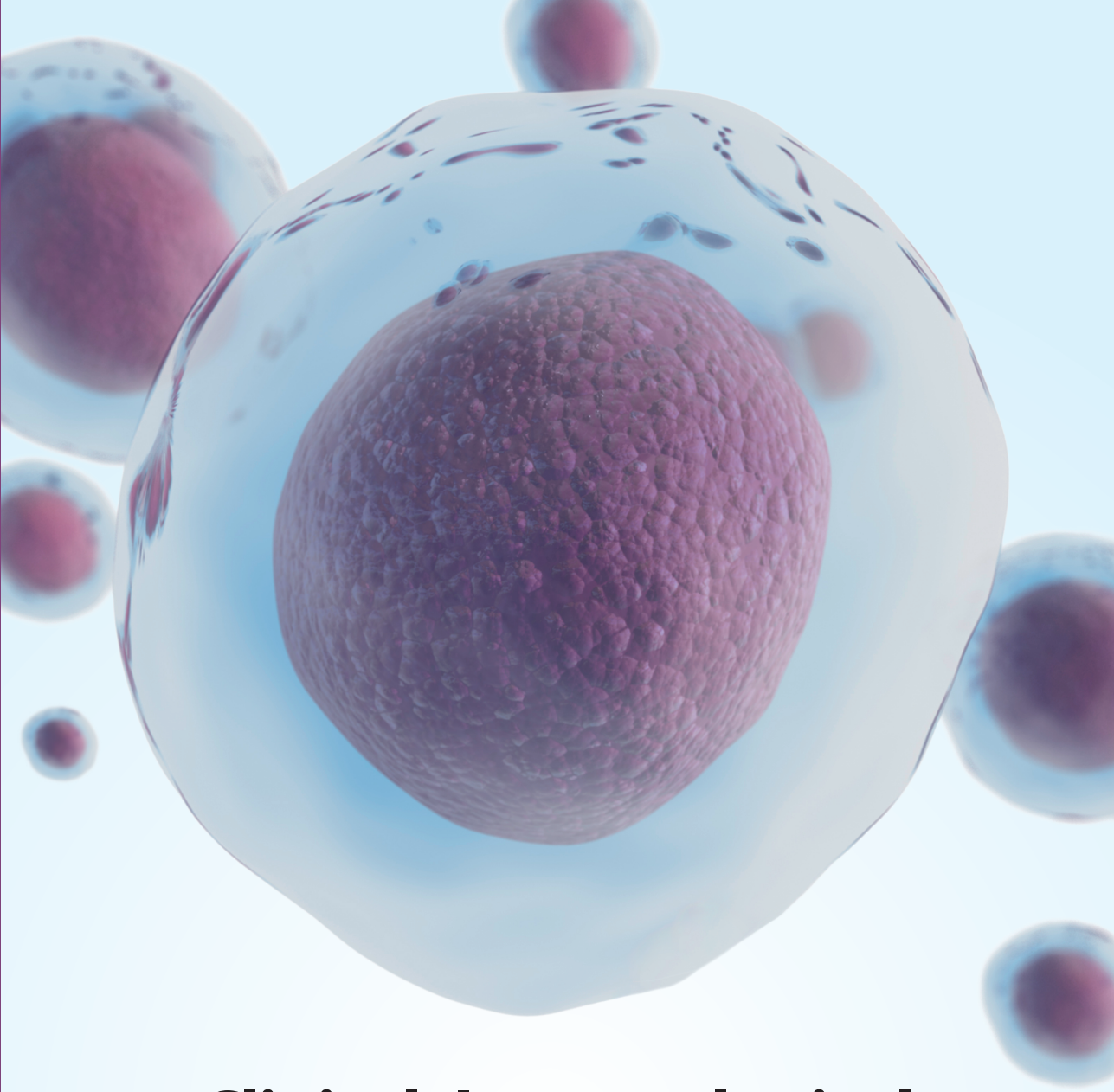

Clinical, Immunological and Genetic Features of Histiocytic Disorders

Timo Zondag

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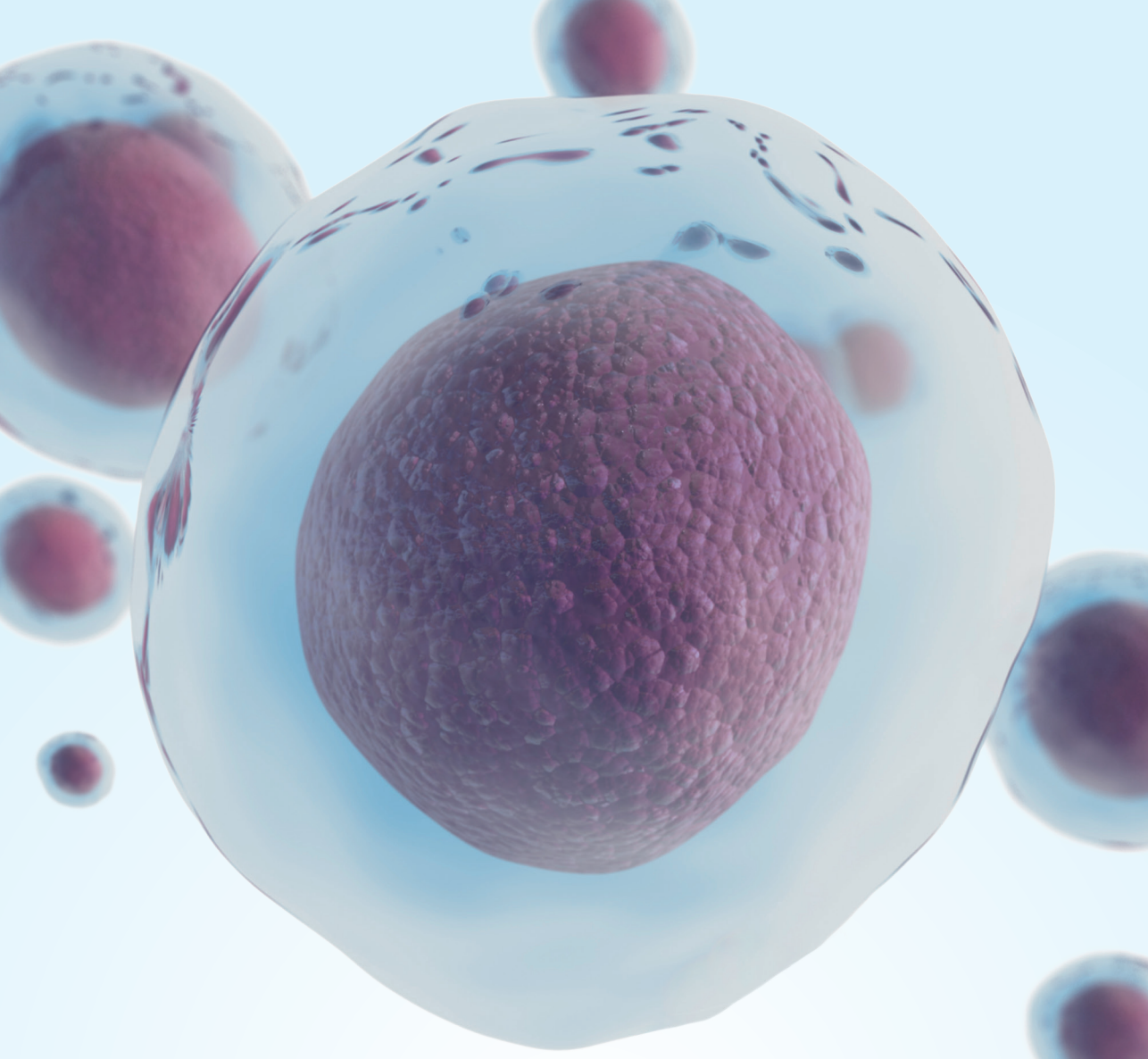
TABLE OF CONTENTS

APPENDIX



SECTION A

GENERAL INTRODUCTION

I

Histiocytic disorders

THE NORMAL HISTIOCYTE

Historically, histiocytes (histo- referring to tissue, and -cyte referring to cell) were considered uncharacterized, heterogeneous, wandering, resting cells residing in numerous tissues.[1] Currently, there is extensive knowledge about these cells. Histiocytes are tissue-resident cells belonging to the mononuclear phagocytic system with the inclusion of their precursors (monocytes). Therefore, histiocytes comprise cells from the macrophage or dendritic lineage (Figure 1). The functions of histiocytes are diverse and consist of clearance of pathogens or tissue debris, antigen presentation for lymphocyte activation, and the production of growth factors associated with tissue regeneration.[2] These functions are crucial for both the innate and adaptive immune responses.

Monocytes are circulating, bone marrow-derived leucocytes descending from the myeloid lineage. These immune cells can migrate into tissues either as part of resting-state immune surveillance or driven by acute or chronic inflammation.[3-6] Monocytes are highly adaptable and adjust to their current microenvironments.[7] They differentiate into a wide range of inflammation-promoting cells, including macrophages and Langerhans cells (LCs).[8-14] These cells undergo tissue-specific specialization and are then termed accordingly (*e.g.,* Kupffer cells in the liver and osteoclasts in the bone). Furthermore, these monocyte-decedent cells retain their flexibility, which is illustrated by their

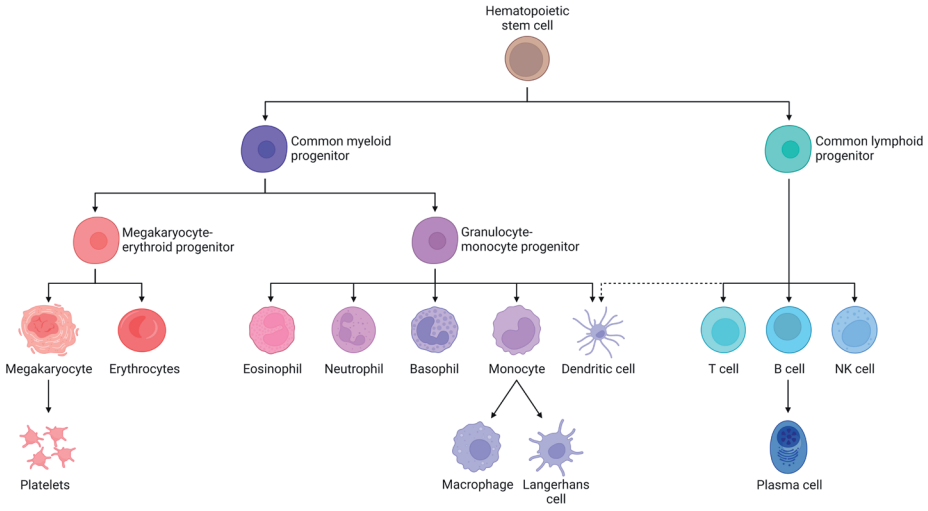


Figure 1 | Overview of hematopoiesis.
This hierarchy of hematopoiesis shows the development from a multipotent stem cell toward either a common myeloid progenitor or a common lymphoid progenitor. The differentiation of the common myeloid progenitor splits into the megakaryocyte-erythroid progenitor or granulocyte-monocyte progenitor. Further differentiation leads to unipotent and specialized cells. Both the myeloid and lymphoid lineage can generate plasmacytoid DCs. Histiocytes comprise monocytes, macrophages, LCs and DCs.

capability of trans-differentiating, for example from dendritic cells (DC) into osteoclasts or into multinucleated giant cells.[15-19]

LCs are a specialized population of antigen-presenting cells residing in epithelial tissues of the skin, gastro-intestinal tract, lungs and urogenital system. LCs are mononuclear cells containing clear, slight eosinophilic cytoplasm with dendritic processes.[20] The nucleus is somewhat folded resembling a coffee bean. Cytoplasmic Birbeck granules are typically visible in LCs with electron microscopy.[21] Birbeck granules are linear organelles with a rounded end, appearing like a tennis racket. LCs are distinctive from DCs as they originate largely from fetal liver monocytes instead of bone marrow-derived progenitors.[22] LCs are seeded in the epithelium during fetal life and are maintained locally after birth.[23] However, in case of inflammatory changes, the LCs repopulate from monocyte precursors (Figure 2).[23,24] They can also initiate an adaptive immune response.[25] Upon penetration of pathogens, the LCs recognize and process antigens, drain them to a lymph node, and present the antigens to T-lymphocytes. Cytotoxic T-cells (CTLs; $CD8^+$ T-cells) will then migrate to the site of the target cells, where they encounter antigens that are presented by human leucocyte antigen (HLA) class I. The antigen-specific T-cell receptors expressed by CTLs will recognize foreign antigens and eliminate the particular target cells. Alternative target cell killing is also possible by natural killer (NK)-cells. These cells are stimulated by the down-regulation of HLA class I

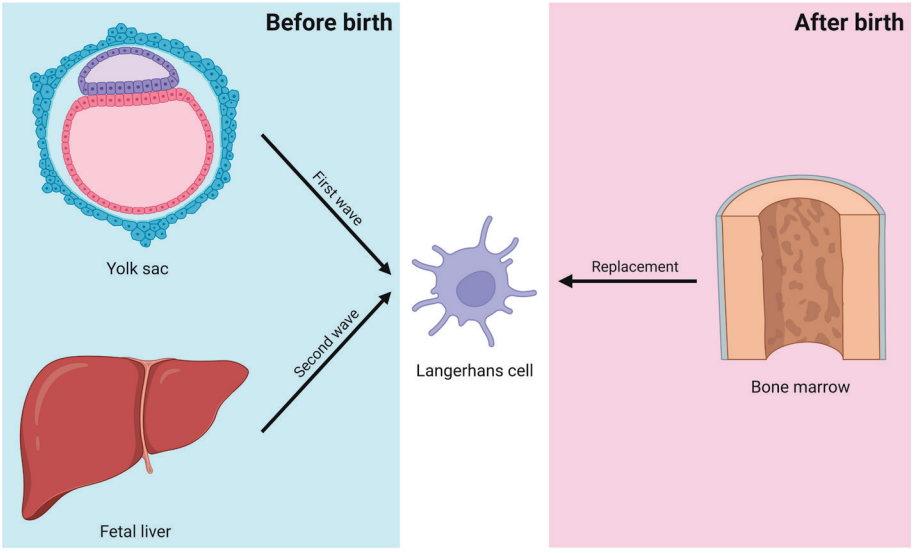


Figure 2 | Development of Langerhans cells.
Before birth, there is a first wave of LCs originating from the yolk sac. Thereafter, the fetal liver produces a second wave of LCs. These Langerhans cells get seeded in various epithelial tissue including that of the skin, lung, gastro-intestinal, and urogenital tract. These LCs are maintained locally throughout life. If necessary, for instance, due to inflammatory conditions, the LCs get replaced by Langerhans-like-cells originating from the bone marrow.

on the surface of a target cell, which is often the case in virus-infected or malignant cells.[26,27]

THE PATHOLOGIC HISTIOCYTE

Aberrantly functioning histiocytes underlie a diverse and wide range of clinical disorders termed histiocytosis. To classify these histiocytic diseases, several classification systems have been proposed. The *WHO committee on histiocytic cell proliferations* typically groups these disorders based on the underlying cell-lineage and biological behavior.[28] They distinguish the pathogenic cells as dendritic or macrophage/monocytic origin and identify non-malignant (termed "Disorders of varied biological behavior") and malignant disorders. However, more recently, Emile *et al.* proposed a revised classification, categorizing histiocytic disorders into 5 distinct groups based on clinical, radiographic, pathological, phenotypic, genetic, and molecular features (Table 1).[29] According to this classification, the L-group clusters
highly related diseases which are collectively driven by mitogen-activated protein kinase (MAPK)-pathway activating mutations and typically present with (partly) overlapping clinical and histological features. The E-group comprises HLH from diverse etiological origins.

Table 1 | Classification of histiocytic disorders according to Emile *et al.*[29]

Group	Histiocytic disorder
L-group	Langerhans Cell Histiocytosis (LCH)
	Indeterminate cell histiocytosis (ICH)
	Erdheim-Chester disease (ECD)
	Mixed LCH/ECD Extracutaneous juvenile xanthogranuloma (JXG)
C-group	Cutaneous non-LCH
	Cutaneous non-LCH with a major systemic component
R-group	Familial Rosai-Dorfman Disease (RDD)
	Sporadic Rosai-Dofman Disease (RDD)
M-group	Primary Malignant Histiocytoses
	Secondary Malignant Histiocytoses
H-group	Primary Hemophagocytic Lymphohistiocytosis (HLH)
	Secondary Hemophagocytic Lymphohistiocytosis (HLH)
	Hemophagocytic Lymphohistiocytosis (HLH) of unknown origin

II

Hemophagocytic lymphohistiocytosis

INTRODUCTION OF HLH

The first report of a series of patients who would probably be diagnosed as hemophagocytic lymphohistiocytosis (HLH) with modern diagnostics dates back to 1939.[30] In this report, Scott *et al.* described a disease characterized by fever, generalized lymphadenopathy, cytopenia and hemophagocytosis. According to modern insights, HLH is a clinical condition in which an immune response is not effectively terminated.[31,32] Consequently, a hyperactive immune state is established, which clinically resembles a systemic inflammatory response syndrome (SIRS).[33,34] Although HLH presentation can be similar to that of SIRS or sepsis, the hyperactive immune state is not a part of the physiological host response. It is rather qualified as a pathological non-termination of the immune response caused by either genetic mutations for familial HLH (FHL) or occurring secondary to specific triggers by a mechanism that is yet to be elucidated (secondary HLH; sHLH).[31,35,36]

PATHOPHYSIOLOGY OF HLH

The pathophysiologic mechanism of HLH is best illustrated for FHL, in which a genetic mutation impairs the killing of target cells by cytotoxic cells (*i.e.,* CTLs or NK-cells). Cytotoxic cells play a crucial role in host defense by eliminating aberrant cells (*e.g.,* virus-infected cells or malignant cells). Subsequent antigen clearance, together with the elimination of antigen-presenting cells (APCs), will terminate antigen presentation. This allows the immune system to return to its resting state ("steady state"). In FHL, target cell killing by cytotoxic cells is impaired or completely lost due to a genetic mutation. Following this impaired cytotoxic function, diseased cells together with the APCs (often the macrophage) cannot be eliminated. Consequently, there is persistent antigen presentation and the immune response is not terminated. This lack of negative feedback results in the production of excessive quantities of (pro-)inflammatory cytokines by the NK cells and CTLs (Figure 3). This "cytokine storm" consists of numerous cytokines including soluble interleukin-2 receptor (sIL-2R or soluble CD25), interferon gamma (IFN-γ), interleukin (IL)-6, IL-10, IL-18, IL-12, and chemokines CXC-motif ligand (CXCL)-9 and CXCL-10.[37-46] These cytokines are the drivers of further HLH-related tissue damage.[39,42] FHL is categorized into five groups, depending on the mutated gene (Figure 4). Type 1 FHL represents a group in which no causal mutation is found. In type 2 FHL, a mutation in the perforin encoding gene, *PFR1*, underlies disease.[35] Cytotoxic cells induce apoptosis in target cells by excreting granules containing granzymes and perforin.[47] Perforin forms pores on target cells through which the granzymes enter and induce apoptosis.[48] Without functional perforin, the cytotoxic cell is unable to

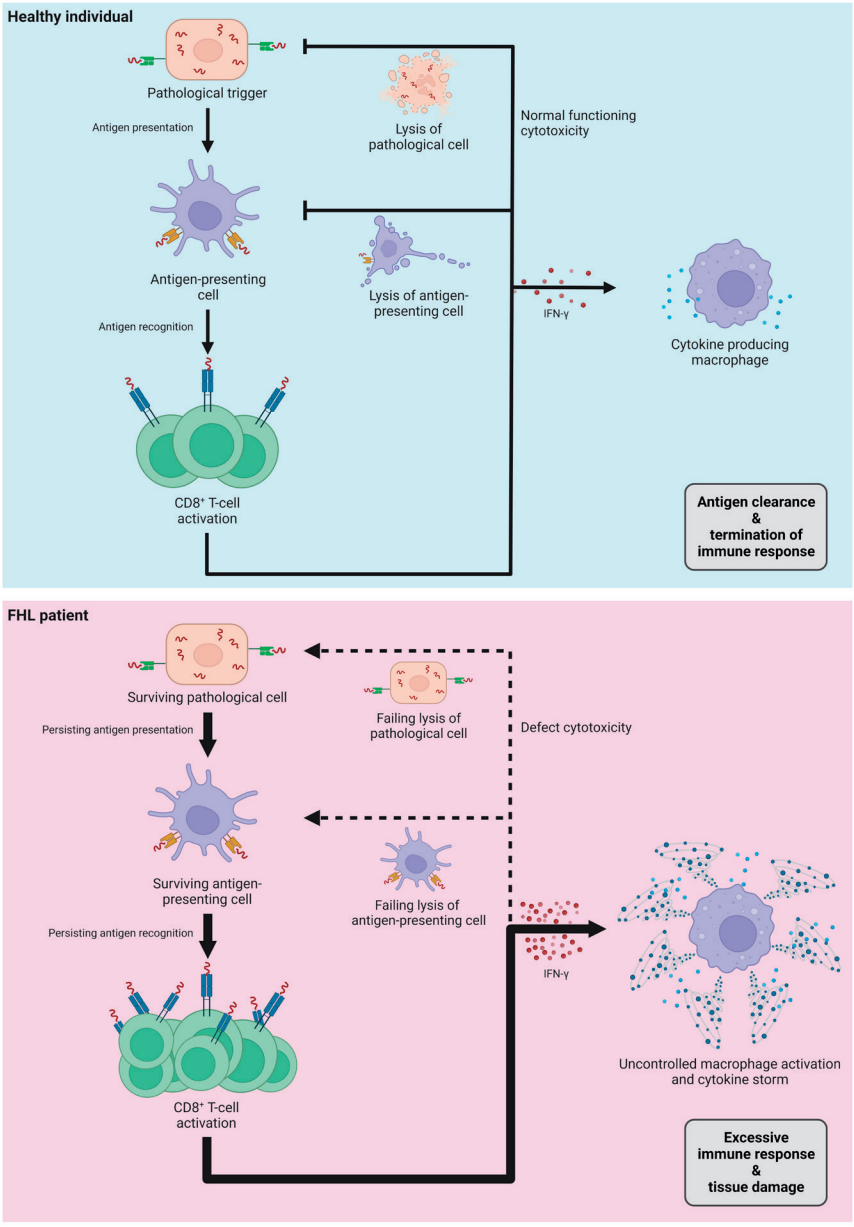


Figure 3 | The pathophysiologic hypothesis of FHL.
The physiological immune defense of a healthy individual is schematically displayed on the top. Antigen-presenting cells (APCs) pick up pathogens or malignant cell fragments in the affected tissue and subsequently activate T-cells. Activated CTLs migrate to affected tissue where they eliminate the pathological cells and clear the antigen. Furthermore, the CTLs eventually eliminate the APCs, which results in the termination of antigen presentation. The immune system will return to its resting state. The pathophysiological situation in FHL patients is displayed at the bottom. A reduced or absent cytotoxic function prohibits the CTLs to eliminate the pathological cells and APCs. The persistent antigen presentation leads to the (over)production of IFN-γ, resulting in an over-activated immune response called HLH.

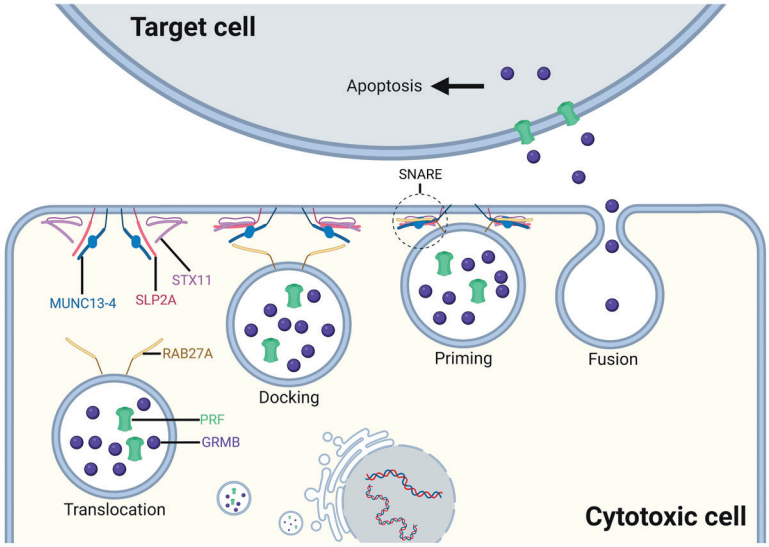


Figure 4 | The cascade of events underlying cytotoxic function.
The cytotoxic function requires accurate interaction by numerous proteins. PRF (perforin) and GZMB (granzymes B) are synthesized and encapsulated in cytotoxic granules. These granules are translocated to the cell membrane where the interaction of several proteins allow docking, priming, and fusion of the granules with the cell membrane. During fusion, the PRF and GZMB are released in the immunological synapse. The PRF will subsequently form holes in the target cell membrane through which GZMB enters and initiates apoptosis.

effectively eliminate virus- or malignantly transformed cells and APCs. FHL2 is the most prevalent form of FHL accounting for approximately 40% of all FHL cases.[49,50] Type 3 FHL is also common, accounting for approximately 30% of cases.[50] Here, a mutation in the gene encoding MUNC13-4 and *UNC13D* is pathognomonic.[51] Upon the activation of the cytotoxic cell, MUNC13-4 plays a crucial role in docking cytotoxic granules onto the cell membrane. To dock and fuse these granules with the cell membrane, the soluble N-ethyl-maleimide-sensitive-factor attachment protein receptor (SNARE) of the granule and the target membrane must interact. MUNC13-4 facilitates the formation of a SNARE complex.[36] Type 4 and 5 FHL are both infrequent and are associated with syntaxin (*STX)11* and syntaxin binding protein (*STXBP)2* mutations, respectively.[50,52,53] The proteins encoded by these genes are part of the SNARE complex and are crucial for vesicle docking. Furthermore, numerous other immunodeficiency entities which are associated with direct and indirect effects on target cell killing, show increased susceptibility to HLH.[54-57]

Other than HLH driven by a mutation, HLH can also occur secondary to an underlying trigger (sHLH). Unlike FHL, the pathophysiology of sHLH remains largely unexplained. Cytotoxic function of NK-cells and cytotoxic T-cells is normal in most patients with sHLH, implying a pathological mechanism different from that of FHL.[58] In sHLH the

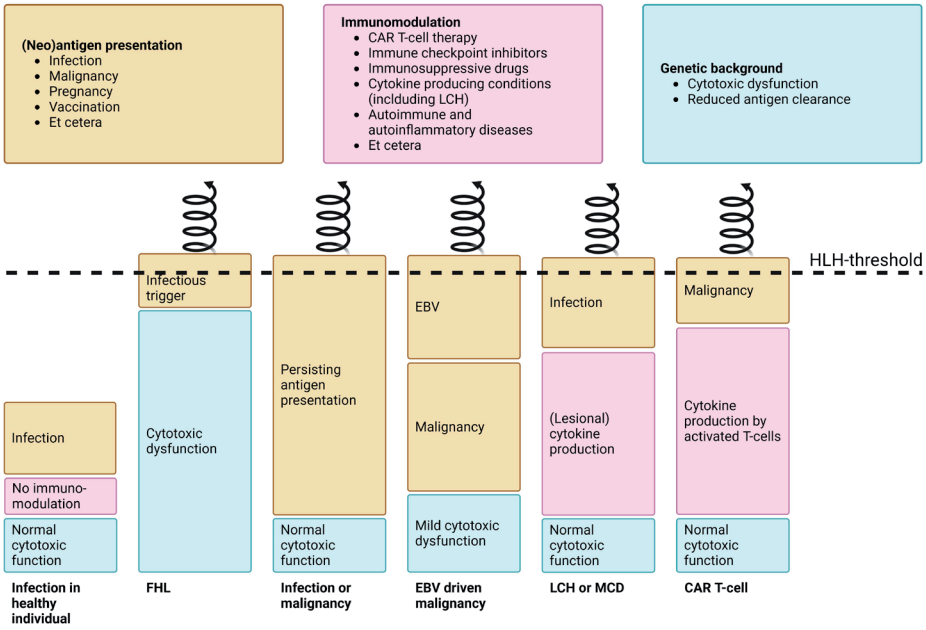


Figure 5 | Hypothetical model for the development of HLH.
HLH results from an accumulation of divers etiologic elements which build up toward an HLH-threshold, beyond which the immune response is no longer effectively regulated and hyperinflammation in the context of HLH develops.

accumulation of several elements builds up to a certain threshold, beyond which the immune response is dysregulated and leads to hyperinflammation in the context of HLH (Figure 5).[36] Background elements such as genetic landscape and chronic inflammatory disorders determine the individual's susceptibility to HLH. A subsequent acquired element, often termed the trigger, can bring the patient up to the HLH-threshold and initiate an HLH episode. Genetic background is illustrated by several studies in which an accumulation of genetic variants (mostly mono-allelic and hypomorphic) is found to underlie sHLH in murine models as well as in humans.[58-62] However, there is no conclusive evidence supporting this hypothesis. Table 2 shows triggers that are associated with HLH. Amongst these triggers, infections account for 41% of the sHLH cases and thereby represent the largest subgroup of sHLH.[63] Viruses account for 69%, bacteria 19%, parasites 5% and fungi 3%.[63] Malignancy- associated hemophagocytic syndrome (MAHS) accounts for 39% of sHLH cases.[63] With 94% caused by lymphomas, B and T cell malignancies are notorious triggers of MAHS.[63-65] Autoimmune diseases including systemic lupus erythematosus (SLE) and adult-onset Still's disease are reported to cause sHLH, which is often termed as macrophage activating syndrome (MAS)-HLH in this subgroup. MAS-HLH represents approximately 10% of all sHLH cases.[63] Idiopathic sHLH accounts for 3% of all sHLH cases.[63] In addition, rare cases like transplantations, drugs, surgery, vaccination, diabetes mellitus and pregnancy also trigger sHLH.[63]

Table 2 | Triggers associated with sHLH. Adapted from Ramos-Casals *et al.*[63]

Trigger	Subgroup	Examples
Infectious	Viruses	EBV
		CMV
		HIV
		HSV
		Hepatitis
		SARS-CoV-2
	Bacteria	Mycobacterium tuberculosis
		Escherichia coli
	Fungi	Histoplasma
	Parasites	Leishmania
		Plasmodium
		Toxoplasma
Malignant	Hematologic	Lymphoma
		Leukemia
	Solid	Melanoma
		Breast cancer
		Prostate cancer
Auto immune		Systemic lupus erythematosus
		Adult-onset Still's disease
		Rheumatoid arthritis
Other		Pregnancy
		Transplantation
		Drugs
		Immune modulatory therapies (including immune checkpoint inhibitors and chimeric antigen receptor T-cell (CART) therapy)
		Diabetes
		Surgery
		Vaccination
Idiopathic		

CLINICAL FEATURES OF HLH

HLH is a rare disease with an estimated incidence of FHL being 1 to 1.5 per 1,000,000 children per year.[66-68] The incidence in adults is unknown. The estimated annual incidence is reported to be 3.6 per million individuals for malignancy associated HLH.[69]

sHLH can present with acute or sub-acute symptoms, mostly emerging in a timeframe of 1–4 weeks.[70-72] Most substantial symptoms of HLH include fever, lymphadenopathy, and hepatosplenomegaly.[73] Further presenting features are numerous and often relate to a specific underlying trigger. Also non-specific presentations are reported, including pulmonary, gastro-intestinal, vascular, dermal and neurological.[63,73-78] Characteristic lab results in HLH cases include cytopenia, hypertriglyceridemia, hypofibrinogenemia, hyperferritinemia, high sIL-2R, impaired *ex vivo*-tested NK cell activity, hyponatremia, coagulopathy, liver dysfunction and elevated alkaline phosphatase.[73,79] Resulting from the cytokine storm, more specifically from high levels of IFN-γ, the macrophages

become hyperactive which leads to hemophagocytosis.[80-83] Despite its nomenclature, hemophagocytosis is seen in just 75–85% of all HLH cases.[63,73] Histopathological investigation frequently reveals an accumulation of macrophages and lymphocytes in the spleen, lymph nodes, liver, and bone marrow.[73,84]

Although depending on the semantics that are adhered to, FHL can be defined as a disease because a cause/etiology is apparent (*i.e.,* genetic alteration effecting cytotoxicity).[85] In the case of sHLH however, there is no definite and identifiable cause, making it a syndrome.[85] Therefore, the general term of HLH could best be seen as a descriptive clinical state with a further classification in FHL or sHLH. A diagnosis of HLH describes the hyper-inflamed clinical state in which the immune response is not adequately terminated/regulated.[31,32] Several tools have been proposed to capture this hyper inflammatory immune state in a list of criteria, and thereby set a diagnosis. The most commonly used criteria were introduced by the HLH04 study.[86] These criteria represent the inclusion criteria of an international prospective clinical trial investigating the treatment of HLH. These inclusion criteria have now been widely adopted as diagnostic criteria in the clinical setting. To fulfill an HLH diagnosis according to this protocol, five out of eight criteria outlined in Table 3 need to be met.[86] Alternatively, a genetic mutation in FHL-related genes is sufficient to fulfill FHL diagnosis. The HLH04 study primarily focused on children, often suffering from FHL. Although the sensitivity and specificity of the provided diagnostic criteria are decent for the adult HLH population, the HLH04 diagnostic criteria should be used cautiously in the case of adult patients.[87] An elevated sIL-2R and ferritin are the most valuable diagnostic parameters for adult HLH patients.[88] Especially the criterion of reduced NK-cell activity

Table 3 | Diagnostic criteria from the HLH-04 study protocol.[86]

Criteria
Fever
Splenomegaly
Cytopenias (affecting ≥2 of 3 lineages) *Hemoglobin <90 g/L* *Platelets <100x10^9/L* *Neutrophils <1.0x10^9/L*
Hypertriglyceridemia and/or hypofibrinogenemia *Fasting triglycerides ≥3.0 mmol/L* *Fibrinogen ≤1.5 g/L*
Hemophagocytosis in bone marrow or spleen or lymph nodes
Low or absent NK-cell activity
Ferritin ≥ 500µg/L
sIL-2 receptor ≥2400 U/ml

The diagnosis is established if five out of eight diagnostic criteria are fulfilled. Alternatively, a diagnose can be set if a genetic mutation is found that is consistent with HLH.

is less applicable to adults, which often concerns sHLH patients.[58] Therefore, HLH in the presence of reduced NK-cell activity should prompt genetic testing, even if an evident trigger or adult presenting age implies sHLH. Another tool designed to diagnose HLH is the HScore. This tool is developed specifically for adults and predicts the probability of sHLH.[89,90] When a HScore cutoff of >168 is applied, there is a diagnostic sensitivity of 95–100%, and specificity of 70–95%.[90,91] Ideally, the HLH04 criteria and HScore should be used in parallel to establish a clinical diagnosis.[90-92]

As soon as HLH is suspected or diagnosed, there should be a thorough workup to determine the underlying mutation or trigger.[87,92] Table 2 outlines the essential triggers of HLH which should be considered. A thorough medical history and physical examination could point toward a possible trigger.[73,79] In addition, the laboratory evaluation should include a complete blood count, C-reactive protein, erythrocyte sedimentation rate, renal function, liver enzymes, liver function, arterial blood gas, triglyceride, fibrinogen, ferritin, and sIL-2 receptor.[79,93-95] Infectious triggers should be evaluated by culturing and performing PCR-based diagnostic assays for panels covering at least Epstein–Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus (HSV) and human immunodeficiency virus (HIV) on peripheral blood.[63] In addition, a positron emission tomography–computed tomography (PET–CT) scan should be considered if a trigger is not directly evident. A biopsy is often essential to establish a diagnosis. Especially bone marrow evaluation could help demonstrate hemophagocytosis or an underlying hematologic malignancy.[95] Alternatively, biopsies elsewhere can identify a trigger such as a malignancy or infection. Moreover, biopsies can demonstrate hemophagocytosis, which is reported in numerous organs including bone marrow, lymph nodes, liver, spleen, and cerebrospinal fluid.[86,96] Genetic testing is crucial when FHL is suspected and should not be limited to FHL-related genes, but rather an extensive DNA sequencing-panel covering a broad set of primary immune deficiencies.[97] Findings that might point toward FHL include familial occurrence, reduced *ex vivo* NK- and T-cell activity, atypical presentation, and lack of an underlying trigger or persistent and recurrent disease.[58] The workup should be quick and aggressive because HLH can quickly lead to life threatening disease.[87,92] An appropriate and swift treatment is then of crucial importance.[92]

HLH TREATMENT

Numerous treatment schedules have been proposed over the years. Depending on the etiology of HLH (*i.e.,* FHL or sHLH) and the underlying trigger, treatment should be customized to each individual patient, preferably in a referral center with experience in HLH diagnosis and treatment. In general, the treatment should control the acute cytokine storm, eliminate pathogenic triggers and eradicate proliferative cells.[98]

Furthermore, maximum supportive measures should always be adequately applied. These can include renal replacement therapy, hemodynamic and respiratory support or blood product replacement.[87] For FHL, permanent cure can only be attained by allogenic stem cell transplantation (SCT).[99-101] For these patients, the HLH94 treatment protocol is widely accepted as the standard of care (Figure 6).[79,102] In this protocol, the initial therapy consists of etoposide, dexamethasone, cyclosporine A (CsA) and possible intrathecal injections with methotrexate, which all should be continued until allogenic SCT. In the more recent HLH04 protocol, CsA was introduced up front during treatment in an attempt to improve the survival in anticipation of SCT.[86] However, long term results show that the HLH04 protocol is not beneficial compared to the HLH94 protocol.[79] Lacking added value of early CsA and considering its potential side effects and toxicities, HLH94 and not HLH04 should be used as standard of care in FHL patients. Thus, CsA should not be introduced before week 9 of the treatment. Other and newer therapeutics including alemtuzumab (anti-CD52), emapalumab (anti-IFN-γ) and ruxolitinib (Janus

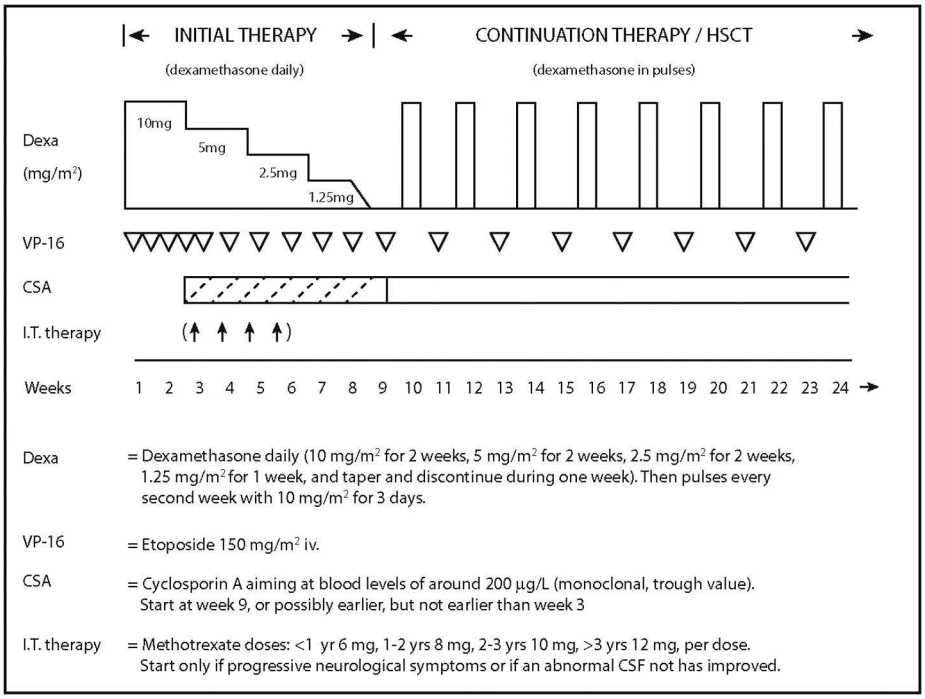


Figure 6 | HLH94 treatment protocol.
The HLH94 study was an international collaboration on the treatment of pediatric HLH. The protocol comprises an initial phase to achieve control of the hyperinflammatory state. The subsequent continuation phase is meant to bridge toward allogenic SCT or to maintain disease control. In the more recent HLH04 protocol, CsA was introduced upfront, but this did not show a beneficial effect on the outcome. Therefore, HLH94 is preferred as the standard of care. Note that the HLH94 study protocol is developed primarily for pediatric patients and has a main focus on FHL. Therefore, the protocol is not applicable to adults, in whom a different therapeutic strategy is warranted with subsequent adjustment of dosages.
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kinase or JAK-inhibitor) are currently being studied. These therapeutic substances seem promising, especially in bridging to SCT.[103-107]

A fixed protocol cannot be devised for sHLH because the disease is very heterogeneous.[92] For sHLH, elimination of the trigger often results in spontaneous regression.[108] Therefore, identifying the underlying trigger and providing adequate treatment thereof should form the basis of disease management. Although no robust prospective trials exist on treatment of sHLH, several agents are considered effective. For tempering the inadequate hyperinflammation, corticosteroids, intravenous immunoglobulin (IVIG), CsA, and immunochemotherapy such as etoposide could be considered.[92,109-111] Concerning adult HLH patients, La Rosée *et al.* provided an up-to-date and broadly accepted treatment guideline which is established by the interdisciplinary group working on adult HLH (Figure 7).[92] This guideline has recently been specified by Hines *et al.* for the critically ill pediatric and adult patients.[87] If FHL is identified in an

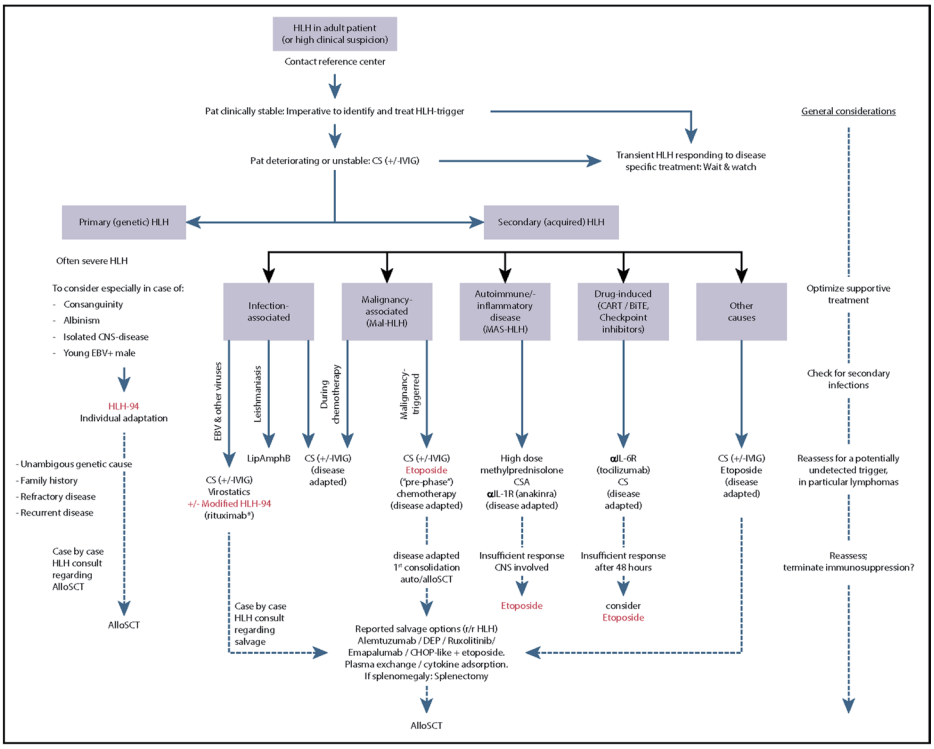

Figure 7 | Treatment guidelines for adult HLH patients.
Since the HLH94 treatment protocol is designed for the pediatric population, it could not simply be adopted for the adult population. Therefore, experts in the field have collaborated to present a joint guideline for the management of adult HLH patients. This flowchart summarizes the most important insights from this guideline. Abbreviations that are not mentioned in the main text: BiTE, bispecific T-cell engager; CART, chimeric antigen receptor T cells; CS, corticosteroids; DEP, doxorubicin, etoposide, methylprednisolone; Pat, patient. *Off-label in EBV-HLH.
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adult patient, the treatment should be guided by the HLH94 protocol, with an allogenic SCT as the mainstay of treatment.[102] In case of sHLH there is a focus on swift diagnosis and vigorous treatment of the underlying trigger. When the patient is clinically stable there is no need for additional HLH-directed therapy, the treatment can be limited to the management of the underlying trigger. However, if the patient is clinically deteriorating or unstable, additional HLH-directed therapy should be initiated. Depending on the underlying trigger, this could consist of corticosteroids, IVIG, rituximab, etoposide, CsA, tocilizumab (anti-IL-6), or anakinra (anti-IL-1). As salvage therapies, there are several regimes proposed including emapalumab (anti-IFN-γ), alemtuzumab (anti-CD52), ruxolitinib (Janus kinase inhibitor), CHOP (cyclophosphamide, hydroxyduanorubicin, vincristine and prednisone), or allogenic SCT. However, experience with these salvage therapies remains poor.[92]

Etoposide-based chemotherapy brings about its effect by inhibiting the topoisomerase II enzyme. Topoisomerase II prevents DNA breaks by releasing tension in under and over winded DNA. Etoposide causes double strand DNA breaks by inhibiting topoisomerase II resulting in apoptosis.[112] Etoposide selectively targets activated T-cells.[113] Although the underlying mechanism remains unclear, etoposide largely spares the quiescent, naive and memory T-cells. Furthermore, etoposide appears to have no direct effect on macrophages and antigen presentation by dendritic cells (DCs).[112-114] T-cells are key in initiating the pathologic immune response in HLH patients. By eliminating activated T-cells with etoposide, the dysregulated immune response and the subsequent cytokine storm can be halted. Additionally, the cytokine storm is diminished by suppressing the tumor necrosis factor (TNF)-α production and thereby suppressing macrophages and cytokine induced tissue damage.[113] In the context of EBV-related HLH, etoposide is suggested to have an additional contribution; the EBV virus is inhibited and thereby the stress of the causative trigger is reduced.[115] The potential role of etoposide in EBV-inhibition is yet to be confirmed. The dose of etoposide according to the HLH94 protocol is 150 mg/m^2 twice weekly. A reduced dose and frequency of 75 mg/m^2 etoposide once weekly is advised for the adult patients.[92]

PROGNOSIS OF HLH

If FHL patients lack appropriate treatment, the survival is approximately 2 months with only 12% surviving the first 6 months.[66,116] However, with adequate therapy, the survival has vastly increased. Following the HLH94 or HLH04 protocol which includes (bridging toward) allogenic SCT, the 5-year survival of FHL patients has increased to 50–60%.[79,117] Even higher rates have been reported with newer therapies such as alemtuzumab, which has been suggested to be effective in bridging toward allogenic SCT.[103]

In adults with sHLH, an overall survival rate of 60% is reported, with a survival rate ranging from approximately 80% in infection- and auto immune disease- associated hemophagocytic syndrome to 28% in hematological malignancies –associated HLH.[63] In contrast to FHL, which is almost always a severe and rapidly fatal disease if left untreated, the clinical course of sHLH can range from a mild to severe.[66,70,116,118]

III

Langerhans cell histiocytosis

INTRODUCTION OF LCH

In the late 1800s and early 1900s, multiple cases of children presenting with eosinophilic granulomas, bone lesions, and diabetes insipidus were reported, probably representing LCH in retrospect. The first reference hereof was in 1893 by Alfred Hand.[119] Together with few other reports it was then termed Hand–Schüller–Christian disease.[120,121] A few years later Erich Letterer and Sture Siwe reported a condition of systemic disease characterized by infiltration of mononuclear phagocytic cells in multiple organs.[122,123] When both Hand–Schüller–Christian disease and Letterer–Siwe disease were identified to concern the same entity, the disease was termed histiocytosis X, referring to the unknown etiology.[124,125] Upon discovery of Birbeck granules in lesional LCH-cells, a dysfunctional LC was assumed to underlie LCH pathogenesis. The term Langerhans cell histiocytosis (LCH) was then introduced.[126] Most recent insights show that the pathognomonic LCH-cells do not originate from the LC.[127-129] Although misleading, the term LCH remains in use.

