B. 5-year-pSu for patients with an affected sibling or genetically verified FHL in HLH-2004 (n=168) and for patients without verified FHL (n=201) (dashed line); (P=0.43, log rank).
C. 5 year-pSu for patients in HLH-2004 that fulfilled the HLH-94 inclusion criteria (n=240) compared with patients in HLH-94 (n=232) (dashed line); (P=0.15, log rank).
D. 5-year pSu for familial patients (affected sibling) in HLH-2004 (n=47) compared with familial patients (affected sibling) in HLH-94 (n=52) (dashed line); (P=0.86, log rank).

FIGURE 3: Analyses of patients that died without HSCT with regard to time of death after onset of therapy and type of patient.
All patients that died without having a hematopoietic stem cell transplant (HSCT) (n=75) were divided into three groups; a) verified FHL (verified biallelic mutations=23, affected sibling=5), b) without verified FHL and ≥1-year old at onset (n=32), and c) without verified FHL and <1-year old at onset (n=15).

Notably, 10/17 (59%) that died days 11-28 were aged ≥1-year and without verified FHL as opposed to 3/17 (18%) that died the first ten days (P=.032), and of these ten patients seven had findings possibly associated with toxicity related to overtreatment. Analyzing the treatment period days 11-42 this pattern was further strengthened (P=.018). In contrast, among the eight (four with verified FHL) that died days 43-60, six (75%) were reported to suffer active HLH, possible suggesting a need of less reduction of initial treatment during this period. Finally, 8/16 (50%) that died after day 120 had verified FHL, many of whom died from disease reactivation, stressing the importance of an early HSCT for this cohort.

FIGURE 4: Survival after HSCT
Survival data after hematopoietic stem cell transplant (HSCT) (n=185). The 5-year probability of survival (pSu) is displayed with a 95 % confidence interval.

A. 5-year-pSu after HSCT for the entire HLH-2004 cohort (n=185).
B. 5-year-pSu for patients with an affected sibling or genetically verified FHL in HLH-2004 (n=133) and for patients without verified FHL (n=52) (dashed line); (P=0.06, log rank).
C. 5-year pSu after HSCT for patients in HLH-2004 that fulfilled the HLH-94 inclusion criteria (n=133), compared with patients in HLH-94 (n=118) (dashed line); (P=0.77, log rank).
D. 5-year pSu after HSCT for familial patients (affected sibling) in HLH-2004 (n=33) compared with familial patients (affected sibling) in HLH-94 (n=40) (dashed line); (P=0.36, log rank).