PATHOPHYSIOLOGY OF LCH

LCH is characterized by inflammatory lesions consisting of various immune cells and often display either fibrosis or tissue destruction. These histologic lesions vary significantly in composition but are all characterized by the presence of pathognomonic LCH-cells. Microscopically, LCH-cells do not resemble classical LCs as they are large oval cells containing eosinophilic cytoplasm but lack extensions typically observed in LCs.[126,129] The nucleus has a grooved coffee bean shape with fine chromatin. LCH-cells stain positively for CD1a, CD207 (langerin, as marker of Birbeck granules), S100, CD33, CD14 (variable), and CD68 (variable) surface markers.[130,131]

Approximately 0.01%–75% of lesional cells concern LCH-cells, with a median of 8.0%.[132] The remainder of lesional cells consist of various inflammatory cells including eosinophils, macrophages, neutrophils and lymphocytes. Historically LCH was thought of as an inflammatory disorder due to its inflammatory histologic appearance and common spontaneous regression after a biopsy was taken. Moreover, increased production of cytokines and chemokines play a crucial role in the development and maintenance of an LCH-lesion.[133,134] Even though X-chromosome inactivation patterns already indicated clonality in the early days, the discovery of a causal BRAF mutation in 2010 brought conclusive evidence for a clonal, neoplastic origin.[135,136] In this study, the authors demonstrated that a *BRAF*p.V600E mutation drives the disease in half of the analyzed cases. BRAF is a protein belonging to the MAPK-pathway; it is crucial for initiation of various cell functions including proliferation and differentiation.[137] The

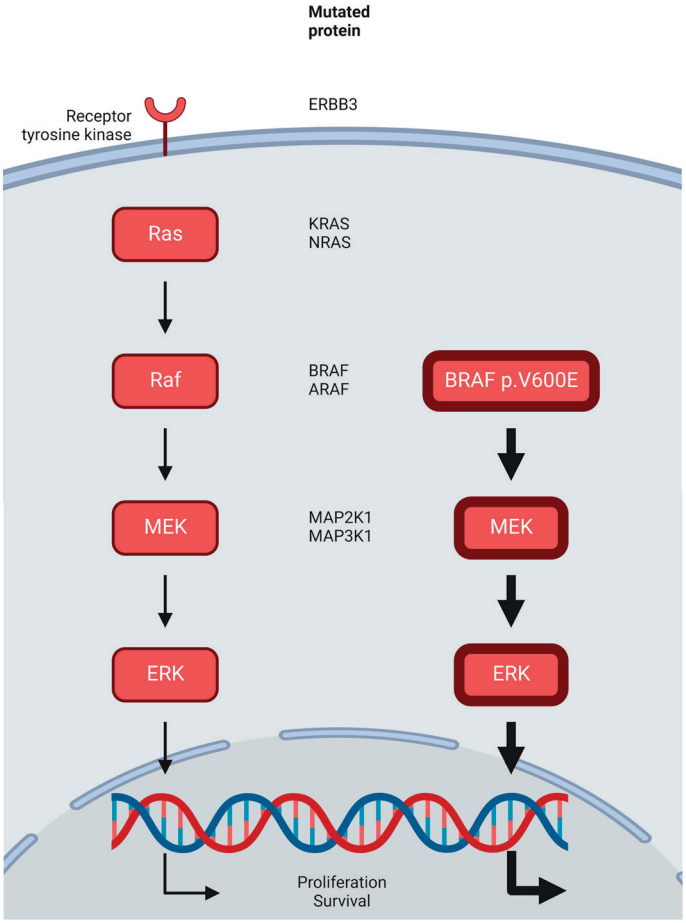


Figure 8 | Activating MAPK-pathway mutations in LCH.
When the receptor tyrosine kinase (RTK) is stimulated, it sets off a cascade resulting in MAPK-pathway activation. Activation of this pathway stimulates the proliferation and survival of the cell. An almost universal finding in LCH is a phosphorylated ERK, resembling the activated status of the MAPK-pathway. Various mutations that can cause this continuous activation are listed next to its corresponding step in the MAPK-pathway. The continued activation of BRAFp.V600E results in persistent activation of downstream proteins and eventually results in proliferation and survival.

activation of *BRAF*p.V600E mutation results in a continuously active MAPK-pathway (Figure 8). In addition, Badalian-Very *et al.* revealed the universal activation of the MAPK-pathway in LCH patients, including those without the *BRAF*p.V600E mutation.[135] This is demonstrated by the universal phosphorylation of the MEK and ERK proteins, which function downstream of the MAPK-pathway.[135,138] The exact mechanism by which this activated MAPK-pathway leads to LCH is yet to be elucidated. However, studies on LCH and melanoma patients indicate that an activated MAPK-pathway not only stimulates the proliferation and differentiation, but also facilitates the upregulation of tumor suppressor proteins such as p16 (INK4a) and senescence-associated acidic beta-

galactosidase (SA-beta-Gal).[139-141] The subsequent state of senescence supports the life span and hyperinflammatory state of the LCH-cell, as demonstrated in a transgenic mouse model wherein BRAFp.V600E is expressed by early multipotent hematopoietic stem cells.[141] It is contradictory that a *BRAF* mutation in LCH-cells induces senescence, because it is a neoplastic disorder. This might be explained by mechanisms seen in other *BRAF*-driven neoplasias, where additional down-regulation or complete loss of tumor suppressor genes outweighs the senescence effects.[142,143] Likewise, LCH-cells might also need to acquire the ability to downregulate additional tumor suppressor genes either by genetic or environmental factors.

Several processes can promote the persistence of an LCH-lesion. In a transgenic mouse model wherein BRAFp.V600E is expressed by CD11c positive myeloid cells, transgene overexpression resulted in the down-regulation of the C-C chemokine receptor 7 (CCR7).[144] This prevents the migration of tissue-resident cells to a draining lymph node and thus prevents the lesion from being eliminated by the adaptive immune system. Additionally, the B-cell lymphoma-2 like protein 1 (BCL2L1) is upregulated, resulting in reduced tendency of LCH-cell to undergo apoptosis.[144]

Although *BRAF*p.V600E is the most common mutation in LCH (accounting for 50–60% of patients), there are numerous alternative activating mutations in the MAPK-pathway (Figure 8).[135,138,145,146] Although all mutations described in literature concern somatic mutations, the role of germ line variants or genetic susceptibility in the pathogenesis could not be ruled out. This is illustrated by reports on familial clustering of LCH in both children and adults.[147-149] Furthermore, ethnic differences in incidence exist, with Hispanics representing the greatest occurrence.[150,151]

Although LCH-cells express the same markers as classical LCs, modern techniques including gene expression profiling show a distinct expression profile of LCH-cells compared to normal cutaneous LCs. [127-129] Lesional LCH-cells show distinct reciprocal expression patterns, making further clustering of LCH-cells possible.[129] LCH-cells express proliferation-related genes such as *SPP1, CCR6, MKI67,* and *CENPF*.[127,129,152] Gene expression profiles in LCH-cells are more closely related to bone marrow-derived immature myeloid dendritic cells than to the regular (dermal) LCs.[127] More recent studies confirm a different cell of origin, with the culprit lying more upstream in hematopoiesis. The cell hit by the causative somatic mutation is variable and can occur in embryonic yolk sac/liver-derived LCs or during the developmental chain of bone marrow-derived LCs.[132,153,154] During embryonic development, the LC precursors are transferred from yolk sac to the future skin in a first wave (Figure 2). Thereafter, a second wave of LC precursors, originating from fetal liver monocytes, is spread throughout the skin. This second wave occurs during late embryonic development and largely replaces the yolk sac-derived LCs.[22,155] After birth this population of LCs is maintained and, if necessary, replaced by Langerhans-like-cells derived from bone marrow precursors.[23,24,156]

It is hypothesized that the extent of LCH disease can be determined by the developmental stage of the cell in which the somatic mutation originates. If the somatic mutation strikes in the developmental chain of bone marrow-derived LCs, the disease presentation is dependent on the exact level at which this mutation occurs (Figure 9).[132,154,157-159] When a mutation arises early during hematopoietic development, this could give rise to multisystem and high-risk disease. The causal mutation will then be apparent in lesional LCH-cells, peripheral blood mononuclear cells (CD11c myeloid dendritic precursor cells and $CD14^+$ monocytes), and/or bone marrow ($CD34^+$) progenitor cells.[132] In fact, the mutation can be found in all cell types, including granulocytes or even B-cells.[160,161] Moreover, evidence obtained from autopsied brain tissue suggests that neurodegenerative LCH (ND-LCH) is caused by the migration of monocytes to the central nerve system by perivascular accumulation and then brain parenchymal infiltration.[153] In the case of a mutation occurring later in the hematopoietic maturation, single-system, low risk disease will appear. However, literature does not unambiguously support this hypothesis. This is illustrated by the conflicting finding that in low-risk, single-system disease the LCH driver mutation is occasionally found in in blood

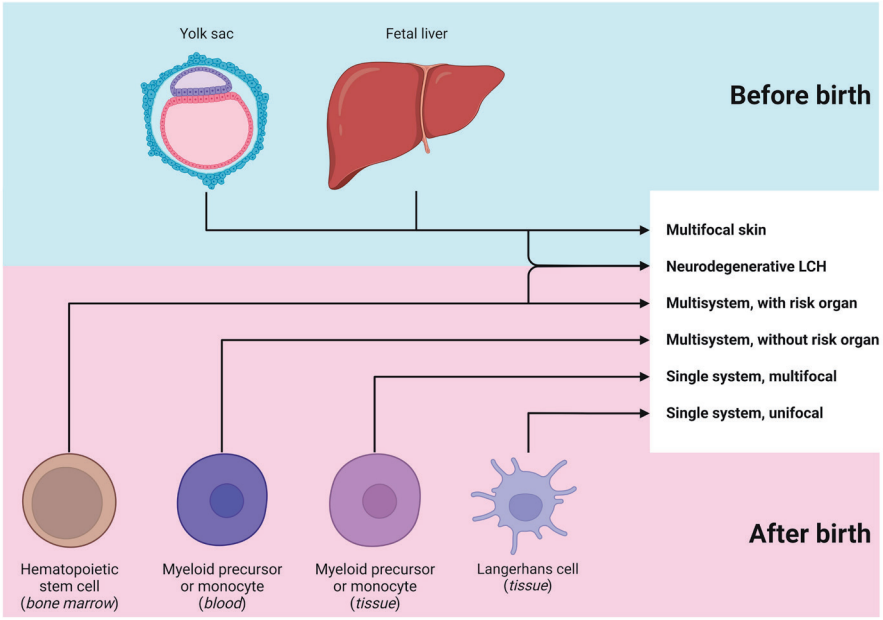


Figure 9 | Hypothesis of divers disease extents.
The developmental stage of the cell that is affected by the somatic mutation affects the extent of the LCH. LCH-cells originating from the yolk sac or fetal liver will result in congenital, self-healing, multifocal skin disease. Alternatively, if the fetal LCH-cell is seeded in the brain, neurodegenerative LCH can arise. When the driver mutation arises in the developmental chain originating from the bone marrow, the disease extent will increase as the mutation occurs earlier during development. This schematic display does not entirely explain the development of the disease extent because the LCH driver mutation is occasionally found in progenitor cells of patients with single-system LCH.

DCs, monocytes, NK-cells or even B-cells, suggesting a mutational hit earlier during hematopoiesis.[160,161] It is highly speculative that LCH-related mutations can also occur in the yolk sac or fetal liver-derived LCs. The subsequent mutated fetal erythro-myeloid progenitor (EMP) cells are seeded in various tissues including brain, skin, liver, and lungs. This might explain occasional, solitary LCH presenting in the central nervous system (CNS).[162] Mouse models indicate that somatic mutations occurring in the fetal EMP cells that are subsequently seeded in the brain, initiate a disease similar to ND-LCH.[162] Moreover, single-system cutaneous LCH that presents shortly after birth could also be attributed to fetal EMP cells. The lesions in these patients often regress spontaneously which could be explained by the mutated LCH-cells being replaced by non-mutated LCs generated by bone marrow-derived precursors not carrying an MAPK-pathway activating mutation.[154,158,163] However, evidence to support this hypothesis is still lacking.

Activating *BRAF* mutations rarely occur as germline mutations, but result in the cardio-facio-cutaneous syndrome. Although reports conflict, these patients seem to only have a slightly increased susceptibility to solid and hematologic malignancies.[164-166] In contrast to germline mutations, somatic *BRAF* mutations are common, which is demonstrated by 8% of all tumors harboring a mutation in this gene.[167,168] A wide range of solid malignancies is known to harbor a *BRAF* mutation.[167,169] Especially neurologic tumors, thyroid carcinomas, lung cancers, colon carcinomas and melanomas have a high prevalence of *BRAF* alterations.[169,170] Additionally, *BRAF* mutations are also reported in hematologic malignancies.[171,172] As LCH-cells share driver mutations with other malignancies, concurrent and clonally related malignancies could be expected.

CLINICAL FEATURES OF LCH

LCH is a heterogeneous disease with a very wide clinical spectrum.[158] Its presentation therefore varies to a great extent between patients. A diagnosis is made based on the pathological evaluation of a lesional biopsy in the context of the patient's clinics. The estimated incidence among children is 3–9 per million per year, decreasing with age.[173-176] The prevalence among adults is unknown, but a previous study reports a prevalence of multisystem disease in adults being 0.07 per million.[177] This will most certainly be an underestimation due to missed diagnoses and lack of a comprehensive (national) registry.[158,178,179] However, awareness, especially in adults is rising and consequently the incidence rate is also increasing.[149]

LCH is best categorized into single-system (SS) and multisystem disease (MS) referring to the number of organ systems involved. Single-system disease is further classified into unifocal or multifocal disease. Multisystem disease accounts for 25–40% of all pediatric LCH cases, gaining in proportion among adults.[146,149,180-182] Almost all organ

systems are reported to harbor LCH-lesions, with bone, skin, and lymph nodes being the most common.[180] Approximately 80% of patients have bone involvement, of which 50% present with unifocal single-system bone lesions.[181] In children, craniofacial lesions (bone with soft tissue extent) are prevalent.[180] These lesions are thought to correspond to higher risk of CNS involvement including diabetes insipidus.[180] Involvement of the skin is seen in approximately 40% of patients.[180] Cutaneous lesions often present as eczematous rash and/or dynamic purple papules of a few millimeters in diameter.[183,184] However, cutaneous presentation is diverse and also includes pustules, petechiae, purpura, and intertrigo with crustae.[183-185] These dermal lesions could arise throughout the whole skin but primarily appear on the trunk and scalp.[183-185]

The liver, spleen, and hematopoietic system are considered risk organs and herald a worse prognosis.[186-188] Approximately 15% of patients have one or more risk organs involved.[181] Liver involvement is defined by hepatomegaly of more than 3 cm below the costal margin and/or liver dysfunction.[189] Splenic involvement is considered when splenomegaly of more than 2 cm under the costal margin is present and hematopoietic involvement when at least one cytopenia is apparent.[189] In patients with hematopoietic involvement, LCH-cells are rarely found in bone marrow biopsies, suggesting a pathogenesis of the cytopenia that is not always related to infiltrating LCH-cells.[187,190] One could hypothesize several contributing factors to this cytopenia. Hemophagocytosis is often seen and could, therefore, directly reduce the number of hematological cells.[187] This is further strengthened by the observation that hemophagocytosis is linked to more pronounced cytopenias and worse prognosis.[187] Alternatively, a direct effect of LCH-cell infiltration or induced elimination by splenomegaly can contribute to cytopenias.[158,187] Other bone marrow involvement-associated findings include myelofibrosis, megakaryocytic dysplasia and emperipolesis.[187]

Pulmonary LCH (pLCH) is considered a special entity within LCH. In single-system pLCH, different etiologic and clinical features are present. Primarily, adults aged 20–40 years are affected by single-system pLCH, and a nearly universal correlation with smoking is reported.[191-193] Moreover, in pLCH patients, only 17% develop extrapulmonary organ involvement.[192] Although these differences indicate a different disease entity, there is pathophysiological similarity. Genetic studies of single-system pLCH reflect the genetic mutations of non-pLCH in similar frequencies.[194,195] Moreover, pLCH patients regularly present with secondary tumors, as do non-pLCH patients.[192] It is still a matter of debate if pLCH should be considered a risk organ.[149,196,197]

TREATMENT OF LCH

There is not a one-treatment-fits-all protocol, and thus treatment should be personalized to the individual patients. Treatment options are diverse and largely depend on risk stratification. Patients should be categorized into those with single-system or multisystem disease and should be evaluated for CNS risk lesions, and risk organ lesions. Therefore, PET-CT evaluation or evaluation via skeletal scintigraphy or magnetic resonance imaging (MRI) should be done after the initial LCH diagnosis in the context of dissemination workup.[198] In the case of unifocal single-system bone LCH, the lesion often regresses spontaneously after biopsy or curettage.[199,200] Therefore, a 'wait-and-see' strategy could be justified for these patients. In the case of CNS risk lesions, a more intensive therapy is required, including agents as prednisone and vinblastine.[180,201] In the case of multisystem disease, the protocol developed for the LCH-III study proves an effective strategy.[189] In this protocol vinblastine in combination with prednisone is the hallmark of the treatment. The latest and still recruiting LCH-IV study set up by the Histiocyte Society prolongs and intensifies maintenance therapy in an attempt to reduce reactivation rates.[202]

In addition to these strategies, targeted therapies are rapidly emerging. Especially in the case of *BRAF* mutations, a targeted BRAF inhibitor such as vemurafenib could benefit the treatment.[203,204] However, several downsides of the treatment make BRAF inhibitors unsuitable for first line therapy. Prolonged use of vemurafenib proves toxic with severe side effects such as secondary tumors and cardiac toxicity.[205,206] As demonstrated in melanoma patients, BRAF inhibitors can result in a paradoxical over-activation of BRAF and CRAF in non-mutated cells.[206] However, this side effect can be overcome by concurrent MEK inhibition by trametinib and thereby blocking the pathway downstream of BRAF in paradoxically over-activated non-mutant cells.[207] Until now, this phenomenon has not been reported in the context of LCH or ECD. Another challenge in the use of pharmacological BRAF inhibition is the unknown duration of treatment. Stopping BRAF inhibition led to 75% relapse in ECD patients, suggesting that the treatment should be long-term or chronic.[208] Lastly BRAF inhibitors are expensive drugs, making this treatment modality inaccessible for a large group of patients.

PROGNOSIS OF LCH

Over the last two decades, the prognosis of pediatric LCH has improved, as demonstrated by the survival rate of approximately 92% before 1997, and up to 98% thereafter.[181] This improved prognosis is especially evident in patients with risk-organ involvement, raising the overall survival from 60% to 92%.[181] The prognosis primarily depends on the risk

stratification of the disease.[149,189] For single-system LCH, especially in the case of unifocal bone disease, survival rates of close to 100% are regularly reported.[149,199,200,209] When one or more risk organs are involved, the survival rate drops to 87% following intensive chemotherapy.[189] For pLCH, a median survival of 12.5 years has been reported.[192] However, reports contradict whether or not pLCH heralds a worse prognosis and should be classified as risk organ.[149,196,197] The prognosis of adult LCH is comparable to that of children.[149] Relapse is common and occurs in approximately one third of all patients. The large majority of these relapses occur within 5 years of initial complete remission.[181] Long-term unfavorable consequences like bone abnormalities, hearing loss, growth retardation, neurologic impairments or hormonal deficiencies including diabetes insipidus are reported in 20–60% of patients. The majority of patients suffering from long-term consequences originally presented with multisystem disease.[181,210,211]

ERDHEIM–CHESTER DISEASE

Erdheim–Chester disease (ECD) is characterized by clonal and lesional accumulation of xantho(granulo)matous histiocytes.[29] ECD shares clinical, histological, and oncogenic features with LCH and is considered to be the macrophage counterpart of LCH.[159,212] The pathognomonic cells of ECD are CD68 and CD163 positive, but lack LC-cell specific markers such as CD1a, Birbeck granules, and CD207 (Langerin).[29,213] Although the phenotype and characteristics of these cells are not exclusively seen in ECD (and are identical, for example in juvenile xanthogranuloma), these cells in the context of ECD are referred to as ECD-cells throughout this thesis. Lesional ECD-cells are surrounded by reactive leucocytes and/or fibrotic tissue.[29] As in LCH, ECD pathophysiology is driven by mutations in the MAPK-pathway, with the *BRAF*p.V600E mutation being the most frequent.[212,214] Cytokine and chemokine levels are altered in ECD patients, indicating a possible pathophysiological role in the disease.[215] Not only histology, but also radiological findings are crucial for an ECD diagnosis.[213] Symmetric sclerotic lesions in the diaphysial and metaphysial compartment of the leg are almost universal features.[213] Although almost all organs can be involved, frequent localizations include the maxillary sinus, large vessels, lungs, central nerve system, skin, perirenal region, and orbitae.[216-218]

IV

Thesis outline

OUTLINE OF THIS THESIS

Although great progress has been made over the past decade regarding the pathophysiological understanding and clinical aspects of histiocytic disorders, knowledge still remains limited. The rarity of histiocytic disorders hampers extensive research. Moreover, a small number of patients and thereby, limited number of study subjects hinder adequately powered studies. Remarkably, there is a significant global interest in this field of research. Hence, numerous research groups make great efforts to advance the field. The aim of this thesis is to address the genetic alterations, (micro) environmental triggers and treatment decisions on the outcome of distinct histiocytic disorders, with a main focus on HLH and LCH.

The clinical characteristics of HLH in children are different from those in adults. Suitable diagnostic and monitoring biomarkers may therefore differ. Clinically, there is a great need for such biomarkers in adults, especially in cases where comorbidities interfere with HLH diagnostics and monitoring. Multicentric Castleman's disease (MCD) is such a condition that can hinder adequate diagnostics or monitoring of HLH. Castleman's disease (CD) is a group of heterogeneous disorders characterized by their shared histopathological feature of lymphoproliferation, but differ in their pathophysiology. CD is classified as unicentric or multicentric and presents with different histological patterns. For MCD, the two most common histologic patterns are hypervascular and plasmacellular, which are often seen simultaneously.[219] MCD presents with inflammatory features including high levels of (pro-) inflammatory cytokines. In Chapter V, we present a case of coinciding plasmacellular MCD and HLH in whom we assessed several serum biomarkers and relate this dataset to the HLH disease activity. By careful exploration of several biomarkers, we present a new potential biomarker that seems to correlate specifically to HLH disease activity.

The pathophysiologic mechanism of FHL has been uncovered to a large extent. Due to a germline mutation, one of the proteins necessary for the cytotoxic machinery of T- and NK-cells is reduced or non-functional. Not all FHL-associated mutations result in the complete absence of the corresponding protein. These patients express reduced or even adequate levels of mutated protein. In these cases, the protein has altered functionality or could even be non-functional. In Chapter VI, we report a case where the RAB27A expression is intact, whereas the corresponding genomic sequence is altered. With functional experiments, we explore the altered protein function and relate this to the patient's clinical features. We further investigate the molecular mechanism behind the altered protein function and gain more insight into protein interaction at a molecular level.

HLH is known to occur secondary to several diseases and syndromes. Clinical conditions leading to a disturbed immune response could prime patients for HLH.

Several primary immunodeficiencies (PIDs) are known to underlie HLH. One of such cases is presented in Chapter VII. Here, we discuss the rationale behind the mechanism of HLH susceptibility in activated PI3K delta syndrome (APDS). APDS is a PID, in which the *PIK3CD* gene undergoes a germ-line gain-of-function mutation, leading to PI3K/AKT/mTOR pathway over-activation. There is a subsequent disturbed immune-balance with exhausted and senescent leucocyte profiles. We not only present a rationale behind HLH susceptibility in this APDS patient, but also discuss a general mechanism of HLH susceptibility for PID patients.

The current treatment recommendations for adult HLH patients are based on observations from pediatric studies combined with alterations made by experts in the field.[87,92] Knowledge is scarce, especially regarding etoposide-based treatment. Therefore, we performed a systematic review in Chapter VIII on the results of etoposide treatment in adult HLH patients. We gathered published data and performed a meta-analysis. Here, we critically evaluate the application of etoposide usage.

MAPK-pathway-activating mutations drive LCH and ECD pathogenesis. In LCH patients there are numerous mutations reported in this pathway. The prevalence of LCH-related MAPK-mutations are rapidly being unraveled but a lot is still to be elucidated on their impact on disease presentation and outcome. Chapters IX and X explore the implications of the different genetic alterations. Chapter IX describes the various clinical implications of the different MAPK-pathway mutations in an international cohort representative of the pediatric LCH population. There is a special focus on the impact of *BRAF*p.V600E on disease extent and prognosis. New insights are gained on the mechanisms by which the *BRAF*p.V600E mutation may affect clinical presentation and outcome. The difference in disease presentation between *BRAF*p.V600E and MAP2K1 mutated patients are described. In addition, we report several new mutations in MAPK-pathway components.

LCH is not limited to the pediatric population, and we therefore investigated adult patients in Chapter X. It is currently unknown if the correlations described in children are also applicable in the adult patient population. In a multicenter study, we analyze several prognostic factors for the adult subgroup, with a main focus on genetic mutations. In this study, we also perform a systematic review of the literature focusing on genetics in adult patients. We display the frequencies and prognostic value of LCH-related mutations. In this study, we pool the data found in literature with our own cohort of adult LCH patients. In addition, Chapter X elaborates on second malignancies occurring in adult LCH patients. We show a high prevalence of second malignancies in this group and present a hypothesis to explain this remarkable observation.

Immunological anti-tumor response is important for clearing or containing neoplasia. In Chapter XI, we explored the feasibility of the presentation of BRAFp.V600E protein-derived peptides and investigated the relationship between BRAFp.V600E and

lesional composition. We studied the relative quantity of $CD8^+$ T-cells to $CD3^+$ T-cells or to LCH-cells. We related this $CD8^+$ T cell density to BRAFp.V600E status and prognosis. During intracellular protein degradation, the BRAFp.V600E protein is broken down into smaller peptide fragments, called neopeptides. For different HLA class I subtypes we explored the presentation capacity concerning these neopeptides.

Finally, a rare case of ECD is presented in Chapter XII. We show that the somatostatin receptor (SSTR) expression *in vivo* appears positive in this patient's ECD-lesions and targeted peptide receptor radionuclide therapy (PRRT) was followed by durable remission. Cells from the monocytic lineage, of which the ECD-cell is assumed to descent, are known to express SSTR. This finding provides a rationale for PRRT as new treatment modality for non-Langerhans cell histiocytosis lesions.

REFERENCES

1. Wiseman BK. THE ORIGIN OF THE WHITE BLOOD CELLS. *Journal of the American Medical Association*. 1934;103(20):1524-1529.
2. Wynn TA, Vannella KM. Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity*. 2016;44(3):450-462.
3. Liao CT, Andrews R, Wallace LE, et al. Peritoneal macrophage heterogeneity is associated with different peritoneal dialysis outcomes. *Kidney Int*. 2017;91(5):1088-1103.
4. Eguiluz-Gracia I, Bosco A, Dollner R, et al. Rapid recruitment of CD14(+) monocytes in experimentally induced allergic rhinitis in human subjects. *J Allergy Clin Immunol*. 2016;137(6):1872-1881 e1812.
5. Grimm MC, Pullman WE, Bennett GM, Sullivan PJ, Pavli P, Doe WF. Direct evidence of monocyte recruitment to inflammatory bowel disease mucosa. *J Gastroenterol Hepatol*. 1995;10(4):387-395.
6. Thurlings RM, Wijbrandts CA, Bennink RJ, et al. Monocyte scintigraphy in rheumatoid arthritis: the dynamics of monocyte migration in immune-mediated inflammatory disease. *PLoS One*. 2009;4(11):e7865.
7. Yang J, Zhang L, Yu C, Yang XF, Wang H. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomark Res*. 2014;2(1):1.
8. Coillard A, Segura E. In vivo Differentiation of Human Monocytes. *Front Immunol*. 2019;10:1907.
9. Collin M, McGovern N, Haniffa M. Human dendritic cell subsets. *Immunology*. 2013;140(1):22-30.
10. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med*. 1973;137(5):1142-1162.
11. Schwentner R, Jug G, Kauer MO, et al. JAG2 signaling induces differentiation of CD14(+) monocytes into Langerhans cell histiocytosis-like cells. *J Leukoc Biol*. 2019;105(1):101-111.
12. Bellmann L, Zelle-Rieser C, Milne P, et al. Notch-Mediated Generation of Monocyte-Derived Langerhans Cells: Phenotype and Function. *J Invest Dermatol*. 2021;141(1):84-94 e86.
13. Watcham S, Kucinski I, Gottgens B. New insights into hematopoietic differentiation landscapes from single-cell RNA sequencing. *Blood*. 2019;133(13):1415-1426.
14. Anderson DA, 3rd, Dutertre CA, Ginhoux F, Murphy KM. Genetic models of human and mouse dendritic cell development and function. *Nat Rev Immunol*. 2021;21(2):101-115.
15. Gallois A, Lachuer J, Yvert G, et al. Genome-wide expression analyses establish dendritic cells as a new osteoclast precursor able to generate bone-resorbing cells more efficiently than monocytes. *J Bone Miner Res*. 2010;25(3):661-672.
16. Coury F, Annels N, Rivollier A, et al. Langerhans cell histiocytosis reveals a new IL-17A-dependent pathway of dendritic cell fusion. *Nat Med*. 2008;14(1):81-87.
17. Grosjean F, Nasi S, Schneider P, et al. Dendritic Cells Cause Bone Lesions in a New Mouse Model of Histiocytosis. *PLoS One*. 2015;10(8):e0133917.
18. da Costa CE, Annels NE, Faaij CM, Forsyth RG, Hogendoorn PC, Egeler RM. Presence of osteoclast-like multinucleated giant cells in the bone and nonostotic lesions of Langerhans cell histiocytosis. *J Exp Med*. 2005;201(5):687-693.
19. Ahmadzadeh K, Vanoppen M, Rose CD, Matthys P, Wouters CH. Multinucleated Giant Cells: Current Insights in Phenotype, Biological Activities, and Mechanism of Formation. *Front Cell Dev Biol*. 2022;10:873226.
20. Langerhans P. Über die Nerven der menschlichen Haut. *Archiv für pathologische Anatomie und Physiologie und für klinische Medicin*. 1868;44(2):325-337.

21. Birbeck MS, Breathnach AS, Everall JD. An Electron Microscope Study of Basal Melanocytes and High-Level Clear Cells (Langerhans Cells) in Vitiligo**From the Chester Beatty Research Institute, Royal Cancer Hospital, London, S.W. 3, and the Departments of Anatomy, and Dermatology, St. Mary's Hospital Medical School (University of London) London, W. 2, England. *Journal of Investigative Dermatology*. 1961;37(1):51-64.
22. Hoeffel G, Wang Y, Greter M, et al. Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J Exp Med*. 2012;209(6):1167-1181.
23. Merad M, Manz MG, Karsunky H, et al. Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat Immunol*. 2002;3(12):1135-1141.
24. Ginhoux F, Tacke F, Angeli V, et al. Langerhans cells arise from monocytes in vivo. *Nat Immunol*. 2006;7(3):265-273.
25. Romani N, Brunner PM, Stingl G. Changing views of the role of Langerhans cells. *J Invest Dermatol*. 2012;132(3 Pt 2):872-881.
26. Boyington JC, Motyka SA, Schuck P, Brooks AG, Sun PD. Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand. *Nature*. 2000;405(6786):537-543.
27. Pende D, Biassoni R, Cantoni C, et al. The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors that is characterized by three immunoglobulin-like domains and is expressed as a 140-kD disulphide-linked dimer. *J Exp Med*. 1996;184(2):505-518.
28. Favara BE, Feller AC, Pauli M, et al. Contemporary classification of histiocytic disorders. The WHO Committee On Histiocytic/Reticulum Cell Proliferations. Reclassification Working Group of the Histiocyte Society. *Med Pediatr Oncol*. 1997;29(3):157-166.
29. Emile JF, Abla O, Fraitag S, et al. Revised classification of histiocytoses and neoplasms of the macrophage-dendritic cell lineages. *Blood*. 2016;127(22):2672-2681.
30. Bodley Scott R, Robb-Smith AHT. HISTIOCYTIC MEDULLARY RETICULOSIS. *The Lancet*. 1939;234(6047):194-198.
31. Jordan MB, Hildeman D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. *Blood*. 2004;104(3):735-743.
32. Brisse E, Wouters CH, Matthys P. Hemophagocytic lymphohistiocytosis (HLH): A heterogeneous spectrum of cytokine-driven immune disorders. *Cytokine Growth Factor Rev*. 2015;26(3):263-280.
33. Farquhar JW, Claireaux AE. Familial haemophagocytic reticulosis. *Arch Dis Child*. 1952;27(136):519-525.
34. Chakraborty RK, Burns B. Systemic Inflammatory Response Syndrome. 2022.
35. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science*. 1999;286(5446):1957-1959.
36. Brisse E, Wouters CH, Matthys P. Advances in the pathogenesis of primary and secondary haemophagocytic lymphohistiocytosis: differences and similarities. *Br J Haematol*. 2016;174(2):203-217.
37. Imashuku S, Hibi S, Sako M, et al. Soluble interleukin-2 receptor: a useful prognostic factor for patients with hemophagocytic lymphohistiocytosis. *Blood*. 1995;86(12):4706-4707.
38. Imashuku S, Hibi S, Fujiwara F, Ikushima S, Todo S. Haemophagocytic lymphohistiocytosis, interferon-gamma-naemia and Epstein-Barr virus involvement. *Br J Haematol*. 1994;88(3):656-658.

39. Osugi Y, Hara J, Tagawa S, et al. Cytokine production regulating Th1 and Th2 cytokines in hemophagocytic lymphohistiocytosis. *Blood*. 1997;89(11):4100-4103.
40. Akashi K, Hayashi S, Gondo H, et al. Involvement of interferon-gamma and macrophage colony-stimulating factor in pathogenesis of haemophagocytic lymphohistiocytosis in adults. *Br J Haematol*. 1994;87(2):243-250.
41. Takada H, Ohga S, Mizuno Y, et al. Oversecretion of IL-18 in haemophagocytic lymphohistiocytosis: a novel marker of disease activity. *Br J Haematol*. 1999;106(1):182-189.
42. Henter JI, Elinder G, Soder O, Hansson M, Andersson B, Andersson U. Hypercytokinemia in familial hemophagocytic lymphohistiocytosis. *Blood*. 1991;78(11):2918-2922.
43. Mazodier K, Marin V, Novick D, et al. Severe imbalance of IL-18/IL-18BP in patients with secondary hemophagocytic syndrome. *Blood*. 2005;106(10):3483-3489.
44. Kessel C, Fall N, Grom A, et al. Definition and validation of serum biomarkers for optimal differentiation of hyperferritinaemic cytokine storm conditions in children: a retrospective cohort study. *The Lancet Rheumatology*. 2021;3(8):e563-e573.
45. Rocco JM, Laidlaw E, Galindo F, et al. Severe mycobacterial IRIS in advanced HIV has features of hemophagocytic lymphohistiocytosis and requires prolonged immune suppression. *Clin Infect Dis*. 2022.
46. Chaturvedi V, Marsh RA, Zoref-Lorenz A, et al. T-cell activation profiles distinguish hemophagocytic lymphohistiocytosis and early sepsis. *Blood*. 2021;137(17):2337-2346.
47. Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. *Nat Rev Immunol*. 2006;6(12):940-952.
48. de Saint Basile G, Sepulveda FE, Maschalidi S, Fischer A. Cytotoxic granule secretion by lymphocytes and its link to immune homeostasis. *F1000Res*. 2015;4(F1000 Faculty Rev):930.
49. Trizzino A, zur Stadt U, Ueda I, et al. Genotype-phenotype study of familial haemophagocytic lymphohistiocytosis due to perforin mutations. *J Med Genet*. 2008;45(1):15-21.
50. Cetica V, Sieni E, Pende D, et al. Genetic predisposition to hemophagocytic lymphohistiocytosis: Report on 500 patients from the Italian registry. *J Allergy Clin Immunol*. 2016;137(1):188-196 e184.
51. Feldmann J, Callebaut I, Raposo G, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell*. 2003;115(4):461-473.
52. zur Stadt U, Schmidt S, Kasper B, et al. Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. *Hum Mol Genet*. 2005;14(6):827-834.
53. zur Stadt U, Rohr J, Seifert W, et al. Familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) is caused by mutations in Munc18-2 and impaired binding to syntaxin 11. *Am J Hum Genet*. 2009;85(4):482-492.
54. Janka GE. Familial and acquired hemophagocytic lymphohistiocytosis. *Annu Rev Med*. 2012;63:233-246.
55. Ménasché G, Pastural E, Feldmann J, et al. Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. *Nat Genet*. 2000;25(2):173-176.
56. Kalinichenko A, Perinetti Casoni G, Dupre L, et al. RhoG deficiency abrogates cytotoxicity of human lymphocytes and causes hemophagocytic lymphohistiocytosis. *Blood*. 2021;137(15):2033-2045.
57. Lam MT, Coppola S, Krumbach OHF, et al. A novel disorder involving dyshematopoiesis, inflammation, and HLH due to aberrant CDC42 function. *J Exp Med*. 2019;216(12):2778-2799.
58. Carvelli J, Piperoglou C, Farnarier C, et al. Functional and genetic testing in adults with HLH reveals an inflammatory profile rather than a cytotoxicity defect. *Blood*. 2020;136(5):542-552.

59. Zhang K, Jordan MB, Marsh RA, et al. Hypomorphic mutations in PRF1, MUNC13-4, and STXBP2 are associated with adult-onset familial HLH. *Blood*. 2011;118(22):5794-5798.
60. Voskoboinik I, Thia MC, Trapani JA. A functional analysis of the putative polymorphisms A91V and N252S and 22 missense perforin mutations associated with familial hemophagocytic lymphohistiocytosis. *Blood*. 2005;105(12):4700-4706.
61. Sepulveda FE, Garrigue A, Maschalidi S, et al. Polygenic mutations in the cytotoxicity pathway increase susceptibility to develop HLH immunopathology in mice. *Blood*. 2016;127(17):2113-2121.
62. Zhang K, Chandrakasan S, Chapman H, et al. Synergistic defects of different molecules in the cytotoxic pathway lead to clinical familial hemophagocytic lymphohistiocytosis. *Blood*. 2014;124(8):1331-1334.
63. Ramos-Casals M, Brito-Zeron P, Lopez-Guillermo A, Khamashta MA, Bosch X. Adult haemophagocytic syndrome. *Lancet*. 2014;383(9927):1503-1516.
64. Falini B, Pileri S, De Solas I, et al. Peripheral T-cell lymphoma associated with hemophagocytic syndrome. *Blood*. 1990;75(2):434-444.
65. Wilson MS, Weiss LM, Gatter KC, Mason DY, Dorfman RF, Warnke RA. Malignant histiocytosis. A reassessment of cases previously reported in 1975 based on paraffin section immunophenotyping studies. *Cancer*. 1990;66(3):530-536.
66. Henter JI, Elinder G, Soder O, Ost A. Incidence in Sweden and clinical features of familial hemophagocytic lymphohistiocytosis. *Acta Paediatr Scand*. 1991;80(4):428-435.
67. Arico M, Danesino C, Pende D, Moretta L. Pathogenesis of haemophagocytic lymphohistiocytosis. *Br J Haematol*. 2001;114(4):761-769.
68. Niece JA, Rogers ZR, Ahmad N, Langevin AM, McClain KL. Hemophagocytic lymphohistiocytosis in Texas: observations on ethnicity and race. *Pediatr Blood Cancer*. 2010;54(3):424-428.
69. Machaczka M, Vaktnas J, Klimkowska M, Hagglund H. Malignancy-associated hemophagocytic lymphohistiocytosis in adults: a retrospective population-based analysis from a single center. *Leuk Lymphoma*. 2011;52(4):613-619.
70. Takahashi N, Chubachi A, Kume M, et al. A clinical analysis of 52 adult patients with hemophagocytic syndrome: the prognostic significance of the underlying diseases. *Int J Hematol*. 2001;74(2):209-213.
71. Takagi S, Masuoka K, Uchida N, et al. High incidence of haemophagocytic syndrome following umbilical cord blood transplantation for adults. *Br J Haematol*. 2009;147(4):543-553.
72. Majluf-Cruz A, Sosa-Camas R, Perez-Ramirez O, Rosas-Cabral A, Vargas-Vorackova F, Labardini-Mendez J. Hemophagocytic syndrome associated with hematological neoplasias. *Leuk Res*. 1998;22(10):893-898.
73. Arico M, Janka G, Fischer A, et al. Hemophagocytic lymphohistiocytosis. Report of 122 children from the International Registry. FHL Study Group of the Histiocyte Society. *Leukemia*. 1996;10(2):197-203.
74. de Kerguenec C, Hillaire S, Molinie V, et al. Hepatic manifestations of hemophagocytic syndrome: a study of 30 cases. *Am J Gastroenterol*. 2001;96(3):852-857.
75. Nakatsuka A, Wada J, Nagase R, Takeda M, Yoshino T, Makino H. Hemophagocytic syndrome associated with fatal veno-occlusive disease in the liver. *Intern Med*. 2007;46(8):495-499.
76. Karras A, Thervet E, Legendre C, Groupe Cooperatif de transplantation d'Ile de F. Hemophagocytic syndrome in renal transplant recipients: report of 17 cases and review of literature. *Transplantation*. 2004;77(2):238-243.
77. Coman T, Dalloz MA, Coolen N, et al. Plasmapheresis for the treatment of acute pancreatitis induced by hemophagocytic syndrome related to hypertriglyceridemia. *J Clin Apher*. 2003;18(3):129-131.

78. Fukaya S, Yasuda S, Hashimoto T, et al. Clinical features of haemophagocytic syndrome in patients with systemic autoimmune diseases: analysis of 30 cases. *Rheumatology (Oxford)*. 2008;47(11):1686-1691.
79. Bergsten E, Horne A, Arico M, et al. Confirmed efficacy of etoposide and dexamethasone in HLH treatment: long-term results of the cooperative HLH-2004 study. *Blood*. 2017;130(25):2728-2738.
80. Pachlopnik Schmid J, Cote M, Menager MM, et al. Inherited defects in lymphocyte cytotoxic activity. *Immunol Rev*. 2010;235(1):10-23.
81. Risma K, Jordan MB. Hemophagocytic lymphohistiocytosis: updates and evolving concepts. *Curr Opin Pediatr*. 2012;24(1):9-15.
82. Egeler RM, Shapiro R, Loechelt B, Filipovich A. Characteristic immune abnormalities in hemophagocytic lymphohistiocytosis. *J Pediatr Hematol Oncol*. 1996;18(4):340-345.
83. Zoller EE, Lykens JE, Terrell CE, et al. Hemophagocytosis causes a consumptive anemia of inflammation. *J Exp Med*. 2011;208(6):1203-1214.
84. Chute DJ, Rawley J, Cox J, Bready RJ. Sudden unexpected death due to hemophagocytic lymphohistiocytosis syndrome. *J Forensic Sci*. 2013;58(4):1080-1084.
85. Pearce JM. Disease, diagnosis or syndrome? *Pract Neurol*. 2011;11(2):91-97.
86. Henter JI, Horne A, Aricó M, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer*. 2007;48(2):124-131.
87. Hines MR, von Bahr Greenwood T, Beutel G, et al. Consensus-Based Guidelines for the Recognition, Diagnosis, and Management of Hemophagocytic Lymphohistiocytosis in Critically Ill Children and Adults. *Crit Care Med*. 2022;50(5):860-872.
88. Zoref-Lorenz A, Murakami J, Hofstetter L, et al. An improved index for diagnosis and mortality prediction in malignancy-associated hemophagocytic lymphohistiocytosis. *Blood*. 2022;139(7):1098-1110.
89. Fardet L, Galicier L, Lambotte O, et al. Development and validation of the HScore, a score for the diagnosis of reactive hemophagocytic syndrome. *Arthritis Rheumatol*. 2014;66(9):2613-2620.
90. Bilston L, Croden J, Taparia M, Karkhaneh M, Grossman J, Sun HL. Validation of the HScore and the HLH-2004 diagnostic criteria for the diagnosis of hemophagocytic lymphohistiocytosis in a multicenter cohort. *Eur J Haematol*. 2022;109(2):129-137.
91. Knaak C, Nyvlt P, Schuster FS, et al. Hemophagocytic lymphohistiocytosis in critically ill patients: diagnostic reliability of HLH-2004 criteria and HScore. *Crit Care*. 2020;24(1):244.
92. La Rosee P, Horne A, Hines M, et al. Recommendations for the management of hemophagocytic lymphohistiocytosis in adults. *Blood*. 2019;133(23):2465-2477.
93. Stapp J, Wilkerson S, Stewart D, Coventry S, Mo JQ, Bove KE. Fulminant neonatal liver failure in siblings: probable congenital hemophagocytic lymphohistiocytosis. *Pediatr Dev Pathol*. 2006;9(3):239-244.
94. Palazzi DL, McClain KL, Kaplan SL. Hemophagocytic syndrome in children: an important diagnostic consideration in fever of unknown origin. *Clin Infect Dis*. 2003;36(3):306-312.
95. Riviere S, Galicier L, Coppo P, et al. Reactive hemophagocytic syndrome in adults: a retrospective analysis of 162 patients. *Am J Med*. 2014;127(11):1118-1125.
96. Majumder A, Sen D. An autopsy series of an oft-missed ante-mortem diagnosis: hemophagocytic lymphohistiocytosis. *Autops Case Rep*. 2021;11:e2021243.
97. Chinn IK, Eckstein OS, Peckham-Gregory EC, et al. Genetic and mechanistic diversity in pediatric hemophagocytic lymphohistiocytosis. *Blood*. 2018;132(1):89-100.
98. Imashuku S. Clinical features and treatment strategies of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis. *Crit Rev Oncol Hematol*. 2002;44(3):259-272.

99. Henter JI, Samuelsson-Horne A, Arico M, et al. Treatment of hemophagocytic lymphohistiocytosis with HLH-94 immunochemotherapy and bone marrow transplantation. *Blood*. 2002;100(7):2367-2373.
100. Durken M, Horstmann M, Bieling P, et al. Improved outcome in haemophagocytic lymphohistiocytosis after bone marrow transplantation from related and unrelated donors: a single-centre experience of 12 patients. *Br J Haematol*. 1999;106(4):1052-1058.
101. Blanche S, Caniglia M, Girault D, Landman J, Griscelli C, Fischer A. Treatment of hemophagocytic lymphohistiocytosis with chemotherapy and bone marrow transplantation: a single-center study of 22 cases. *Blood*. 1991;78(1):51-54.
102. Henter JI, Arico M, Egeler RM, et al. HLH-94: a treatment protocol for hemophagocytic lymphohistiocytosis. HLH study Group of the Histiocyte Society. *Med Pediatr Oncol*. 1997;28(5):342-347.
103. Moshous D, Briand C, Castelle M, et al. Alemtuzumab as First Line Treatment in Children with Familial Lymphohistiocytosis. *Blood*. 2019;134:80.
104. Vallurupalli M, Berliner N. Emapalumab for the treatment of relapsed/refractory hemophagocytic lymphohistiocytosis. *Blood*. 2019;134(21):1783-1786.
105. Marsh RA, Allen CE, McClain KL, et al. Salvage therapy of refractory hemophagocytic lymphohistiocytosis with alemtuzumab. *Pediatr Blood Cancer*. 2013;60(1):101-109.
106. Jacqmin P, Laveille C, Snoeck E, et al. Emapalumab in primary haemophagocytic lymphohistiocytosis and the pathogenic role of interferon gamma: A pharmacometric model-based approach. *Br J Clin Pharmacol*. 2022;88(5):2128-2139.
107. Keenan C, Nichols KE, Albeituni S. Use of the JAK Inhibitor Ruxolitinib in the Treatment of Hemophagocytic Lymphohistiocytosis. *Front Immunol*. 2021;12:614704.
108. Brastianos PK, Swanson JW, Torbenson M, Sperati J, Karakousis PC. Tuberculosis-associated haemophagocytic syndrome. *Lancet Infect Dis*. 2006;6(7):447-454.
109. Emmenegger U, Spaeth PJ, Neftel KA. Intravenous immunoglobulin for hemophagocytic lymphohistiocytosis? *J Clin Oncol*. 2002;20(2):599-601.
110. Emmenegger U, Frey U, Reimers A, et al. Hyperferritinemia as indicator for intravenous immunoglobulin treatment in reactive macrophage activation syndromes. *Am J Hematol*. 2001;68(1):4-10.
111. Imashuku S, Kuriyama K, Teramura T, et al. Requirement for etoposide in the treatment of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis. *J Clin Oncol*. 2001;19(10):2665-2673.
112. Hande KR. Etoposide: four decades of development of a topoisomerase II inhibitor. *Eur J Cancer*. 1998;34(10):1514-1521.
113. Johnson TS, Terrell CE, Millen SH, Katz JD, Hildeman DA, Jordan MB. Etoposide selectively ablates activated T cells to control the immunoregulatory disorder hemophagocytic lymphohistiocytosis. *J Immunol*. 2014;192(1):84-91.
114. Ferraro C, Quemeneur L, Fournel S, Prigent AF, Revillard JP, Bonnefoy-Berard N. The topoisomerase inhibitors camptothecin and etoposide induce a CD95-independent apoptosis of activated peripheral lymphocytes. *Cell Death Differ*. 2000;7(2):197-206.
115. Kikuta H, Sakiyama Y. Etoposide (VP-16) inhibits Epstein-Barr virus determined nuclear antigen (EBNA) synthesis. *Br J Haematol*. 1995;90(4):971-973.
116. Janka GE. Familial hemophagocytic lymphohistiocytosis. *Eur J Pediatr*. 1983;140(3):221-230.
117. Trottestam H, Horne A, Arico M, et al. Chemoimmunotherapy for hemophagocytic lymphohistiocytosis: long-term results of the HLH-94 treatment protocol. *Blood*. 2011;118(17):4577-4584.

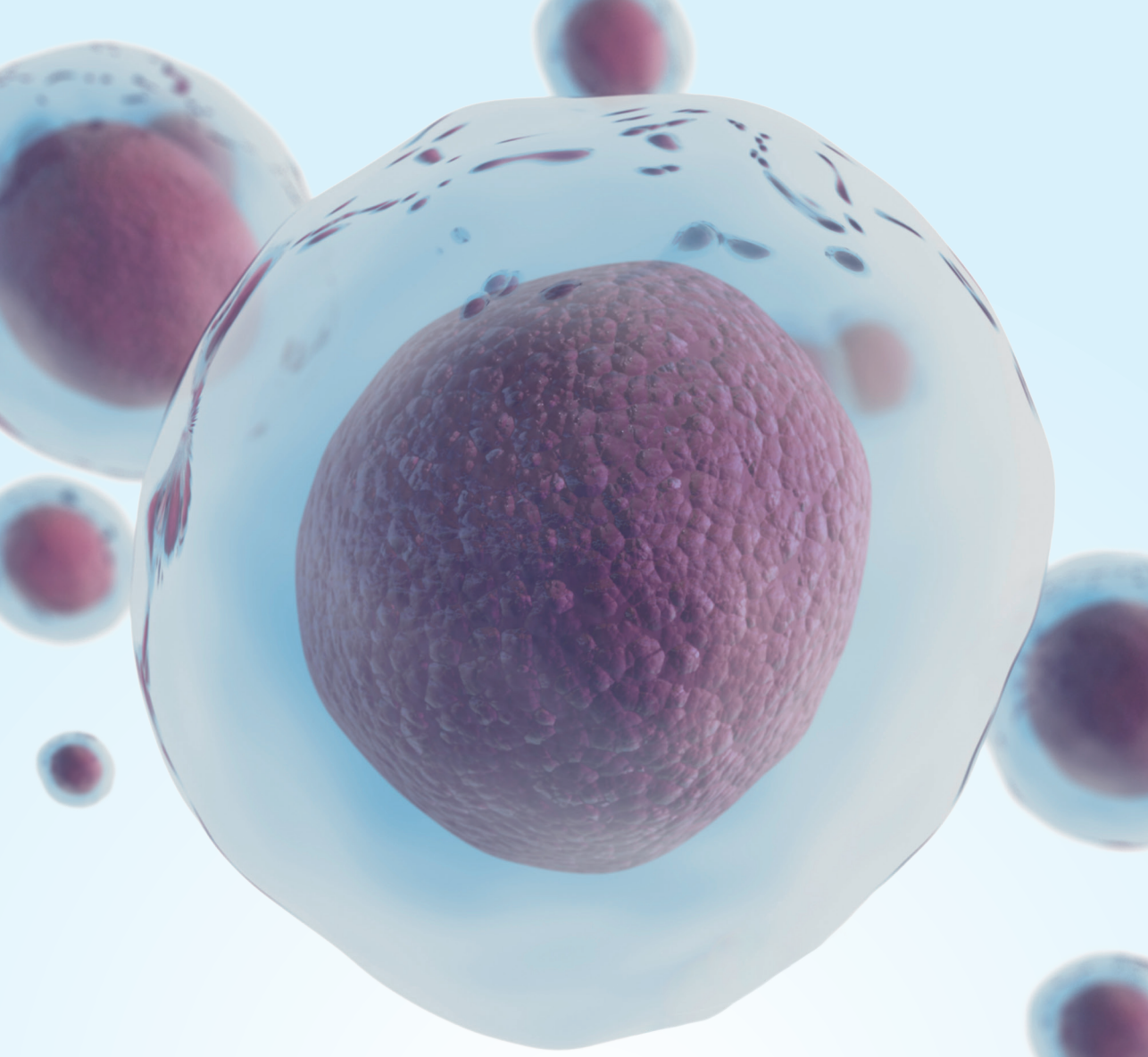
118. Shirono K, Tsuda H. Virus-associated haemophagocytic syndrome in previously healthy adults. *Eur J Haematol*. 1995;55(4):240-244.
119. Hand A. Polyuria and tuberculosis. *Arch Pediatr*. 1893;10:673-675.
120. Schüller A. Über eigenartige schädeldefekte im jugendalter. *Fortschr Röntgenstr*. 1915;23(12):1916.
121. Christian HA. Defect of membranous bones, exophthalmos and diabetes insipidus: an unusual syndrome of dyspituitarism. *Med Clin North Am*. 1920;3:849-871.
122. Letterer E. Aleukamische Retiku lose (ein Beitrag zu den proliferation Erkran kungen des Retikuloendothelial apparates). *Frankfurt Ztschr Pathol*. 1924;30:377.
123. Siwe SA. Die Reticuloendotheliose-ein neues Krankheitsbild unter den Hepatosplenomegalien. *Z Kinderheilkd*. 1933;55:21.22.
124. Farber S. The nature of" solitary granuloma" of bone. *Am J Path*. 1941;17:625-629.
125. Lichtenstein L. Histiocytosis X; integration of eosinophilic granuloma of bone, Letterer-Siwe disease, and Schuller-Christian disease as related manifestations of a single nosologic entity. *AMA Arch Pathol*. 1953;56(1):84-102.
126. Nezelof C, Basset F, Rousseau MF. Histiocytosis X histogenetic arguments for a Langerhans cell origin. *Biomedicine*. 1973;18(5):365-371.
127. Allen CE, Li L, Peters TL, et al. Cell-specific gene expression in Langerhans cell histiocytosis lesions reveals a distinct profile compared with epidermal Langerhans cells. *J Immunol*. 2010;184(8):4557-4567.
128. Hutter C, Kauer M, Simonitsch-Klupp I, et al. Notch is active in Langerhans cell histiocytosis and confers pathognomonic features on dendritic cells. *Blood*. 2012;120(26):5199-5208.
129. Shi H, He H, Cui L, et al. Transcriptomic landscape of circulating mononuclear phagocytes in Langerhans cell histiocytosis at the single-cell level. *Blood*. 2021;138(14):1237-1248.
130. Valladeau J, Ravel O, Dezutter-Dambuyant C, et al. Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity*. 2000;12(1):71-81.
131. Goyal G, Heaney ML, Collin M, et al. Erdheim-Chester disease: consensus recommendations for evaluation, diagnosis, and treatment in the molecular era. *Blood*. 2020;135(22):1929-1945.
132. Berres ML, Lim KP, Peters T, et al. BRAF-V600E expression in precursor versus differentiated dendritic cells defines clinically distinct LCH risk groups. *J Exp Med*. 2014;211(4):669-683.
133. Laman JD, Leenen PJ, Annels NE, Hogendoorn PC, Egeler RM. Langerhans-cell histiocytosis 'insight into DC biology'. *Trends Immunol*. 2003;24(4):190-196.
134. Egeler RM, Favara BE, van Meurs M, Laman JD, Claassen E. Differential In situ cytokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expression of cytokines relevant to disease and treatment. *Blood*. 1999;94(12):4195-4201.
135. Badalian-Very G, Vergilio JA, Degar BA, et al. Recurrent BRAF mutations in Langerhans cell histiocytosis. *Blood*. 2010;116(11):1919-1923.
136. Yu RC, Chu C, Buluwela L, Chu AC. Clonal proliferation of Langerhans cells in Langerhans cell histiocytosis. *Lancet*. 1994;343(8900):767-768.
137. Liu D, Liu Z, Condouris S, Xing M. BRAF V600E maintains proliferation, transformation, and tumorigenicity of BRAF-mutant papillary thyroid cancer cells. *J Clin Endocrinol Metab*. 2007;92(6):2264-2271.
138. Chakraborty R, Hampton OA, Shen X, et al. Mutually exclusive recurrent somatic mutations in MAP2K1 and BRAF support a central role for ERK activation in LCH pathogenesis. *Blood*. 2014;124(19):3007-3015.

139. Cangi MG, Biavasco R, Cavalli G, et al. BRAFV600E-mutation is invariably present and associated to oncogene-induced senescence in Erdheim-Chester disease. *Ann Rheum Dis*. 2015;74(8):1596-1602.
140. Michaloglou C, Vredeveld LC, Soengas MS, et al. BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature*. 2005;436(7051):720-724.
141. Bigenwald C, Le Berichel J, Wilk CM, et al. BRAF(V600E)-induced senescence drives Langerhans cell histiocytosis pathophysiology. *Nat Med*. 2021;27(5):851-861.
142. Patel PL, Suram A, Mirani N, Bischof O, Herbig U. Derepression of hTERT gene expression promotes escape from oncogene-induced cellular senescence. *Proc Natl Acad Sci U S A*. 2016;113(34):E5024-5033.
143. Damsky WE, Bosenberg M. Melanocytic nevi and melanoma: unraveling a complex relationship. *Oncogene*. 2017;36(42):5771-5792.
144. Hogstad B, Berres ML, Chakraborty R, et al. RAF/MEK/extracellular signal-related kinase pathway suppresses dendritic cell migration and traps dendritic cells in Langerhans cell histiocytosis lesions. *J Exp Med*. 2018;215(1):319-336.
145. Berres ML, Allen CE, Merad M. Pathological consequence of misguided dendritic cell differentiation in histiocytic diseases. *Adv Immunol*. 2013;120:127-161.
146. Heritier S, Emile JF, Barkaoui MA, et al. BRAF Mutation Correlates With High-Risk Langerhans Cell Histiocytosis and Increased Resistance to First-Line Therapy. *J Clin Oncol*. 2016;34(25):3023-3030.
147. Arico M, Nichols K, Whitlock JA, et al. Familial clustering of Langerhans cell histiocytosis. *Br J Haematol*. 1999;107(4):883-888.
148. Shahla A, Parvaneh V, Hossein HD. Langerhans cells histiocytosis in one family. *Pediatr Hematol Oncol*. 2004;21(4):313-320.
149. Arico M, Girschikofsky M, Genereau T, et al. Langerhans cell histiocytosis in adults. Report from the International Registry of the Histiocyte Society. *Eur J Cancer*. 2003;39(16):2341-2348.
150. Ribeiro KB, Degar B, Antoneli CB, Rollins B, Rodriguez-Galindo C. Ethnicity, race, and socioeconomic status influence incidence of Langerhans cell histiocytosis. *Pediatr Blood Cancer*. 2015;62(6):982-987.
151. Peckham-Gregory EC, McClain KL, Allen CE, Scheurer ME, Lupo PJ. The role of parental and perinatal characteristics on Langerhans cell histiocytosis: characterizing increased risk among Hispanics. *Ann Epidemiol*. 2018;28(8):521-528.
152. Fleming MD, Pinkus JL, Fournier MV, et al. Coincident expression of the chemokine receptors CCR6 and CCR7 by pathologic Langerhans cells in Langerhans cell histiocytosis. *Blood*. 2003;101(7):2473-2475.
153. McClain KL, Picarsic J, Chakraborty R, et al. CNS Langerhans cell histiocytosis: Common hematopoietic origin for LCH-associated neurodegeneration and mass lesions. *Cancer*. 2018;124(12):2607-2620.
154. Allen CE, Merad M, McClain KL. Langerhans-Cell Histiocytosis. *N Engl J Med*. 2018;379(9):856-868.
155. Gomez Perdiguero E, Klapproth K, Schulz C, et al. Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature*. 2015;518(7540):547-551.
156. Liu Z, Gu Y, Chakarov S, et al. Fate Mapping via Ms4a3-Expression History Traces Monocyte-Derived Cells. *Cell*. 2019;178(6):1509-1525 e1519.
157. Durham BH, Roos-Weil D, Baillou C, et al. Functional evidence for derivation of systemic histiocytic neoplasms from hematopoietic stem/progenitor cells. *Blood*. 2017;130(2):176-180.
158. Rodriguez-Galindo C, Allen CE. Langerhans cell histiocytosis. *Blood*. 2020;135(16):1319-1331.
159. Milne P, Bigley V, Bacon CM, et al. Hematopoietic origin of Langerhans cell histiocytosis and Erdheim-Chester disease in adults. *Blood*. 2017;130(2):167-175.

160. Xiao Y, van Halteren AGS, Lei X, et al. Bone marrow-derived myeloid progenitors as driver mutation carriers in high- and low-risk Langerhans cell histiocytosis. *Blood*. 2020;136(19):2188-2199.
161. Eder SK, Schwentner R, Ben Soussia P, et al. Vemurafenib acts as a molecular on-off switch governing systemic inflammation in Langerhans cell histiocytosis. *Blood Adv*. 2022;6(3):970-975.
162. Mass E, Jacome-Galarza CE, Blank T, et al. A somatic mutation in erythro-myeloid progenitors causes neurodegenerative disease. *Nature*. 2017;549(7672):389-393.
163. Simko SJ, Garmezy B, Abhyankar H, et al. Differentiating skin-limited and multisystem Langerhans cell histiocytosis. *J Pediatr*. 2014;165(5):990-996.
164. Kratz CP, Rapisuwon S, Reed H, Hasle H, Rosenberg PS. Cancer in Noonan, Costello, cardiofaciocutaneous and LEOPARD syndromes. *Am J Med Genet C Semin Med Genet*. 2011;157C(2):83-89.
165. Kratz CP, Franke L, Peters H, et al. Cancer spectrum and frequency among children with Noonan, Costello, and cardio-facio-cutaneous syndromes. *Br J Cancer*. 2015;112(8):1392-1397.
166. Niemeyer CM. RAS diseases in children. *Haematologica*. 2014;99(11):1653-1662.
167. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature*. 2002;417(6892):949-954.
168. Pratilas CA, Xing F, Solit DB. Targeting oncogenic BRAF in human cancer. *Curr Top Microbiol Immunol*. 2012;355:83-98.
169. Sholl LM. A narrative review of BRAF alterations in human tumors: diagnostic and predictive implications. *Precision Cancer Medicine*. 2020;3.
170. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. 2012;2(5):401-404.
171. Abdel-Wahab O, Park CY. BRAF-mutant hematopoietic malignancies. *Oncotarget*. 2014;5(18):7980-7981.
172. Machnicki MM, Stoklosa T. BRAF--a new player in hematological neoplasms. *Blood Cells Mol Dis*. 2014;53(1-2):77-83.
173. Alston RD, Tatevossian RG, McNally RJ, Kelsey A, Birch JM, Eden TO. Incidence and survival of childhood Langerhans cell histiocytosis in Northwest England from 1954 to 1998. *Pediatr Blood Cancer*. 2007;48(5):555-560.
174. Guyot-Goubin A, Donadieu J, Barkaoui M, Bellec S, Thomas C, Clavel J. Descriptive epidemiology of childhood Langerhans cell histiocytosis in France, 2000-2004. *Pediatr Blood Cancer*. 2008;51(1):71-75.
175. Salotti JA, Nanduri V, Pearce MS, Parker L, Lynn R, Windebank KP. Incidence and clinical features of Langerhans cell histiocytosis in the UK and Ireland. *Arch Dis Child*. 2009;94(5):376-380.
176. Stalemark H, Laurencikas E, Karis J, Gavhed D, Fadeel B, Henter JI. Incidence of Langerhans cell histiocytosis in children: a population-based study. *Pediatr Blood Cancer*. 2008;51(1):76-81.
177. Goyal G, Shah MV, Hook CC, et al. Adult disseminated Langerhans cell histiocytosis: incidence, racial disparities and long-term outcomes. *Br J Haematol*. 2018;182(4):579-581.
178. Arico M. Langerhans cell histiocytosis in adults: more questions than answers? *Eur J Cancer*. 2004;40(10):1467-1473.
179. Pierro J, Vaiselbuh SR. Adult Langerhans Cell Histiocytosis As a Diagnostic Pitfall. *J Clin Oncol*. 2016;34(6):e41-45.
180. Grois N, Potschger U, Prosch H, et al. Risk factors for diabetes insipidus in langerhans cell histiocytosis. *Pediatr Blood Cancer*. 2006;46(2):228-233.
181. Rigaud C, Barkaoui MA, Thomas C, et al. Langerhans cell histiocytosis: therapeutic strategy and outcome in a 30-year nationwide cohort of 1478 patients under 18 years of age. *Br J Haematol*. 2016;174(6):887-898.

182. Kemps PG, Zondag TC, Arnardottir HB, et al. Clinicogenomic associations in childhood Langerhans cell histiocytosis: an international cohort study. *Blood Adv*. 2022.
183. Gianotti F, Caputo R. Histiocytic syndromes: a review. *J Am Acad Dermatol*. 1985;13(3):383-404.
184. Newman B, Hu W, Nigro K, Gilliam AC. Aggressive histiocytic disorders that can involve the skin. *J Am Acad Dermatol*. 2007;56(2):302-316.
185. Crickx E, Bouaziz JD, Lorillon G, et al. Clinical Spectrum, Quality of Life, BRAF Mutation Status and Treatment of Skin Involvement in Adult Langerhans Cell Histiocytosis. *Acta Derm Venereol*. 2017;97(7):838-842.
186. Gao YJ, Su M, Tang JY, Pan C, Chen J. Treatment Outcome of Children With Multisystem Langerhans Cell Histiocytosis: The Experience of a Single Children's Hospital in Shanghai, China. *J Pediatr Hematol Oncol*. 2018;40(1):e9-e12.
187. Galluzzo ML, Braier J, Rosenzweig SD, Garcia de Davila MT, Rosso D. Bone marrow findings at diagnosis in patients with multisystem langerhans cell histiocytosis. *Pediatr Dev Pathol*. 2010;13(2):101-106.
188. Gadner H, Grois N, Arico M, et al. A randomized trial of treatment for multisystem Langerhans' cell histiocytosis. *J Pediatr*. 2001;138(5):728-734.
189. Gadner H, Minkov M, Grois N, et al. Therapy prolongation improves outcome in multisystem Langerhans cell histiocytosis. *Blood*. 2013;121(25):5006-5014.
190. Minkov M, Potschger U, Grois N, Gadner H, Dworzak MN. Bone marrow assessment in Langerhans cell histiocytosis. *Pediatr Blood Cancer*. 2007;49(5):694-698.
191. Colby TV, Lombard C. Histiocytosis X in the lung. *Hum Pathol*. 1983;14(10):847-856.
192. Vassallo R, Ryu JH, Schroeder DR, Decker PA, Limper AH. Clinical outcomes of pulmonary Langerhans'-cell histiocytosis in adults. *N Engl J Med*. 2002;346(7):484-490.
193. Howarth DM, Gilchrist GS, Mullan BP, Wiseman GA, Edmonson JH, Schomberg PJ. Langerhans cell histiocytosis: diagnosis, natural history, management, and outcome. *Cancer*. 1999;85(10):2278-2290.
194. Mourah S, How-Kit A, Meignin V, et al. Recurrent NRAS mutations in pulmonary Langerhans cell histiocytosis. *Eur Respir J*. 2016;47(6):1785-1796.
195. Kamionek M, Ahmadi Moghaddam P, Sakhdari A, et al. Mutually exclusive extracellular signal-regulated kinase pathway mutations are present in different stages of multi-focal pulmonary Langerhans cell histiocytosis supporting clonal nature of the disease. *Histopathology*. 2016;69(3):499-509.
196. Braier J, Latella A, Balancini B, et al. Outcome in children with pulmonary Langerhans cell Histiocytosis. *Pediatr Blood Cancer*. 2004;43(7):765-769.
197. Ronceray L, Potschger U, Janka G, et al. Pulmonary involvement in pediatric-onset multisystem Langerhans cell histiocytosis: effect on course and outcome. *J Pediatr*. 2012;161(1):129-133 e121-123.
198. Phillips M, Allen C, Gerson P, McClain K. Comparison of FDG-PET scans to conventional radiography and bone scans in management of Langerhans cell histiocytosis. *Pediatr Blood Cancer*. 2009;52(1):97-101.
199. Jubran RF, Marachelian A, Dorey F, Malogolowkin M. Predictors of outcome in children with Langerhans cell histiocytosis. *Pediatr Blood Cancer*. 2005;45(1):37-42.
200. Postini AM, Andreacchio A, Boffano M, Pagano M, Brach Del Prever A, Fagioli F. Langerhans cell histiocytosis of bone in children: a long-term retrospective study. *J Pediatr Orthop B*. 2012;21(5):457-462.

201. Medicine NUSNLo. H-9926-LCH III: Treatment Protocol of the Third International Study for Langerhans Cell Histiocytosis. Vol. 2022; 2022.
202. Medicine NUSNLo. LCH-IV, International Collaborative Treatment Protocol for Children and Adolescents With Langerhans Cell Histiocytosis. Vol. 2022; 2022.
203. Haroche J, Cohen-Aubart F, Emile JF, et al. Dramatic efficacy of vemurafenib in both multisystemic and refractory Erdheim-Chester disease and Langerhans cell histiocytosis harboring the BRAF V600E mutation. *Blood*. 2013;121(9):1495-1500.
204. Diamond EL, Subbiah V, Lockhart AC, et al. Vemurafenib for BRAF V600-Mutant Erdheim-Chester Disease and Langerhans Cell Histiocytosis: Analysis of Data From the Histology-Independent, Phase 2, Open-label VE-BASKET Study. *JAMA Oncol*. 2018;4(3):384-388.
205. Sloot S, Fedorenko IV, Smalley KS, Gibney GT. Long-term effects of BRAF inhibitors in melanoma treatment: friend or foe? *Expert Opin Pharmacother*. 2014;15(5):589-592.
206. Gibney GT, Messina JL, Fedorenko IV, Sondak VK, Smalley KS. Paradoxical oncogenesis--the long-term effects of BRAF inhibition in melanoma. *Nat Rev Clin Oncol*. 2013;10(7):390-399.
207. Flaherty KT, Infante JR, Daud A, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med*. 2012;367(18):1694-1703.
208. Cohen Aubart F, Emile JF, Carrat F, et al. Targeted therapies in 54 patients with Erdheim-Chester disease, including follow-up after interruption (the LOVE study). *Blood*. 2017;130(11):1377-1380.
209. Tazi A, Lorillon G, Haroche J, et al. Vinblastine chemotherapy in adult patients with langerhans cell histiocytosis: a multicenter retrospective study. *Orphanet J Rare Dis*. 2017;12(1):95.
210. Haupt R, Nanduri V, Calevo MG, et al. Permanent consequences in Langerhans cell histiocytosis patients: a pilot study from the Histiocyte Society-Late Effects Study Group. *Pediatr Blood Cancer*. 2004;42(5):438-444.
211. Chow TW, Leung WK, Cheng FWT, et al. Late outcomes in children with Langerhans cell histiocytosis. *Arch Dis Child*. 2017;102(9):830-835.
212. Chetritt J, Paradis V, Dargere D, et al. Chester-Erdheim disease: a neoplastic disorder. *Hum Pathol*. 1999;30(9):1093-1096.
213. Diamond EL, Dagna L, Hyman DM, et al. Consensus guidelines for the diagnosis and clinical management of Erdheim-Chester disease. *Blood*. 2014;124(4):483-492.
214. Haroche J, Charlotte F, Arnaud L, et al. High prevalence of BRAF V600E mutations in Erdheim-Chester disease but not in other non-Langerhans cell histiocytoses. *Blood*. 2012;120(13):2700-2703.
215. Stoppacciaro A, Ferrarini M, Salmaggi C, et al. Immunohistochemical evidence of a cytokine and chemokine network in three patients with Erdheim-Chester disease: implications for pathogenesis. *Arthritis Rheum*. 2006;54(12):4018-4022.
216. Arnaud L, Gorochov G, Charlotte F, et al. Systemic perturbation of cytokine and chemokine networks in Erdheim-Chester disease: a single-center series of 37 patients. *Blood*. 2011;117(10):2783-2790.
217. Surabhi VR, Menias C, Prasad SR, Patel AH, Nagar A, Dalrymple NC. Neoplastic and non-neoplastic proliferative disorders of the perirenal space: cross-sectional imaging findings. *Radiographics*. 2008;28(4):1005-1017.
218. Cavalli G, Guglielmi B, Berti A, Campochiaro C, Sabbadini MG, Dagna L. The multifaceted clinical presentations and manifestations of Erdheim-Chester disease: comprehensive review of the literature and of 10 new cases. *Ann Rheum Dis*. 2013;72(10):1691-1695.
219. Wu D, Lim MS, Jaffe ES. Pathology of Castleman Disease. *Hematol Oncol Clin North Am*. 2018;32(1):37-52.



SECTION B

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

V

Cytokine and viral load kinetics in human herpesvirus 8-associated multicentric Castleman's disease complicated by hemophagocytic lymphohistiocytosis: a case report

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ABSTRACT

Human herpes virus 8 (HHV-8)-associated secondary hemophagocytic lymphohistiocytosis (HLH) is a rare but critical immuno-hematological entity in immunocompetent patients. Establishing a diagnosis is challenging as is the monitoring of disease activity and therapeutic effects. We report a case of a HHV-8-associated hemophagocytic lymphohistiocytosis in a HIV-negative adult patient with multicentric Castleman's disease. As a novel finding, we report the use of certain inflammatory parameters, primarily interleukin-10 combined with viral load monitoring of the causative infectious agent in this case HHV-8 to monitor the clinical course of the hemophagocytic lymphohistiocytosis in the setting of bacterial septic complications.

BACKGROUND

Hemophagocytic lymphohistiocytosis (HLH) in adults represents a potentially fatal hyperinflammatory syndrome. HLH is either primary due to a genetic defect or secondary to underlying infectious, rheumatologic or malignant disorders.[1,2] The estimated incidence is 1.2 cases per million individuals per year resulting in mortality rates of approximately 41%, depending on underlying cause and treatment.[3,4] The immunopathology in primary (familial) HLH is explained by various genetic defects in the perforin-granzyme pathway resulting in the inability to terminate the immune response.[5] The causal mechanism of secondary HLH is unknown. Previous observations have shown an uncontrollable immune activation often described as a "cytokine storm" characterized by massive elevation of cytokines including interferon -gamma (IFN-γ), interleukin (IL)-6, IL10 and soluble IL-2 receptor (sIL-2R).[1,2,6] This severe clinical condition mimics a septic state with fever, shock, hepatosplenomegaly, elevated inflammatory parameters and pancytopenia and may often require intensive chemo-immunotherapy on top of treating both the underlying disorder and septic complications during the treatment.[1,2,7] The Epstein-Barr virus (EBV), cytomegalovirus (CMV) and human immunodeficiency virus (HIV) have commonly been reported as viral triggers for secondary HLH. The human herpes virus type 8 (HHV-8) is an infrequently described viral cause of secondary HLH in predominantly immunocompromised and, rarely, immunocompetent adults. Here, we report a case of a HIV-negative patient without known medical history with a HHV-8-associated multicentric Castleman's disease (MCD) inducing HLH. We relate the viral load and cytokine profiles to the clinical course which provides a novel proof of concept method for discriminating HLH activity from bacterial complications during the course of MCD.

CASE PRESENTATION

A 61-year-old woman of Turkish descent, with no significant medical history apart from mild obstructive uncomplicated pulmonary disease, presented with fluctuating fever, generalized lymphadenopathy and fatigue for the last 7 months. Her blood results revealed pancytopenia, without abnormalities of electrolytes, creatinine, creatin kinase, fibrinogen, lipids or liver function. HIV, EBV, CMV and tuberculosis were excluded as causative micro-organisms. Repetitive blood cultures remained negative. Further evaluations of the lymphadenopathy revealed HHV-8 positive plasmacellular MCD (Figure 1). An additional bone marrow biopsy showed phagocytosis of intact erythrocytes and thrombocytes by macrophages. Excessive ferritin-and sIL-2R levels supported the diagnosis of HLH. Retrospectively, cytokines and HHV-8 levels were studied (Table 1).

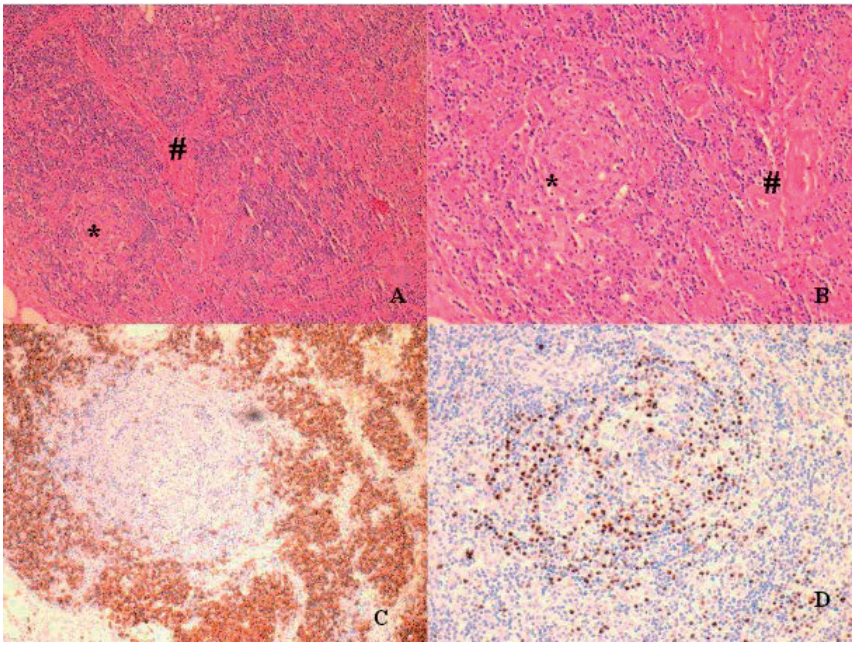

Figure 1 | Lymph node section of a patient with hemophagocytic lymphohistiocytosis and Castleman's disease.

(A, B) HE section of a lymph node. Note the regressed follicles (*) and the vessels (#) between which (C) there is a large increase of the number of CD138 positive plasma cells (brown staining) and (D) cells in which HHV-8 (brown staining) is demonstrated.

Original object lens magnification was x 20, (A,C) and x 40 (B, D) with the use of an Olympus, Color View, BX40 microscope, using a Color View digital camera.

Detailed immunological evaluations included normal immunoglobulin (Ig) levels, normal humoral/acquired defense against EBV (IgG) and HHV-8 (IgG), evidence of a prior but cleared hepatitis B virus infection (antiHBc present, HBsAg negative), normal bone marrow lymphocyte counts (percentage of 7.5%), and normal B and T-cell distribution (CD113 74%, CD14 10%, CD15 59%, CD19 6%, DCD34 < 1% with normal Smk/l ration (1.5). The patient was using 5mg of prednisone and had slightly decreased T-cells (CD4 and CD8; 0.18 and 0.16 x 10^9/L, respectively, measured at the admission for relapse of the MCD accompanied by hemophagocytosis). At the same time point, the B- and NK-cells remained within normal ranges. Clinical deterioration accompanied by increasing HLH-related cytokines (IFN-γ, sIL-2R, IL-6, IL-10 and IL-12) prompted MCD directed chemotherapy (Vincristine, Doxorubicin, Dexamethasone) 2 months after admission. The volume of inguinal lymph nodes, HHV-8 viral load and HLH-related cytokines (IFN-γ, sIL-2R and IL-10) decreased. However, fatal bacterial complications (presented by septic shock and increasing CRP and IL-6) prevented a second course of chemotherapy. The patient died of bacterial sepsis and multiple organ failure 3 months after admission.

Table 1 | Laboratory evaluations in a patient with Castleman's disease and HLH.

	Day of admission	Day 30	Day 60	Day 90
Leukocyte, x10^9/L	4.4	5.4	4.2	1.6
Hemoglobulin, mmol/L	5.2	4.8	3.4	4.1
Thrombocyte, x10^9/L	77	247	14	29
CRP, mg/L	23	80	185	352
Ferritin, mcg/l	2627	4964	8852	58257
IL-1B, pg/ml	<5	<5	<5	<5
IL-2, pg/ml	<5	<5	<5	<5
IL-4, pg/ml	<5	<5	<5	ND
IL-5, pg/ml	<5	<5	<5	ND
IL-6, pg/ml	6	37	42	3862
IL-8, pg/ml	9	96	31	1144
IL-10, pg/ml	134	>5000	>5000	385
IL-12p70, pg/ml	<5	<5	10	6
IFN-G, pg/ml	10	30	5	8
TNF-A, pg/ml	<5	<5	<5	<5
sIL2-R, pg/ml	37106	68687	104037	55327
HHV-8, copiesx10^5/mL	1.17	21.1	24.2	4.04

CRP indicates C-reactive protein; IL, interleukin; IFN-G, interferon gamma; TNF-A, tumor necrosis factor alpha; sIL2-R, soluble interleukin receptor 2; HHV-8, human herpes virus 8 and ND, not determined.

DISCUSSION

We describe a rare case of MCD-associated HLH in a HIV-negative patient with no relevant immunocompromised history and propose to add IL-10 and HHV-8 viral load as relevant clinical parameters to monitor disease activity and differentiate from septic complications.

Reports on HHV-8-associated MCD complicated by HLH in immunocompetent patients, without concomitant EBV or CMV, are rare and concern almost exclusively immunocompromised HIV-positive patients.[8] So far, only one large case series describes HLH occurring in 8 out of 18 patients in HIV-seronegative patients with HHV-8 related MCD.[9] However, in this study the EBV and CMV statuses are unknown which could have been important confounders. Two related case reports include 2 HIV negative patients with HHV-8-associated MCD complicated by HLH (one patient with a plasmablastic variant and one with Kaposi sarcoma), although both studies omitted information on CMV and only 1 patient was proven to be EBV negative.[10,11] Our patient is apparently immunocompetent. However, it can be proposed that the low T-cells, probably due to the septic state and prolonged low doses of steroids, might be a driving force in the

HHV-8 induced MCD in our patient. On the other hand, decreased CD4 cell counts are regarded not related to the development of MCD since there is no relation between CD4 levels and flares of MCD or decrease on prevalence of MCD after the initiation of successful anti-HIV therapy[12]. One could also consider that CD4 and CD8 cells in our patient have been skewed towards the localization of MCD.

Both MCD and infection induced familial HLH are hypothesized to be involved in a similar immunopathological pathway since comparable cytokine patterns are described.[8,13] There are no studies on cytokines of immunocompetent adult MCD patients with HLH. We postulate that the clinical picture of HLH in our patient is caused by progression of HHV-8 induced MCD subsequently leading to uncontrollable cytokine production consisting of IFN- γ, IL-10 and IL-6. The overproduction of the latter two by B-lymphoid cells in MCD might trigger a dysfunctional cascade of cytokine production with subsequent uncontrolled accumulation of activated T-lymphocytes and macrophages leading to hemophagocytosis.[1,6,8,14]

The clinical situation of our patient represents the classical debate how to distinguish HLH from sepsis. IL-6 is difficult to interpret since it is elevated in both sepsis and MCD. The observation in paediatric HLH patients with MCD by Tang *et al* might indicate a common pathway in primary HLH with MCD and our patient with secondary HLH. Their study demonstrates that IL-10 and IFN-γ levels may differentiate between HLH and sepsis which becomes apparent by the relatively more elevated IL-6 than IL-10 and IFN-γ in sepsis in a similar pattern as we observed in our patient.[15] The fatal septic complications in our patient are thus illustrated by increasing IL-6, IL-8, ferritin and CRP and the MCD-HLH related symptoms by IL-10, IFN-γ and HHV-8 viral load.

The specific HLH-associated cytokine overproduction in association with HHV-8 infected polyclonal B cells are comparable to observations in other patients with HLH.[1,2,6] Viral load monitoring in EBV-associated HLH predicts severity and outcome.[7] Whether HHV-8 viral load and specific cytokines can predict the course of HHV-8-associated MCD complicated by HLH is currently unknown. In analogy with the observation in EBV-associated HLH, we provide proof of principle for the first time that a similar strategy could be applicable to monitor HLH activity in HHV-8-associated MCD patients by observing the HHV-8 load together with IL-10, IFN-γ and sIL-2R, in which higher values correspond to a more active HLH disease condition. This approach might be of value in the active monitoring and management of patients with HHV-8-associated HLH hence improving the clinical outcome.

CONCLUSION

In conclusion we present unique data from an apparently immunocompetent patient with HIV/EBV/CMV negative MCD-associated HLH in which HHV-8 viral load, IL-10, IFN-γ and sIL-2R kinetics correspond with the clinical course of the HLH. These kinetics, more specifically IL-10 levels and HHV-8 viral load are proposed as new parameters that might be of use in monitoring the disease activity in HHV-8-associated MCD complicated by HLH.

REFERENCES

1. Osugi Y, Hara J, Tagawa S, et al. Cytokine production regulating Th1 and Th2 cytokines in hemophagocytic lymphohistiocytosis. *Blood*. 1997;89(11):4100-4103.
2. Imashuku S, Hibi S, Fujiwara F, Todo S. Hyper-interleukin (IL)-6-naemia in haemophagocytic lymphohistiocytosis. *Br J Haematol*. 1996;93(4):803-807.
3. Henter JI, Elinder G, Soder O, Ost A. Incidence in Sweden and clinical features of familial hemophagocytic lymphohistiocytosis. *Acta Paediatr Scand*. 1991;80(4):428-435.
4. Ramos-Casals M, Brito-Zeron P, Lopez-Guillermo A, Khamashta MA, Bosch X. Adult haemophagocytic syndrome. *Lancet*. 2014;383(9927):1503-1516.
5. Goransdotter Ericson K, Fadeel B, Nilsson-Ardnor S, et al. Spectrum of perforin gene mutations in familial hemophagocytic lymphohistiocytosis. *Am J Hum Genet*. 2001;68(3):590-597.
6. Henter JI, Elinder G, Soder O, Hansson M, Andersson B, Andersson U. Hypercytokinemia in familial hemophagocytic lymphohistiocytosis. *Blood*. 1991;78(11):2918-2922.
7. van Laar JA, Buysse CM, Vossen AC, et al. Epstein-Barr viral load assessment in immunocompetent patients with fulminant infectious mononucleosis. *Arch Intern Med*. 2002;162(7):837-839.
8. Bower M, Veraitch O, Szydlo R, et al. Cytokine changes during rituximab therapy in HIV-associated multicentric Castleman disease. *Blood*. 2009;113(19):4521-4524.
9. Dossier A, Meignin V, Fieschi C, Boutboul D, Oksenhendler E, Galicier L. Human herpesvirus 8-related Castleman disease in the absence of HIV infection. *Clin Infect Dis*. 2013;56(6):833-842.
10. Li CF, Ye H, Liu H, Du MQ, Chuang SS. Fatal HHV-8-associated hemophagocytic syndrome in an HIV-negative immunocompetent patient with plasmablastic variant of multicentric Castleman disease (plasmablastic microlymphoma). *Am J Surg Pathol*. 2006;30(1):123-127.
11. Kim B, Jeon YK, Kim CW. Kaposi sarcoma herpes virus-associated hemophagocytic syndrome complicated by multicentric castleman disease and kaposi sarcoma in a HIV-negative immunocompetent patient: an autopsy case. *J Korean Med Sci*. 2009;24(5):970-974.
12. Casper C. The aetiology and management of Castleman disease at 50 years: translating pathophysiology to patient care. *Br J Haematol*. 2005;129(1):3-17.
13. Grossman WJ, Radhi M, Schauer D, Gerday E, Grose C, Goldman FD. Development of hemophagocytic lymphohistiocytosis in triplets infected with HHV-8. *Blood*. 2005;106(4):1203-1206.
14. Dupin N, Diss TL, Kellam P, et al. HHV-8 is associated with a plasmablastic variant of Castleman disease that is linked to HHV-8-positive plasmablastic lymphoma. *Blood*. 2000;95(4):1406-1412.
15. Tang Y, Xu X, Song H, et al. Early diagnostic and prognostic significance of a specific Th1/Th2 cytokine pattern in children with haemophagocytic syndrome. *Br J Haematol*. 2008;143(1):84-91.

VI

Novel RAB27A variant associated with late-onset hemophagocytic lymphohistiocytosis alters effector protein binding

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ABSTRACT

Autosomal recessive mutations in RAB27A are associated with Griscelli syndrome type 2 (GS2), characterized by hypopigmentation and development of early-onset, potentially fatal hemophagocytic lymphohistiocytosis (HLH). We describe a 35-year old male who presented with recurrent fever, was diagnosed with Epstein-Barr virus-driven chronic lymphoproliferation, fulfilled clinical HLH criteria, and who carried a novel homozygous RAB27A c.551G > A p.(R184Q) variant. We aimed to evaluate the contribution of the identified RAB27A variant in regard to the clinical phenotype as well as cellular and biochemical function. The patient displayed normal pigmentation as well as RAB27A expression in blood-derived cells. However, patient NK and CD8+ T cell exocytosis was low. Ectopic expression of the RAB27A p.R184Q variant rescued melanosome distribution in mouse Rab27a-deficient melanocytes, but failed to increase exocytosis upon reconstitution of human RAB27A-deficient CD8+ T cells. Mechanistically, the RAB27A p.R184Q variant displayed reduced binding to SLP2A but augmented binding to MUNC13-4, two key effector proteins in immune cells. MUNC13-4 binding was particularly strong to an inactive RAB27A p.T23N/p.R184Q double mutant. RAB27A p.R184Q was expressed and could facilitate melanosome trafficking, but did not support lymphocyte exocytosis. The HLH-associated RAB27A variant increased Munc13-4 binding, potentially representing a novel mode of impairing RAB27A function selectively in hematopoietic cells.

INTRODUCTION

Griscelli syndrome type 2 (GS2) is a pigmentation disorder associated with autosomal recessive mutations in RAB27A.[1] In contrast to other forms of GS, GS2 patients typically develop early-onset, life-threatening hemophagocytic lymphohistiocytosis (HLH), a hyperinflammatory syndrome.[2] Familial forms of HLH are associated with defective lymphocyte cytotoxicity, which requires exocytosis of cytotoxic granules, a form of specialized lysosomes.[3]

RAB27A encodes RAB27A, a 25 kDa member of the Rab family of small GTPases.[4] The C-terminus can be prenylated by Rab geranylgeranyltransferase (RGGTase) acting on cysteine-containing motifs, thereby anchoring RAB27A to the membrane.[5-7] GTPase are activated by guanin exchange factors (GEFs), which induce an active, effector protein-binding conformation through exchange of GDP for GTP. In turn, GTPase-activating proteins (GAPs) inactivate GTPases.[8] These forms are mimicked by RAB27A Q78L (active) and T23N (inactive) substitutions. In melanocytes, the dispersal of pigment-containing melanosomes is driven by RAB27A, which coordinates the melanophilin-myosin-Va motor complex and an actin filament assembly complex, as a prelude to melanin exocytosis.[2,9] In hematopoietic cells, secretory lysosome trafficking, docking, and exocytosis is mediated by RAB27A interactions with SLP2A and MUNC13-4.[10,11] The RAB27A/SLP2A complex has been crystalized, revealing that the RAB27A α5-helix interacts with SLP2A.[12] In contrast, HLH-associated RAB27A missense variants that disrupt MUNC13-4 binding have been mapped to the RAB27A α4-helix.[13] The RAB27A interaction with MELANOPHILIN has not been mapped but does not interfere with MUNC13-4 binding.[13] These observations can explain how certain previously reported RAB27A variants specifically impair MUNC13-4 binding and exocytosis in hematopoietic cells, without affecting pigmentation in melanocytes.[13,14]

We describe an adult-onset HLH patient from consanguineous parents harboring a novel homozygous RAB27A c.551G > A p.(R184Q) variant. Our results suggest a novel mode of selective disruption of RAB27A function in hematopoietic cells.

METHODS

Patient and Control Samples

This study was approved by the ethic committees of the Board of Stockholm. Informed consents from the individuals included in the study were obtained according to the Declaration of Helsinki. The patient was diagnosed according to the HLH-2004 criteria. Clinical data, laboratory findings, and genetics were collected from the patient's medical

records. Peripheral blood mononuclear cells (PBMCs) and hair were collected and analyzed. Six siblings were unavailable or did not consent to genetic analyses.

DNA Extraction, Amplification, and Sequence Analysis

DNA was enriched using Agilent SureSelect Clinical Research Exome V2 capture and paired-end sequenced on the Illumina platform. The aim was to obtain 8.1 Giga base pairs per exome with a mapped fraction of 0.99. The average coverage of the exome was ~ 50×. Duplicate reads were excluded. Data were demultiplexed with bcl2fastq Conversion Software from Illumina. Reads were mapped to the genome using the BWA-MEM algorithm (reference: http://bio-bwa.sourceforge.net/). Variant detection was performed by the Genome Analysis Toolkit HaplotypeCaller (reference: http://www.broadinstitute.org/gatk/). The detected variants were filtered and annotated with Cartagenia software and classified with Alamut Visual.

Sequence variants were searched in a primary immunodeficiency panel covering > 300 genes. Homozygous VPS13B c.2471C > T p.(S824F) and heterozygous CARD11 c.2711G > A p.(S904N) variants of uncertain significance were also identified. No known pathogenic variants were identified.

Immunophenotyping and Cytotoxic Lymphocyte Function Analysis

Lymphocyte subset numbers were quantified by flow cytometry (FACS Symphony instrument, BD Biosciences) using BD IMK kit with TruCount tubes (BD Biosciences) according to the manufacturer's instructions. Lymphocyte phenotype and function were further assessed upon stimulation and staining of freshly isolated PBMC.[15] Briefly, fluorochrome-conjugated anti-CD3 (BioLegend), anti-CD4 (Invitrogen), anti-CD8 (BioLegend), anti-CD16 (BD Bioscience), anti-CD56 (BD Bioscience), and anti-CD107a (BioLegend) monoclonal antibodies were used. Functional testing of cytotoxic lymphocytes was performed incubating PBMC in vitro with murine P815 cells together with anti-CD16 or anti-CD3 antibodies for stimulation of NK cells and T cells, respectively. Natural cytotoxicity was tested using K562 cells. Exocytosis was quantified using CD107a+ surface expression. Flowjo v.9.9 (BD Biosciences) was used for analysis of the flow data.

Western Blot for RAB27A in Primary Cells

One million PBMCs per donor were lysed in RIPA buffer supplemented with 1× protease inhibitor cocktail (Santa Cruz Biotechnology) for 30 min on ice. Supernatants were mixed with 4× NuPage loading buffer (Invitrogen) added 10 mM DTT (Invitrogen), run on a 4–12% Bis–Tris gel (Invitrogen), and transferred to a nitrocellulose membrane (iBlot, Invitrogen). Rabbit polyclonal anti-RAB27A (Proteintech Group) and HRP-conjugated goat anti-rabbit secondary antibodies (Invitrogen) were used for detection. A directly

HRP-conjugated mouse anti-actin antibody (Sigma) was used as loading control. Blocking buffer and antibodies were diluted in 5% non-fat dry milk (Biorad) in TBS-Tween 0.2%.

Sequence Alignment and 3D Structure Visualization

RAB27A protein sequences of different organisms were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/) and aligned using CLC Main Workbench software (version 7.0, Qiagen). The 3D structure of RAB27A interacting with SLP2A (PDB 3BC1) was downloaded and visualized using Chimera 1.13 software.

Plasmid Constructs

Plasmids encoding RAB27A WT, p.Q78L, and p.T23N were kindly provided by Dr. Genevieve de Saint Basile.[16] PCR amplification was performed to shuttle cDNA to a modified pMax backbone with an N-terminal 3xFLAG tag using NheI and AgeI restriction sites. The RAB27A c. 551G > A (p.R184Q) variants were generated by site-directed mutagenesis. The plasmid sequences were confirmed by Sanger sequencing. A SLP2A-hem containing vector was kindly provided by Dr. G. de Saint Basile and transferred to a vector with an N-terminal MYC-tag.[17] N-terminal MYC-tagged MUNC13-4 and MELANOPHILIN constructs were also generated. Adenovirus vectors allowing expression of the RAB27A p.R184Q mutant as a fusion to monomeric red fluorescent protein (mRFP) were generated as previously described.[18] For the lentiviral constructs, RAB27A WT and p.R184Q were cloned into pLeGO-G2 (Addgene plasmid #25,917) using BamHI and EcoRI restriction sites. Later, viral particles (VSV-G) from supernatant of packing HEK-293FT cells were added to stimulated CD8+ T cells from healthy individuals or GS2 patients.

Melanosome Distribution

Immortal Rab27a-deficient murine ashen melanocytes were cultured as previously described.[19] For analysis of melanosome distribution, 2.5 × 104 cells were plated on 13-mm glass coverslips. Twenty-four hours later, the cells were transduced with adenovirus expression vectors, and after a further 24 h of incubation, these were fixed and stained to detect the localization of RAB27A wild-type and p.R184Q proteins as previously described.[19] Intracellular distribution of melanosomes and RAB27A was recorded as previously described.[19]

Reconstitution Experiments in CD8.+ T Cells from RAB27A-Deficient Patients

CD8+ T cells were isolated from PBMC of selected GS2 patients by negative magnetic selection (Miltenyi Biotech), stimulated with 10 µL anti-CD3/CD28 immunocomplexes (STEMCELL Technologies) and 100 IU/mL of recombinant IL-2 for 48 h. Cells were thereafter transduced with VSV-G viral particles containing constructs encoding either N-terminally mCherry tagged RAB27A wild-type or p.R184Q. The next day, the cells were

washed and cultured in complete medium supplemented with 100 UI/mL IL-2. After 3 days of culture, cells were assessed for exocytosis by surface expression of CD107a as previously described. Cells were cultured in RPMI medium (Hyclone) supplemented with 10% FCS at 37 °C, 5% CO2.

Co-immunoprecipitation of Effector Proteins

HEK-293FT cells were chemically transfected (Lipofectamine 2000, Invitrogen) according to the manufacturers' protocol. After 24 h, cells were lysed in 25 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 × protease inhibitor cocktail (HALT), 250 U/mL benzonase (Invitrogen), 10 mM DTT, 1% TritonX-100, 5 mM EDTA, and 0.5 × sodium orthovanadate. A goat anti-FLAG tag antibody (Abcam) was used for immunoprecipitation, with magnetic protein G beads used to harvest the immunocomplexes (Dynabeads, Thermo). This was then eluted in the presence of NuPAGE LDS buffer (Invitrogen), run on a 4–12% Bis–Tris gel (Invitrogen), and transferred to a nitrocellulose membrane (iBlot, invitrogen). Mouse anti-Myc (Invitrogen) and mouse anti-FLAG (Sigma) antibodies were used to blot for the recombinant proteins.

Statistical Analysis

Mean values, standard deviation, and p-values (paired parametric t-test) were calculated using GraphPad Prism 7.0 software (GraphPad Prism Inc.). The threshold for statistical significance was set at $p \leq 0.05$.

RESULTS

A Novel Homozygous RAB27A p.(R184Q) Variant Identified in a HLH Patient

A 35-year-old male with a history of recurrent sinopulmonary infections and schizophrenia initially presented with recurrent fever and dry coughs. He was from consanguineous parents of Turkish origin and had eight siblings (Figure 1A). He was initially diagnosed with EBV-driven lymphoproliferation based on high EBV copy numbers (9929 IU/mL) and pathology. In spite of rituximab therapy, the fever persisted. Initially, only a mild anemia was present and ferritin levels were low. However, 3 months after the diagnosis of chronically active EBV disease, the patient developed overt inflammation, fulfilling the HLH-2004 criteria (Table 1).[20] Ferritin peaked at 67,938 µg/L. Despite extensive efforts, lymphoma was excluded, and no other underlying cause of HLH besides EBV infection was identified. The patient was treated with corticosteroids, intravenous immunoglobulin, etoposide, rituximab, and alemtuzumab, but the HLH repeatedly relapsed. Almost 2 years after initial presentation with EBV-driven lymphoproliferation,

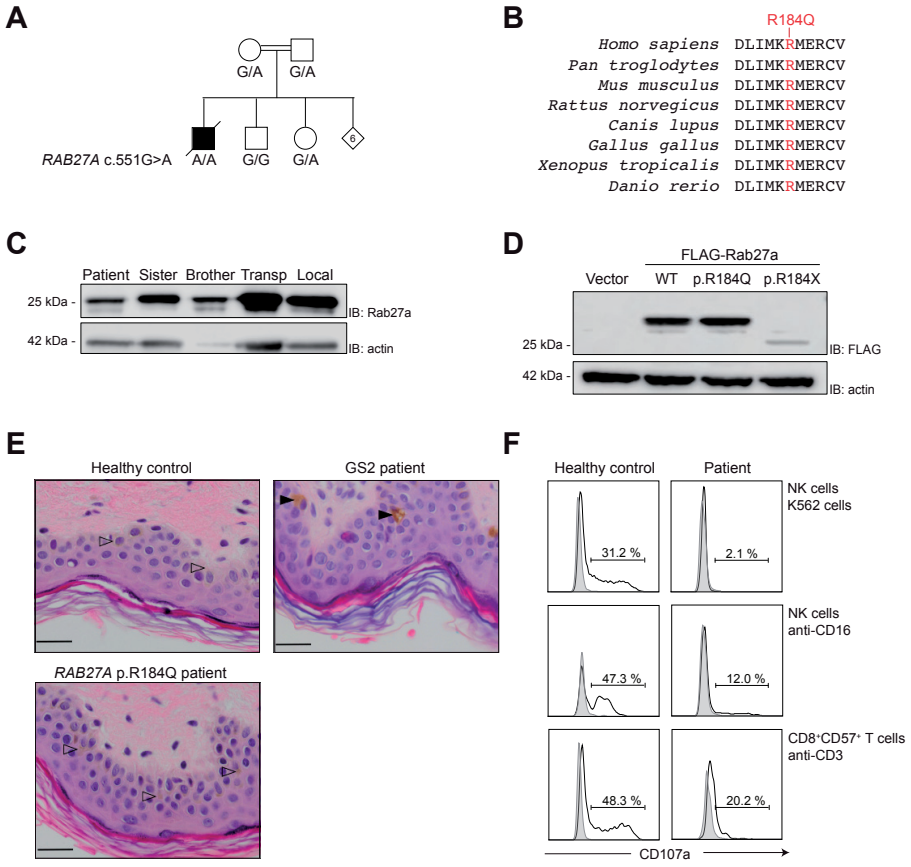

Figure 1 | A novel homozygous RAB27A c.551G > A (p.R184Q) variant in a fatal HLH case.
A Pedigree of family. Six out of eight siblings were not available for genetic analysis. B RAB27A amino acid evolutionary conservation in mammals, birds, frogs, and fish of the sequence surrounding the p.R184Q variant. C Expression of RAB27A determined by western blot of freshly isolated PBMC lysates from the patient, siblings, and healthy controls, as indicated. Actin was used as loading control. Blots are representative of two independent experiments. D Western blot of HEK-293FT cells transiently transfected with plasmids encoding FLAG-RAB27A wild-type (WT), patient-derived p.R184Q, or truncating p.R184X variants. Results are representative of three independent experiments. E Hematoxylin–eosin staining of skin biopsies from a healthy control (indicating normal melanocytes with arrowheads), a typical GS patient (displaying characteristic hyperpigmented oval melanocytes indicated with filled arrowheads), and the patient. Bars indicate 20 mm. F Histograms show exocytosis (quantified on the basis of CD107a surface expression) of cytotoxic lymphocyte subsets from the patient as well as a healthy transport control, as specified. PBMCs were stimulated with target cells and antibodies as indicated, for 2 h. The cells were analyzed by flow cytometry, gating on CD3–CD56+ NK cells or CD3+CD8+CD57.+ T cells. Data are representative of two independent experiments.

the patient developed pulmonary aspergillosis and died of pulmonary insufficiency in anticipation of a hematopoietic stem cell transplant.

Whole-exome sequencing uncovered a homozygous missense variant in RAB27A:NM_004580.4 (RAB27A):c.551G > A, p.(R184Q) (Figure 1A), which has a population frequency of < 0.0001 according to public databases (gnomAD v3.1.1)[21], is

Table 1 | HLH-2004 criteria at diagnosis.

Criteria	Fulfilled
Fever	Yes
Splenomegaly	Yes
Cytopenias (affecting ≥2 of 3 lineages)	
Haemoglobin <90g/L	*77*
Platelets <100x10^9/L	*75*
Neutrophils <1.0x10^9/L	*2.17*
Hypertriglyceridemia and/or hypofibrinogenemia	
Fasting triglycerides ≥3.0mmol/L	*3.07*
Fibrinogen ≤1.5g/L	*5.4*
Hemophagocytosis in bone marrow or spleen or lymph nodes	Yes
Low or absent NK-cell activity	Yes
Ferritin ≥ 500µg/L	2135
sIL-2 receptor ≥ 2400U/ml	82606

predicted damaging (CADD score 25.20)[22,23], and has not previously been associated with HLH. Representing a change from a positively to a negatively charged amino acid in the C-terminal α5-helix of RAB27A, the R184 position is highly conserved among vertebrates (Figure 1B). In addition, rare homozygous VPS13B c.2471C > T p.(S824F) and a heterozygous CARD11 c.2711G > A p.(S904N) variants of uncertain significance were also identified (CADD scores 3.54 and 23.0, respectively). Autosomal recessive VPS13B variants cause Cohen syndrome, characterized by obesity, hypotonia, mental deficiency, and facial, oral, ocular, and limb anomalies.[24] Leukopenia, especially neutropenia, is also a feature of Cohen syndrome.[25] Apart from mild cognitive impairment, the patient did not present clinical features characteristic of Cohen syndrome illustrated by a body mass index (BMI) between 20 and 25, normal muscle tone, absent psychomotor retardation, and no syndromic appearances/anomalies. Germline CARD11 mutations are associated with different primary immune disorders in humans.[26] The patient's history of recurrent sinopulmonary infections and persistent EBV infection overlaps with clinical manifestations of heterozygous CARD11 mutations causing B-cell expansion with NF-κB and T-cell anergy (BENTA). Of note, heterozygous CARD11 variants associated with BENTA are typically located in the N-terminal CARD and LATCH domains and not in the C-terminus as was the case in this patient. In addition, B-cell expansions were not observed in our patient. Given a paucity of features associated with Cohen syndrome, yet association of autosomal recessive RAB27A variants with HLH, we focused further on evaluating the potential contribution of the predicted damaging RAB27A variant to disease.

Rab27a Expression and Patient Characteristics

In order to examine the expression of the RAB27A variant protein, we performed western blots of peripheral blood mononuclear cell lysates. The patient expressed RAB27A (Figure 1C), indicating that the protein was not degraded. Furthermore, ectopic expression of RAB27A wild-type (WT), p.R184Q, and p.R184X constructs in 293FT cells also revealed comparable expression of RAB27A WT and p.R184Q, whereas the p.R184X was degraded (Figure 1D). The RAB27A p.R184X mutant cannot be C-terminally prenylated and hence is unstable.[27]

Our patient developed gray hair from age 20 years, but microscopic examination lacked typical GS features (large uneven clumps of pigment) (data not shown). Furthermore, in contrast to typical GS patients that display hyperpigmented oval melanocytes without adjacent tissue pigmentation[28], a skin biopsy from the patient indicated normal distribution of melanin throughout the epidermis (Figure 1E). RAB27A-deficiency is associated with defective cytotoxic lymphocyte exocytosis.[1,29] Patient NK cells as well as CD8+CD57+ T cells displayed reduced exocytosis (Figure 1F; normal range (mean ± 2SD) for induction of CD107a on NK cells was for K562 cell or anti-CD16 stimulation 9–41% and 30–66%, respectively, and for that on CD8+CD57+ T cells 28–76%, in healthy adults), but not abolished as frequently observed in FHL.[15,30] Furthermore, in the patient, both NK cells and CD8+CD57+ T cells undergoing exocytosis displayed low intensity of CD107a surface expression, as previously reported in a patient with hypomorphic UNC13D variants associated with late-onset HLH.[31]

Thus, the RAB27A p.R184Q was expressed at the protein level. Furthermore, evaluation of the patient suggested that the RAB27A variant may not affect melanosome trafficking of pigment but impair lymphocyte exocytosis.

Rab27a p.R184Q Displays Unperturbed Function in Melanocytes While It Leads to Defective Cytotoxic Function in Lymphocytes

To understand if the RAB27A p.R184Q variant could cause disease, we evaluated its function in melanocytes and lymphocytes. Adenoviral RAB27A wild-type or p.R184Q variant constructs with an N-terminal mRFP fluorescent tag were generated for expression of RAB27A in melanocytes. These constructs were expressed in melanocytes from ashen mice that are homozygous for a Rab27a variant that disrupts exon splicing.[32] The RAB27A p.R184Q variant rescued pigment dispersion in Rab27a-deficient melanocytes in a manner comparable to RAB27A wild-type constructs (Figure 2A). Furthermore, to evaluate if the patient-derived RAB27A variant could rescue lymphocyte exocytosis, we selected GS2 patients with biallelic RAB27A variants that resulted in defective RAB27A expression (Suppl Table 1)[33] and isolated peripheral blood CD8+ T cells and transduced them with lentiviral constructs encoding either N-terminal mCherry tagged RAB27A wild-type or p.R184Q proteins. After transduction, exocytosis was evaluated

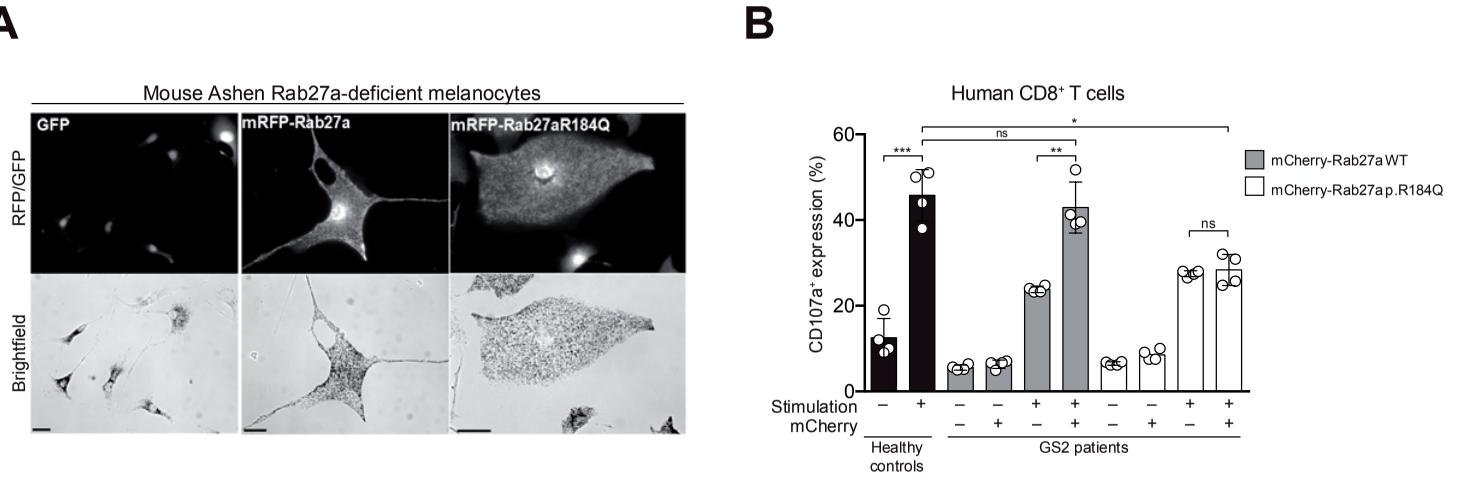

Figure 2 | Reconstitution of RAB27A-deficient melanocytes and T cells with RAB27A WT and p.R184Q variants.

A Rab27a-deficient mouse ashen melanocytes transduced with adenoviruses encoding mRFP-tagged RAB27A WT or p.R184Q variants. Fluorescence images show expression of vector control GFP or mRFP-RAB27A constructs and brightfield images melanosome distribution in transduced cells (bar indicates 20 µm for GFP and 10 µm for RFP images). B Unmanipulated CD8+ T cells from healthy control or RAB27A-deficient GS2 patients transduced with lentiviruses encoding mCherry-RAB27A WT or p.R184Q variants. Untransduced CD8+ T cells from healthy donors represent controls. For GS2 patient cells, the graph depicts the frequency of CD8.+ T cells with surface CD107a expression according to gating on mCherry expression, as indicated. Dots represent individual patients, bars represent mean values with SD. Statistics: ns non-significant $P > 0.05$; *$P \leq 0.05$, **$P \leq 0.01$.

Taken together, these results support the notion that RAB27A p.R184Q facilitates melanosome pigmentation but does not efficiently support cytotoxic lymphocyte exocytosis.

following anti-CD3 antibody stimulation. Untransduced CD8+ T cells from healthy volunteers demonstrated a robust increase in exocytosis upon anti-CD3 stimulation. The transduction efficiency of the mCherry-RAB27A WT constructs was higher in GS2 patient CD8+ T cells in all individuals (Suppl Figure S1A, S1B, S1C). The expression levels of the mCherry-RAB27A WT relative to mCherry-RAB27A p.R184Q were also consistently higher in GS2 patient CD8+ T cells in all individuals (Suppl Figure S1D). Importantly, anti-CD3 antibody stimulation significantly increased exocytosis by GS2 patient CD8+ T cells transduced with RAB27A wild-type, but not those with RAB27A p.R184Q constructs (Figure 2B).

Altered Effector SLP2A/MUNC13-4 Binding Affinity for RAB27A p.R184Q

To determine how the patient-derived RAB27A variant might interfere with lymphocyte exocytosis, we assessed the capacity of the RAB27A p.R184Q variant to interact with the effector proteins expressed in immune cells. FLAG-tagged RAB27A wild-type, "active" p.Q78L, or "inactive" p.T23N constructs, encoding the wild-type or p.R184Q variant, were co-expressed with plasmids encoding MYC-tagged, full-length SLP2A, MUNC13-4 in HEK-293FT cells. Co-immunoprecipitation of SLP2A, MUNC13-4 with tagged RAB27A variants was quantified in cell lysates (Figure 3A). Relative to RAB27A wild type, the RAB27A p.R184Q variant displayed around 25% reduced binding to SLP2A (Figure 3A, B). A reduction of more than 30% was observed when the RAB27A p.R184Q variant also carried the constitutive p.Q78L mutation (Figure 3A, C). Furthermore, relative to RAB27A wild type, the RAB27A p.R184Q variant displayed tenfold increased binding to MUNC13-4 (Figure 3D, E). The RAB27A p.R184Q variant also carrying the p.T23N mutation displayed 100-fold greater MUNC13-4 binding, whereas the p.Q78L mutation construct displayed only mildly increased MUNC13-4 binding (Figure 3D, E). In contrast to previously published reports, the inactive RAB27A p.T23N mutant bound MUNC13-4 with higher propensity than the active p.Q78L mutant in our experimental setting (Figure 3D, E). Similar co-immunoprecipitation experiments of MUNC13-4 in cells expressing melanophilin revealed equal binding of RAB27A WT and p.R184Q to melanophilin (Figure 3F), whereas the constitutive active RAB27A p.Q78L variant displayed increased and the inactive p.T23N variant displayed decreased binding, respectively (Figure 3F).

In summary, relative to RAB27A WT, the RAB27A p.R184Q variant displayed decreased binding to SLP2A and increased binding to MUNC13-4. This data suggests that the RAB27A p.R184Q variant displays an imbalance in effector binding, specifically disrupting MUNC13-4-mediated exocytosis.

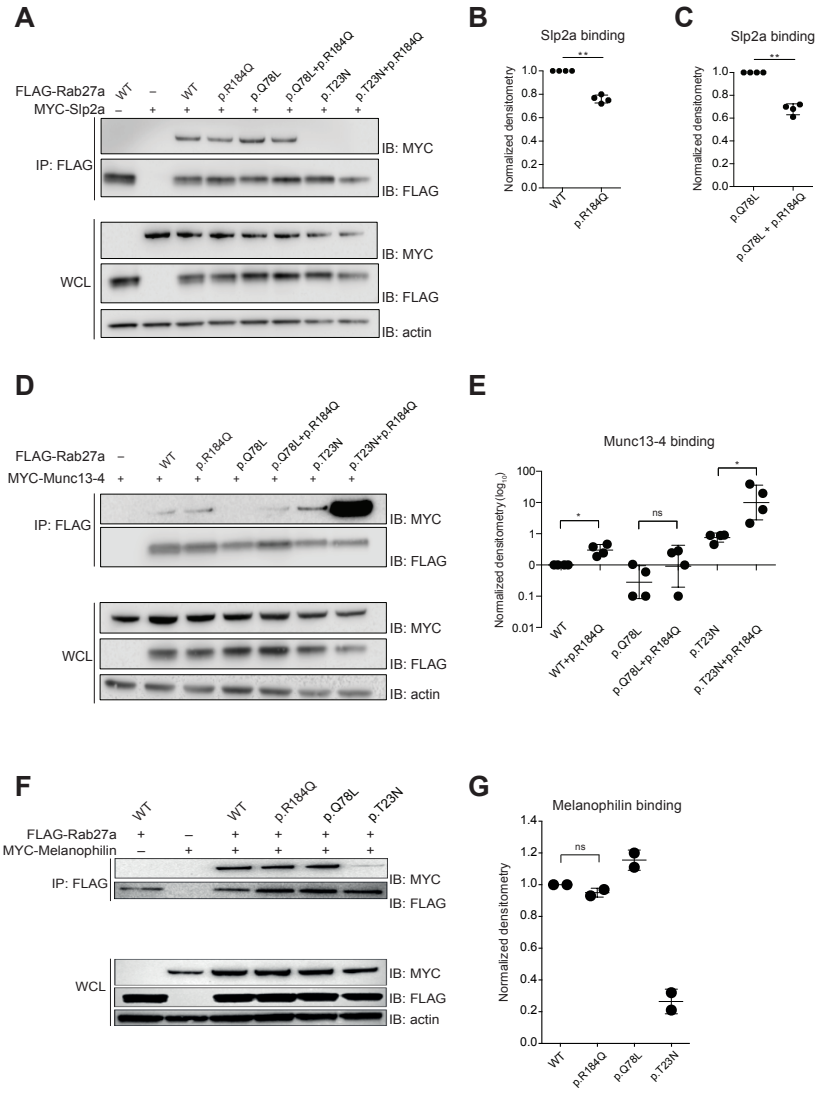

Figure 3 | RAB27A p.R184Q displays altered binding to effector proteins present in immune cells.
A 293FT cells co-transfected with MYC-SLP2A and FLAG-RAB27A WT p.Q78L (active mutant) or p.T23N (inactive mutant) in combination with patient-derived p.R184Q variant, as indicated. Immunoprecipitates (IPs) or whole cell lysates (WCLs) were probed by western blotting (IB) with antibodies, as indicated. B Quantification of SLP2A binding following anti-FLAG immunoprecipitation for RAB27A p.R184Q variant. C Quantification of Slp2a binding following anti-FLAG immunoprecipitation for Rab27a p.Q78L + p.184Q constructs. D 293FT cells co-transfected with MYC-MUNC13-4 and FLAG-RAB27A WT, p.Q78L (active mutant), or p.T23N (inactive mutant) in combination with patient-derived p.R184Q variant, as indicated. E Quantification of MUNC13-4 binding following anti-FLAG immunoprecipitation for the different RAB27A constructs in transfected HEK-293FT cells. F 293FT cells co-transfected with MYC-MELANOPHILIN and FLAG-RAB27A WT, p.Q78L (active mutant) or p.T23N (inactive mutant) in combination with patient-derived p.R184Q variant, as indicated. G Quantification of MELANOPHILIN binding following anti-FLAG immunoprecipitation for different RAB27A constructs in transfected HEK-293FT cells. Data are representative of at least three independent experiments, except in G, which displays results from two independent experiments. Statistics: ns, non-significant $P > 0.05$; *$P \leq 0.05$, **$P \leq 0.01$.

DISCUSSION

Biallelic loss-of-function variants in RAB27A cause hypopigmentation and development of HLH[1], but atypical forms of GS2 lacking hypopigmentation have also been described. RAB27A missense mutations that selectively impair RAB27A binding to MUNC13-4 or non-coding rearrangements affecting a lymphocyte-specific promoter have previously been identified in GS2 patients, selectively displaying immunological features of the disease.[13,14,34,35] We describe an adult-onset HLH patient from consanguineous parents harboring a novel homozygous RAB27A c.551G>A p.(R184Q) variant. Our results suggest a novel mode of selective disruption of RAB27A function in hematopoietic cells, leaving pigment dispersion intact.

The structure of RAB27A p.Q78L variant in complex with the SLP2A has been solved (Figure 4)[12], while RAB27A/MELANOPHILIN and RAB27A/MUNC13-4 complexes have not been reported. SLP2A interacts with the RAB27A α5-helix where the R184 residue is located.[12] The RAB27A R184 residue maintains electrostatic stability required for Slp2a binding, potentially explaining why exchange of charge impaired SLP2A binding in our experiments. The N-terminus of RAB27A can bind MELANOPHILIN, with the Rab27b/melanophilin structure indicating that the β1/β2-sheets and α2-helix of the closely structurally related RAB27A likely mediate binding of MELANOPHILIN.[23] A few HLH-associated RAB27A variants in GS2 patients with normal pigmentation selectively abolish MUNC13-4 but not MELANOPHILIN binding (Figure 4).[13,14,34] The RAB27A p.R141_V142delinsI and p.Y159C variants have indicated that the α4-helix may interact with MUNC13-4.[13] Remarkably, our data indicates that the RAB27A p.R184Q variant binds MUNC13-4 significantly more strongly than RAB27A WT, with the affinity further increased by combination with the RAB27A p.T23N mutation predicted to mimic a

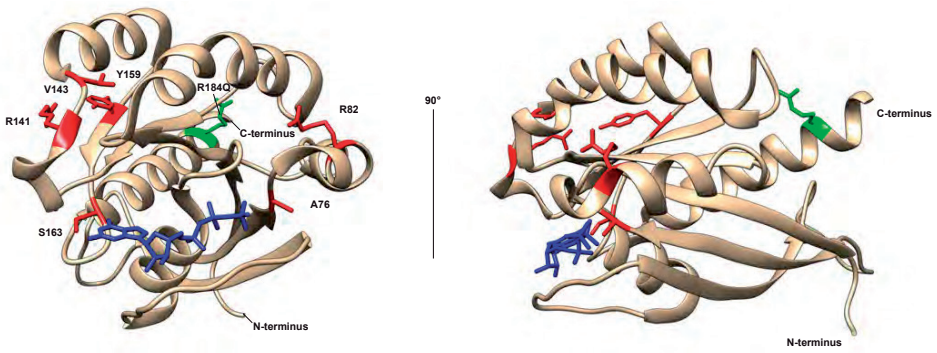

Figure 4 | Contribution of the RAB27A α5-helix to effector protein interactions.
Model of the RAB27A structure highlighting the R184 residue (green) located in α5-helix as well as other disease-causing RAB27A variants selectively associated with defective lymphocyte cytotoxicity but normal pigmentation that disrupt MUNC13-4 binding (red).[11,19,27] GTP is colored in blue.

GDP-bound inactive confirmation. MUNC13-4 was originally identified as an effector of GTP-bound RAB27A,[36] and active RAB27A p.Q78L bound MUNC13-4 more strongly than inactive RAB27A p.T23N in the NK cell line YTS.[37] In our experiments in transfected HEK-293FT cells, RAB27A p.Q78L displayed higher binding to MELANOPHILIN and SLP2A than to RAB27A p.T23N, as expected. However, surprisingly, MUNC13-4 displayed higher binding to RAB27A p.T23N than to RAB27A p.Q78L. The combination of the RAB27A p.T23N and p.R184Q variants leads to a dramatic increase in binding, suggesting that an inactive, patient-derived RAB27A variant may be exceedingly efficient at binding and potentially sequestering MUNC13-4. Our results warrant further studies into the interplay between RAB27A binding to effectors SLP2A versus MUNC13-4 in the context of nucleotide binding, and how the affinities of these interactions may determine the efficiency of cytotoxic granule exocytosis and lymphocyte cytotoxicity.

Our results show an inability of patient-derived RAB27A p.R184Q to rescue exocytosis by RAB27A-deficient CD8+ T cells. RAB27A is required for docking and priming of the cytotoxic granules via interactions with SLP2A and MUNC13-4.[10,11] It is not clear what may contribute the most to the patient phenotype, (i) reduced expression of RAB27A p.R184Q relative to RAB27A WT in lymphocytes, (ii) decreased binding to SLP2A, (iii) increased binding to MUNC13-4, or a combination of these three factors. In 293FT cells, RAB27A WT and p.R184Q were similarly expressed, while RAB27A p.R184Q displayed lower expression in primary human CD8+ T cells. These data suggest a reduced stability of the patient-derived RAB27A variant in a physiological setting. Still, the reduced level of RAB27A is unlikely to fully explain the severe reduction in lymphocyte exocytosis. Overexpression of a SLP2A Slp homology domain construct has revealed an important role for RAB27A–SLP family protein interactions for CD8+ T cell granule exocytosis.[11,17] In our biochemical experiments, the reduction of RAB27A binding to SLP2A was quite modest and may thus not explain the strong impairment in cytotoxic lymphocyte degranulation. Ménasché and colleagues demonstrated that overexpression of RAB27A p.Q78L in a CD8+ T cell line diminished granule exocytosis.[16] Thus, active RAB27A or strong RAB27A-MUNC13-4 interactions may result in decreased granule exocytosis and target cell killing. A priori, strong binding between RAB27A and MUNC13-4 leading to sequestration of MUNC13-4 might be expected to cause dominant forms of disease. The observations in this family, so far, do however not suggest a dominant mode of inheritance. Hopefully, identification of additional patients and families with this RAB27A variant can shed light on this important question. These results hopefully can spur further studies of the interaction of RAB27A with its distinct effectors.

Presenting at 35 years of age, to the best of our knowledge, this patient may represent the latest onset of GS2 reported to date.[35,38] Directions on clinical penetrance of the RAB27A c.551G>A p.(R184Q) variant are lacking in this late-onset HLH patient. The family encompassed eight siblings, six of which did not consent or were not available

to genetic testing. Further analyses of this family or other individuals homozygous for this variant that impairs RAB27A function in lymphocytes can hopefully provide further insights into the clinical penetrance. Nonetheless, the low cytotoxic T and NK cell exocytosis in the patient, the failure of the patient-derived RAB27A variant to reconstitute T cell exocytosis, and the degree of aberrant binding of RAB27A to MUNC13-4 suggest a significant impact of this variant on attenuating lymphocyte cytotoxicity and causing hyperinflammation. Similarly, autosomal loss-of-function PRF1 missense mutations that severely impair perforin expression and lymphocyte cytotoxicity have been associated with development of HLH.[39] Notably, the patient also carried rare homozygous VPS13B missense and heterozygous CARD11 missense variants of uncertain significance. The patient did however not display typical clinical features of Cohen syndrome associated with autosomal recessive VPS13B deficiency.[40] The clinical phenotype of heterozygous gain of function CARD11 variants causes BENTA, a disease with susceptibility to viral infections and occasionally HLH,[26] but CARD11 variants previously associated with BENTA have been localized to the N-terminal domains of the protein whereas the CARD11 variant in our patient was located at the C-terminus. Nonetheless, we cannot exclude that these variants in genes also expressed in immune cells might have modified disease in our patient.

In conclusion, our results indicate that the HLH patient–derived RAB27A p.R184Q variant maintains melanin distribution, yet displays dysregulated interactions with MUNC13-4 and SLP2A that impaired lymphocyte cytotoxicity. As such, this variant represents the first disease-associated RAB27A variant with increased MUNC13-4 binding. Together, these results suggest that the RAB27A p.R184Q variant can predispose to disease, potentially explain late-onset HLH in our patient, and advance insight into protein interactions causing pathophysiology. In addition, this case highlights the relevance of genetic testing in adults for relapsing HLH patients, especially when associated with a chronically active EBV infection or other immune anomalies. Further studies are warranted to develop rationale for targeted drug therapy.

SUPPLEMENTARY FILES

The supplementary files can be found online at
https://link.springer.com/article/10.1007/s10875-022-01315-4.

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REFERENCES

1. Ménasché G, Pastural E, Feldmann J, et al. Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. *Nat Genet*. 2000;25(2):173-176.
2. Van Gele M, Dynoodt P, Lambert J. Griscelli syndrome: a model system to study vesicular trafficking. *Pigment Cell Melanoma Res*. 2009;22(3):268-282.
3. de Saint Basile G, Ménasché G, Fischer A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nat Rev Immunol*. 2010;10(8):568-579.
4. Nagata K, Satoh T, Itoh H, et al. The ram: a novel low molecular weight GTP-binding protein cDNA from a rat megakaryocyte library. *FEBS Lett*. 1990;275(1-2):29-32.
5. Larijani B, Hume AN, Tarafder AK, Seabra MC. Multiple factors contribute to inefficient prenylation of Rab27a in Rab prenylation diseases. *J Biol Chem*. 2003;278(47):46798-46804.
6. Pereira-Leal JB, Seabra MC. The mammalian Rab family of small GTPases: definition of family and subfamily sequence motifs suggests a mechanism for functional specificity in the Ras superfamily. *J Mol Biol*. 2000;301(4):1077-1087.
7. Rak A, Pylypenko O, Niculae A, Pyatkov K, Goody RS, Alexandrov K. Structure of the Rab7:REP-1 complex: insights into the mechanism of Rab prenylation and choroideremia disease. *Cell*. 2004;117(6):749-760.
8. Boguski MS, McCormick F. Proteins regulating Ras and its relatives. *Nature*. 1993;366(6456):643-654.
9. Alzahofi N, Welz T, Robinson CL, et al. Rab27a co-ordinates actin-dependent transport by controlling organelle-associated motors and track assembly proteins. *Nat Commun*. 2020;11(1):3495.
10. Elstak ED, Neeft M, Nehme NT, et al. The munc13-4-rab27 complex is specifically required for tethering secretory lysosomes at the plasma membrane. *Blood*. 2011;118(6):1570-1578.
11. Holt O, Kanno E, Bossi G, et al. Slp1 and Slp2-a localize to the plasma membrane of CTL and contribute to secretion from the immunological synapse. *Traffic*. 2008;9(4):446-457.
12. Chavas LM, Ihara K, Kawasaki M, et al. Elucidation of Rab27 recruitment by its effectors: structure of Rab27a bound to Exophilin4/Slp2-a. *Structure*. 2008;16(10):1468-1477.
13. Cetica V, Hackmann Y, Grieve S, et al. Patients with Griscelli syndrome and normal pigmentation identify RAB27A mutations that selectively disrupt MUNC13-4 binding. *J Allergy Clin Immunol*. 2015;135(5):1310-1318 e1311.
14. Ohishi Y, Ammann S, Ziaee V, et al. Griscelli Syndrome Type 2 Sine Albinism: Unraveling Differential RAB27A Effector Engagement. *Front Immunol*. 2020;11:612977.
15. Chiang SC, Theorell J, Entesarian M, et al. Comparison of primary human cytotoxic T-cell and natural killer cell responses reveal similar molecular requirements for lytic granule exocytosis but differences in cytokine production. *Blood*. 2013;121(8):1345-1356.
16. Menasche G, Feldmann J, Houdusse A, et al. Biochemical and functional characterization of Rab27a mutations occurring in Griscelli syndrome patients. *Blood*. 2003;101(7):2736-2742.
17. Menasche G, Menager MM, Lefebvre JM, et al. A newly identified isoform of Slp2a associates with Rab27a in cytotoxic T cells and participates to cytotoxic granule secretion. *Blood*. 2008;112(13):5052-5062.
18. Hume AN, Tarafder AK, Ramalho JS, Sviderskaya EV, Seabra MC. A coiled-coil domain of melanophilin is essential for Myosin Va recruitment and melanosome transport in melanocytes. *Mol Biol Cell*. 2006;17(11):4720-4735.
19. Hume AN, Collinson LM, Hopkins CR, et al. The leaden gene product is required with Rab27a to recruit myosin Va to melanosomes in melanocytes. *Traffic*. 2002;3(3):193-202.

20. Henter JI, Horne A, Aricó M, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer*. 2007;48(2):124-131.
21. Karczewski KJ, Francioli LC, Tiao G, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature*. 2020;581(7809):434-443.
22. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet*. 2014;46(3):310-315.
23. Kukimoto-Niino M, Sakamoto A, Kanno E, et al. Structural basis for the exclusive specificity of Slac2-a/melanophilin for the Rab27 GTPases. *Structure*. 2008;16(10):1478-1490.
24. Kolehmainen J, Black GC, Saarinen A, et al. Cohen syndrome is caused by mutations in a novel gene, COH1, encoding a transmembrane protein with a presumed role in vesicle-mediated sorting and intracellular protein transport. *Am J Hum Genet*. 2003;72(6):1359-1369.
25. Norio R, Raitta C, Lindahl E. Further delineation of the Cohen syndrome; report on chorioretinal dystrophy, leukopenia and consanguinity. *Clin Genet*. 1984;25(1):1-14.
26. Lu HY, Biggs CM, Blanchard-Rohner G, Fung SY, Sharma M, Turvey SE. Germline CBM-opathies: From immunodeficiency to atopy. *J Allergy Clin Immunol*. 2019;143(5):1661-1673.
27. Meschede IP, Santos TO, Izidoro-Toledo TC, Gurgel-Gianetti J, Espreafico EM. Griscelli syndrome-type 2 in twin siblings: case report and update on RAB27A human mutations and gene structure. *Braz J Med Biol Res*. 2008;41(10):839-848.
28. Klein C, Philippe N, Le Deist F, et al. Partial albinism with immunodeficiency (Griscelli syndrome). *J Pediatr*. 1994;125(6 Pt 1):886-895.
29. Haddad EK, Wu X, Hammer JA, 3rd, Henkart PA. Defective granule exocytosis in Rab27a-deficient lymphocytes from Ashen mice. *J Cell Biol*. 2001;152(4):835-842.
30. Bryceson YT, Pende D, Maul-Pavicic A, et al. A prospective evaluation of degranulation assays in the rapid diagnosis of familial hemophagocytic syndromes. *Blood*. 2012;119(12):2754-2763.
31. Rudd E, Bryceson YT, Zheng C, et al. Spectrum, and clinical and functional implications of UNC13D mutations in familial haemophagocytic lymphohistiocytosis. *J Med Genet*. 2008;45(3):134-141.
32. Wilson SM, Yip R, Swing DA, et al. A mutation in Rab27a causes the vesicle transport defects observed in ashen mice. *Proc Natl Acad Sci U S A*. 2000;97(14):7933-7938.
33. Patiroglu T, Akar HH, Unal E, et al. PARTIAL OCULOCUTANEOUS ALBINISM AND IMMUNODEFICIENCY SYNDROMES: TEN YEARS EXPERIENCE FROM A SINGLE CENTER IN TURKEY. *Genet Couns*. 2016;27(1):67-76.
34. Netter P, Chan SK, Banerjee PP, et al. A novel Rab27a mutation binds melanophilin, but not Munc13-4, causing immunodeficiency without albinism. *J Allergy Clin Immunol*. 2016;138(2):599-601 e593.
35. Tesi B, Rascon J, Chiang SCC, et al. A RAB27A 5' untranslated region structural variant associated with late-onset hemophagocytic lymphohistiocytosis and normal pigmentation. *J Allergy Clin Immunol*. 2018;142(1):317-321 e318.
36. Shirakawa R, Higashi T, Tabuchi A, et al. Munc13-4 is a GTP-Rab27-binding protein regulating dense core granule secretion in platelets. *J Biol Chem*. 2004;279(11):10730-10737.
37. Menager MM, Menasche G, Romao M, et al. Secretory cytotoxic granule maturation and exocytosis require the effector protein hMunc13-4. *Nat Immunol*. 2007;8(3):257-267.
38. Sepulveda FE, Debeurme F, Menasche G, et al. Distinct severity of HLH in both human and murine mutants with complete loss of cytotoxic effector PRF1, RAB27A, and STX11. *Blood*. 2013;121(4):595-603.

39. Chia J, Yeo KP, Whisstock JC, Dunstone MA, Trapani JA, Voskoboinik I. Temperature sensitivity of human perforin mutants unmasks subtotal loss of cytotoxicity, delayed FHL, and a predisposition to cancer. *Proc Natl Acad Sci U S A*. 2009;106(24):9809-9814.
40. Kivitie-Kallio S, Norio R. Cohen syndrome: essential features, natural history, and heterogeneity. *Am J Med Genet*. 2001;102(2):125-135.

VII

Hemophagocytic lymphohistiocytosis in activated PI3K delta syndrome, an illustrative case report

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TO THE EDITOR,

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening condition characterized by a dysregulated immune system. HLH is driven by impaired antigen clearance that leads to uncontrolled immune activation.[1] In primary HLH, a genetic mutation impairs the cytotoxic function of natural killer (NK) cells and cytotoxic T-cells, resulting in persistent antigen presentation and dysregulation of histiocyte activation. In secondary HLH (sHLH), unknown or non-genetic triggers result in a state of hyperinflammation. The most common triggers associated with HLH include infections, such as Epstein–Barr virus (EBV), hematological malignancies, and autoimmune diseases.

Activated PI3K delta syndrome (APDS) is a primary immunodeficiency (PID) caused by a gain-of-function mutation in *PIK3CD*. As a result of the of the PI3K/AKT/mTOR pathway over-activation, T and B cell senescence is induced.[2-4] APDS is characterized by recurrent sinopulmonary infections, non-malignant lymphoproliferation, (persistent) viral infections, autoimmunity and increased risk of lymphomas.[5]

In APDS patients, HLH might be expected. However, it is only scarcely reported. Here, we describe a young adult with APDS who developed persistent EBV-related HLH. We subsequently discuss the association between HLH and APDS.

Case Report

A 19-year-old man of Indian descent was referred for the evaluation of chronic active EBV disease. The patient's medical history revealed congenital stenosis of the left bronchus, recurrent sinopulmonary infections, and autism spectrum disorder with an IQ of 80–86. EBV seroconversion was identified at 7 years of age when the patient presented idiopathic abdominal lymphadenopathy. Furthermore, a previous laboratory examination revealed IgA deficiency and low IgG2 and IgG4 levels without an overarching diagnosis. Four months before presentation, the patient developed fatigue, fever, night sweats, hepatosplenomegaly, anemia, hepatitis, weight loss of 10 kg and lymphadenopathy (Figure 1). Serum EBV copy numbers were repeatedly >1,500/mL. Histology of the three lymph nodes did not reveal any evidence of malignant lymphoma, but rather a markedly positive EBV-encoded RNA (EBER). Chronically active EBV was diagnosed, and rituximab therapy of 500mg weekly during 4 weeks was initiated. In the workup of PID, whole exome sequencing (confirmed by Sanger sequencing) revealed a heterozygous c.3074A>C, p.Glu1025Gly mutation in the *PIK3CD* gene (NM_005026.3), which is associated with APDS (Figure 2).[6] Neither parent carried this mutation, suggesting a *de novo* origin.

A stable condition without any additional immunosuppressive drugs was achieved for 3 months after which he developed high spiking fever, pneumonia, progressive lymphadenopathy, severe unexplained cardiomyopathy with progressive anemia,

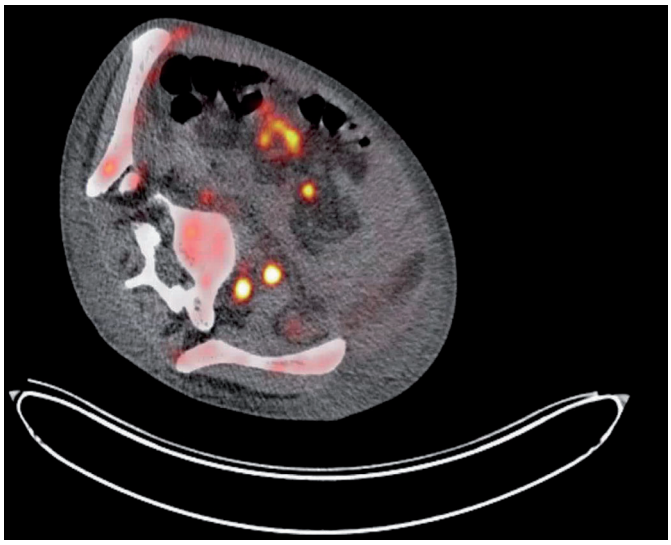

Figure 1 | High glucose metabolism in 18F-fluorodeoxyglucose positron emission tomography positive lymph nodes.

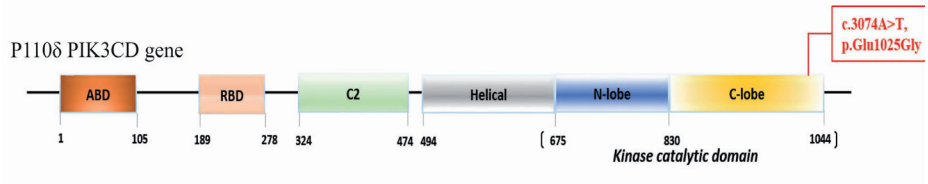

Figure 2 | Heterozygous c.3074A>C, p.Glu1025Gly mutation in PIK3CD identified in the patient.
Linear representation of P110δ(PIK3CD) protein domains. The mutation identified in the patient is shown in red.
Abbreviations: ABD, adaptor-binding domain; RBD, Ras-binding domain; C2, putative membrane-binding domain.

thrombocytopenia, and hyperferritinemia (Table 1). Malignant lymphoma was excluded and hemophagocytosis was detected in the bone marrow (Figure 3A, B). The patient therefore fulfilled six out of eight diagnostic HLH-04 criteria (Table 2).[7] His clinical status quickly deteriorated, and he developed acute respiratory distress syndrome and multiple organ failure. Salvage therapy was initiated with high-dose steroids, sirolimus, and intravenous immunoglobulin. PI3K delta inhibitors were withheld due to heart and respiratory failure. Etoposide was withheld due to leucopenia and severe sepsis with systemic infection (hospital acquired pneumonia with H. influenza, not responding to treatment). Within 1 month, the patient died from cardiac failure with refractory pulmonary edema.

HLH in APDS

APDS is caused by a heterozygous gain-of-function mutation in *PIK3CD*. This mutation is known to activate leucocyte development, pushing immune cells towards differentiation

Table 1 | Laboratory investigations.

Laboratory investigations	At admission	At time of clinical deterioration	Reference
Hemoglobulin (g/dL)	6.28	5.8	13.9–16.9
Leukocytes (x10^9/L)	4.4	5.0	3.5–10
Neutrophils (x10^9/L)	2.5	3.84	1.4–8.0
Platelets (x10^9/L)	177	24	150–370
CRP (mg/L)	190	321	< 10
ASAT (U/L)	40	312	< 35
ALAT (U/L)	19	46	< 45
Lactate dehydrogenase (U/L)	263	798	< 248
Gamma-GT (U/L)	230	391	< 55
Alkaline phosphatase (U/L)	918	1301	< 115
Total bilirubin (µmol/L)	26	66	< 17
NT-pro-BNP (pmol/L)		1028	< 15
IgA (g/L)	0.76		0.76–3.9
IgG (g/L)	5.70		7.0–16.0
IgM (g/L)	1.24		0.45–2.30
IgG1 (g/L)	4.67		4.9–11.4
IgG2 (g/L)	0.62		1.5–6.4
IgG3 (g/L)	0.32		0.2–1.1
IgG4 (g/L)	< 0.08		0.08–1.4
Ferritin (µg/L)	1985	10364	30 – 240
sIL2R (pg/mL)	>110000	>110000	< 2,500
Triglycerides (mmol/L)	2.41	6.58	< 2
EBV DNA (IU/mL)	2.54E4/P	1.17E6/P	

Abbreviations: CRP: C-reactive protein, ASAT: aspartate aminotransferase, ALAT: alanine aminotransferase, Gamma-GT: Gamma-glutamyltransferase, NT-pro-BNP: N-terminal prohormone of brain natriuretic peptide, Ig: Immunoglobulin, sIL2R: soluble interleukin-2 receptor

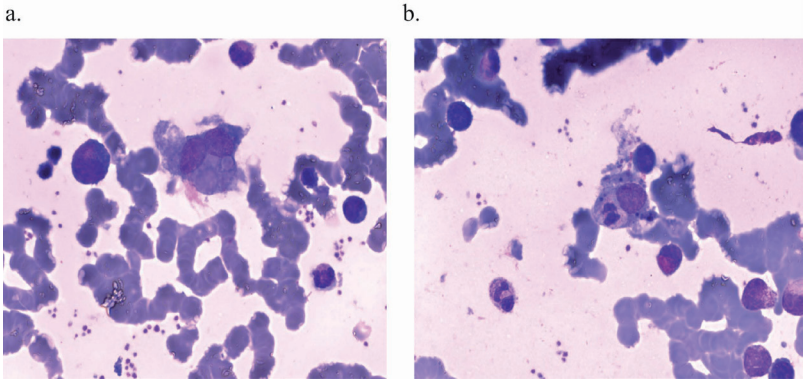

Figure 3 | Bone marrow biopsies from the patient with mutated *PIK3CD*.
A. Hematoxylin and eosin (H&E) staining shows the hemophagocytosis of erythroid cells by a macrophage. B. May–Grünwald–Giemsa staining shows hemophagocytosis of a polynuclear granulocyte by a macrophage.

Table 2 | HLH criteria.

HLH-04 criteria [7]	Patient finding
Fever	Present
Splenomegaly	Present
Cytopenias (affecting ≥2 of 3 lineages in the peripheral blood) *Hemoglobin <90g/L* *Platelets <100 x10^9/L* *Neutrophils <1.0 x10^9/L*	Present *58 g/L* *24 x10^9/L* *3.84 x10^9/L*
Hypertriglyceridemia and/or hypofibrinogenaemia *Fasting triglycerides ≥3.0 mmol/L* *Fibrinogen ≤1.5 g/L*	Not present *6.58 mmol/L* *Not done*
Hemophagocytosis in bone marrow or spleen or lymph nodes	Present
Low or absent NK-cell activity	Not done
Ferritin ≥500 µg/L	Present (10364 µg/L)
Soluble IL-2 receptor ≥2,400 U/ml	Present (>110000 pg/mL)

and exhaustion by raising $CD8^+CD57^+$ and $CD8^+$ effector memory cells.[2,3] This is indicative of a defective immune response that is incapable of effective elimination of pathogens. Persisting antigen presentation can then evoke HLH. Especially in the case of persistent EBV, a virus that is already able to trigger HLH, APDS patients are theoretically more prone to develop hyperinflammation and thus HLH.[8] Although chronically active EBV and EBV-associated lymphomas are common in APDS, HLH has only been reported in two APDS patients, who are summarized in Table 3.[9,10] Both patients were children at the time of APDS diagnosis, which suggests that genetic diagnosis is more often considered in pediatric HLH. Because a high index of suspicion is necessary to identify both HLH and APDS, underdiagnosis is likely.

This case emphasizes the importance of genetic testing for PIDs (including HLH genes) in patients with the first episode of HLH, regardless of age. An increasing number of genetic mutations in older patients with HLH have been reported.[11] In particular, a medical history of recurrent infections, autoimmunity, developmental delay, and EBV-related HLH in young adults should indicate PIDs. Correct genetic diagnosis enables personalized treatment and thereby improving survival. Appropriate treatment of APDS patients developing HLH is a great therapeutic challenge. In our opinion the mainstay of treatment should be appropriate management of APDS, such as hematopoietic stem cell transplantation or mTOR inhibitors, especially the new promising PI3K inhibitor leniolisib. By doing so, the patient is less susceptible to developing HLH. If HLH arises, an additional trigger (e.g. infectious or malignant) should be sought thoroughly and treated rigorously. In severe cases, HLH directed therapy could be considered to halt the hyperinflammation. This therapy should consist of corticosteroids, with the addition of etoposide in life threatening cases.[12] This case illustrates that earlier diagnosis of APDS

Table 3 | Previously published cases on HLH in APDS patients.

Characteristic	Patient 1 [9]	Patient 2 [10]
Sex	Male	Male
Age at the time of APDS diagnosis	12 years	2 years
Age at the time of HLH onset	12 years	5 months
Trigger of HLH	Idiopathic	Hodgkin lymphoma
Presentation	Diffuse lymphadenopathy, hepatosplenomegaly, arthritis, rash	Diffuse lymphadenopathy, hepatosplenomegaly, autoimmune hemolytic anemia.
PIK3D mutation	c.323C>G p.R108L	c.3061G>A p.E1021K
Concurrent mutation	None noted	PRF1 p.L236F
Functional NK and T-cell test	Normal	Normal function. Lower (but not deficient) perforin levels in NK cells.
White blood cell count ($x10^3/mm^3$)	6.14 (4.0–10.8)	9.08
Neutrophils ($x10^3/mm^3$)	4.58 (1.5–8.0)	2.39
Lymphocytes ($x10^3/mm^3$)	1.21 (0.9–4.0)	4.89
IgG (mg/dL)	2119 (700–1600)	426 (270–792)
IgA (mg/dL)	207 (70–400)	47 (5.8–58)
IgM (mg/dL)	127 (40–230)	258 (18.4–145)

could have resulted in more adequate monitoring and targeted treatment before the occurrence of multi-organ failure.

CORRECTION TO: HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS IN ACTIVATED PI3K DELTA SYNDROME, AN ILLUSTRATIVE CASE REPORT

The original version of this article contains an error in the nucleotide change of the patients mutation. The correct nucleotide change is a heterozygous c.3074A>G mutation in the *PIK3CD* gene (NM_005026.3). This was incorrectly stated in the case description, Figure 2 and in the title of Figure 2. Although the nucleotide change was stated incorrectly, the subsequent amino acid change (p.Glu1025Gly) was correct throughout the text. Therefore, our further findings and conclusions are unaffected.

REFERENCES

1. Jordan MB, Hildeman D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. *Blood*. 2004;104(3):735-743.
2. Lucas CL, Kuehn HS, Zhao F, et al. Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110delta result in T cell senescence and human immunodeficiency. *Nat Immunol*. 2014;15(1):88-97.
3. Wentink MWJ, Mueller YM, Dalm V, et al. Exhaustion of the CD8(+) T Cell Compartment in Patients with Mutations in Phosphoinositide 3-Kinase Delta. *Front Immunol*. 2018;9:446.
4. Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. *Nat Rev Immunol*. 2003;3(4):317-330.
5. Coulter TI, Chandra A, Bacon CM, et al. Clinical spectrum and features of activated phosphoinositide 3-kinase delta syndrome: A large patient cohort study. *J Allergy Clin Immunol*. 2017;139(2):597-606 e594.
6. Dulau Florea AE, Braylan RC, Schafernak KT, et al. Abnormal B-cell maturation in the bone marrow of patients with germline mutations in PIK3CD. *J Allergy Clin Immunol*. 2017;139(3):1032-1035 e1036.
7. Henter JI, Horne A, Arico M, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer*. 2007;48(2):124-131.
8. Ramos-Casals M, Brito-Zeron P, Lopez-Guillermo A, Khamashta MA, Bosch X. Adult haemophagocytic syndrome. *Lancet*. 2014;383(9927):1503-1516.
9. Tessarin G, Rossi S, Baronio M, et al. Activated Phosphoinositide 3-Kinase Delta Syndrome 1: Clinical and Immunological Data from an Italian Cohort of Patients. *J Clin Med*. 2020;9(10).
10. Cansever M, Zietara N, Chiang SCC, et al. A Rare Case of Activated Phosphoinositide 3-Kinase Delta Syndrome (APDS) Presenting With Hemophagocytosis Complicated With Hodgkin Lymphoma. *J Pediatr Hematol Oncol*. 2020;42(2):156-159.
11. Miao Y, Zhu HY, Qiao C, et al. Pathogenic Gene Mutations or Variants Identified by Targeted Gene Sequencing in Adults With Hemophagocytic Lymphohistiocytosis. *Front Immunol*. 2019;10:395.
12. La Rosee P, Horne A, Hines M, et al. Recommendations for the management of hemophagocytic lymphohistiocytosis in adults. *Blood*. 2019;133(23):2465-2477.

VIII

The role of etoposide in the treatment of adult patients with hemophagocytic lymphohistiocytosis

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Experimental hematology & oncology 2023 Jan 9;12(1):2

ABSTRACT

Hemophagocytic lymphohistiocytosis (HLH) is a potentially fatal inflammatory clinical condition, in which an exaggerated immune response is ineffectively regulated. Although etoposide-containing regimens are generally recommended for children with HLH, the exact role of etoposide in the adult setting remains unclear. We performed a systematic review of the literature on the use of etoposide in adults with HLH. All articles written in English on the use of etoposide in adults with HLH published on or before July 2021 and available in seven databases were analyzed. Thirteen studies were found to be relevant to the search results. Ten of these studies report a statistical analysis on the effect of etoposide, of which five found etoposide-containing regimens superior to non-etoposide-containing regimens. Seven studies provided sufficient data to be included in the meta-analysis. For these data, the estimated logit relative risk of etoposide on survival was 1.06 (95% confidence interval: 0.92–1.21, standard error: 2.06). The pooled data of the meta-analysis did thus not support a beneficial effect of etoposide. It should be taken into account that the presented results are highly susceptible to bias and that the effect of etoposide differs between HLH-triggers. Although the meta-analysis does not support the effect of etoposide, we do not recommend abandoning etoposide as treatment modality. The limitations of the meta-analysis, together with several individual articles confirming the benefit of etoposide, justify etoposide for select cases in adults with HLH such as refractory or severe disease with (threatening) multiorgan failure.

TO THE EDITOR,

HLH is a severe and life-threatening immunological dysregulation either caused by genetic mutation (familial HLH; FHL) or secondary to various triggers (secondary HLH; sHLH). The estimated incidence of FHL is 1 to 1.5 per million children per year.[1]

Although etoposide-containing regimens are generally recommended for children with HLH, the exact role of etoposide in the adult patient remains unclear. The treatment strategy in adult patients with sHLH, as recommended by the interdisciplinary working group on adult HLH of the Histiocyte Society, does include etoposide in its treatment strategy.[2] Following this recommendation, etoposide could be considered for sHLH with all underlying triggers, although its use in auto-immune and immunotherapy associated HLH is more restricted.[2] However, evidence to support the use of etoposide in adult sHLH patients is scarce. Therefore we performed a systematic literature review and meta-analysis on the clinical use and effectiveness of etoposide in adult HLH patients. A detailed description of the methods including the search strategy is available in the supplemental material.

The seven studies that are included in the meta-analysis (Table 1) show an estimated logit relative risk (RR_L) of 1.06 (standard error: 2.06; 95% CI: 0.92–1.21) (Figure 1). The survival probability of the etoposide-treated patients did thus not differ significantly from the survival probability of the non-etoposide-treated patients. As detailed in the supplemental data, the homogeneity was not rejected. Five individual studies show an analysis that is significantly in favor of etoposide [3-7] whereas five other papers report no additional benefit of etoposide [8-12] (Table 1). Similar to a study by Imashuku *et al*.,[13] Song *et al*.[6] also analyzed patients receiving etoposide within 4 weeks after diagnosis and compared this group with a group of patients receiving etoposide 4 weeks after diagnosis or who did not receive etoposide. No significant difference was observed in survival between the two groups ($p = 0.163$).

The presented results should be interpreted with caution. All studies concern retrospective cohort studies and used different statistical methods. In our meta-analysis we used the risk ratio for addressing the outcome. Due to a lack of provided data by the articles, we could not use a more suitable time-to-event measure such as a hazard ratio. Moreover, there is a high risk of bias in all studies (Table 1). In particular, confounding by indication should be noted since patients receiving etoposide generally concern more severe cases and consequently have a prior survival probability which is lower. As the confounding by indication is in favor of non-etoposide-treated patients, a stronger benefit of etoposide than the calculated effect size could be assumed.

The seven studies included in the meta-analysis were homogeneous based on the findings of the χ^2 homogeneity test (supplemental material). However, within individual groups (*i.e.* etoposide and non-etoposide-treated), a high degree of heterogeneity

Table 1 | Articles reporting the effect of etoposide in adults with hemophagocytic lymphohistiocytosis.

Article	Trigger	Total number of adults	Inclusion in meta-analysis	Survival of etoposide-treated patients, % (n)	Survival of non-etoposide-treated patients, % (n)	Additional information	Supporting the effect of etoposide	Risk of bias according to ROBINS-I
Song *et al.*, 2019 [14]	Pregnancy	13	Yes	100% (6)	71% (5)		NS	Critical
Knaak *et al.*, 2020 [15]	Various	40	Yes	14% (1)	45% (15)		NS	Critical
Naymagon *et al.*, 2021 [12]	Various	90	Yes	21% (9)	33% (16)	Log-rank test for difference in the survival distribution (p = 0.41)	No	Critical
Diack *et al.*, 2020 [8]	Various	26	Yes	29% (2)	26% (5)	p = 0.9	No	Critical
Ahn *et al.*, 2010 [16]	Various	26	Yes	31% (4)	69% (9)		NS	Critical
Barba *et al.*, 2015 [9]	Various	71	Yes	54% (15)	67% (29)	p = 0.3	No	Critical
Arca *et al.*, 2015 [3]	Various	162	Yes	85% (69)	74% (60)	p = 0.079, aOR: 0.21, p = 0.04	Yes	Serious
Bigenwald *et al.*, 2018 [4]	Malignancy	71	No			uHR: 0.55 (p = 0.04), aHR: 0.50 (p = 0.04)	Yes	Critical
Bubik *et al.*, 2020 [5]	Various	31	No			HR: 0.22 for ≥5 doses of etoposide (p = 0.003)	Yes	Critical
Li *et al.*, 2020 [7]	B-cell lymphoma	31	No			Log-rank test for difference in survival distribution (p = 0.0183)	Yes	Critical
Song *et al.*, 2019 [6]	EBV	58	No			Etoposide as 1st line therapy vs no etoposide or 2nd line therapy (p = <0.001)	Yes	Critical
Buyse *et al.*, 2010 [10]	Various	56	No			EIT for non-survivors 6 h vs survivors 4 h (p = 0.19)	No	Serious
Schram *et al.*, 2015 [11]	Various	68	No			OS etoposide: 9.5 months, OS no etoposide: 1.9 months (p = 0.78)	No	Critical

Abbreviations: aHR, adjusted hazard ratio; aOR, adjusted odds ratio; EBV, Epstein–Barr virus; EIT, etoposide initiation time (time from intensive care unit admission to etoposide initiation); HR, hazard ratio; NS, not stated; OS, overall survival; uHR, unadjusted hazard ratio

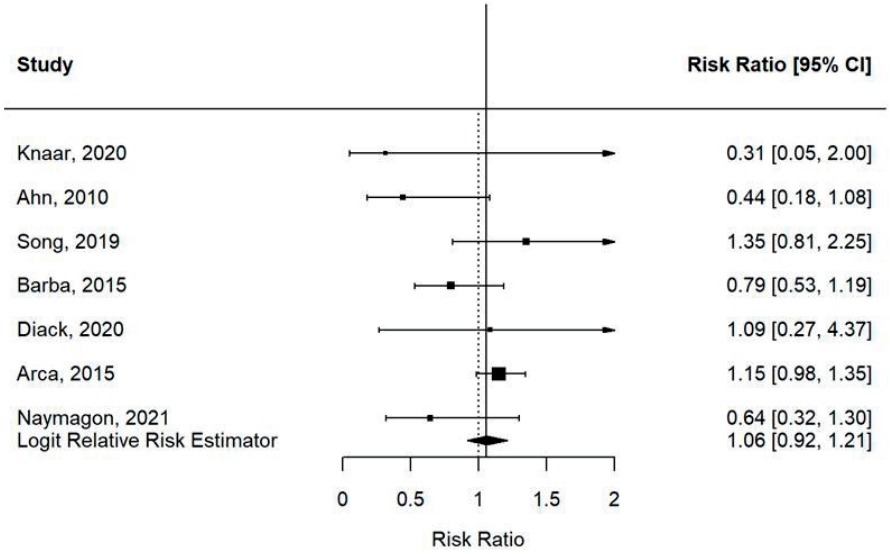


Figure 1 | The estimated relative risks of seven studies, which provided data on an etoposide-treated group and a non-etoposide-treated group.
The black vertical line represents the logit relative risk estimator. Abbreviation: CI, confidence interval

is assumed to be present. For example, the studies included patients with diverse etiological HLH triggers, all having a different a priori survival rate.[17] Etoposide may have a different effect among patients with these different etiological triggers. Moreover, several confounders are assumed to effect outcome and should ideally be taken into account. Therefore, it is highly favorable to perform an alternative/additional analysis taking (baseline) confounders into account. In this regard, it would be of particular interest to sub-analyze groups by HLH trigger, since our data suggests that etoposide might be especially beneficial in EBV and lymphoma associated HLH (Table 1).[4,6,7] Owing to the lack of data, we were unable to perform such analysis. However, assuming an equal degree of heterogeneity among the groups (*i.e.* etoposide and non-etoposide-treated), the data will be averaged out and will thus bring forward a pooled data set that might be compared, although with caution. Given the available data, we believe that this approach is the best available method to address the research question but we also emphasize its limitation.

It is important to note that the studies included in the meta-analysis primarily concern studies that do not present data that support the effect of etoposide (one out of seven studies showing benefit, Table 1). On the contrary, the studies that are not included in the meta-analysis primarily concern studies that do show a benefit of etoposide (four out of seven studies showing benefit, Table 1). Only taking the meta-analysis into account might thus underestimate the effect of etoposide.

The data presented by the meta-analysis should not lead to abandoning etoposide as a treatment modality. The limitations of the meta-analysis that generally lead to an underestimation of the effect size of etoposide, together with several individual articles confirming the benefit of etoposide, justify etoposide for individualized cases of adult HLH. These data support the recent management recommendations by the interdisciplinary working group on adult HLH of the histiocyte society.[2] According to this guideline, it is proposed to initiate a monitored step-up approach starting with corticosteroids and IVIG, especially in patients with mild or moderate disease. Etoposide can be considered for individualized treatment of cases of refractory or severe disease with (threatening) multiorgan failure.

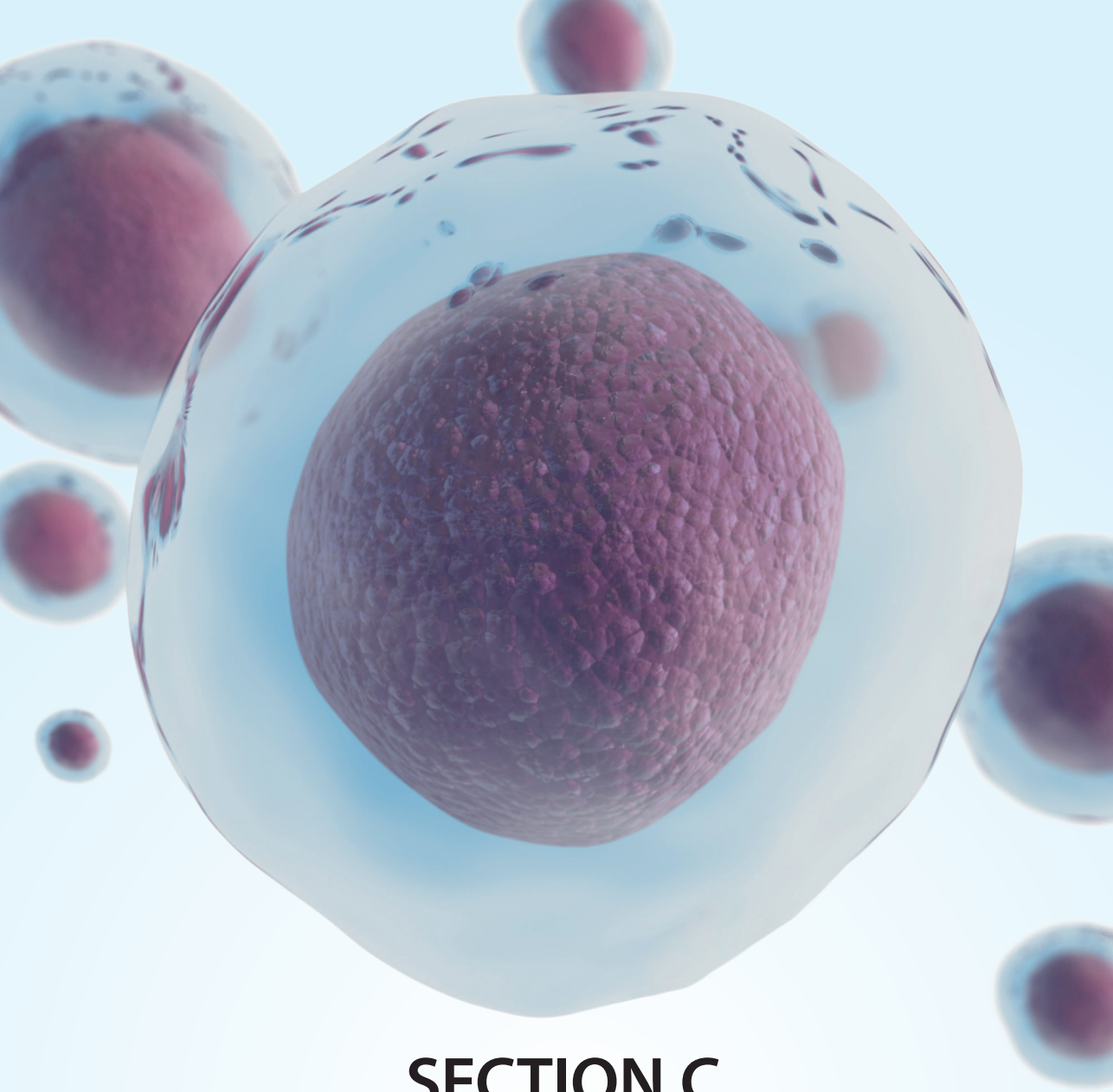
Conclusive studies on etoposide as a treatment modality in adults are not available. To make definitive conclusions on etoposide and its timely administration, a collaboration between HLH treatment centers is needed to initiate a prospective randomized controlled trial. Currently, no definite evidence is available to guide which HLH patients may benefit from etoposide. Thus, etoposide should be administered after careful consideration.

SUPPLEMENTARY FILES

The supplementary files can be found online at
https://ehoonline.biomedcentral.com/articles/10.1186/s40164-022-00362-2.

REFERENCES

1. Meeths M, Horne A, Sabel M, Bryceson YT, Henter JI. Incidence and clinical presentation of primary hemophagocytic lymphohistiocytosis in Sweden. *Pediatric Blood and Cancer*. 2015;62(2):346-352.
2. La Rosée P, Horne A, Hines M, et al. Recommendations for the management of hemophagocytic lymphohistiocytosis in adults. *Blood*. 2019;133(23):2465-2477.
3. Arca M, Fardet L, Galicier L, et al. Prognostic factors of early death in a cohort of 162 adult haemophagocytic syndrome: impact of triggering disease and early treatment with etoposide. *Br J Haematol*. 2015;168(1):63-68.
4. Bigenwald C, Fardet L, Coppo P, et al. A comprehensive analysis of Lymphoma-associated haemophagocytic syndrome in a large French multicentre cohort detects some clues to improve prognosis. *Br J Haematol*. 2018;183(1):68-75.
5. Bubik RJ, Barth DM, Hook C, et al. Clinical outcomes of adults with hemophagocytic lymphohistiocytosis treated with the HLH-04 protocol: a retrospective analysis. *Leuk Lymphoma*. 2020;61(7):1592-1600.
6. Song Y, Wang Y, Wang Z. Requirement for etoposide in the initial treatment of Epstein-Barr virus-associated haemophagocytic lymphohistiocytosis. *Br J Haematol*. 2019;186(5):717-723.
7. Li B, Guo J, Li T, et al. Clinical characteristics of hemophagocytic lymphohistiocytosis associated with non-hodgkin B-cell lymphoma: a multicenter retrospective study. *Clin Lymphoma Myeloma Leuk*. 2021;21(2):e198-e205.
8. Diack ND, Kane BS, Fall S, et al. Adult hemophagocytic lymphohistiocytosis in Sub-Saharan area: A retrospective study of 26 cases. *Cureus*. 2020;12(3):e7258.
9. Barba T, Maucort-Boulch D, Iwaz J, et al. Hemophagocytic lymphohistiocytosis in intensive care unit: a 71-case strobe-compliant retrospective study. *Medicine (Baltim)*. 2015;94(51):e2318.
10. Buyse S, Teixeira L, Galicier L, et al. Critical care management of patients with hemophagocytic lymphohistiocytosis. *Intensive Care Med*. 2010;36(10):1695-1702.
11. Schram AM, Comstock P, Campo M, et al. Haemophagocytic lymphohistiocytosis in adults: a multicentre case series over 7 years. *Br J Haematol*. 2016;172(3):412-419.
12. Naymagon L, Tremblay D, Mascarenhas J. The efficacy of etoposide-based therapy in adult secondary hemophagocytic lymphohistiocytosis. *Acta Haematol*. 2021;144(5):560-568.
13. Imashuku S, Kuriyama K, Sakai R, et al. Treatment of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis (EBV-HLH) in young adults: a report from the HLH study center. *Med Pediatr Oncol*. 2003;41(2):103-109.
14. Song Y, Wang Z, Hao Z, et al. Requirement for etoposide in the treatment of pregnancy related hemophagocytic lymphohistiocytosis: a multicenter retrospective study. *Orphanet J Rare Dis*. 2019;14(1):50.
15. Knaak C, Schuster FS, Spies C, et al. Hemophagocytic Lymphohistiocytosis in critically ill patients. *Shock*. 2020;53(6):701-709.
16. Ahn JS, Rew SY, Shin MG, et al. Clinical significance of clonality and Epstein-Barr virus infection in adult patients with hemophagocytic lymphohistiocytosis. *Am J Hematol*. 2010;85(9):719-722.
17. Ramos-Casals M, Brito-Zerón P, López-Guillermo A, Khamashta MA, Bosch X. Adult haemophagocytic syndrome. *Lancet*. 2014;383(9927):1503-1516.



SECTION C

LANGERHANS CELL HISTIOCYTOSIS

IX

Clinicogenomic associations in childhood Langerhans cell histiocytosis: an international cohort study

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ABSTRACT

Langerhans cell histiocytosis (LCH) is a rare neoplastic disorder caused by somatic genetic alterations in hematopoietic precursor cells differentiating into $CD1a^{+}/CD207^{+}$ histiocytes. LCH clinical manifestation is highly heterogeneous. *BRAF* and *MAP2K1* mutations account for approximately 80% of genetic driver alterations in neoplastic LCH-cells. However, their clinical associations remain incompletely understood. Here, we present an international clinicogenomic study of childhood LCH, investigating 377 patients genotyped for at least $BRAF^{V600E}$. MAPK pathway gene alterations were detected in 300 (79.6%) patients, including 191 (50.7%) with $BRAF^{V600E}$, 54 with *MAP2K1* mutations, 39 with *BRAF* exon 12 mutations, 13 with rare *BRAF* alterations, and 3 with *ARAF* or *KRAS* mutations. Our results confirm that $BRAF^{V600E}$ associates with lower age at diagnosis and higher prevalence of multisystem LCH, high-risk disease, and skin involvement. Furthermore, $BRAF^{V600E}$ appeared to correlate with a higher prevalence of central nervous system (CNS)-risk bone lesions. In contrast, *MAP2K1* mutations associated with a higher prevalence of SS-bone LCH, and *BRAF* exon 12 deletions seemed to correlate with more lung involvement. Although $BRAF^{V600E}$ correlated with reduced event-free survival (EFS) in the overall cohort, neither *BRAF* nor *MAP2K1* mutations associated with EFS when patients were stratified by disease extent. Thus, the correlation of $BRAF^{V600E}$ with inferior clinical outcome is (primarily) driven by its association with disease extents known for high rates of progression or relapse, including multisystem LCH. These findings advance our understanding of factors underlying the remarkable clinical heterogeneity of LCH, but also question the independent prognostic value of lesional $BRAF^{V600E}$ status.

INTRODUCTION

Langerhans cell histiocytosis (LCH) is a rare hematologic neoplasm characterized by the accumulation of myeloid-differentiated cells with characteristic CD1a and CD207 expression in various tissues and organs[1–3]. The disease primarily affects children, with an incidence similar to pediatric Hodgkin lymphoma[4]. LCH clinical manifestation is highly heterogeneous, ranging from self-healing skin lesions or a solitary bone lesion to a life-threatening disease involving multiple organ systems (Supplementary Figure 1)[5]. Disease extent is an established prognostic factor in pediatric LCH[6–8]. The most severe clinical form of the disease tends to affect young children (age < 2 years) and typically involves risk organs (RO), including the hematopoietic system, liver and spleen[9,10]. LCH with RO involvement is often refractory to chemotherapy and associated with an increased risk of death; therefore, it is called "high-risk LCH"[11–13]. Although overall mortality of LCH patients is low, a significant proportion of them experiences disease relapses[14] and/or develops permanent consequences, such as diabetes insipidus (DI) or neurodegenerative (ND)-LCH. Currently, the biological mechanisms underlying the heterogeneous clinical presentation and outcome of LCH remain incompletely understood.

In 2010, Rollins and colleagues discovered recurrent somatic *BRAF*^{V600E} mutations in LCH[15]. Subsequently, other groups confirmed the presence of *BRAF*^{V600E} mutations in approximately 50-60% of LCH-patients[10,16–20], and identified alternative MAPK pathway activating genetic alterations in patients without *BRAF*^{V600E} (Supplementary Figure 2)[17,21–28]. Most notably, somatic mutations in *MAP2K1* exon 2 or 3 and small insertions and/or deletions (indels) in *BRAF* exon 12 were recurrently detected[17,22–25]. Together, *BRAF* and *MAP2K1* mutations seem to account for approximately 80% of genetic driver alterations in pediatric LCH[29]. *BRAF*^{V600E}, *MAP2K1* and *BRAF* exon 12 mutations are essentially mutually exclusive[22], with only very rare cases having co-occurring mutations[30,31].

In 2016, Héritier *et al.* reported that *BRAF*^{V600E} associated with high-risk disease and increased rates of first-line therapy resistance and relapse in 315 pediatric LCH patients[10], including 173 children (54.6%) with *BRAF*^{V600E}. Since then, no study has been published with similar or more LCH patients genotyped for *BRAF*^{V600E}. Hence, their observations still need to be duly confirmed. Moreover, large cohort studies with molecular data beyond *BRAF*^{V600E} are needed to determine the clinical impact of recurrent mutations in *MAP2K1* or *BRAF* exon 12, which remain largely unknown[29]. Accordingly, here we describe an international clinicogenomic study of childhood LCH, investigating 377 patients genotyped for at least *BRAF*^{V600E}, including 300 (79.6%) patients with a detected MAPK pathway gene alteration.

MATERIALS AND METHODS

Study design

We performed an international observational cohort study of clinical associations of somatic genetic alterations in pediatric LCH. Patients were included between 2014 and 2021 in a retrospective cohort study of LCH patients in four academic children's hospitals in The Netherlands and Canada, or they were enrolled between 2013 and 2021 in the prospective LCH-IV clinical study (NCT02205762/EudraCT 2011-001699-20) in The Netherlands, Italy, Austria, Czech Republic, Sweden or Denmark. The study was performed in accordance with the Declaration of Helsinki, and ethical approval or a waiver of consent for retrospective research was obtained from the Institutional Review Boards at participating institutions. Written informed consent was obtained from patients and/or their legal representatives when required. Inclusion of patients from the LCH-IV study was approved by the Data Safety Monitoring Committee. Inclusion stopped April 30, 2021.

Patient selection and data collection

Patients were identified through registries of involved institutions. Eligibility criteria were (i) a definitive diagnosis of LCH, (ii) age at LCH diagnosis below eighteen years, and (iiia) available LCH-lesional *BRAF*^{V600E} status by PCR or sequencing through routine molecular diagnostics or (iiib) availability of formalin-fixed paraffin-embedded (FFPE) LCH tissue for molecular analysis in the specific context of this study. Diagnosis of LCH was confirmed by a combination of clinical findings and the presence of CD1a^{+} histiocytes in the lesional tissue sample(s). For patients with isolated skin disease, LCH diagnosis also required confirmation of CD207 co-expression by CD1a^{+} histiocytes, to rule out indeterminate cell histiocytosis – a diagnostic pitfall[32]. Patients enrolled in the prospective LCH-IV study were staged, treated and followed-up according to study protocol; other patients were managed according to standard-of-care. Importantly, first-line treatment and follow-up of patients were irrespective of mutational status; no patient received first-line therapy with BRAF and/or MEK inhibitors. Details of data collection are provided in the Supplementary Methods.

Disease extent was categorized according to the classification of the LCH Study Group of the Histiocyte Society[9], differentiating between single-system (SS) and multisystem (MS) disease. Within these categories, detailed subtypes were distinguished based on the type of organ(s) involved and/or the manifestation as uni- or multifocal disease. The hematopoietic system, liver and spleen were considered risk organs (RO)[9,33], and bone lesions affecting the orbital, temporal/mastoid, sphenoidal, zygomatic, or ethmoidal bones, the maxilla, paranasal sinuses, or anterior or middle cranial fossa were considered central nervous system (CNS)-risk lesions[9,34–36]. LCH with RO involvement

was termed "high-risk LCH". Event was defined as disease progression, LCH relapse or death from any cause. Disease progression was defined as (i) insufficient response and/or progression of existing LCH manifestations requiring the start or a second- or further-line of chemotherapy, targeted therapy and/or radiotherapy, or (ii) the development of new lesions in the presence of active disease. Disease relapse was defined as the development of new lesions after complete remission for prior LCH manifestations.

Molecular pathologic analysis

Molecular analysis was performed as part of routine diagnostics and/or in the specific context of this study. Importantly, none of the tissue samples were analyzed by BRAF-VE1 immunohistochemistry alone. For (additional) molecular analysis performed in the context of this study, manual microdissection of LCH-lesional FFPE tissue sections was performed based on a CD1a stained reference slide to obtain representative tissue parts enriched for neoplastic LCH-cells[37], thereby increasing the success rate of mutation detection[38]. Automated DNA isolation from the microdissected tissue fragments and *BRAF*^{V600E} allele-specific real-time PCR and/or droplet digital PCR were performed as previously described[39–41]. When possible, cases without *BRAF*^{V600E} were further analyzed using Sanger sequencing and/or a custom-designed AmpliSeq next-generation-sequencing (NGS) panel containing primers to detect mutations in *MAP2K1* (NM_002755.3) exon 2-3 and *BRAF* (NM_004333.6) exon 12+15, as well as in *ARAF*, *MAP3K1*, *N/KRAS* and many other cancer-associated genes (Supplementary Methods)[42]. Finally, a small proportion of *BRAF*^{V600E} negative patients was analyzed for alternative *BRAF* alterations by FFPE-targeted locus capture (FFPE-TLC) NGS[43,44] – a DNA-based technique able to identify both small variants (e.g. single nucleotide variants or small indels) and structural variants (e.g. gene rearrangements). Details are available in the Supplementary Methods.

Statistical analysis

Comparisons of (sub)groups were performed using the two-sided Mann-Whitney U or Kruskal-Wallis test for continuous data and two-sided Fisher's or Fisher-Freeman-Halton exact test for categorical data. In general, threshold for significance was $P < 0.05$; however, in univariable analysis of LCH presentation according to mutational status $P < 0.00125$ was considered statistically significant (Bonferroni correction for multiple testing)[10]. In addition to significant results, findings insignificant after Bonferroni correction but with P values < 0.05 were highlighted. These represent potential associations, but without sufficient statistical evidence in this study, and will require further careful evaluation to determine their potential clinical relevance. Variables significant after correction were included in a multivariable binary logistic regression analysis to identify the factor(s) most associated with *BRAF*^{V600E} after adjustment for the other variables. For hierarchical

categorical variables (e.g. bone – bone subtypes), the primary variable was considered for inclusion (e.g. bone). Because risk organ involvement was restricted to MS-LCH, liver and hematopoietic system involvement – both significant in univariable analysis – were not included as independent variables, but instead MS-RO+ LCH was added as disease extent category to the regression model. Univariable survival analyses were performed using the Kaplan-Meier method, and survival curves were compared using the log-rank test. Event-free survival (EFS) was defined as the time from diagnosis until the first event or – for cases without an event – the date of last follow-up. To investigate how much of the effect of *BRAF*^{V600E} on EFS was mediated by disease extent, univariable survival analyses were stratified by disease extent and multivariable survival analysis was performed using Cox regression. Median follow-up was estimated using the reverse Kaplan-Meier method[45]. Incidences of DI, ND-LCH, specific sites of disease, chemotherapy, and second-line systemic therapy were indicated by proportions[10], because of incomplete time-to-event data for cumulative incidence calculations. Statistical analyses were performed using Graphpad Prism 9.0.1 or IBM SPSS Statistics 25.

RESULTS

A total of 377 patients with childhood LCH and available *BRAF*^{V600E} status were included. This cohort comprised 198 (52.5%) patients from the prospective LCH-IV study and 179 (47.5%) patients from a Dutch-Canadian retrospective study, with comparable clinical characteristics (Supplementary Table 1). The combined cohort comprised 222 (58.9%) males and 155 (41.1%) females. Median age at diagnosis was 3.6 years (range 0.0 – 17.9 years). Patients could be classified into 288 (76.4%) patients with SS-LCH and 89 (23.6%) patients with MS-LCH. Patients with SS-LCH could be categorized into 184 patients with unifocal bone disease (SS-UFB), 64 patients with multifocal bone disease (SS-MFB), 32 patients with isolated skin disease (SS-skin), and 8 patients with isolated involvement of another organ system, including the lungs (N = 3), lymph nodes (N = 3), CNS (N = 1) or soft tissue (N = 1). Patients with MS-LCH could be divided into 55 patients without risk organ involvement (MS-RO-) and 34 patients with risk organ involvement (MS-RO+ or high-risk LCH).

MAPK pathway gene alterations were detected in 300/377 (79.6%) patients, including 191 (50.7%) with *BRAF*^{V600E} (Table 1). In the subgroup without *BRAF*^{V600E}, *MAP2K1* mutations were identified in 54 patients and *BRAF* exon 12 indels were detected in 39 children (Table 2). *MAP2K1* mutations occurred in exon 2 in 36/54 (66.7%) patients and in exon 3 in 18/54 (33.3%) patients. *BRAF* exon 12 indels included small in-frame deletions at the beginning of exon 12 affecting the β3-αC loop in 27/39 (69.2%) patients and in-frame insertions of nine nucleotides at the end of exon 12 affecting the αC-β4 loop in

Table 1 | Clinical characteristics according to *BRAF*^{V600E} status.

	BRAFV600E positive	BRAFV600E negative	OR (95% CI)	P value	
Patients	191	186			
Age at diagnosis, median (range)	2.6 years (0.0 – 17.6)	5.7 years (0.0 – 17.9)	N/A	<0.001	**
Age <3 years	105 (55.0%)	66 (35.5%)	2.22 (1.47-3.36)	<0.001	**
Age ≥3 years	86 (45.0%)	120 (64.5%)			
Sex					
Male	113 (59.2%)	109 (58.6%)	1.02 (0.68-1.54)	0.92	
Female	78 (40.8%)	77 (41.4%)			
Disease extent at diagnosis					
Multisystem	64 (33.5%)	25 (13.4%)	3.25 (1.93-5.45)	<0.001	**
Single system	127 (66.5%)	161 (86.6%)			
Detailed subtype[$]					
MS, RO+	27 (14.1%)	7 (3.8%)	4.21 (1.79-9.93)	<0.001	**
MS, RO-	37 (19.4%)	18 (9.7%)	2.24 (1.23-4.10)	0.009	*
SS, bone	109 (57.1%)	139 (74.7%)	0.45 (0.29-0.70)	<0.001	**
SS, UFB	87 (45.5%)	97 (52.2%)	0.77 (0.51-1.15)	0.22	
SS, UFB, CNS-risk[$$]	16 (8.4%)	19 (10.2%)	0.80 (0.40-1.62)	0.60	
SS, MFB	22 (11.5%)	42 (22.6%)	0.45 (0.26-0.78)	0.006	*
SS, skin	18 (9.4%)	14 (7.5%)	1.28 (0.62-2.65)	0.58	
SS, other	0 (0.0%)	8[§§] (4.3%)	N/A	0.003	*
Disease site(s) at diagnosis					
Bone	157 (82.2%)	159 (85.5%)	0.78 (0.45-1.36)	0.41	
Multifocal bone lesions	56 (29.3%)	55 (29.6%)	0.99 (0.63-1.54)	1	
CNS-risk bone lesion(s)[$$]	64 (33.5%)	40 (21.5%)	1.84 (1.16-2.92)	0.011	*
Spinal column lesion(s)	18 (9.4%)	28 (15.1%)	0.59 (0.31-1.10)	0.12	
Skin	69 (36.1%)	24 (12.9%)	3.82 (2.27-6.43)	<0.001	**
Liver[#]	24 (12.6%)	5 (2.7%)	5.20 (1.94-13.95)	<0.001	**
Hematopoietic system#	19 (9.9%)	3 (1.6%)	6.74 (1.96-23.18)	0.001	**
Spleen[#]	15 (7.9%)	4 (2.2%)	3.88 (1.26-11.91)	0.017	*
Lymph node	17 (8.9%)	17 (9.1%)	0.97 (0.48-1.97)	1	
Lung	10 (5.2%)	14 (7.5%)	0.68 (0.29-1.57)	0.40	
Central nervous system[##]	8 (4.2%)	4 (2.2%)	1.99 (0.59-6.72)	0.38	
Gastrointestinal tract	7 (3.7%)	0 (0.0%)	N/A	0.015	*
Follow-up, median (range)	4.0 years (0.0 – 38.8)	3.8 years (0.0 – 36.0)	N/A	0.61†	
Permanent consequences developed during follow-up					
Diabetes insipidus	23 (12.0%)	14 (7.5%)	1.68 (0.84-3.38)	0.17	
Neurodegenerative LCH[§]	5 (2.6%)	0 (0.0%)	N/A	0.06	
Death	4	4	N/A	0.97†	

Symbols: $ Fisher's exact tests comparing patients with vs. without a disease extent subtype are shown. Fisher-Freeman-Halton exact test comparing proportions in all different subgroups (a contingency table larger than 2x2) is not shown, but was significant (P <0.001). $$ Bone lesions affecting the orbital, temporal/mastoid, sphenoidal, zygomatic, or ethmoidal bones, the maxilla, paranasal sinuses, or anterior or middle cranial fossa. # These organs are considered risk organs. ## Given that the posterior pituitary and pituitary stalk are direct extensions of the hypothalamus, pituitary tumors are classified as CNS involvement. All twelve patients with CNS involvement at diagnosis had tumorous lesions, e.g. pituitary stalk lesions. § Only patients with both clinical and radiologic neurodegenerative LCH are reported. §§ These eight patients comprised three patients with SS-lung LCH, three patients with SS-lymph node LCH, and single cases with SS-soft tissue or (tumorous) SS-CNS disease. † Obtained with the log-rank test. * P <0.05. ** P <0.00125 (Bonferroni correction for multiple testing; tests are shown in this table and Supplementary Table 3). Abbreviations: SS, single-system; UFB, unifocal bone; CNS, central nervous system; MFB, multifocal bone; MS, multisystem; RO, risk organ; N/A, not available.

Table 2 Clinical characteristics of patients with *MAP2K1* or *BRAF* exon 12 mutations.

	MAP2K1 mutated			BRAF exon 12 mutated		
	MAP2K1 exon 2	**MAP2K1 exon 3**	**All patients**	**In-frame deletions**	**In-frame insertions**	**All patients**
Patients	36	18	54	27	12	39
Age at diagnosis, median (range)	2.7 years (0.0-15.1)	8.3 years (1.7-17.1)	4.8 years (0.0 – 17.1)	3.4 years (0.2-17.9)	6.2 years (0.5-14.4)	5.9 years (0.2 - 17.9)
Age <3 years	19 (52.8%)	1 (5.6%)	20 (37.0%)	12 (44.4%)	3 (25.0%)	15 (38.5%)
Age ≥3 years	17 (47.2%)	17 (94.4%)	34 (63.0%)	15 (55.6%)	9 (75.0%)	24 (61.5%)
Sex						
Male	23 (63.9%)	10 (55.6%)	33 (61.1%)	16 (59.3%)	5 (41.7%)	21 (53.8%)
Female	13 (36.1%)	8 (44.4%)	21 (38.9%)	11 (40.7%)	7 (58.3%)	18 (46.2%)
Disease extent at diagnosis						
Multisystem	3 (8.3%)	1 (5.6%)	4 (7.4%)	7 (25.9%)	1 (8.3%)	8 (20.5%)
Single system	33 (91.7%)	17 (94.4%)	50 (92.6%)	20 (74.1%)	11 (91.7%)	31 (79.5%)
Detailed subtype						
MS, RO+	1 (2.8%)	0 (0.0%)	1 (1.9%)	3 (11.1%)	0 (0.0%)	3 (7.7%)
MS, RO-	2 (5.6%)	1 (5.6%)	3 (5.6%)	4 (14.8%)	1 (8.3%)	5 (12.8%)
SS, bone	30 (83.3%)	16 (88.9%)	46 (85.2%)	15 (55.6%)	11 (91.7%)	26 (66.7%)
SS, UFB	22 (61.1%)	13 (72.2%)	35 (64.8%)	11 (40.7%)	8 (66.7%)	19 (48.7%)
SS, UFB, CNS-risk	4 (11.1%)	1 (5.6%)	5 (9.3%)	4 (14.8%)	2 (16.7%)	6 (15.4%)
SS, MFB	8 (22.2%)	3 (16.7%)	11 (20.4%)	4 (14.8%)	3 (25.0%)	7 (17.9%)
SS, skin	3 (8.3%)	0 (0.0%)	3 (5.6%)	2 (7.4%)	0 (0.0%)	2 (5.1%)
SS, other	0 (0.0%)	1 (5.6%)	1 (1.9%)	3 (11.1%)	0 (0.0%)	3 (7.7%)
Disease site(s) at diagnosis						
Bone	33 (91.7%)	17 (94.4%)	50 (92.6%)	20 (74.1%)	12 (100%)	32 (82.1%)
Multifocal bone lesions	11 (30.6%)	3 (16.7%)	14 (25.9%)	7 (25.9%)	3 (25.0%)	10 (25.6%)
CNS-risk bone lesion(s)	8 (22.2%)	1 (5.6%)	9 (16.7%)	8 (29.6%)	3 (25.0%)	11 (28.2%)
Spinal column lesion(s)	6 (16.7%)	2 (11.1%)	8 (14.8%)	5 (18.5%)	2 (16.7%)	7 (17.9%)
Skin	5 (13.9%)	0 (0.0%)	5 (9.3%)	6 (22.2%)	0 (0.0%)	6 (15.4%)
Liver	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (11.1%)	0 (0.0%)	3 (7.7%)
Hematopoietic system	1 (2.8%)	0 (0.0%)	1 (1.9%)	1 (3.7%)	0 (0.0%)	1 (2.6%)
Spleen	0 (0.0%)	0 (0%)	0 (0.0%)	2 (7.4%)	0 (0.0%)	2 (5.1%)
Lymph node	2 (5.6%)	2 (11.1%)	4 (7.4%)	4 (14.8%)	1 (8.3%)	5 (12.8%)
Lung	1 (2.8%)	0 (0.0%)	1 (1.9%)	6 (22.2%)	0 (0.0%)	6 (15.4%)
CNS	1 (2.8%)	0 (0.0%)	1 (1.9%)	1 (3.7%)	0 (0.0%)	1 (2.6%)
Follow-up, median (range)	2.7 years (0.2-15.2)	4.3 years (1.4-30.4)	3.7 years (0.2 - 30.4)	6.3 years (0.4-27.0)	3.5 years (0.7-9.4)	5.3 years (0.4 – 27.0)
Permanent consequences during follow-up						
Diabetes insipidus	1 (2.8%)	1 (5.6%)	2 (3.7%)	5 (18.5%)	0 (0.0%)	5 (12.8%)
Death	2	0	2	2	0	2

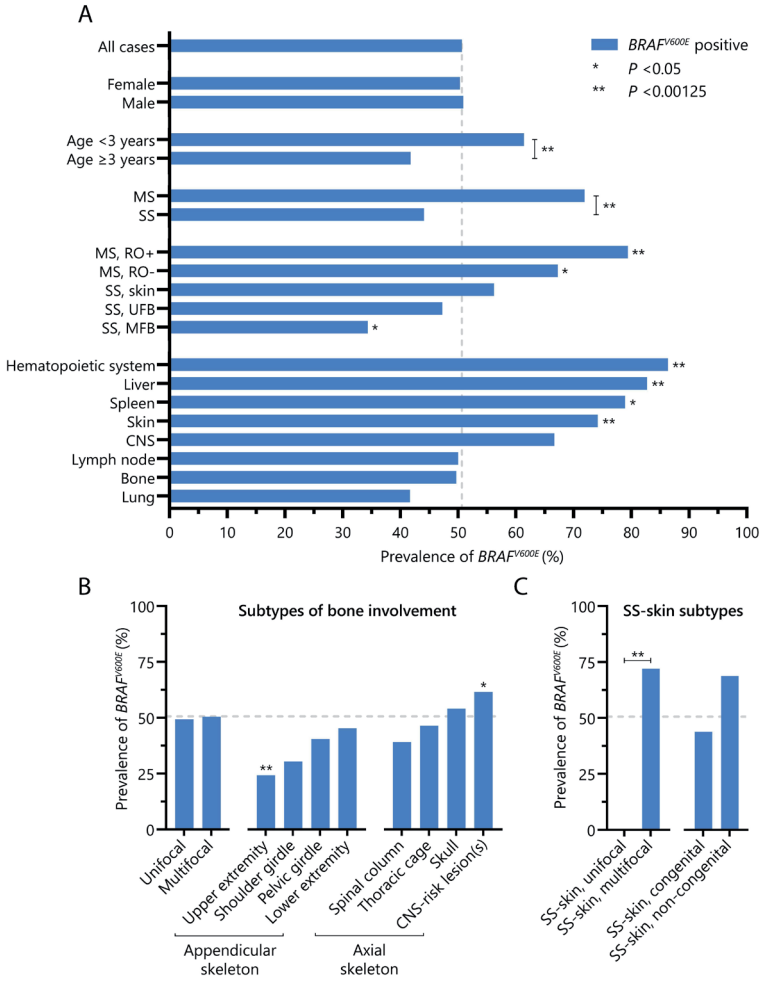

Figure 1 | Clinical features at LCH diagnosis according to BRAFV600E status.
(A) Prevalence of *BRAF*^{V600E} in patients with specific clinical characteristics at LCH diagnosis. **(B)** Prevalence of *BRAF*^{V600E} in patients with specific types of bone involvement at diagnosis. This figure depicts all patients with osseous lesions, irrespective of single- or multisystemic disease extent. Bones are grouped according to the classification used by the National Cancer Institute. Upper extremity: humerus, radius, ulna, carpals, metacarpals, phalanges. Shoulder girdle: clavicle, scapula. Pelvic girdle: coxal, innominate and hip bones (incl. ilium, ischium, acetabulum, and pubis). Lower extremity: femur, tibia, fibula, patella, tarsals, metatarsals, phalanges. Spinal column: cervical, thoracic and lumbar vertebrae, sacrum, coccyx. Thoracic cage: ribs and sternum. CNS-risk lesions are bone lesions affecting the orbital, temporal/mastoid, sphenoidal, zygomatic, or ethmoidal bones, the maxilla, paranasal sinuses, or anterior or middle cranial fossa, according to LCH Study Group definitions[9,34–36]. **(C)** Prevalence of *BRAF*^{V600E} in patients with specific presentations of single-system skin LCH at diagnosis. Numbers of patients are provided in Table 1 and Supplementary Table 3. Dashed lines indicate the prevalence of *BRAF*^{V600E} in all cases (51%). Statistical tests with *P* values <0.05 are shown. Symbols: **, *P* <0.00125; *, *P* <0.05. Abbreviations: MS, multisystem; SS, single-system; RO, risk organ; UFB, unifocal bone; MFB, multifocal bone; CNS, central nervous system.

12/39 (30.8%) patients[25,46–48]. In the remaining *BRAF*^{V600E} negative patients, *BRAF* exon 15 mutations other than *BRAF*^{V600E} (*N*=10), *BRAF* fusions (*N*=3), *ARAF* mutations (*N*=2), and a *KRAS* mutation (*N*=1) were detected (Supplementary Table 2).

BRAFV600E status in relation to clinical presentation

BRAF^{V600E} correlated with demographic characteristics, disease extent and specific sites of disease at diagnosis (Figure 1; Table 1; Supplementary Table 3). Patients with *BRAF*^{V600E} were significantly younger than patients without *BRAF*^{V600E} (median age 2.6 years vs. 5.7 years; *P* <0.001). In addition, *BRAF*^{V600E} positive patients more often had MS-LCH (33.5% vs. 13.4%; *P* <0.001; Figure 1A) and high-risk disease (14.1% vs. 3.8%; *P* <0.001). Regarding sites of disease, *BRAF*^{V600E} significantly associated with more involvement of the skin (*P* < 0.001), liver (*P* < 0.001) and hematopoietic system (*P* = 0.001), and with less involvement of the upper extremity bones (*P* = 0.001; Figure 1B). Within SS-skin LCH, *BRAF*^{V600E} was significantly associated with multifocal skin involvement (*P* = 0.001; Figure 1C).

Concerning potential associations, *BRAF*^{V600E} seemed associated with less SS-MFB disease (*P* = 0.006; Figure 1A). Furthermore, *BRAF*^{V600E} appeared to correlate with a higher prevalence of spleen involvement (*P* = 0.017), gastrointestinal involvement (*P* = 0.015; Table 1), and CNS-risk bone lesions (*P* = 0.011; Figure 1B). When analyzing sites of disease during entire follow-up – including at LCH progression and/or relapse, these potential associations remained apparent (Supplementary Figure 3; Supplementary Table 4).

In multivariable analysis with age, disease extent (categorized as SS/MS-RO-/MS-RO+ LCH) and skin involvement as independent variables (Supplementary Table 5A), *BRAF*^{V600E} was significantly associated with skin involvement (OR 2.23, 95% CI 1.16-4.29, *P* = 0.017). However, odds ratio was highest for MS-RO+ disease extent (OR 2.54, 95% CI 0.99-6.54, *P* = 0.05). In a regression model with age, multisystem disease (irrespective of RO status) and skin involvement as independent variables, *BRAF*^{V600E} was significantly associated with both multisystem disease and skin involvement (Supplementary Table 5B).

MAP2K1 and BRAF exon 12 mutations in relation to clinical presentation

MAP2K1 mutations also correlated with clinical features at diagnosis (Figure 2; Supplementary Table 6). Compared to children with *BRAF*^{V600E}, patients with *MAP2K1* mutations had significantly more SS-bone LCH (85.2% vs. 57.1%; *P* <0.001; Figure 2D), and less MS-LCH (7.4% vs. 33.5%; *P* <0.001; Figure 2E) and skin involvement (9.3% vs. 36.1%; *P* <0.001; Figure 2G). *MAP2K1* mutations also appeared to correlate with more SS-bone disease when compared to *BRAF* exon 12 mutations (*P* = 0.045; Figure 2D); particularly compared to *BRAF* exon 12 deletions affecting the β3-αC loop (*P* = 0.006; Supplementary Figure 4B). Regarding subtypes of bone involvement, *MAP2K1* mutated patients had the lowest prevalence of CNS-risk bone lesions, despite having the most bone involvement (Figure 2F). Children with *MAP2K1* mutations did have highest prevalence of bone lesions

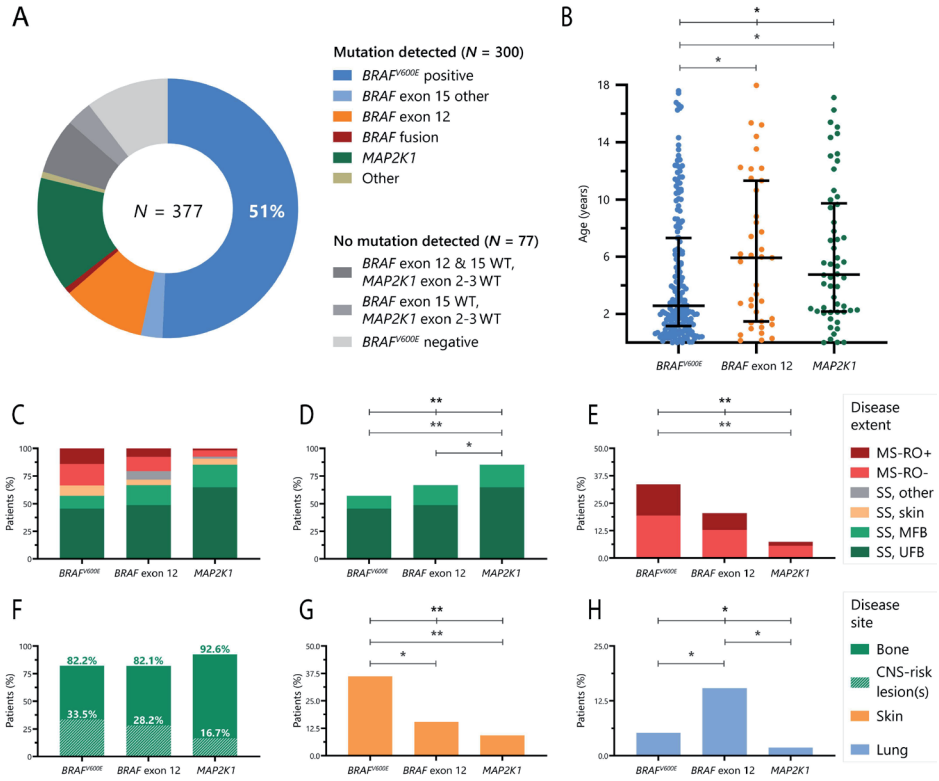

Figure 2 | Clinical features at LCH diagnosis of children with BRAFV600E, BRAF exon 12 or MAP2K1 mutations.

(A) Pie chart showing the mutational status of the 377 patients from our cohort. **(B)** Dot plot showing age at diagnosis of patients with *BRAF*^{V600E}, *BRAF* exon 12 or *MAP2K1* mutations. Error bars depict medians with interquartile ranges. **(C-E)** Bar charts depicting the percentage of patients with *BRAF*^{V600E}, *BRAF* exon 12 or *MAP2K1* mutations having specific disease extents at LCH diagnosis. Statistical comparisons were performed for SS-bone disease in panel D and multisystem disease in panel E. **(F-H)** Bar charts depicting the percentage of patients with *BRAF*^{V600E}, *BRAF* exon 12 or *MAP2K1* mutations having specific disease sites at LCH diagnosis. Statistical tests with *P* values <0.05 are depicted. Numbers of patients are provided in Tables 1-2. Symbols: **, *P* <0.00125; *, *P* <0.05. New abbreviation: WT, wildtype.

in the upper extremities and/or shoulder girdle (Supplementary Figure 5). Regarding demographic characteristics, *MAP2K1* mutations appeared to correlate with older age at diagnosis when compared to *BRAF*^{V600E} ($P = 0.011$; Figure 2B).

Similar to what was observed for *MAP2K1* mutations, *BRAF* exon 12 indels seemed associated with older age at diagnosis ($P = 0.049$; Figure 2B) and less skin involvement ($P = 0.014$; Figure 2G) when compared to *BRAF*^{V600E}. Furthermore, *BRAF* exon 12 mutations appeared to correlate with a higher prevalence of lung involvement when compared to *BRAF*^{V600E} ($P = 0.035$) or *MAP2K1* mutations ($P = 0.020$; Figure 2H). Pulmonary involvement was particularly frequent in patients with *BRAF* exon 12 deletions affecting the β3-αC loop (6/27, 22.2%; Supplementary Figure 4F). Notably, *BRAF* exon 12 deletions were also

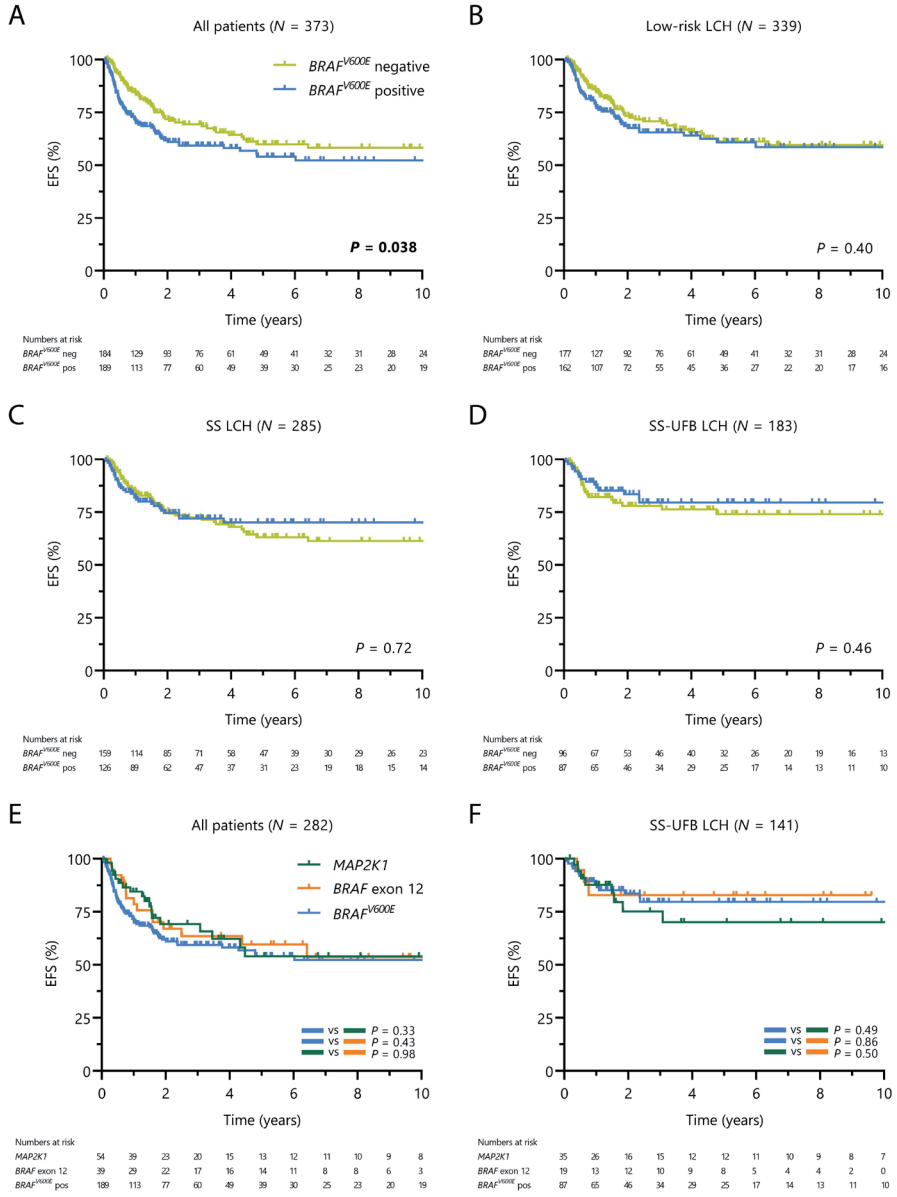

Figure 3 | Clinical outcome of pediatric LCH patients according to mutational status and disease extent. **(A-D)** Kaplan-Meier curves showing event-free survival (EFS) according to lesional *BRAF*^{V600E} status for all 373 patients (panel A), 339 patients with low-risk LCH (panel B), 285 patients with single-system LCH (panel C) or 183 patients with single-system unifocal bone LCH at diagnosis. Patients with low-risk LCH comprise all patients except those with high-risk (= MS-RO+) disease. **(E-F)** Kaplan-Meier curves showing EFS of patients with *BRAF*^{V600E}, *BRAF* exon 12 or *MAP2K1* mutations. Curves are shown for all 282 patients (panel E) and for 141 patients with single-system unifocal bone LCH at diagnosis. Four patients without clinical follow-up were not included in these survival analyses.

detected in 3/7 (43%) patients with thymic involvement[49–51]; the other four patients had alternative *BRAF* alterations (Supplementary Figure 6).

BRAFV600E status in relation to clinical outcome

Follow-up was similar for *BRAF*^{V600E} positive and negative patients (median 4.0 years vs. 3.8 years; $P = 0.61$). In the overall cohort, children with *BRAF*^{V600E} had significantly reduced EFS (5-year EFS 54.1%, SE ±4.4% vs. 59.9%, SE ±4.4%; $P = 0.038$; Figure 3A), but not reduced overall survival (Supplementary Figure 7). *BRAF*^{V600E} mutated patients also more frequently received second-line systemic therapy (29.1% vs. 15.8%; Supplementary Table 7). When patients were stratified by disease extent, however, *BRAF*^{V600E} no longer associated with outcome parameters like EFS (Figure 3B-D). *BRAF*^{V600E} mutated children particularly had noninferior EFS in the subgroups of patients with SS-UFB disease (Figure 3D) or SS-MFB LCH (Supplementary Figure 8) – representing almost two-thirds (66%) of all patients in our cohort. Additionally, disease extent but not lesional *BRAF*^{V600E} status was associated with EFS in multivariable survival analysis of the overall cohort (*BRAF*^{V600E} HR 1.08, 95% CI 0.75-1.55, $P = 0.67$; Supplementary Table 8).

MAP2K1 and BRAF exon 12 mutations in relation to clinical outcome

Follow-up was similar for *BRAF*^{V600E}, *MAP2K1* and *BRAF* exon 12 mutated patients (median 4.0 years vs. 3.7 years vs. 5.3 years, respectively; $P = 0.87$). No significant differences in EFS were observed between the three molecular subgroups – particularly after patient stratification by disease extent (Figure 3E-F; Supplementary Figures 8-9). Incidence of DI was similar among children with *BRAF*^{V600E} or *BRAF* exon 12 mutations (12.0% vs. 12.8%), and higher than among children with *MAP2K1* mutations (3.7%) – although not statistically significant (Tables 1-2; Supplementary Table 6).

Clinical features of LCH patients with alternative MAPK pathway gene alterations

Rare MAPK pathway gene alterations were identified in sixteen *BRAF*^{V600E} negative patients (Supplementary Tables 2 and 11). Ten children had a *BRAF* exon 15 mutation other than *BRAF*^{V600E}, including six with a *BRAF*^{V600D} mutation[52] and single cases with a *BRAF* p.T599_V600insEAT, *BRAF* p.T599_V600insEKST, *BRAF* p.V600_R603delinsEKSQ, or *BRAF* p.V600_W604delinsESRG mutation. All six patients with *BRAF*^{V600D} had SS-bone LCH (either UFB or MFB); none of them developed disease in another organ system during follow-up (median 4.7 years; range 0.6 – 12.6 years). However, several *BRAF*^{V600D} mutated patients had uncommon abscess-like soft tissue extension through the skin (Figure 4E; Supplementary Table 2)[53]. The patient with the *BRAF* p.V600_R603delinsEKSQ mutation had MS-RO+ disease and required second- and third-line chemotherapy. *BRAF* fusions were identified in three patients using FFPE-TLC NGS (Figure 4A-D; Supplementary Figure 10)[44]. All three cases had different *BRAF* fusion partners – including *TMEM106B*,

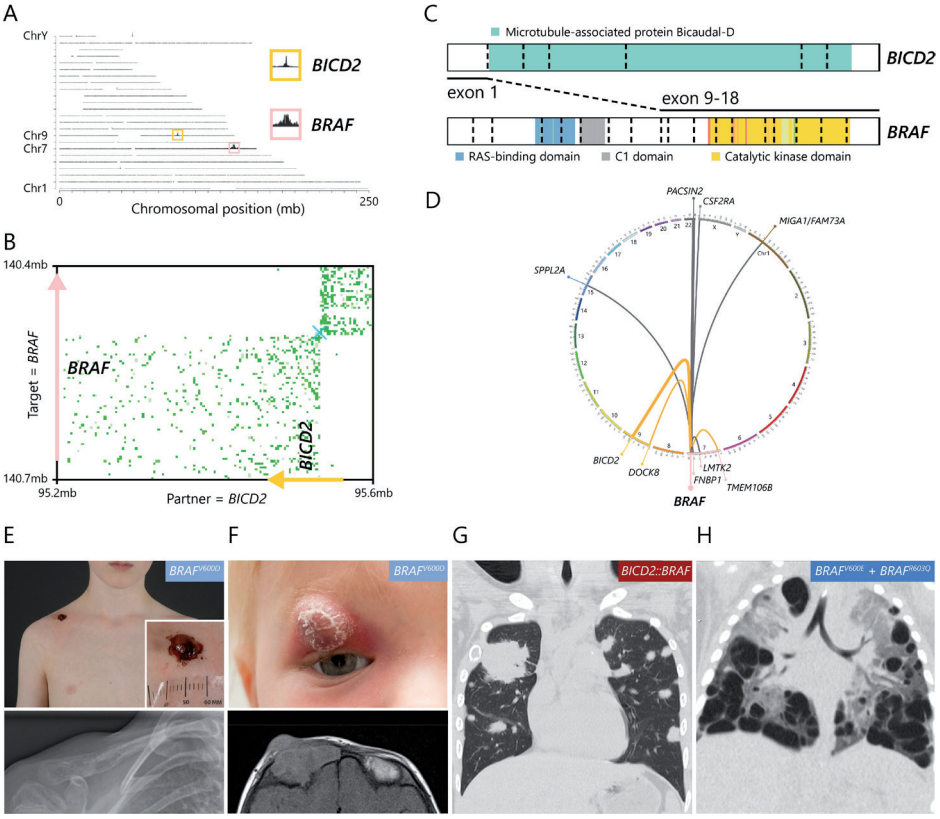

Figure 4 | Molecular and clinical findings in patients with rare *BRAF* alterations.
(A) Genome-wide coverage of fragments retrieved from a FFPE-TLC experiment targeting *BRAF* on a FFPE tissue sample from a patient from our cohort. A rearranged region to the *BRAF* gene (pink box) on chromosome 7 was identified by the concentration of fragments clustered around the *BICD2* gene (yellow box) on chromosome 9. **(B)** Butterfly plot uncovering the reciprocal *BICD2*::*BRAF* rearrangement. Proximity-ligation products between the target gene (*BRAF*) and rearrangement partner (*BICD2*) are depicted in green. Strand directions are indicated by arrows. See Supplementary Figure 10 for details about FFPE-TLC technology. **(C)** Illustration of the identified *BICD2*::*BRAF* fusion, made with ProteinPaint software[83]. **(D)** Circos plot depicting the three distinct *BRAF* rearrangements identified in patients from our cohort in orange, as well as other *BRAF* rearrangements previously identified in LCH patients in grey[17,25,86–88]. **(E)** Clinical and conventional radiography images of a patient from our cohort with $BRAF^{V600D}$ mutated SS-UFB LCH, who had a single osteolytic lesion in the right clavicle with remarkable abscess-like soft tissue extension through the skin. **(F)** Clinical and magnetic resonance imaging (MRI) images of a patient from our cohort with $BRAF^{V600D}$ mutated SS-MFB LCH, who had a relapse of multifocal bone disease with a remarkable orbital lesion with clear skin changes. **(G)** Coronal image of a chest computed tomography (CT) scan showing atypical pulmonary lesions in the patient with a *BICD2*::*BRAF* fusion. Shown are multiple solid nodules in both lungs, including a very large tumor in the right upper lobe measuring 55 x 18 x 15 mm. A biopsy of this tumor excluded co-occurrence of lymphoma or another disease and revealed clusters of $CD1a^{+}$ $CD207^{+}$ cells, compatible with LCH. **(H)** Coronal image of a chest CT scan showing many large cystic lung lesions in a child with high-risk LCH harboring both $BRAF^{V600E}$ and $BRAF^{R603Q}$ mutations.

DOCK8 and *BICD2*. Two of three *BRAF*-rearranged patients had SS-bone LCH without progression or relapse during their follow-up (5.4 or 27.5 years); the remaining patient with a *BICD2*::*BRAF* fusion had MS-RO- disease with uncommon large tumors in both lungs (Figure 4G) – instead of small nodules and cysts typical for pulmonary LCH[54–56]. This patient had progressive disease despite two lines of chemotherapy and recently received MEK inhibition (trametinib) with a complete metabolic response after six months of treatment. The *ARAF* and *KRAS* mutations were confined to patients with SS-bone LCH.

Additional MAPK pathway gene alterations were identified in three $BRAF^{V600E}$ positive cases, including one previously reported patient with a *MAP3K1* mutation[24]. The other two cases had an additional *BRAF* p.R603Q mutation, previously also reported in another child with $BRAF^{V600E}$ mutated LCH[17]. One of our patients had SS-UFB LCH with multiple bone relapses during follow-up – requiring multiple lines of chemotherapy. The other child had MS-RO+ disease and required second-line chemotherapy with cladribine because of progression of lung lesions. Eventually, all patients reached complete remission (Supplementary Table 2).

DISCUSSION

Through an international collaborative effort, we present here a large clinicogenomic study of childhood LCH. We confirm findings of previous research, most notably by Héritier *et al.*[10], but also reveal new associations of *BRAF* and *MAP2K1* mutations with clinical features at presentation (Table 3). In addition, we highlight that lesional $BRAF^{V600E}$ status did not correlate with inferior clinical outcome after patient stratification by disease extent – a prognostic factor known for decades[6–8].

Regarding $BRAF^{V600E}$, our study points at a potential association with a higher prevalence of gastrointestinal involvement. Interestingly, gastrointestinal involvement was recently shown to provide additive unfavorable prognostic impact in patients with high-risk LCH[57]. Together, these data further support that $BRAF^{V600E}$ associates with the most severe clinical presentations of pediatric LCH. $BRAF^{V600E}$ also seemed more prevalent in patients with CNS-risk bone lesions; thus, $BRAF^{V600E}$ now appears to associate with all disease characteristics known to correlate with clinical ND-LCH, including multisystem disease, skin and pituitary involvement, and CNS-risk bone lesions[10,34,35,58]. Accordingly, all five patients with clinical ND-LCH in our cohort harbored $BRAF^{V600E}$ (Table 1) and had at least one of these disease characteristics. Although we confirm that $BRAF^{V600E}$ correlates with reduced event-free survival in the overall cohort[10], we show that this is (primarily) driven by the association of $BRAF^{V600E}$ with disease extents known for high rates of progression or relapse (Supplementary Figure 11), including MS-LCH and SS-

Table 3 | Summary of key findings.

Mutational status in relation to clinical presentation					
Significant associations($P < 0.00125$)			Potential associations($P \geq 0.00125 - P < 0.05$)		
BRAF^{V600E}	↑ Multisystem disease ↑ Multifocal SS-skin disease ↑ Risk organ involvement ↑ Skin involvement ↓ Age at diagnosis	Confirming prior findings[10]	*BRAF*^{V600E}	↑ CNS-risk bone involvement ↑ Gastrointestinal involvement ↓ SS-MFB disease	New findings
	↓ Upper extremity bone involvement	New findings	*BRAF* exon 12	↑ Lung involvement	
MAP2K1	↑ SS-bone disease				

Mutational status in relation to clinical outcome					
Significant associations($P < 0.05$)			No evidence for associations($P \geq 0.05$)		
BRAF^{V600E}	↓ EFS when analyzing the overall cohort ↑ 2nd line systemic therapy (overall cohort)	Confirming prior findings[10]	*BRAF*^{V600E}	Outcome in clinical subgroups, e.g. SS-UFB	Highlighted negative findings
			BRAF exon 12 *MAP2K1*	Outcome in overall cohort and subgroups	

For associations of *BRAF*^{V600E}, *BRAF*^{V600E} positive (N = 191) vs. *BRAF*^{V600E} negative (N = 186) patients were compared. For associations of *MAP2K1* or *BRAF* exon 12 mutations, assignment is based on the statistical comparison of *MAP2K1* mutated patients (N = 54) or *BRAF* exon 12 mutated patients (N = 39) with *BRAF*^{V600E} positive patients, respectively. Associations of *MAP2K1* or *BRAF* exon 12 mutations required 1) a difference with *BRAF*^{V600E} positive patients resulting in a Fisher's Exact test P value <0.05 (for potential associations) or <0.00125 (for significant associations), and 2) a difference between *MAP2K1* and *BRAF* exon 12 mutated patients resulting in a Fisher's Exact test P value <0.05.

multifocal skin disease (Supplementary Figure 9). This insight did not emerge from the study by Héritier *et al.*[10], because the univariable survival analyses presented in their study were only stratified by risk organ involvement – with MS-RO- and SS-multifocal skin disease still overrepresented in *BRAF*^{V600E} mutated patients in the low-risk disease subgroup (Figure 3B). Interestingly, lesional *BRAF*^{V600E} status also did not correlate with inferior clinical outcome in recent molecular studies of adult LCH[31,59–61]. Thus, the quest for independent prognostic factors in LCH continues.

Our study also confirms that *MAP2K1* mutations in pediatric LCH predominantly occur in exon 2, affecting the αA-helix of MEK1 (Figure 5; Supplementary Figure 12)[62,63], with p.Q58_E62del as the most detected mutation (Supplementary Table 9)[19]. The distinction between *MAP2K1* exon 2 or 3 mutations is important, because the mutations in exon 2 are RAF-regulated, whereas the deletions in exon 3 are RAF- and phosphorylation-independent[64]. Consequently, these *MAP2K1* exon 3 deletions are less sensitive to allosteric MEK inhibitors, such as trametinib or cobimetinib[63,64]. Additionally, these so-called "class 3 *MAP2K1* mutants" result in higher activation of downstream ERK *in vitro* when compared to *MAP2K1* exon 2 mutations[64]. Therefore, one could hypothesize that *MAP2K1* exon 3 deletions might associate with a more severe clinical phenotype;

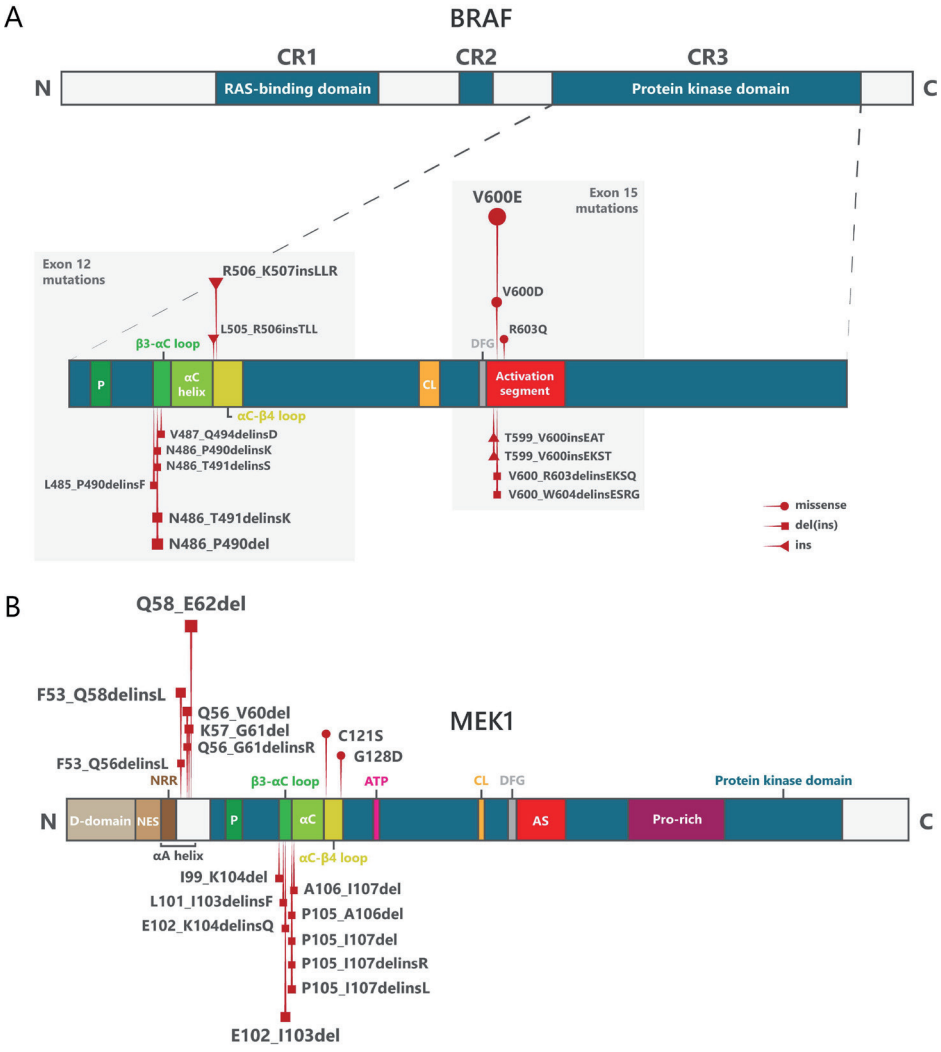

Figure 5 | Identified BRAF and MEK1 alterations.
(A-B) Schematic representations of BRAF and MEK1 proteins with alterations detected in patients from our cohort. MEK1 is encoded by the gene *MAP2K1*. Figures not entirely to scale. Abbreviations: N, N-terminus; C, C-terminus; CR, conserved region; D, docking; NES, nuclear export signal; NRR, negative regulatory region; P, phosphate-binding; ATP, ATP binding site; CL, catalytic loop; DFG, DFG motif; AS, activation segment; Pro, proline.

however, only 1/17 children in our cohort with these mutations had multisystem LCH (Table 2). In addition, their event-free survival was not significantly worse than of children with *MAP2K1* exon 2 mutations (Supplementary Figure 13). In general, our study points out that *MAP2K1* mutations typically correlate with a less severe clinical presentation of pediatric LCH, with significantly more SS-bone LCH and less multisystem disease compared to $BRAF^{V600E}$ (Figure 2).

BRAF exon 12 mutations predominantly comprised small in-frame deletions affecting the β3-αC loop of the BRAF protein (Figure 5; Supplementary Figure 12)[25]. Like in adult LCH[59], *BRAF* p.N486_P490del was the most detected mutation (Supplementary Table 10). Additionally, insertions at the end of *BRAF* exon 12 affecting the αC-β4 loop (Figure 5) were detected in twelve patients[48]. These patients comprised eleven children with the *BRAF* p.R506_K507insLLR mutation, first described in two cases by Héritier *et al.*[28], and one child with a *BRAF* p.L505_R506insTLL mutation. Importantly, both β3-αC and αC-β4 loop mutations are resistant to first-generation BRAF inhibitors like vemurafenib[25,28,46,48]. In accordance with the two cases reported by Héritier *et al.*[28], the αC-β4 loop insertions predominantly occurred in children with SS-UFB LCH (Table 2). Yet, we also identified this mutation in one child with multisystem LCH that had multiple bone relapses and required four lines of chemotherapy – again demonstrating that (recurrent) mutations are not completely specific to one clinical form of the disease. Interestingly, *BRAF* exon 12 deletions affecting the β3-αC loop seemed to associate with a high prevalence of lung involvement. This is in accordance with molecular studies of adult LCH, which described the frequent presence of these mutations in adult LCH patients with pulmonary involvement[31,59,60] – often in the context of multisystem disease. Thus, molecular analysis of *BRAF* exon 12 should be particularly applied in patients with pulmonary lesions[54,56], which may inform LCH diagnosis and enable rational targeted therapy.

Finally, alternative MAPK pathway gene alterations seemed related to some uncommon disease manifestations, since we observed rare abscess-like soft tissue extension in several $BRAF^{V600D}$ mutated patients and atypical solid lung lesions in our case with a *BICD2*::*BRAF* fusion. Notably, all three cases with a *BRAF* fusion had different fusion partners, which stands in contrast to the high prevalence of one specific *ALK* fusion partner (*KIF5B*) in patients with ALK-positive histiocytosis[65]. Thus, comprehensive molecular techniques are essential for detection of these rare genetic variants; their identification may have clinical consequences, as illustrated by our *BICD2*::*BRAF*-positive case that received third-line systemic therapy with trametinib and obtained a complete metabolic response (Supplementary Table 2).

Altogether, these data indicate that oncogenic mutation subtype appears an important – but not the sole – driver of heterogeneity in clinical presentation of pediatric LCH. With increasing access to targeted therapies, identification of the precise somatic driver alteration in patients that could benefit from these agents is important, as mutation subtype influences responsiveness to BRAF and MEK inhibitors. Factors other than mutation subtype seem more involved in driving heterogeneous outcomes within clinical subgroups. To this end, (longitudinal) assessment of mutant alleles in cellular or cell-free DNA derived from peripheral blood and/or bone marrow represents an interesting opportunity for prognostic staging and monitoring response to therapy[66–75].

Why specific mutations associate with distinct LCH clinical presentations remains an interesting and important issue for further investigation[76]. Notably, specific genetic alterations also associate with distinct histiocytic entities[17,65,77], again demonstrating the intimate relationship between molecular pathogenesis and clinical histiocytosis phenotype. Differential ERK activation inflicted by the different genetic alterations may play a role[5,64], although this was not apparent for *MAP2K1* exon 2 vs. exon 3 mutations in our cohort. Additionally, the association of *BRAF*^{V600E} with severe clinical forms of LCH may rely on (thus far unknown) mediators or confounders, such as additional (epi)genomic alterations, tissue- or context-specific factors, and the mutated cell-of-origin (Supplementary Figure 11). Although previous whole-exome-sequencing studies have revealed infrequent additional genomic alterations in LCH[17,22,25], several studies have indicated a distinct impact of *BRAF*^{V600E} on the LCH-lesional immune microenvironment[37,78,79]. Furthermore, several studies have suggested an important role for the mutated cell-of-origin in governing LCH clinical phenotype – with high-risk disease caused by mutations in multipotent hematopoietic stem/progenitor cells and low-risk disease caused by the same mutations in more committed myeloid precursors[16,80,81]. However, also this simplified model does not fully explain LCH clinical phenotype, since recent studies have identified mutation-carrying (myeloid and lymphoid) cells in the blood from patients with low-risk or even single-system LCH[41,80,82]. Therefore, it remains important to elucidate how somatic mutations in multipotent progenitors can cause both single- and multisystemic LCH, and why progenitor cells active in children with high-risk LCH often harbor *BRAF*^{V600E}.

Limitations of our study include the fact that not all *BRAF*^{V600E} negative patients were analyzed beyond *BRAF*^{V600E} (Figure 2A). Consequently, our study does not provide exact incidence rates of *MAP2K1*, *BRAF* exon 12, and rare MAPK pathway gene alterations. However, these alterations were detected in 109 children with LCH, allowing analysis of their clinical associations at unprecedented scale. Furthermore, because of the retrospective design, we cannot rule out some selection bias influencing the clinical spectrum of our cohort. However, our cohort included 288 (76.4%) patients with SS-LCH, compared to <70% in the study by Héritier *et al.*[10], strongly arguing against overrepresentation of patients with severe disease. Instead, we regard the relatively unbiased composition of our cohort as one of the strengths of our study. Nevertheless, our findings should be confirmed by sufficiently powered cohort studies; particularly the potential associations require further investigation (Table 3).

Overall, we present an international clinicogenomic study of childhood LCH, defining the clinical impact of recurrent *BRAF* and *MAP2K1* mutations. We demonstrate distinct associations of these driver mutation subtypes with demographic characteristics, disease extent and specific sites of disease. Another key finding is that mutational status did not associate with event-free survival when patients were stratified by disease

extent. These findings advance our understanding of factors underlying the remarkable clinical heterogeneity of pediatric LCH, and may guide molecular diagnostics beyond *BRAF*^{V600E} – e.g. in children with (severe) lung involvement[56].

SUPPLEMENTARY FILES

The supplementary files can be found online at
https://ashpublications.org/bloodadvances/article/7/4/664/486556.

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REFERENCES

1. Maarten Egeler R, van Halteren AGS, Hogendoorn PCW, Laman JD, Leenen PJM. Langerhans cell histiocytosis: fascinating dynamics of the dendritic cell-macrophage lineage. *Immunol. Rev.* 2010;234(1):213–232.
2. Emile J-F, Abla O, Fraitag S, et al. Revised classification of histiocytoses and neoplasms of the macrophage-dendritic cell lineages. *Blood*. 2016;127(22):2672–2681.
3. Allen CE, Beverley PCL, Collin M, et al. The coming of age of Langerhans cell histiocytosis. *Nat. Immunol.* 2020;21(1):1–7.
4. Allen CE, Merad M, McClain KL. Langerhans-Cell Histiocytosis. *N. Engl. J. Med.* 2018;379(9):856–868.
5. Rodriguez-Galindo C, Allen CE. Langerhans cell histiocytosis. *Blood*. 2020;135(16):1319–1331.
6. Lahey ME. Prognostic factors in histiocytosis X. *Am. J. Pediatr. Hematol. Oncol.* 1981;3(1):57–60.
7. Ladisch S, Gadner H. Treatment of Langerhans cell histiocytosis--evolution and current approaches. *Br. J. Cancer. Suppl.* 1994;23:S41-6.
8. Sterlich K, Minkov M. Childhood Langerhans Cell Histiocytosis: Epidemiology, Clinical Presentations, Prognostic Factors, and Therapeutic Approaches. *Rare Dis. - Diagnostic Ther. Odyssey*. 2021; Chapter 4.
9. Haupt R, Minkov M, Astigarraga I, et al. Langerhans cell histiocytosis (LCH): Guidelines for diagnosis, clinical work-up, and treatment for patients till the age of 18 years. *Pediatr. Blood Cancer*. 2013;60(2):175–184.
10. Héritier S, Emile J-F, Barkaoui M-A, et al. BRAF Mutation Correlates With High-Risk Langerhans Cell Histiocytosis and Increased Resistance to First-Line Therapy. *J. Clin. Oncol.* 2016;34(25):3023–3030.
11. Gadner H, Grois N, Pötschger U, et al. Improved outcome in multisystem Langerhans cell histiocytosis is associated with therapy intensification. *Blood*. 2008;111(5):2556–2562.
12. Gadner H, Minkov M, Grois N, et al. Therapy prolongation improves outcome in multisystem Langerhans cell histiocytosis. *Blood*. 2013;121(25):5006–5014.
13. Donadieu J, Bernard F, van Noesel M, et al. Cladribine and cytarabine in refractory multisystem Langerhans cell histiocytosis: results of an international phase 2 study. *Blood*. 2015;126(12):1415–1423.
14. Egeler RM, Katewa S, Leenen PJM, et al. Langerhans cell histiocytosis is a neoplasm and consequently its recurrence is a relapse: In memory of Bob Arceci. *Pediatr. Blood Cancer*. 2016;63(10):1704–1712.
15. Badalian-Very G, Vergilio J-A, Degar BA, et al. Recurrent BRAF mutations in Langerhans cell histiocytosis. *Blood*. 2010;116(11):1919–1923.
16. Berres M-L, Lim KPH, Peters T, et al. BRAF-V600E expression in precursor versus differentiated dendritic cells defines clinically distinct LCH risk groups. *J. Exp. Med.* 2014;211(4):669–683.
17. Durham BH, Lopez Rodrigo E, Picarsic J, et al. Activating mutations in CSF1R and additional receptor tyrosine kinases in histiocytic neoplasms. *Nat. Med.* 2019;25(12):1839–1842.
18. Hayase T, Saito S, Shioda Y, et al. Analysis of the BRAF and MAP2K1 mutations in patients with Langerhans cell histiocytosis in Japan. *Int. J. Hematol.* 2020;112(4):560–567.
19. Yang Y, Wang C, Wang D, et al. Clinical study of MAP2K1-mutated Langerhans cell histiocytosis in children. *J. Cancer Res. Clin. Oncol.* 2021. [Epub ahead of print]
20. Feng S, Han L, Yue M, et al. Frequency detection of BRAF V600E mutation in a cohort of pediatric langerhans cell histiocytosis patients by next-generation sequencing. *Orphanet J. Rare Dis.* 2021;16(1):272.
21. Nelson DS, Quispel W, Badalian-Very G, et al. Somatic activating ARAF mutations in Langerhans cell histiocytosis. *Blood*. 2014;123(20):3152–3155.

22. Chakraborty R, Hampton OA, Shen X, et al. Mutually exclusive recurrent somatic mutations in MAP2K1 and BRAF support a central role for ERK activation in LCH pathogenesis. *Blood*. 2014;124(19):3007–3015.
23. Brown N, Furtado L, Betz B, Kiel M. High prevalence of somatic MAP2K1 mutations in BRAF V600E negative Langerhans cell histiocytosis. *Blood*. 2014;124(10):1655–1659.
24. Nelson DS, van Halteren A, Quispel WT, et al. MAP2K1 and MAP3K1 mutations in langerhans cell histiocytosis. *Genes, Chromosom. Cancer*. 2015;54(6):361–368.
25. Chakraborty R, Burke TM, Hampton OA, et al. Alternative genetic mechanisms of BRAF activation in Langerhans cell histiocytosis. *Blood*. 2016;128(21):2533–2537.
26. Diamond EL, Durham BH, Haroche J, et al. Diverse and Targetable Kinase Alterations Drive Histiocytic Neoplasms. *Cancer Discov.* 2016;6(2):154–165.
27. Lee LH, Gasilina A, Roychoudhury J, et al. Real-time genomic profiling of histiocytoses identifies early-kinase domain BRAF alterations while improving treatment outcomes. *JCI Insight*. 2017;2(3):e89473.
28. Héritier S, Hélias-Rodzewicz Z, Chakraborty R, et al. New somatic BRAF splicing mutation in Langerhans cell histiocytosis. *Mol. Cancer*. 2017;16(1):115.
29. Héritier S, Emile J-F, Hélias-Rodzewicz Z, Donadieu J. Progress towards molecular-based management of childhood Langerhans cell histiocytosis. *Arch. Pédiatrie*. 2019;26(5):301–307.
30. McGinnis LM, Nybakken G, Ma L, Arber DA. Frequency of MAP2K1, TP53, and U2AF1 Mutations in BRAF-mutated Langerhans Cell Histiocytosis. *Am. J. Surg. Pathol.* 2018;42(7):885–890.
31. Jouenne F, Chevret S, Bugnet E, et al. Genetic landscape of adult Langerhans cell histiocytosis with lung involvement. *Eur. Respir. J.* 2020;55(2):1901190.
32. Kemps PG, Hebeda KM, Pals ST, et al. Spectrum of histiocytic neoplasms associated with diverse haematological malignancies bearing the same oncogenic mutation. *J. Pathol. Clin. Res.* 2021;7(1):10–26.
33. Ronceray L, Pötschger U, Janka G, Gadner H, Minkov M. Pulmonary Involvement in Pediatric-Onset Multisystem Langerhans Cell Histiocytosis: Effect on Course and Outcome. *J. Pediatr.* 2012;161(1):129-133.e3.
34. Grois N, Pötschger U, Prosch H, et al. Risk factors for diabetes insipidus in langerhans cell histiocytosis. *Pediatr. Blood Cancer*. 2006;46(2):228–233.
35. Grois N, Fahrner B, Arceci RJ, et al. Central Nervous System Disease in Langerhans Cell Histiocytosis. *J. Pediatr.* 2010;156(6):873-881.e1.
36. Chellapandian D, Shaikh F, van den Bos C, et al. Management and Outcome of Patients With Langerhans Cell Histiocytosis and Single-Bone CNS-Risk Lesions: A Multi-Institutional Retrospective Study. *Pediatr. Blood Cancer*. 2015;62(12):2162–2166.
37. Kemps PG, Zondag TC, Steenwijk EC, et al. Apparent Lack of BRAFV600E Derived HLA Class I Presented Neoantigens Hampers Neoplastic Cell Targeting by CD8+ T Cells in Langerhans Cell Histiocytosis. *Front. Immunol.* 2020;10:3045.
38. Melloul S, Hélias-Rodzewicz Z, Cohen-Aubart F, et al. Highly sensitive methods are required to detect mutations in histiocytoses. *Haematologica*. 2019;104(3):e97–e99.
39. van Eijk R, Licht J, Schrumpf M, et al. Rapid KRAS, EGFR, BRAF and PIK3CA Mutation Analysis of Fine Needle Aspirates from Non-Small-Cell Lung Cancer Using Allele-Specific qPCR. *PLoS One*. 2011;6(3):e17791.
40. van Eijk R, Stevens L, Morreau H, van Wezel T. Assessment of a fully automated high-throughput DNA extraction method from formalin-fixed, paraffin-embedded tissue for KRAS, and BRAF somatic mutation analysis. *Exp. Mol. Pathol.* 2013;94(1):121–125.

41. Xiao Y, van Halteren AGS, Lei X, et al. Bone marrow–derived myeloid progenitors as driver mutation carriers in high- and low-risk Langerhans cell histiocytosis. *Blood*. 2020;136(19):2188–2199.
42. Cohen D, Hondelink LM, Solleveld-Westerink N, et al. Optimizing Mutation and Fusion Detection in NSCLC by Sequential DNA and RNA Sequencing. *J. Thorac. Oncol.* 2020;15(6):1000–1014.
43. de Vree PJP, de Wit E, Yilmaz M, et al. Targeted sequencing by proximity ligation for comprehensive variant detection and local haplotyping. *Nat. Biotechnol.* 2014;32(10):1019–1025.
44. Allahyar A, Pieterse M, Swennenhuis J, et al. Robust detection of translocations in lymphoma FFPE samples using targeted locus capture-based sequencing. *Nat. Commun.* 2021;12(1):3361.
45. Schemper M, Smith TL. A note on quantifying follow-up in studies of failure time. *Control. Clin. Trials*. 1996;17(4):343–346.
46. Chen S-H, Zhang Y, Van Horn RD, et al. Oncogenic BRAF Deletions That Function as Homodimers and Are Sensitive to Inhibition by RAF Dimer Inhibitor LY3009120. *Cancer Discov.* 2016;6(3):300–315.
47. Foster SA, Whalen DM, Özen A, et al. Activation Mechanism of Oncogenic Deletion Mutations in BRAF, EGFR, and HER2. *Cancer Cell*. 2016;29(4):477–493.
48. Yap J, Deepak RNVK, Tian Z, et al. The stability of R-spine defines RAF inhibitor resistance: A comprehensive analysis of oncogenic BRAF mutants with in-frame insertion of αC-β4 loop. *Sci. Adv.* 2021;7(24):eabg0390.
49. Ducassou S, Seyrig F, Thomas C, et al. Thymus and mediastinal node involvement in childhood langerhans cell histiocytosis: Long-term follow-up from the French national cohort. *Pediatr. Blood Cancer*. 2013;60(11):1759–1765.
50. Picarsic J, Egeler RM, Chikwava K, Patterson K, Jaffe R. Histologic Patterns of Thymic Involvement in Langerhans Cell Proliferations: A Clinicopathologic Study and Review of the Literature. *Pediatr. Dev. Pathol.* 2015;18(2):127–138.
51. Yao J-F, Wang D, Ma H-H, et al. Characteristics and Treatment Outcomes of Pediatric Langerhans Cell Histiocytosis with Thymic Involvement. *J. Pediatr.* 2022;244(0):194-202.e5.
52. Kansal R, Quintanilla-Martinez L, Datta V, et al. Identification of the V600D mutation in Exon 15 of the BRAF oncogene in congenital, benign langerhans cell histiocytosis. *Genes, Chromosom. Cancer*. 2013;52(1):99–106.
53. Janssen LJF, Brons PPT, Schreuder HWB, et al. Image Gallery: A rare abscess-like presentation of Langerhans cell histiocytosis. *Br. J. Dermatol.* 2017;176(4):e33–e33.
54. Kambouchner M, Emile J-F, Copin M-C, et al. Childhood pulmonary Langerhans cell histiocytosis: a comprehensive clinical-histopathological and BRAFV600E mutation study from the French national cohort. *Hum. Pathol.* 2019;89:51–61.
55. Della Valle V, Donadieu J, Sileo C, et al. Chest computed tomography findings for a cohort of children with pulmonary Langerhans cell histiocytosis. *Pediatr. Blood Cancer*. 2020;67(10):e28496.
56. Le Louet S, Barkaoui M-A, Miron J, et al. Childhood Langerhans cell histiocytosis with severe lung involvement: a nationwide cohort study. *Orphanet J. Rare Dis.* 2020;15(1):241.
57. Minkov M, Pötschger U, Thacker N, et al. Additive Prognostic Impact of Gastrointestinal Involvement in Severe Multisystem Langerhans Cell Histiocytosis. *J. Pediatr.* 2021;237:65-70.e3.
58. Héritier S, Barkaoui M-A, Miron J, et al. Incidence and risk factors for clinical neurodegenerative Langerhans cell histiocytosis: a longitudinal cohort study. *Br. J. Haematol.* 2018;183(4):608–617.
59. Chen J, Zhao A-L, Duan M-H, et al. Diverse kinase alterations and myeloid-associated mutations in adult histiocytosis. *Leukemia*. 2022;36(2):573–576.
60. Cao X, Duan M, Zhao A, et al. Treatment outcomes and prognostic factors of patients with adult Langerhans cell histiocytosis. *Am. J. Hematol.* 2022;97(2):203–208.

61. Stathi D, Yavropoulou MP, Allen CE, et al. Prevalence of the BRAF V600E mutation in Greek adults with Langerhans cell histiocytosis. *Pediatr. Hematol. Oncol.* 2022;1–9.
62. Yuan J, Ng WH, Tian Z, et al. Activating mutations in MEK1 enhance homodimerization and promote tumorigenesis. *Sci. Signal.* 2018;11(554):eaar6795.
63. Hanrahan AJ, Sylvester BE, Chang MT, et al. Leveraging Systematic Functional Analysis to Benchmark an In Silico Framework Distinguishes Driver from Passenger MEK Mutants in Cancer. *Cancer Res.* 2020;80(19):4233–4243.
64. Gao Y, Chang MT, McKay D, et al. Allele-Specific Mechanisms of Activation of MEK1 Mutants Determine Their Properties. *Cancer Discov.* 2018;8(5):648–661.
65. Kemps PG, Picarsic J, Durham BH, et al. ALK-positive histiocytosis: a new clinicopathologic spectrum highlighting neurologic involvement and responses to ALK inhibition. *Blood*. 2022;139(2):256–280.
66. Hyman DM, Diamond EL, Vibat CRT, et al. Prospective Blinded Study of BRAF V600E Mutation Detection in Cell-Free DNA of Patients with Systemic Histiocytic Disorders. *Cancer Discov.* 2015;5(1):64–71.
67. Héritier S, Hélias-Rodzewicz Z, Lapillonne H, et al. Circulating cell-free BRAF V600E as a biomarker in children with Langerhans cell histiocytosis. *Br. J. Haematol.* 2017;178(3):457–467.
68. Schwentner R, Kolenová A, Jug G, et al. Longitudinal assessment of peripheral blood BRAFV600E levels in patients with Langerhans cell histiocytosis. *Pediatr. Res.* 2019;85(6):856–864.
69. Eckstein OS, Visser J, Rodriguez-Galindo C, Allen CE. Clinical responses and persistent BRAF V600E+ blood cells in children with LCH treated with MAPK pathway inhibition. *Blood*. 2019;133(15):1691–1694.
70. Donadieu J, Larabi IA, Tardieu M, et al. Vemurafenib for Refractory Multisystem Langerhans Cell Histiocytosis in Children: An International Observational Study. *J. Clin. Oncol.* 2019;37(31):2857–2865.
71. Cui L, Zhang L, Ma H-H, et al. Circulating cell-free *BRAF* V600E during chemotherapy is associated with prognosis of children with Langerhans cell histiocytosis. *Haematologica*. 2020;105(9):e444-447.
72. Wang C-J, Cui L, Ma H-H, et al. BRAF V600E Mutation in Cell-Free DNA, Rather than in Lesion Tissues, at Diagnosis Is An Independent Prognostic Factor in Children with Langerhans Cell Histiocytosis. *Mol. Cancer Ther.* 2021;20(7):1316–1323.
73. Poch R, Le Louet S, Hélias-Rodzewicz Z, et al. A circulating subset of BRAF V600E -positive cells in infants with high-risk Langerhans cell histiocytosis treated with BRAF inhibitors. *Br. J. Haematol.* 2021;194(4):745–749.
74. Eder SK, Schwentner R, Ben Soussia P, et al. Vemurafenib acts as a molecular on-off switch governing systemic inflammation in Langerhans cell histiocytosis. *Blood Adv.* 2022;6(3):970–975.
75. Kudo K, Toki T, Kanezaki R, et al. BRAF V600E-positive cells as molecular markers of bone marrow disease in pediatric Langerhans cell histiocytosis. *Haematologica*. 2022;107(7):1719–1725.
76. McClain KL, Chakraborty R. BRAF V600E vs cell of origin: what governs LCH? *Blood*. 2021;138(14):1203–1204.
77. McClain KL, Bigenwald C, Collin M, et al. Histiocytic disorders. *Nat. Rev. Dis. Prim.* 2021;7(1):73.
78. Zeng K, Wang Z, Ohshima K, et al. BRAF V600E mutation correlates with suppressive tumor immune microenvironment and reduced disease-free survival in Langerhans cell histiocytosis. *Oncoimmunology*. 2016;5(7):e1185582.
79. Sengal A, Velazquez J, Hahne M, et al. Overcoming T-cell exhaustion in LCH: PD-1 blockade and targeted MAPK inhibition are synergistic in a mouse model of LCH. *Blood*. 2021;137(13):1777–1791.

80. Milne P, Bigley V, Bacon CM, et al. Hematopoietic origin of Langerhans cell histiocytosis and Erdheim-Chester disease in adults. *Blood*. 2017;130(2):167–175.
81. Milne P, Abhyankar HA, Scull BP, et al. Cellular distribution of mutations and association with disease risk in Langerhans cell histiocytosis without BRAFV600E. *Blood Adv.* 2022. [Epub ahead of print]
82. Halbritter F, Farlik M, Schwentner R, et al. Epigenomics and Single-Cell Sequencing Define a Developmental Hierarchy in Langerhans Cell Histiocytosis. *Cancer Discov.* 2019;9(10):1406–1421.
83. Zhou X, Edmonson MN, Wilkinson MR, et al. Exploring genomic alteration in pediatric cancer using ProteinPaint. *Nat. Genet.* 2016;48(1):4–6.
84. Cerami E, Gao J, Dogrusoz U, et al. The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data: Figure 1. *Cancer Discov.* 2012;2(5):401–404.
85. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative Analysis of Complex Cancer Genomics and Clinical Profiles Using the cBioPortal. *Sci. Signal.* 2013;6(269):pl1.
86. Zarnegar S, Durham BH, Khattar P, et al. Novel activating BRAF fusion identifies a recurrent alternative mechanism for ERK activation in pediatric Langerhans cell histiocytosis. *Pediatr. Blood Cancer*. 2018;65(1):e26699.
87. Zanwar S, Abeykoon JP, Dasari S, et al. Clinical and therapeutic implications of BRAF fusions in histiocytic disorders. *Blood Cancer J.* 2022;12(6):97.
88. Zhang R, Wang C-J, Zhao Y-Z, Ma H-H, Wang D, Zhang L, Lian H-H, Li W-J, Zhang Q, Li N, Yang Y, Wang T-Y, Cui L LZ-G. Genetic Landscape and its Prognostic Significance in Children with Langerhans cell Histiocytosis. *Pediatr. Blood Cancer*. 2022;69(S1):e29453.

XI

Apparent lack of *BRAF*p.V600E derived HLA class I presented neoantigens hampers neoplastic cell targeting by CD8+ T cells in Langerhans cell histiocytosis

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ABSTRACT

Langerhans Cell Histiocytosis (LCH) is a neoplastic disorder of hematopoietic origin characterized by inflammatory lesions containing clonal histiocytes (LCH-cells) intermixed with various immune cells, including T cells. In 50-60% of LCH-patients, the somatic *BRAF*^{V600E} driver mutation, which is common in many cancers, is detected in these LCH-cells in an otherwise quiet genomic landscape. Nonsynonymous mutations like *BRAF*^{V600E} can be a source of neoantigens capable of eliciting effective antitumor CD8^{+} T cell responses. This requires neopeptides to be stably presented by Human Leukocyte Antigen (HLA) class I molecules and sufficient numbers of CD8^{+} T cells at tumor sites. Here, we demonstrate substantial heterogeneity in CD8^{+} T cell density in n=101 LCH-lesions, with *BRAF*^{V600E} mutated lesions displaying significantly lower CD8^{+} T cell:CD1a^{+} LCH-cell ratios (p=0.01) than *BRAF* wildtype lesions. Because LCH-lesional CD8^{+} T cell density had no significant impact on event-free survival, we investigated whether the intracellularly expressed *BRAF*^{V600E} protein is degraded into neopeptides that are naturally processed and presented by cell surface HLA class I molecules. Epitope prediction tools revealed a single HLA class I binding *BRAF*^{V600E} derived neopeptide (KIGDFGLAT**E**K), which indeed displayed strong to intermediate binding capacity to HLA-A*03:01 and HLA-A*11:01 in an *in vitro* peptide-HLA binding assay. Mass spectrometry-based targeted peptidomics was used to investigate the presence of this neopeptide in HLA class I presented peptides isolated from several *BRAF*^{V600E} expressing cell lines with various HLA genotypes. While the HLA-A*02:01 binding *BRAF* wildtype peptide KIGDFGLATV was traced in peptides isolated from all five cell lines expressing this HLA subtype, KIGDFGLAT**E**K was not detected in the HLA class I peptidomes of two distinct *BRAF*^{V600E} transduced cell lines with confirmed expression of HLA-A*03:01 or HLA-A*11:01. These data indicate that the *in silico* predicted HLA class I binding and proteasome-generated neopeptides derived from the *BRAF*^{V600E} protein are not presented by HLA class I molecules. Given that the *BRAF*^{V600E} mutation is highly prevalent in chemotherapy refractory LCH-patients who may qualify for immunotherapy, this study therefore questions the efficacy of immune checkpoint inhibitor therapy in LCH.

INTRODUCTION

Langerhans Cell Histiocytosis (LCH) is a rare neoplastic disorder of hematopoietic origin that primarily affects children, but also involves adults (1). Its clinical manifestation varies from a single bone lesion or benign skin rash to a widely disseminated and life-threatening condition, similar to acute myeloid leukemia (2). The histopathological hallmark of LCH are phenotypically aberrant $CD1a^+$ $CD207^+$ histiocytes (LCH-cells), although not all pathological $CD1a^+$ histiocytes co-express CD207 (3). Typically, these cells are accompanied by a diverse inflammatory infiltrate, often including T cells (2). These T cells have been shown to frequently make intimate contact with LCH-cells (4,5). While patients with high $CD8^+$ T cell density in the tumor infiltrate have a more favorable prognosis across many other neoplastic diseases (6), little is still known about the presence and clinical impact of $CD8^+$ T cells in LCH-lesions (7–9).

Naive ($CD8^+$) T cells require antigen binding by their T cell receptor and co-stimulatory signals for (proper) activation. Previous studies have already demonstrated that LCH-cells express the co-stimulatory receptors CD40 (10–12), CD80 (3,11–13), ICOS ligand (ICOSL) (14) and, although variably, CD86 (3,11,12) *in situ*. Moreover, transcriptome analyses revealed that LCH-cells express similar levels of CD40, CD80 and CD86 messenger RNA when compared to normal epidermal $CD207^+$ Langerhans cells (15,16), and that they confer high expression of genes relevant for antigen presentation (including CD1E) and genes encoding members of the HLA (class II) complex (17). Thus, LCH-cells do not appear to have an intrinsic defect in their capacity to elicit a T cell immune response (12). This may explain why a proportion of LCH-lesional T cells have been shown to express cell surface markers indicative of recent activation (2), including CD40L (10), ICOS (14), CXCR3 (7), CD25 (5,14), PD-1 (18,19), RANKL (20) and CD45RO (7). In addition, marked monoclonal expansion of LCH-lesion infiltrating $CD3^+$ T cells has been observed (19), suggesting that T cell receptor activation occurred *in situ*. The antigen-specificity of activated LCH-lesional T cells is, however, unknown (2).

In 2010, universal activation of the mitogen-activated protein kinase (MAPK) signaling pathway in LCH-cells was demonstrated (21,22). Since then, recurrent somatic mutations in genes of the MAPK signaling pathway have been identified in approximately 85% of LCH-patients (23,24). Oncogenic driver mutations are essential for tumorigenesis and tend to be clonally conserved. This makes neoantigens derived from proteins encoded by oncogenes highly attractive targets for immunotherapy. In addition, the natural T cell pool should contain T cells expressing high affinity T cell receptors for these neoantigens (25), which may exert potent antitumor function (26–29). This requires, however, neoantigens to be stably presented by Human Leukocyte Antigen (HLA) class I molecules and sufficient numbers of $CD8^+$ T cells at tumor sites. Over the past years, several HLA class I presented "public" neoantigens resulting from recurrent hotspot

mutations in driver oncogenes have been discovered (30–38). Approximately 50-60% of LCH-patients carry the somatic *BRAF*^{V600E} driver mutation (1,21). CD8^{+} T cells specific for *BRAF*^{V600E} derived neopeptides have already been reported *in vitro* and in murine models (39–42). Thus, activation of LCH-lesional *BRAF*^{V600E} neoantigen-specific CD8^{+} T cells could hypothetically lead to the eradication of *BRAF*^{V600E} expressing LCH-cells. Moreover, the concurrent formation of long-lasting bone-marrow homing memory CD8^{+} T cells could control new outgrowth of residual *BRAF*^{V600E} mutated histiocyte precursor cells (43). Immunotherapy specifically aimed at enhancing the number and effector function of these *BRAF*^{V600E}-specific CD8^{+} T cells could offer great promise in the treatment of high-risk LCH-patients, given that these patients often bear the *BRAF*^{V600E} mutation and fail first-line chemotherapy (44). Importantly, the *BRAF* gene is mutated in ~7% of human cancers, with the *BRAF*^{V600E} mutation accounting for >90% of all genetic variations (45,46). Hence, the identification of HLA class I presented "public" neoantigens derived from the *BRAF*^{V600E} protein would offer great therapeutic opportunity for many patients with other *BRAF*^{V600E} mutated neoplasms as well (47).

The aim of this study was therefore to (i) assess the presence and clinical impact of lesional CD8^{+} T cells in (HLA) genotyped LCH-patients, and (ii) to investigate whether *BRAF*^{V600E} derived neopeptides are presented by HLA class I molecules and could be recognized by such CD8^{+} T cells.

MATERIALS AND METHODS

Patients and samples

Patient accrual started after approval of the study protocol (CCMO NL33428.058.10) by each local Institutional Review Board. Only patients of whom formalin-fixed-paraffin-embedded (FFPE) first disease onset (FDO) LCH tissue biopsies were available were asked to participate in the study. Informed consent was provided by n=135 patients and/or their parents/legal guardians. LCH diagnosis was confirmed by a combination of clinical findings and the presence of phenotypically aberrant CD1a^{+} histiocytes in the tissue biopsy. The tissue samples were handled according to the code of conduct for proper secondary use of human tissue of the Federation of Dutch Medical Scientific Societies (FEDERA). Clinical information was collected by each participating center separately using a standardized Case Report Form (CRF) and anonymized data were provided to the researchers of the LUMC. Events were defined as LCH disease progression or reactivation. Progression was defined as (i) progression of existing lesions requiring start or intensification of systemic chemotherapy and/or radiotherapy, or (ii) the development of new lesions when Non-Active Disease (NAD) state had not yet been

attained. LCH reactivation was defined as the development of new lesions after NAD had been attained for LCH FDO.

Flow cytometric analysis of LCH tissue biopsies

Fresh LCH tissue was dissociated using a gentle MACS tissue dissociator (Miltenyi Biotec) and single cells were cryopreserved in DMSO and albumin containing Roswell Park Memorial Institute (RPMI) culture medium. Before flow cytometric analysis, cells were thawed in RPMI + 20% fetal calf serum (FCS) + Penicillin-Streptomycin (P/S) containing 1600 IU/ml DNAase (Sigma-Aldrich). After washing, the cells were stained with a mixture of different antibodies: CD45 (2D1, 1:50, BD Biosciences), CD1a (HI149, 1:50, BD Biosciences), CD207 (DCGM4, 1:25, Beckman Coulter), CD14 (MØP9, 1:20, BD Biosciences), CD3 (UCHT1, 1:200, BD Biosciences), CD8 (SK1, 1:100, BD Biosciences), HLA-DR (G46-6, 1:200, BD Biosciences) and panHLA class I (G46-2.6, 1:40, BD Biosciences). The cells were then re-washed and immediately analyzed on a FACS ARIA3 or FACS Fusion cell sorter (BD Biosciences)

HLA genotyping and analysis

High-resolution HLA genotyping was performed by DKMS Life Sciences Lab on DNA extracted from buccal swabs obtained from n=104 LCH-patients using an ampliqon sequencing-based approach, as previously described (48,49). For n=14 additional patients, low-resolution HLA genotype data were acquired using a sequence specific oligoprimer-based approach (50). Hardy-Weinberg Equilibrium testing and HLA association analyses were performed using the HLA genotype data of Dutch LCH-patients. To evaluate statistical significance, two-sided Fisher's exact tests were carried out. The p-values were corrected for multiple comparisons conform the Šidák method (51). Odds ratios and corresponding 95% confidence intervals were calculated according to the method of Woolf with the Haldane correction (52,53). Since a large control group could lead to significant differences that are clinically irrelevant, p-values were standardized to a smaller control sample size following the method of Good (54). The smaller control sample size was obtained using the following calculation: the total number of LCH-patients plus 3 times the number of patients as maximum allowed size for the control group.

Immunohistochemical staining of LCH tissue sections

FFPE tissue sections (4-10µm) were deposited on Superfrost™ (Thermo Fisher Scientific) glass slides, dried overnight at 37°C and stored at 4°C. Prior to immunohistochemical (IHC) staining, selected 4µm slides were preheated at 66°C for 1 hour and deparaffinized in xylol. For enzymatic CD1a IHC staining, endogenous peroxidase was blocked using Methanol/0.3% H_2O_2 for 20 minutes, before slides were rehydrated in ethanol and

demineralized in water baths. Antigen retrieval was performed in boiling citrate buffer (pH 6.0) for 10 minutes and sections were incubated overnight with mouse IgG1-anti-human CD1a antibody (Clone 010, 1:800, DAKO) diluted in phosphate buffered saline (PBS)/0.5% bovine serum albumin (BSA). The next day, Envision+ System-HRP labelled polymer anti-mouse (DAKO) was applied for 30 minutes and color development was attained using commercial DAB+ (DAKO) for 10 minutes in the dark. This reaction was stopped using demineralized water and slides were counterstained with Mayer's hematoxylin (Klinipath) for 5 seconds prior to mounting with Pertex (Leica Microsystems).

An earlier published protocol was used for triple CD1a/CD3/CD8 fluorescent IHC staining (14). In brief, antigen retrieval was performed in boiling EDTA buffer (pH 8.0) for 10 minutes followed by a blocking step using 10% Normal Goat Serum in PBS/0.5% BSA for 15 minutes at room temperature. Slides were incubated overnight with the following primary antibody mix: rabbit IgG-anti-human CD3 (polyclonal, 1:300, DAKO), mouse IgG2b-anti-human CD8 (clone 4B11, 1:100, Novocastra, via Leica Microsystems) and mouse IgG1-anti-human CD1a (Clone 010, 1:400, DAKO). The next day, tissue slides were incubated for 30 minutes in the dark with 1:300 diluted goat-anti-mouse IgG1 Alexa Fluor 488, goat-anti-mouse IgG2b Alexa Fluor 546 and goat-anti-mouse IgG2a Alexa Fluor 647 antibodies (all from Invitrogen, via Thermofisher Life Technologies Europe). After washing in PBS, the sections were mounted with Mowiol (homemade) or Prolong Gold (Thermo Fisher Scientific) and stored in the dark at 4°C.

BRAF^{V600E} mutation analysis

CD1a^{+} enriched tissue parts were marked by a blinded pathologist on enzymatically CD1a stained LCH tissue slides. Based on these reference slides, CD1a^{+} enriched tissue parts were manually microdissected from multiple consecutively cut 10µm tissue sections prepared from the remainder of the LCH tissue blocks. Total nucleic acid was automatically isolated from microdissected tissue using the Siemens Tissue Preparation System (Siemens Healthcare) robot (55). Presence of the *BRAF^{V600E}* mutation was assessed by allele-specific real-time qPCR, as previously described (56). Of the n=54 *BRAF^{V600E}* negative samples, absence of the *BRAF^{V600E}* mutation was confirmed in 46 samples (85%) by next-generation sequencing (n=39), whole exome sequencing (n=1) (57) or *BRAF^{V600E}* droplet digital PCR (n=6).

Quantification of T cell density in LCH-lesions

For the manual cell counting method, multiple representative images were taken of each tissue slide at 400x magnification using a conventional Leica DM5500 fluorescent microscope equipped with LAS AF software (Leica Microsystems). Images were solely taken of representative areas containing phenotypically aberrant CD1a^{+} LCH-cells. Using Image J software (version 1.47v) with the public Cell Counter plugin, fluorescently

stained $CD1a^+$, $CD3^+CD8^-$ and $CD3^+CD8^+$ cells were manually counted in all images by two independent researchers (PGK and ECS) who were unaware of patient identity and outcome data. The cell counts of the individual images were added to form total $CD1a^+$, $CD3^+CD8^-$, $CD3^+CD8^+$ cell counts. When total cell counts differed more than 10% between the two researchers, a third researcher (AGSH) reviewed the cell counting results and selected the most appropriate scoring (19/101 cases). Total $CD3^+$ cell counts were obtained by adding total $CD3^+CD8^-$ and $CD3^+CD8^+$ cell counts. To adjust for substantial differences in biopsy size between different patients, which may lead to profound disparities in absolute numbers of counted cells, ratios between the final numbers of total $CD3^+$ and $CD3^+CD8^+$ T cells and $CD1a^+$ LCH-cells were calculated for each patient.

For the manual semi-quantitative eyeball estimation method, whole slide images were taken of the same immunostained tissue slides at 400x magnification using a Pannoramic 250 Flash II slidescanner (3DHISTECH). These images were scored semi-quantitatively for LCH-lesional $CD3^+$ and $CD3^+CD8^+$ T cell density as has been previously described (58,59): 1+, no, or sporadic T cells; 2+, moderate number of T cells; 3+, abundant occurrence of T cells; and 4+, highly abundant occurrence of T cells. Scoring examples are shown in Supplementary Figure 1. Unfortunately, n=21/101 (21%) of the tissue slides could not be reanalyzed due to considerable photobleaching of the fluorophores, induced by the earlier collection of high-power images for the manual cell counting analysis. Slides were scored independently by three researchers (PGK, ECS and AGSH). When scorings between two or more researchers differed more than 1 value (15/80 cases), the scoring was reviewed by all three researchers collectively and a consensus score was attained. Otherwise, the average score of the three scorings determined the final result, rounded to the nearest whole value (1-4+).

Whole slide images of sufficient quality (without significant color casts and/or folded tissue parts that are highly autofluorescent and/or out of focus) from n=48 LCH-patients were analyzed using a quantitative automated digital image analysis method (Figure S2). First, the LCH-lesion and its directly adjacent T cells were encircled in the whole slide image in CaseViewer software and exported. In this way, cells that clearly did not belong to the microenvironment of the $CD1a^+$ LCH-cells were excluded. Using a custom in-house developed macro in ImageJ software, a white balance was then set for each individual exported image by designating background, foreground and autofluorescence. Next, uniform color thresholds for green ($CD1a^+$), red ($CD3^+CD8^-$) and purple ($CD3^+CD8^+$) were applied to all images, so that only green, red and purple areas with color intensities higher than the threshold remained. Since automated quantification of individual cells was not feasible, the cumulative area of the remaining green, red and purple areas was measured for each image, representing the total quantity of $CD1a^+$, $CD3^+CD8^-$ and $CD3^+CD8^+$ cells. Purple&Red ($CD3^+$) area/Green ($CD1a^+$) area and Purple ($CD3^+CD8^+$) area/Green ($CD1a^+$) area ratios could then be calculated for each patient. Comparison

of the results obtained using our three separate analysis methods showed substantial concordance (Figure S3), supporting the validity of the findings in this study.

In vitro peptide-HLA class I binding analysis

Competition-based peptide-HLA class I binding assays were performed as previously described (60). The HLA binding affinities of the target peptides and strong binding reference peptides are expressed as the concentration that inhibits 50% binding of a fluorescently-labeled standard peptide (IC_{50}). The standard peptides were FLPSDCFPSV for HLA-A*02:01 and KVFPCALINK for HLA-A*03:01 and HLA-A*11:01. Notably, the ratio between the IC_{50} of a target peptide and the IC_{50} of an established strong binding reference peptide (for example 260:250 vs. 100:5) provides superior information about the true HLA class I binding capacity of the target peptide than the absolute IC_{50} of the target peptide.

Generation of $BRAF^{V600E}$ expressing EBV-LCLs

The full length $BRAF^{V600E}$ sequence incorporated in a pBABE-Puro-BRAF-V600E plasmid was re-cloned into a LZRS-ires-Green Fluorescent Protein (GFP) retroviral vector by introducing the SwaI restriction site and a kozak sequence in front of the ATG start codon at the 5'end of the $BRAF^{V600E}$ sequence using Phusio DNA polymerase. In addition, a stop codon and NotI restriction site was introduced at the 3'end of the $BRAF^{V600E}$ sequence. The original pBABE-Puro-BRAF-V600E plasmid was kindly provided by William Hahn (Addgene plasmid #15269; http://n2t.net/addgene:15269; RRID: Addgene_15269) (61). Ligation of the *BRAF* PCR product in the LZRS vector digested with SwaI and NotI was performed overnight at 16 degrees Celcius. Prior to spin inoculation of Phoenix packaging cells, the correct sequence of the re-cloned $BRAF^{V600E}$ gene was confirmed by Sanger sequencing (data not shown). Retrovirus containing supernatant was subsequently used to transduce Epstein-Barr virus-immortalized B cell lines (EBV-LCLs) with either a control empty LZRS vector (mock transduced EBV-LCL) or with the new $BRAF^{V600E}$ containing LZRS vector ($BRAF^{V600E}$ transduced EBV-LCL). Stably transduced GFP^{high} cells were purified using an ARIA3 flowcytometer prior to bulk expansion in RPMI medium containing 10% bovine serum.

Mass spectrometry-based targeted peptidomics

Cells were lysed at a concentration of 100e6 cells/ml lysis buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Zwittergent 3-12 (N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate) and protease inhibitor (Complete, Roche Applied Science)) for 2 h at 0°C (62). Lysates were successively centrifuged for 10 min at 2500 × g and for 45 min at 31,000 x g to remove nuclei and other insoluble material, respectively. Next, lysates were cleared through a CL-4B Sepharose column (1 ml/1e9 cells) and passed through

an anti-panHLA class I column containing 2.5 mg W6/32 IgG per ml protein A Sepharose (62). The W6/32 column was washed three times each with 1 ml of lysis buffer, 3 ml of low salt buffer (20 mM Tris-Cl pH 8.0, 120 mM NaCl), 1 ml of high salt buffer (20 mM Tris-Cl pH 8.0, 1 M NaCl), and finally with 3 ml of low salt buffer. Peptides were eluted with 5 ml of 10% acetic acid per ml column, diluted with 10 ml of 0.1% formic acid and purified by SPE (Oasis HLB, Waters) using 20% and 30% acetonitrile in 0.1% formic acid to elute the peptides.

For parallel reaction monitoring (PRM) analyses, the samples were lyophilized and resuspended in buffer A. HLA-eluates were injected together with a mix of 40 fmol of each heavy labeled peptide. The Orbitrap Fusion LUMOS mass spectrometer was operated in PRM-mode. Peptides KIGDFGLAT**E**, KIGDFGLATV, KIGDFGLATEK, and KIGDFGLATVK were monitored. Selected peptides, the transitions and collision energies can be found in Supplementary Table 1. The isolation width of Q1 was 1.2 Da. MS2 resolution was 35,000 at an AGC target value of 1 million at a maximum fill time of 100 ms. The gradient was run from 2% to 36% solvent B (20/80/0.1 water/acetonitrile/formic acid (FA) v/v) in 120 min. The nano-HPLC column was drawn to a tip of ~5 µm and acted as the electrospray needle of the MS source. PRM data analysis and data integration were performed in Skyline 3.6.0.10493. Peptide abundances were calculated by comparing the peak area of the eluted (light) and the peak area of the spiked-in heavy peptides.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0.1 and IBM SPSS Statistics version 25. Comparisons of (sub)groups were performed with the Mann-Whitney U test for continuous data and the Fisher exact test for categorical data. The Cox proportional hazards model was used for univariate analysis. Notably, log transformation of the widely differing $CD8^+$ T cell:$CD1a^+$ LCH-cell ratios was performed to increase the validity of the univariate analysis. Survival curves were estimated with the Kaplan-Meier method and compared with the Log-rank test. A p-value of <0.05 was considered statistically significant.

RESULTS

LCH-cells express normal levels of HLA class I and II molecules at their cell surface

Since loss or downregulation of HLA expression has been shown to be a major tumor escape mechanism from T lymphocytes in a wide variety of cancers (63), we first evaluated by flow cytometric analysis the levels of HLA class I and HLA-DR expression on the surface of $CD1a^+$ (LCH-)cells present in n=6 LCH-biopsies. The gating strategy applied is shown in Supplementary Figure 4. The mean fluorescent intensity (MFI) of

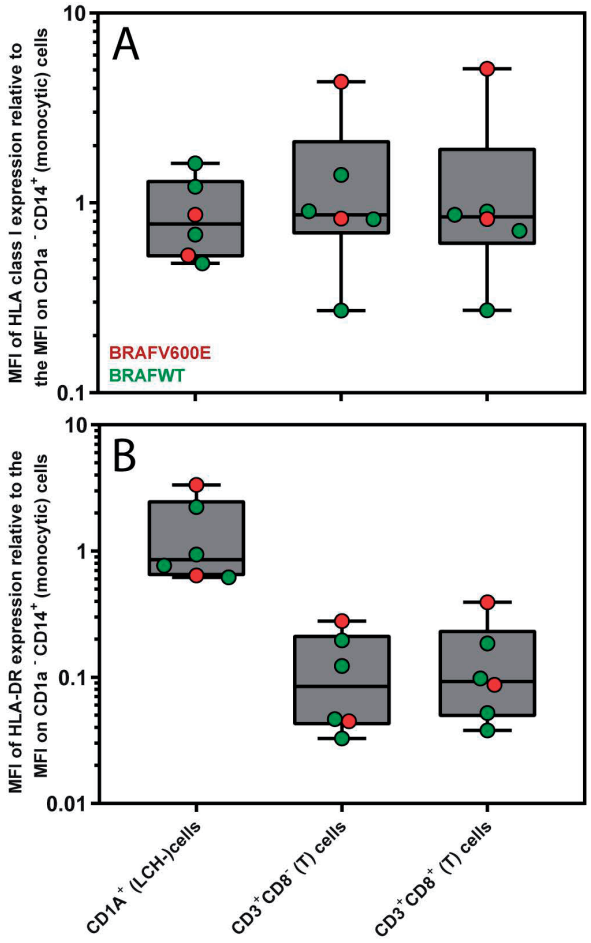

Figure 1 | Flow cytometric measurements of HLA class I and HLA-DR expression by LCH biopsy-derived hematopoietic cells.

Mean Fluorescent Intensity (MFI) of (A) HLA class I (W6/32) and (B) HLA-DR expression by live $CD1a^+$ (LCH-)cells, $CD3^+CD8^-$ (T) cells and $CD3^+CD8^+$ (T) cells relative to the MFI of HLA class I or HLA-DR expression by $CD1a^-$ $CD14^+$ (monocytic) cells. The gating strategy applied is shown in Supplementary Figure 1. The box extends from the 25^{th} to 75^{th} percentiles. The line in the middle of the box is plotted at the median. The whiskers go down to the smallest value and up to the largest. Each individual value is plotted as a point superimposed on the graph.

HLA class I and HLA-DR expression by $CD1a^+$ (LCH-)cells was comparable to MFI levels of HLA class I and HLA-DR expression by $CD1a^-$ $CD14^+$ (monocytic) cells present in the same LCH-biopsies (Figure 1; HLA class I, p=0.69; HLA-DR, p=0.94).

The HLA genotype of LCH-patients does not differ from healthy controls

Besides HLA expression, HLA subtype is a crucial factor influencing whether a (neo) antigen is actually presented at the surface of nucleated cells. Several earlier published studies have suggested associations between particular HLA subtypes and LCH disease (extension) (64–67). To investigate this, we compared HLA genotype data from n=94

Dutch LCH-patients to the HLA genotypes of 5604 healthy Dutch blood donors reflecting the HLA genotype of the Dutch population (50). To maintain sufficient statistical power, HLA genotype was compared at low resolution level. No significant differences between Dutch LCH-patients and the Dutch reference population were observed (Table S2). Thus, our data do not support previous reports describing excess frequency of HLA-Bw61 and HLA-Cw7 (64), HLA-B7 and HLA-DR2 (65) and HLA-DR4 and/or HLA-Cw7 (66) genotypes in LCH-patients (Table S2 and S3). Moreover, our results neither confirm that LCH-patients with unifocal bone disease have significantly more often HLA-DR4 and/or HLA-Cw7 (66) subtypes nor that patients with single-system LCH have an increased prevalence of HLA-DRB1*03 (67) when compared to patients with multisystem LCH (Table S4 and Figure S5, respectively).

BRAF^{V600E} mutation correlates with decreased CD8+ T cell density in LCH-lesions

Assured that LCH-cells express HLA class I (and II) molecules and that there is a normal HLA subtype distribution among LCH-patients, we next investigated the presence of $CD8^+$ T cells in LCH-lesions. Various methods for the quantification of cell numbers in (specific areas of) tissue sections exist, including eyeball estimation, manual cell counting and automated digital image analysis. Although automated digital image analysis is increasingly being applied, manual cell counting is still considered the golden standard (68). Accordingly, we first determined the relative number of total $CD3^+$ and $CD3^+CD8^+$ T cells in LCH-lesions using this method. Fluorescently stained $CD1a^+$, $CD3^+CD8^-$ and $CD3^+CD8^+$ cells (Figure 2A) were manually counted in LCH-biopsies from n=101 patients collected at first disease onset using the public ImageJ Cell Counter plugin. A median of 1810 cells (range: 188-9301) were counted in a median of 16 representative images (range: 2-56) taken at 400x magnification of tissue areas containing phenotypically aberrant $CD1a^+$ LCH-cells. Large inter- and intrapatient heterogeneity was seen in the relative number of LCH-lesional $CD3^+$ and $CD8^+$ T lymphocytes (Figure S6 and 2B, respectively). Calculated $CD8^+$ T cell:$CD1a^+$ LCH-cell ratios (CD8 ratios) ranged from 0.00–4.96. The median CD8 ratio was 0.06, corresponding to 1 $CD8^+$ T cell per 16 $CD1a^+$ LCH-cells. No significant difference in LCH-lesional CD8 ratios was observed between bone and skin biopsies (p=0.37) nor between patients with single- or multisystem LCH disease (p=0.55). Yet, *BRAF^{V600E}* mutated patients displayed significantly lower LCH-lesional CD8 ratios when compared to *BRAF* wildtype (*BRAF^{WT}*) patients (p= 0.01; Figure 2C). *BRAF^{V600E}* mutated LCH-lesions had a median CD8 ratio of 0.0316, corresponding to 1 $CD8^+$ T cell per 32 $CD1a^+$ LCH-cells. In contrast, *BRAF^{WT}* lesions had a median CD8 ratio of 0.0775, corresponding to 1 $CD8^+$ T cell per 13 $CD1a^+$ LCH-cells. *BRAF^{V600E}* mutated lesions also had significantly lower total $CD3^+$ T cell:$CD1a^+$ LCH-cell ratios than *BRAF^{WT}* lesions (p= 0.001; Figure S7). As manual selection of representative tissue areas may introduce bias, we also analyzed whole slide images taken from a subset of immunostained tissue

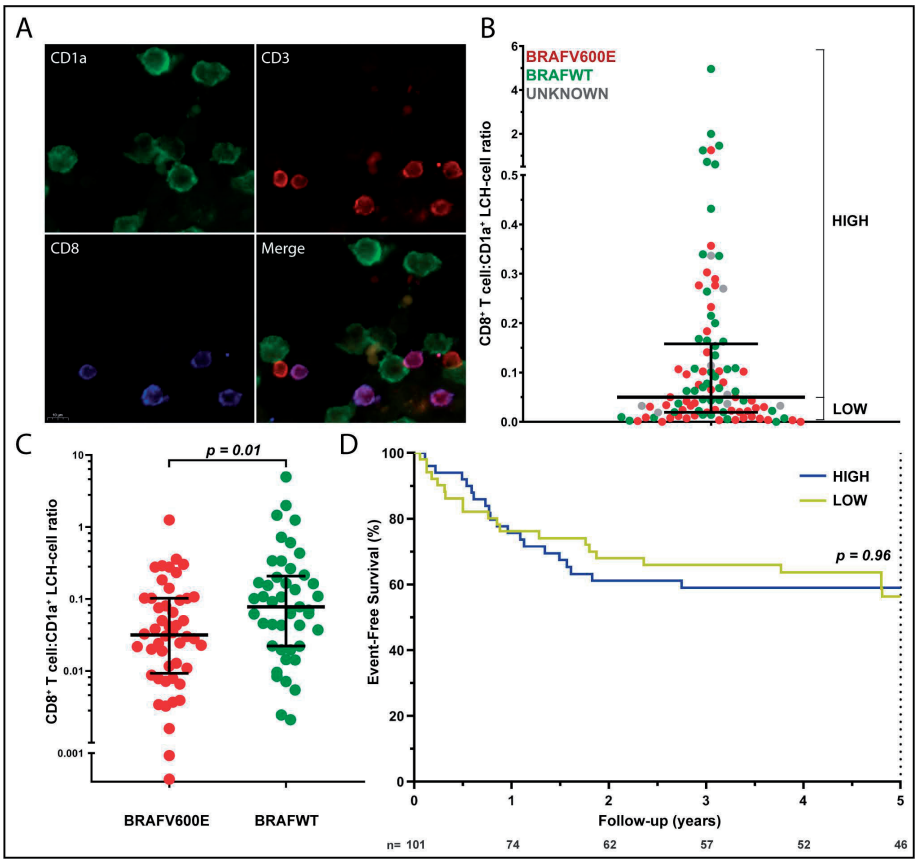

Figure 2 | LCH-lesional CD8+ T cell densities in first disease onset tissue biopsies of LCH-patients.
(A) Representative images of immunostained phenotypically aberrant CD1a$^+$ LCH-cells (green), CD3$^+$CD8$^-$ T cells (red) and CD3$^+$CD8$^+$ T cells (purple) that were manually counted. (B) Distribution of LCH-lesional CD8$^+$ T cell:CD1a+ LCH-cell ratios (CD8 ratios) in first-disease onset tissue biopsies of n=101 LCH-patients. For Kaplan-Meier survival analysis (shown in panel D), patients were divided by a median split and grouped in patients with HIGH or LOW CD8 ratios. (C) Distribution of CD8 ratios in *BRAF*^{V600E} mutated (n=48) and *BRAF* wildtype (n=45) LCH-lesions. (D) Kaplan-Meier survival analysis of patients with HIGH (n=50) and LOW (n=51) CD8 ratios. Event was defined as LCH disease progression or reactivation. N, number of patients at risk.

sections using a previously described semi-quantitative eyeball estimation method (58,59) (Figure S1) and a quantitative automated digital image analysis method (Figure S2). The correlation between the *BRAF*^{V600E} mutation and decreased LCH-lesional CD3$^+$ and CD8$^+$ T cell density was confirmed by these two additional analysis methods (Figure S8).

Lesional CD8+ T cell density does not correlate with event-free survival in LCH

We subsequently assessed whether lesional CD8$^+$ T cell density is of prognostic value in LCH. Using univariate cox regression analysis, no significant association was observed between LCH-lesional CD8 ratio and event-free survival (p=0.46; Hazard Ratio= 0.89;

Table 1 | Characteristics of LCH-patients from whom biopsies were analyzed for LCH-lesional CD8+ T cell density.

	All patients	High LCH-lesional CD8 ratio	Low LCH-lesional CD8 ratio	P-value
Patients	101	50 (50%)	51 (50%)	
Gender				
Male	53 (52%)	29 (58%)	24 (47%)	0.32
Female	48 (48%)	21 (42%)	27 (53%)	
Age distribution				
Pediatric patients	85 (84%)	40 (80%)	45 (88%)	0.29
Adult patients	16 (16%)	10 (10%)	6 (12%)	
Disease extension				
SS	78 (77%)	39 (78%)	39 (76%)	1
MS RO-	12 (12%)	5 (10%)	7 (14%)	0.76
MS RO+	11 (11%)	6 (12%)	5 (10%)	0.76
Mutation status				
$BRAF^{V600E}$ positive	48 (48%)	18 (36%)	30 (59%)	0.02
$BRAF^{V600E}$ negative	45 (45%)	28 (56%)	17 (33%)	
Unknown	8 (8%)	4 (8%)	4 (8%)	
Chemotherapy for FDO	34 (34%)	15 (30%)	19 (37%)	0.53
Follow-up (median)	10.1 years	8.5 years	11.3 years	0.36

SS, single-system LCH disease; MS RO-, multisystem LCH disease without risk organ (bone marrow, liver and/or spleen) involvement; MS RO+, multisystem LCH disease with risk organ involvement; FDO, first disease onset; CD8 ratio, CD8+ T cell:CD1a+ LCH-cell ratio.

95% Confidence Interval= 0.66-1.21). In addition, no significant difference was present when patients were divided by a median split, grouped in patients with HIGH or LOW CD8 ratios (Figure 2B; Table 1) and compared with regard to event-free survival (p=0.96, Figure 2D). Thus, LCH-lesional $CD8^+$ T cell density did not correlate with disease outcome in this retrospective patient cohort.

*The $BRAF^{V600E}$ derived neopeptide KIGDFGLATEK binds to HLA-A*03:01 and HLA-A*11:01*

To investigate the immunogenicity of the *$BRAF^{V600E}$* mutation, we used the online NetMHC 4.0 server (69) to explore putative HLA class I binding 8-12 amino acid long (8-12mer) neopeptides derived from the *$BRAF^{V600E}$* protein. In addition, NetCHOP 3.1 software (70) was used to predict proteasomal cleavage motifs and thereby identify peptides that are presumably generated by the human proteasome. From all 8-12mer *$BRAF^{V600E}$* derived neopeptides that are generated by the human proteasome according to NetCHOP, only a single neopeptide, the 11mer KIGDFGLAT**E**K, is predicted to bind to one or more of the analyzed HLA class I molecules (Table S5). According to NetMHC, KIGDFGLAT**E**K binds weakly to HLA-A*11:01 and HLA-A*03:01, expressed by respectively n=11/104 (11%) and n=25/104 (24%) LCH-patients in our cohort. The remainder of the 8-12mer *$BRAF^{V600E}$* derived neopeptides are all considered not be generated by the

Table 2 | *In silico* and *in vitro* HLA class I binding affinities of *BRAF*^{V600E} and *BRAF* wildtype protein-derived peptides and two strong binding reference peptides.

Peptide	Predicted proteasomal cleavage	Predicted HLA binding affinity (IC_{50}, nM)						*In vitro* HLA binding affinity (IC_{50}, nM)		
		NetMHC 3.4			NetMHC 4.0			Peptide-HLA binding assay[56]		
	NetCHOP 3.1	A*02 :01	A*03 :01	A*11 :01	A*02 :01	A*03 :01	A*11 :01	A*02 :01	A*03 :01	A*11 :01
KIGDFGLATV	YES	38	17310	23823	107	13515	19725	40	NT	NT
KIGDFGLATE	NO	15997	20112	23609	19485	22113	23743	86918	NT	NT
KIGDFGLATVK	YES	25719	448	163	3347	191	363	NT	415	32
KIGDFGLATEK	YES	23298	622	98	29947	278	345	NT	672	45
QVPLRPMTYK	NR	31545	77	62	32523	21	37	NT	297	36
FLPSDFFPSV	NR	4	24267	27281	4	19261	22553	8	NT	NT

500nM
50nM
0nM

IC50, the concentration that inhibits 50% binding of a fluorescently-labeled standard peptide; nM, nanomolar; NR, not relevant, because these are the strong binding reference peptides; NT, not tested.

human proteasome and/or to be non-binders. Additional *in vitro* peptide-HLA binding studies however demonstrated that KIGDFGLAT**E**K binds with comparable affinity to HLA-A*11:01 as the strong binding reference peptide (QVPLRPMTYK) that was used in our competition-based peptide-HLA binding assay (60). In line with the predicted binding affinity, this neopeptide was shown to also bind, albeit less efficiently, to HLA-A*03:01 (Table 2), as evidenced by the small difference in nanomolar concentration that inhibited 50% binding (IC_{50}) of the fluorescently-labelled standard peptide (KVFPCALINK) between KIGDFGLAT**E**K and QVPLRPMTYK (672 nM vs. 297 nM, respectively). Notably, NetMHCstab 1.0 software (71) predicts that the KIGDFGLAT**E**K-HLA-A*11:01 complex is highly stable (predicted half-life: 8.87 hours) and that the KIGDFGLAT**E**K-HLA-A*03:01 complex is weakly stable (predicted half-life: 3.29 hours). We also assessed the *in vitro* HLA binding affinity of the 11mer KIGDFGLATVK and 10mer KIGDFGLATV *BRAF* wildtype peptides and of the 10mer KIGDFGLAT**E** neopeptide (Table 2). In accordance with the predictions made by NetMHC, KIGDFGLATVK was shown to bind with comparable affinity to HLA-A*11:01 as the strong binding reference peptide QVPLRPMTYK, and to confer weaker binding to HLA-A*03:01, just like KIGDFGLAT**E**K. Moreover, the 10mer *BRAF* wildtype peptide KIGDFGLATV was shown to bind with comparable affinity to HLA-A*02:01 as the strong binding reference peptide (FLPSDFFPSV) used in our assay. In contrast, its mutant counterpart KIGDFGLAT**E** does not bind at all to this particular HLA class I molecule.

*HLA-A*11:01 and/or HLA-A*03:01 genotype is not associated with increased event-free survival in BRAF**V600E** mutated LCH-patients*

Having established that the *BRAF*^{V600E} derived neopeptide KIGDFGLAT**E**K can bind to two HLA class I molecules that are relatively frequent in the Caucasian population, we

evaluated whether *BRAF*^{V600E} mutated LCH-patients expressing HLA-A*03:01 and/or HLA-A*11:01 had increased event-free survival as compared to LCH-patients without these HLA genotypes. High-resolution HLA genotype data was available for n=48 *BRAF*^{V600E} mutated LCH-patients. Patient characteristics are shown in Supplementary Table 6. No significant difference in event-free survival was observed between *BRAF*^{V600E} mutated LCH-patients with and without HLA-A*03:01 and/or HLA-A*11:01 (p=0.32, Figure S8).

KIGDFGLATEK is not detected in the HLA class I peptidome of BRAF^{V600E} *expressing cells*

To assess whether KIGDFGLAT**E**K is actually presented on the surface of cells that express *BRAF*^{V600E} and HLA-A*03:01 and/or HLA-A*11:01**,** we performed mass spectrometry-based targeted peptidomics of HLA class I presented peptides isolated from various EBV-LCL transduced with a LZRS-retroviral vector containing full length *BRAF*^{V600E} protein and reporter Green Fluorescent Protein (GFP) encoding DNA sequences. Based on the results of the *in silico* analysis and *in vitro* peptide-HLA binding assays, three different EBV-LCL were selected for the transduction experiments with HLA-A*03:01/HLA-A*02:01 (SB), HLA-A*11:01/HLA-A*02:01 (MLA) and HLA-A*02:01/HLA-A*02:01 (JY) genotypes. Extended HLA genotypes are shown in Supplementary Table 7. After retroviral transduction, GFP^{high} cells were sorted and expanded in bulk. JY and MLA cell lines that were mock transduced with a control (empty-)GFP retroviral vector were analyzed in parallel. Flow cytometric analysis demonstrated that neither retroviral transduction with the *BRAF*^{V600E} containing vector (Figure S10) nor transduction with the control empty vector (data not shown) altered HLA class I (W6/32) and HLA-DR expression at the cell surface. Moreover, HLA subtype-specific antibodies (kindly provided by Dr. D.L. Roelen, HLA genotyping laboratory LUMC, Leiden) confirmed normal HLA subtype expression by *BRAF*^{V600E} transduced SB, MLA (Figure S10) and JY cells (data not shown). We also included an HLA-A*01/HLA-A*24 bearing *BRAF*^{V600E} mutated colon carcinoma cell line (HT29) with earlier confirmed HLA (72–74) and *BRAF*^{V600E} protein (75,76) expression in our analysis. Using parallel reaction monitoring (PRM)-based targeted peptidomics (77), the 11mer neopeptide KIGDFGLATEK was not detected in the HLA class I peptidomes of both BRAFV600E expressing cell lines expressing HLA-A*03:01 or HLA-A*11:01 (Table 3). Notably, neither the 11mer BRAF wildtype peptide KIGDFGLATVK was detected in HLA class I peptides isolated from the mock and or BRAFV600E transduced SB and MLA EBV-LCL. In contrast, the 10mer BRAF wildtype peptide KIGDFGLATV was detected in the HLA class I peptidomes of 3/3 BRAFV600E transduced and 2/2 mock transduced cell lines expressing HLA-A*02:01 (Table 3).

Table 3 | Peptides detected using mass-spectrometry based targeted peptidomics of HLA class I peptides isolated from multiple *BRAF* wildtype or *BRAF*^{V600E} expressing cell lines.

	Cell line											
	JY Mock		JY BRAF^{V600E}		MLA mock		MLA BRAF^{V600E}		SB BRAF^{V600E}		HT29	
	1900x10e6		51x10e6		158x10e6		170x10e6		28x10e6		1800x10e6	
Peptide	A*02 :01	A*02 :01	A*02 :01	A*02 :01	A*02 :01	A*11 :01	A*02 :01	A*11 :01	A*02 :01	A*03 :01	A*01	A*24
KIGDFGLATV	+		+		+		+		+		-	
KIGDFGLATE	-		-		-		-		-		-	
KIGDFGLATVK	-		-		-		-		-		-	
KIGDFGLATEK	-		-		-		-		-		-	

Mock, transduced with a control (empty-)GFP retroviral vector; *BRAF*^{V600E}, transduced with a *BRAF*^{V600E}-GFP retroviral vector; HT29, colon carcinoma cell line harboring the heterozygous *BRAF*^{V600E} mutation; +, peptide detected; -, peptide not detected.

DISCUSSION

A large number of studies have demonstrated a positive association between overall CD8$^+$ T cell density in the tumor infiltrate and a favorable clinical prognosis in many different types of cancers (6). In this study, we did not observe such an association in a substantial cohort of LCH-patients with well-documented clinical outcome. This dissimilarity between LCH and other neoplastic disorders may be due to their vast differences in mutational load and, correspondingly, the number of T cell activating neoantigens that can arise from this mutational burden. Furthermore, the immune suppressive microenvironment in LCH-lesions (5,14,15,18,78–81) may hamper CD8$^+$ T cell infiltration, (non-mutated) antigen recognition and cytolytic function.

In line with an earlier undetailed observation (7), the relative number of LCH-lesional CD8$^+$ T cells appears low in this study. Moreover, we demonstrate with three separate analysis methods that *BRAF*^{V600E} mutated LCH-patients display lower lesional CD3$^+$ and CD8$^+$ T cell densities than *BRAF* wildtype patients. Although the clinical significance of this latter observation is not immediately apparent, it does point out that the different MAPK pathway mutations expressed by neoplastic LCH-cells seem to have a distinct impact on their immune microenvironment. A number of studies on *BRAF*^{V600E} positive melanoma have already suggested that the *BRAF*^{V600E} mutation promotes immune evasion by upregulating the transcription of many immunomodulatory chemokine and cytokine genes as well as the internalization of cell surface HLA class I molecules (82,83). The presence of many of these immunomodulatory chemokines and cytokines in LCH-lesions has been extensively demonstrated (2). Notably, we did however observe normal HLA class I expression by CD1a$^+$ (LCH-)cells in two *BRAF*^{V600E} positive LCH-

biopsies analyzed by flow cytometry (Figure 1A), and showed that transduction of EBV-immortalized B cells with a *BRAF*^{V600E} encoding retroviral vector does not impair HLA class I expression. Zeng and colleagues recently described that *BRAF*^{V600E} mutated LCH-patients have significantly higher numbers of lesional Foxp3^{+} regulatory T cells and increased PD-L1 expression by LCH-cells when compared to *BRAF*^{WT} patients (80). In accordance with this study, a preliminary report by Chakraborty and others also describes that *BRAF*^{V600E} expressing LCH-cells display higher expression levels of ligands for inhibitory receptors, including PD-L1/L2 and Galectin-9, when compared to *BRAF*^{WT} patients (19). Notably, the presence of PD-1 expressing T cells in LCH-lesions has been reported as well (18,19), and was confirmed in (*BRAF*^{V600E} positive) patients from our cohort (Figure S11). PD-L1 blockade has been shown to induce expansion of tumor-infiltrating CD8^{+} T cells (84). Thus, the reported increased PD-L1 expression by *BRAF*^{V600E} positive LCH-cells (19,80) could explain the decreased LCH-lesional CD8^{+} T cell density in *BRAF*^{V600E} mutated patients from our study. In addition, the immune suppressive microenvironment in LCH-lesions (5,14,15,18,78–81) may clarify why the rare CD8^{+} T cells that did make it into these lesions had no significant clinical impact. This is supported by our own observation of low numbers of HLA-DR^{pos} LCH-lesional CD8^{+} T cells (Figure 1), low numbers of 'licenced-to-kill' CD8^{+} T cells co-expressing the cytolytic enzymes Perforin and Granzyme B (85) (Figure S12), and rare presence of Caspase 3 expressing LCH-cells (data not shown). HLA-DR is widely recognized as a marker of T cell activation (86), and Caspase 3 is the hallmark marker of efficient target cell apoptosis induced by activated CD8^{+} T cells (87). In line with the recently reported defective response of LCH-lesion infiltrating T cells to allogeneic stimulation (19), these observations collectively suggest that CD8^{+} T cells in LCH-lesions are often dysfunctional. Future studies using (imaging) mass cytometry, which allows the simultaneous detection of a multitude of cellular markers (with spatial context), are needed to study the phenotypic characteristics of LCH-lesional (CD8^{+}) T cells in more detail. Moreover, the alleged distinct impact of the different MAPK pathway mutations on the immune microenvironment of neoplastic LCH-cells should ideally be investigated in a LCH mouse model.

Encouraged by published results suggesting that *BRAF*^{V600E} protein-derived neopeptides can trigger antitumor immunity (41,82), we used the most recent version of publicly accessible NetMHC software to explore putatively HLA class I binding neoantigens derived from the *BRAF*^{V600E} protein. Surprisingly, from all 8-12mer *BRAF*^{V600E} derived neopeptides that are predicted to be generated by the human proteasome by NetCHOP 3.1 software, only a single neopeptide (KIGDFGLAT**E**K) was predicted to bind to one or more of the analyzed HLA class I molecules. *In vitro* peptide-HLA binding assays confirmed the predicted binding capacity of KIGDFGLAT**E**K to HLA-A*03:01 and HLA-11*01. In contrast to the results generated with an earlier version of Syphpeiti software (41), the NetMHC 4.0 server did not qualify the two (putatively HLA-A*02:01 binding)

neopeptides L**A**T**E**KSRW**S**G and L**A**T**E**KSRWS to be HLA-binders**.** Using PRM-based targeted peptidomics**,** KIGDFGLAT**E**K was not detected in the HLA class I peptidomes of 2/2 *BRAF*^{V600E} expressing EBV-LCL (MLA *BRAF*^{V600E} and SB *BRAF*^{V600E}) that expressed normal levels of HLA-A*03:01 or HLA-A*11:01. In contrast, the HLA-A*02:01 binding *BRAF* wildtype peptide KIGDFGLATV was traceable in HLA class I peptides isolated from 5/5 cell lines expressing this HLA subtype, verifying normal antigen processing in these cells and adequate sensitivity of our peptidomics approach. Since the 11mer *BRAF* wildtype peptide KIGDFGLATVK was not detected in mock (empty-GFP) nor *BRAF*^{V600E} transduced EBV-LCL as well, the apparent lack of KIGDFGLAT**E**K presentation at the cell surface seems not due to a competitive HLA binding disadvantage relative to its wildtype counterpart (88). Instead, both KIGDFGLAT**E**K and KIGDFGLATVK peptides may not generated by the human proteasome. This could be explained by the fact that both HLA-A*03:01 and HLA-A*11:01 molecules exclusively bind peptides with lysine as the C-terminal anchor residue (89). NetCHOP software only produces neural network predictions for proteosomal cleavage. Protein cleavage yielding C-terminal lysine residues is, however, not readily accomplished by the human proteasomes alone. Instead, this process requires the cytosolic endopeptidases nardilysin and thimet oligopeptidase as well (89,90). Another possibility is that the 11mer KIGDFGLATVK and KIGDFGLAT**E**K peptides are expressed at the cell surface, but that they are underrepresented among the large pool of naturally presented ligands eluted from peptide-HLA class I complexes, because of a common peptide length distribution including mostly 9mer peptides and far less 8mer, 10mer and longer peptides (91), as also demonstrated by the list of peptides that were detected using data-dependent acquisition-based peptidomics in the HLA class I peptide pools isolated from the mock transduced JY and MLA EBV-LCL (Table S8). The high sensitivity of targeted peptidomics makes this option however less probable, although it must be noted that lower numbers (28-170x10e6) of *BRAF*^{V600E} transduced SB, MLA and JY B cells were subjected to analysis as compared to mock transduced B cells (158-1900x10e6). This was because GFP^{high} *BRAF*^{V600E} transduced cells displayed intrinsically higher apoptosis rates leading to substantially lower yields (data not shown).

In addition to the importance of CD8$^+$ T cells, multiple studies have highlighted the importance of CD4$^+$ T cells in tumor rejection (34,92–96). Notably, one study identified *BRAF*^{V600E}-specific CD4$^+$ T cells after repetitive peptide stimulation of peripheral blood mononuclear cells from three melanoma patients whose metastatic tumors harbored the *BRAF*^{V600E} mutation(40). Moreover, Veatch and colleagues recently identified HLA-DQB1*03-restricted *BRAF*^{V600E}-specific CD4$^+$ T cells in an acral melanoma patient, who nonetheless developed metastases under ipilimumab (anti-CTLA-4) immunotherapy (97). Unfortunately, the precise amino acid sequence of the recognized neoantigen was not reported. Available software tools to predict HLA class II binding peptides are known to be significantly less accurate than available algorithms for predicting HLA

class I binding peptides. Moreover, the yield of *BRAF*^{V600E} transduced B cells expressing HLA-DQB1*03:02 (SB EBV-LCL) was far too small to elute sufficient quantities of peptide-HLA class II complexes needed for successful data-dependent acquisition-based peptidomics. We could, therefore, not confirm that this recently identified *BRAF*^{V600E} protein-derived HLA-DQB1*03 binding neopeptide is naturally processed and presented at the cell surface of our *BRAF*^{V600E} transduced HLA-DQB1*03 expressing EBV-LCL. We did however investigate whether *BRAF*^{V600E} mutated LCH-patients expressing HLA-DQB1*03 in general, or HLA-DQB1*03:02 and/or HLA-DQB1*03:03 in particular (due to their putative strongest binding and/or peptide-HLA complex stability (97)), had increased event-free survival when compared to *BRAF*^{V600E} mutated patients without these HLA genotypes. Notably, HLA class I subtype has already been demonstrated to influence response to checkpoint blockade immunotherapy in patients with diverse cancers (98). Neither *BRAF*^{V600E} mutated LCH-patients with HLA-DQB1*03 (n=30, 62.5%) nor with HLA-DQB1*03:02 and/or HLA-DQB1*03:03 (n=18, 37.5%) however displayed increased event-free survival when compared to patients without these HLA alleles (p=0.78 and p=0.57, respectively; data not shown). Thus, although we agree that adoptive cell therapy with T cell receptor-engineered *BRAF*^{V600E}-specific CD4$^+$ T cells may offer great therapeutic potential, the clinical impact of potentially present *BRAF*^{V600E}-specific CD4$^+$ T cells in HLA-DQB1*03 bearing *BRAF*^{V600E} mutated LCH-patients is questionable. Of note, the rare CD4$^+$ *BRAF*^{V600E}-specific T cells reported in the acral melanoma patient by Veatch *et al.* were not paralleled by *BRAF*^{V600E}-specific CD8$^+$ T cells, but by diverse CD8$^+$ T cells reactive to multiple melanoma-associated self-antigens. Whether such non-mutated tumor-associated antigens are also expressed by LCH-cells is of great interest and remains to be determined. This will however be challenging given the (relatively) low numbers of neoplastic LCH-cells that can be obtained for peptidome analysis from fresh or frozen LCH tissue samples, which are in addition very scarce due the rarity of the disease.

Since the generation of neoantigens is a probabilistic process (47), we can of course not rule out that other somatic mutations in LCH-cells are a source of neoantigens that are naturally processed and presented in (stable) peptide-HLA class I complexes. Based on recent insights, this probability is however very low. With the development of deep-sequencing technologies, comprehensive analyses of neoantigen-specific T cell responses have been carried out for a substantial number of cancer patients since 2013 (25,26,29). The striking conclusion that can now be drawn from these studies is that only a very small fraction of nonsynonymous mutations leads to the formation of a neoantigen for which CD4$^+$ or CD8$^+$ T cell reactivity can be detected (25). Most melanomas and a sizable fraction of other high-prevalence cancers in adults have a mutational load above 10 somatic mutations per Mb, corresponding to approximately 150 nonsynonymous mutations within expressed genes (25,99,100). Even in melanoma patients, neoantigen-specific T cell reactivity is however not always observed (95).

Thus, there is a growing awareness that tumor types with a mutational load below 10, and especially below 1 mutation(s) per Mb, are less likely to express neoantigens that can be recognized by autologous T cells (25). Although the total number of LCH samples analyzed by whole-exome sequencing (WES) is still small (101), a remarkably low frequency of somatic mutations in LCH-cells was found in the largest WES analysis to date (n=41), with a median of 1 somatic mutation per patient (0.03 mutations per Mb) (22). Thus, the likelihood of neoantigen formation and concurrent induction of protective neoantigen-specific T cell responses in LCH-patients seems very low (25). Notably, Goyal and others recently demonstrated a low mutational burden in other histiocytic neoplasms as well (102). We therefore question the usefulness of classical immune checkpoint inhibitors for the treatment of relapsed or refractory LCH (or other histiocytic neoplasms), especially given that these LCH-patients often bear the *BRAF*^{V600E} mutation (44), and that pretherapy intratumoral CD8^{+} T cell density has been shown to positively correlate with mutational burden, neoantigen load and response to immune checkpoint inhibition in many other neoplastic diseases (103,104).

SUPPLEMENTARY FILES

The supplementary files can be found online at
https://www.frontiersin.org/articles/10.3389/fimmu.2019.03045.

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REFERENCES

1. Haroche J, Cohen-Aubart F, Rollins BJ, Donadieu J, Charlotte F, Idbaih A, Vaglio A, Abdel-Wahab O, Emile J-F, Amoura Z. Histiocytoses: emerging neoplasia behind inflammation. *Lancet Oncol* (2017) 18:e113–e125. doi:10.1016/S1470-2045(17)30031-1
2. Maarten Egeler R, van Halteren AGS, Hogendoorn PCW, Laman JD, Leenen PJM. Langerhans cell histiocytosis: fascinating dynamics of the dendritic cell-macrophage lineage. *Immunol Rev* (2010) 234:213–232. doi:10.1111/j.0105-2896.2009.00883.x
3. Sahm F, Capper D, Preusser M, Meyer J, Stenzinger A, Lasitschka F, Berghoff A-S, Habel A, Schneider M, Kulozik A, et al. BRAFV600E mutant protein is expressed in cells of variable maturation in Langerhans cell histiocytosis. *Blood* (2012) 120:e28–e34. doi:10.1182/blood-2012-06-429597
4. Egeler RM, Favara BE, van Meurs M, Laman JD, Claassen E. Differential In situ cytokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expression of cytokines relevant to disease and treatment. *Blood* (1999) 94:4195–201. doi:10.1182
5. Senechal B, Elain G, Jeziorski E, Grondin V, De Serre NPM, Jaubert F, Beldjord K, Lellouch A, Glorion C, Zerah M, et al. Expansion of regulatory T cells in patients with langerhans cell histiocytosis. *PLoS Med* (2007) 4:1374–1384. doi:10.1371/journal.pmed.0040253
6. Fridman WH, Zitvogel L, Sautès-Fridman C, Kroemer G. The immune contexture in cancer prognosis and treatment. *Nat Rev Clin Oncol* (2017) 14:717–734. doi:10.1038/nrclinonc.2017.101
7. Annels NE, da Costa CET, Prins FA, Willemze A, Hogendoorn PCW, Egeler RM. Aberrant Chemokine Receptor Expression and Chemokine Production by Langerhans Cells Underlies the Pathogenesis of Langerhans Cell Histiocytosis. *J Exp Med* (2003) 197:1385–1390. doi:10.1084/jem.20030137
8. Grois N, Prayer D, Prosch H, Lassmann H, CNS LCH Co-operative Group. Neuropathology of CNS disease in Langerhans cell histiocytosis. *Brain* (2005) 128:829–838. doi:10.1093/brain/awh403
9. Alexandrescu S, Tatevian N, Czerniak BA, Covinsky MH, Burns NK, Brown RE. Morphoproteomics provides support for TGF-β pathway signaling in the osteoclastogenesis and immune dysregulation of osteolytic Langerhans cell histiocytosis. *Int J Clin Exp Pathol* (2012) 5:503–11.
10. Egeler RM, Favara BE, Laman JD, Claassen E. Abundant expression of CD40 and CD40-ligand (CD154) in paediatric Langerhans cell histiocytosis lesions. *Eur J Cancer* (2000) 36:2105–2110. doi:10.1016/S0959-8049(00)00296-3
11. Tazi A, Moreau J, Bergeron A, Dominique S, Hance AJ, Soler P. Evidence that Langerhans cells in adult pulmonary Langerhans cell histiocytosis are mature dendritic cells: importance of the cytokine microenvironment. *J Immunol* (1999) 163:3511–5.
12. Geissmann F, Lepelletier Y, Fraitag S, Valladeau J, Bodemer C, Debré M, Leborgne M, Saeland S, Brousse N. Differentiation of Langerhans cells in Langerhans cell histiocytosis. *Blood* (2001) 97:1241–1248. doi:10.1182/blood.V97.5.1241
13. Emile J-F, Fraitag S, Leborgne M, de Prost Y, Brousse N. Langerhans' cell histiocytosis cells are activated langerhans' cells. *J Pathol* (1994) 174:71–76. doi:10.1002/path.1711740202
14. Quispel WT, Stegehuis-Kamp JA, Santos SJ, Egeler RM, van Halteren AGS. Activated Conventional T-Cells Are Present in Langerhans Cell Histiocytosis Lesions Despite the Presence of Immune Suppressive Cytokines. *J Interf Cytokine Res* (2015) 35:831–839. doi:10.1089/jir.2014.0190
15. Allen CE, Li L, Peters TL, Leung HE, Yu A, Man T-K, Gurusiddappa S, Phillips MT, Hicks MJ, Gaikwad A, et al. Cell-Specific Gene Expression in Langerhans Cell Histiocytosis Lesions Reveals a Distinct Profile Compared with Epidermal Langerhans Cells. *J Immunol* (2010) 184:4557–4567. doi:10.4049/jimmunol.0902336

16. Hutter C, Kauer M, Simonitsch-Klupp I, Jug G, Schwentner R, Leitner J, Bock P, Steinberger P, Bauer W, Carlesso N, et al. Notch is active in Langerhans cell histiocytosis and confers pathognomonic features on dendritic cells. *Blood* (2012) 120:5199–208. doi:10.1182/blood-2012-02-410241
17. Halbritter F, Farlik M, Schwentner R, Jug G, Fortelny N, Schnöller T, Pisa H, Schuster LC, Reinprecht A, Czech T, et al. Epigenomics and Single-Cell Sequencing Define a Developmental Hierarchy in Langerhans Cell Histiocytosis. *Cancer Discov* (2019) 9:1406–1421. doi:10.1158/2159-8290.CD-19-0138
18. Gatalica Z, Bilalovic N, Palazzo JP, Bender RP, Swensen J, Millis SZ, Vranic S, Hoff D Von, Arceci RJ. Disseminated histiocytoses biomarkers beyond BRAFV600E: frequent expression of PD-L1. *Oncotarget* (2015) 6:19819–19825. doi:10.18632/oncotarget.4378
19. Chakraborty R, Allen CE, Sengal A, Gaikwad AS, Abhyankar H, Scull B, Eckstein OS, Merad M, McClain KL. Immune Checkpoint Inhibitors Restore Function of Lesion Infiltrating T Lymphocytes in Langerhans Cell Histiocytosis. *Blood* (2017) 130:2280.
20. da Costa CET, Annels NE, Faaij CMJM, Forsyth RG, Hogendoorn PCW, Egeler RM. Presence of osteoclast-like multinucleated giant cells in the bone and nonostotic lesions of Langerhans cell histiocytosis. *J Exp Med* (2005) 201:687–693. doi:10.1084/jem.20041785
21. Badalian-Very G, Vergilio J. Recurrent BRAF mutations in Langerhans cell histiocytosis. *Blood* (2010) 116:6–8. doi:10.1182/blood-2010-04-279083.
22. Chakraborty R, Hampton OA, Shen X, Simko SJ, Shih A, Abhyankar H, Lim KPH, Covington KR, Trevino L, Dewal N, et al. Mutually exclusive recurrent somatic mutations in MAP2K1 and BRAF support a central role for ERK activation in LCH pathogenesis. *Blood* (2014) 124:3007–3015. doi:10.1182/blood-2014-05-577825
23. Chakraborty R, Burke TM, Hampton OA, Zinn DJ, Lim KPH, Abhyankar H, Scull B, Kumar V, Kakkar N, Wheeler DA, et al. Alternative genetic mechanisms of BRAF activation in Langerhans cell histiocytosis. *Blood* (2016) 128:2533–2537. doi:10.1182/blood-2016-08-733790
24. Durham BH. Molecular Characterization of the Histiocytoses: Neoplasia of Dendritic Cells and Macrophages. *Semin Cell Dev Biol* (2019) 86:62–76. doi:10.1016/j.semcdb.2018.03.002
25. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Science* (2015) 348:69–74. doi:10.1126/science.aaa4971
26. van Rooij N, van Buuren MM, Philips D, Velds A, Toebes M, Heemskerk B, van Dijk LJA, Behjati S, Hilkmann H, el Atmioui D, et al. Tumor Exome Analysis Reveals Neoantigen-Specific T-Cell Reactivity in an Ipilimumab-Responsive Melanoma. *J Clin Oncol* (2013) 31:e439–e442. doi:10.1200/JCO.2012.47.7521
27. Robbins PF, Lu Y-C, El-Gamil M, Li YF, Gross C, Gartner J, Lin JC, Teer JK, Cliften P, Tycksen E, et al. Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat Med* (2013) 19:747–752. doi:10.1038/nm.3161
28. Wick DA, Webb JR, Nielsen JS, Martin SD, Kroeger DR, Milne K, Castellarin M, Twumasi-Boateng K, Watson PH, Holt RA, et al. Surveillance of the Tumor Mutanome by T Cells during Progression from Primary to Recurrent Ovarian Cancer. *Clin Cancer Res* (2014) 20:1125–1134. doi:10.1158/1078-0432.CCR-13-2147
29. Yarchoan M, Johnson BA, Lutz ER, Laheru DA, Jaffee EM. Targeting neoantigens to augment antitumour immunity. *Nat Rev Cancer* (2017) 17:209–222. doi:10.1038/nrc.2016.154
30. Wolfel T, Hauer M, Schneider J, Serrano M, Wolfel C, Klehmann-Hieb E, De Plaen E, Hankeln T, Meyer zum Buschenfelde K, Beach D. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science* (1995) 269:1281–1284. doi:10.1126/science.7652577

31. Kvistborg P, Shu CJ, Heemskerk B, Fankhauser M, Thrue CA, Toebes M, van Rooij N, Linnemann C, van Buuren MM, Urbanus JHM, et al. TIL therapy broadens the tumor-reactive CD8 + T cell compartment in melanoma patients. *Oncoimmunology* (2012) 1:409–418. doi:10.4161/onci.18851
32. Leisegang M, Kammertoens T, Uckert W, Blankenstein T. Targeting human melanoma neoantigens by T cell receptor gene therapy. *J Clin Invest* (2016) 126:854–8. doi:10.1172/JCI83465
33. Chheda ZS, Kohanbash G, Okada K, Jahan N, Sidney J, Pecoraro M, Yang X, Carrera DA, Downey KM, Shrivastav S, et al. Novel and shared neoantigen derived from histone 3 variant H3.3K27M mutation for glioma T cell therapy. *J Exp Med* (2018) 215:141–157. doi:10.1084/jem.20171046
34. Tran E, Ahmadzadeh M, Lu Y-C, Gros A, Turcotte S, Robbins PF, Gartner JJ, Zheng Z, Li YF, Ray S, et al. Immunogenicity of somatic mutations in human gastrointestinal cancers. *Science* (2015) 350:1387–1390. doi:10.1126/science.aad1253
35. Wang QJ, Yu Z, Griffith K, Hanada K, Restifo NP, Yang JC. Identification of T-cell Receptors Targeting KRAS-Mutated Human Tumors. *Cancer Immunol Res* (2016) 4:204–14. doi:10.1158/2326-6066.CIR-15-0188
36. Tran E, Robbins PF, Lu Y-C, Prickett TD, Gartner JJ, Jia L, Pasetto A, Zheng Z, Ray S, Groh EM, et al. T-Cell Transfer Therapy Targeting Mutant KRAS in Cancer. *N Engl J Med* (2016) 375:2255–2262. doi:10.1056/NEJMoa1609279
37. Inderberg EM, Wälchli S, Myhre MR, Trachsel S, Almåsbak H, Kvalheim G, Gaudernack G. T cell therapy targeting a public neoantigen in microsatellite instable colon cancer reduces in vivo tumor growth. *Oncoimmunology* (2017) 6:e1302631. doi:10.1080/2162402X.2017.1302631
38. van der Lee DI, Reijmers RM, Honders MW, Hagedoorn RS, de Jong RCM, Kester MGD, van der Steen DM, de Ru AH, Kweekel C, Bijen HM, et al. Mutated nucleophosmin 1 as immunotherapy target in acute myeloid leukemia. *J Clin Invest* (2019) 129:774–785. doi:10.1172/JCI97482
39. Andersen MH, Fensterle J, Ugurel S, Reker S, Houben R, Guldberg P, Berger TG, Schadendorf D, Trefzer U, Bröcker E-B, et al. Immunogenicity of Constitutively Active V599E BRaf. *Cancer Res* (2004) 64:5456–5460. doi:10.1158/0008-5472.CAN-04-0937
40. Sharkey MS, Lizée G, Gonzales MI, Patel S, Topalian SL. CD4 + T-Cell Recognition of Mutated B-RAF in Melanoma Patients Harboring the V599E Mutation. *Cancer Res* (2004) 64:1595–1599. doi:10.1158/0008-5472.CAN-03-3231
41. Somasundaram R, Swoboda R, Caputo L, Otvos L, Weber B, Volpe P, van Belle P, Hotz S, Elder DE, Marincola FM, et al. Human Leukocyte Antigen-A2–Restricted CTL Responses to Mutated BRAF Peptides in Melanoma Patients. *Cancer Res* (2006) 66:3287–3293. doi:10.1158/0008-5472.CAN-05-1932
42. Cintolo JA, Datta J, Xu S, Gupta M, Somasundaram R, Czerniecki BJ. Type I-polarized BRAF-pulsed dendritic cells induce antigen-specific CD8+ T cells that impact BRAF-mutant murine melanoma. *Melanoma Res* (2016) 26:1–11. doi:10.1097/CMR.0000000000000203
43. Berres M-L, Lim KPH, Peters T, Price J, Takizawa H, Salmon H, Idoyaga J, Ruzo A, Lupo PJ, Hicks MJ, et al. BRAF-V600E expression in precursor versus differentiated dendritic cells defines clinically distinct LCH risk groups. *J Exp Med* (2014) 211:669–83. doi:10.1084/jem.20130977
44. Héritier S, Emile J-F, Barkaoui M-A, Thomas C, Fraitag S, Boudjemaa S, Renaud F, Moreau A, Peuchmaur M, Chassagne-Clément C, et al. BRAF Mutation Correlates With High-Risk Langerhans Cell Histiocytosis and Increased Resistance to First-Line Therapy. *J Clin Oncol* (2016) 34:3023–3030. doi:10.1200/JCO.2015.65.9508
45. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, et al. Mutations of the BRAF gene in human cancer. *Nature* (2002) 417:949–954. doi:10.1038/nature00766

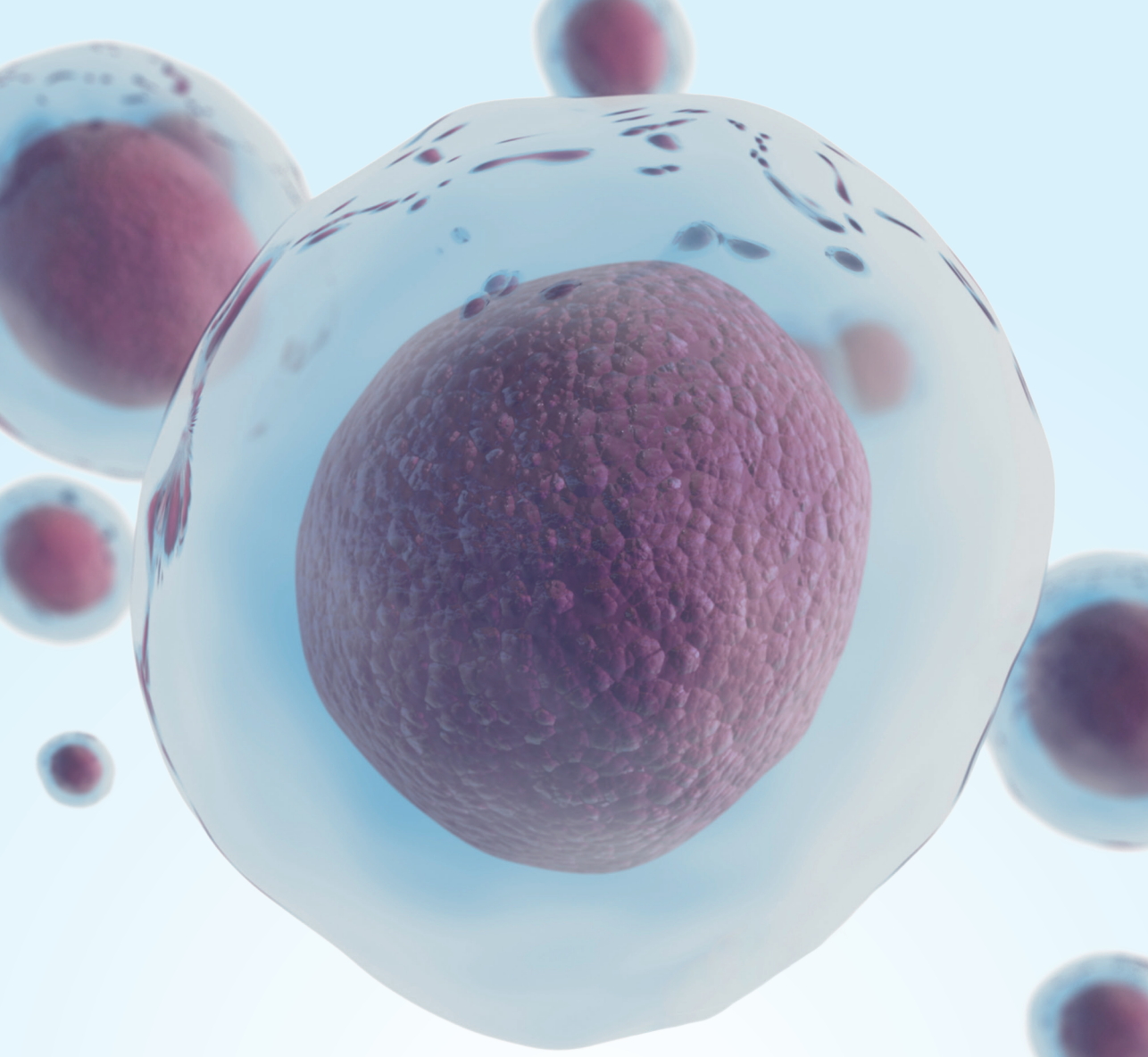
46. Dhomen N, Marais R. New insight into BRAF mutations in cancer. *Curr Opin Genet Dev* (2007) 17:31–39. doi:10.1016/j.gde.2006.12.005
47. Klebanoff CA, Wolchok JD. Shared cancer neoantigens: Making private matters public. *J Exp Med* (2018) 215:5–7. doi:10.1084/jem.20172188
48. Schöfl G, Lang K, Quenzel P, Böhme I, Sauter J, Hofmann JA, Pingel J, Schmidt AH, Lange V. 2.7 million samples genotyped for HLA by next generation sequencing: lessons learned. *BMC Genomics* (2017) 18:161. doi:10.1186/s12864-017-3575-z
49. Lange V, Böhme I, Hofmann J, Lang K, Sauter J, Schöne B, Paul P, Albrecht V, Andreas JM, Baier DM, et al. Cost-efficient high-throughput HLA typing by MiSeq amplicon sequencing. *BMC Genomics* (2014) 15:63. doi:10.1186/1471-2164-15-63
50. van Sonderen A, Roelen DL, Stoop JA, Verdijk RM, Haasnoot GW, Thijs RD, Wirtz PW, Schreurs MWJ, Claas FHJ, Sillevis Smitt PAE, et al. Anti-LGI1 encephalitis is strongly associated with HLA-DR7 and HLA-DRB4. *Ann Neurol* (2017) 81:193–198. doi:10.1002/ana.24858
51. Šidák Z. Rectangular Confidence Regions for the Means of Multivariate Normal Distributions. *J Am Stat Assoc* (1967) 62:626–633. doi:10.1080/01621459.1967.10482935
52. Woolf B. On estimating the relation between blood group and disease. *Ann Hum Genet* (1955) 19:251–253. doi:10.1111/j.1469-1809.1955.tb01348.x
53. Haldane JB. The estimation and significance of the logarithm of a ratio of frequencies. *Ann Hum Genet* (1956) 20:309–311. doi:10.1111/j.1469-1809.1955.tb01285.x
54. Good IJ. C140. Standardized tail-area prosabilities. *J Stat Comput Simul* (1982) 16:65–66. doi:10.1080/00949658208810607
55. van Eijk R, Stevens L, Morreau H, van Wezel T. Assessment of a fully automated high-throughput DNA extraction method from formalin-fixed, paraffin-embedded tissue for KRAS, and BRAF somatic mutation analysis. *Exp Mol Pathol* (2013) 94:121–125. doi:10.1016/J.YEXMP.2012.06.004
56. da Costa CET, Szuhai K, van Eijk R, Hoogeboom M, Sciot R, Mertens F, Björgvinsdóttir H, Debiec-Rychter M, de Krijger RR, Hogendoorn PCW, et al. No genomic aberrations in Langerhans cell histiocytosis as assessed by diverse molecular technologies. *Genes, Chromosom Cancer* (2009) 48:239–249. doi:10.1002/gcc.20634
57. Nelson DS, Quispel W, Badalian-Very G, van Halteren AGS, van den Bos C, Bovee JVMG, Tian SY, Van Hummelen P, Ducar M, MacConaill LE, et al. Somatic activating ARAF mutations in Langerhans cell histiocytosis. *Blood* (2014) 123:3152–3155. doi:10.1182/blood-2013-06-511139
58. De Meulenaere A, Vermassen T, Aspeslagh S, Deron P, Duprez F, Laukens D, Van Dorpe J, Ferdinande L, Rottey S. Tumor PD-L1 status and CD8+tumor-infiltrating T cells: markers of improved prognosis in oropharyngeal cancer. *Oncotarget* (2017) 8:80443–80452. doi:10.18632/oncotarget.19045
59. De Meulenaere A, Vermassen T, Aspeslagh S, Zwaenepoel K, Deron P, Duprez F, Rottey S, Ferdinande L. Prognostic markers in oropharyngeal squamous cell carcinoma: focus on CD70 and tumour infiltrating lymphocytes. *Pathology* (2017) 49:397–404. doi:10.1016/j.pathol.2017.02.002
60. Tan TL, Geluk A, Toebes M, Ottenhoff TH, Drijfhout JW. A novel, highly efficient peptide-HLA class I binding assay using unfolded heavy chain molecules: identification of HIV-1 derived peptides that bind to HLA-A*0201 and HLA-A*0301. *J Immunol Methods* (1997) 205:201–9. doi:10.1016/S0022-1759(97)00086-0
61. Boehm JS, Zhao JJ, Yao J, Kim SY, Firestein R, Dunn IF, Sjostrom SK, Garraway LA, Weremowicz S, Richardson AL, et al. Integrative Genomic Approaches Identify IKBKE as a Breast Cancer Oncogene. *Cell* (2007) 129:1065–1079. doi:10.1016/j.cell.2007.03.052

62. Hassan C, Kester MGD, de Ru AH, Hombrink P, Drijfhout JW, Nijveen H, Leunissen JAM, Heemskerk MHM, Falkenburg JHF, van Veelen PA. The Human Leukocyte Antigen–presented Ligandome of B Lymphocytes. *Mol Cell Proteomics* (2013) 12:1829–1843. doi:10.1074/mcp.M112.024810
63. Garrido F, Aptsiauri N, Doorduijn EM, Garcia Lora AM, van Hall T. The urgent need to recover MHC class I in cancers for effective immunotherapy. *Curr Opin Immunol* (2016) 39:44–51. doi:10.1016/j.coi.2015.12.007
64. Tomooka Y, Torisu M, Miyazaki S, Goya N. Immunological studies on histiocytosis X. I. Special reference to the chemotactic defect and the HLA antigen. *J Clin Immunol* (1986) 6:355–362. doi:10.1007/BF00915374
65. YU RC, CHU AC. Langerhans Cell Histiocytosis - Clinicopathological Reappraisal and Human Leucocyte Antigen Association. *Br J Dermatol* (1996) 135:36–41. doi:10.1046/j.1365-2133.1996.d01-929.x
66. McClain KL, Laud P, Wu W-S, Pollack MS. Langerhans cell histiocytosis patients have HLA Cw7 and DR4 types associated with specific clinical presentations and no increased frequency in polymorphisms of the tumor necrosis factor alpha promoter. *Med Pediatr Oncol* (2003) 41:502–507. doi:10.1002/mpo.10366
67. Bernstrand C, Carstensen H, Jakobsen B, Svejgaard A, Henter J-I, Olerup O. Immunogenetic Heterogeneity in Single-System and Multisystem Langerhans Cell Histiocytosis. *Pediatr Res* (2003) 54:30–36. doi:10.1203/01.PDR.0000069844.50684.7D
68. Miksch RC, Hao J, Schoenberg MB, Dötzer K, Schlüter F, Weniger M, Yin S, Ormanns S, D'Haese JG, Guba MO, et al. Development of a reliable and accurate algorithm to quantify the tumor immune stroma (QTiS) across tumor types. *Oncotarget* (2017) 8:114935–114944. doi:10.18632/oncotarget.22932
69. Andreatta M, Nielsen M. Gapped sequence alignment using artificial neural networks: application to the MHC class I system. *Bioinformatics* (2016) 32:511–517. doi:10.1093/bioinformatics/btv639
70. Nielsen M, Lundegaard C, Lund O, Keşmir C. The role of the proteasome in generating cytotoxic T-cell epitopes: insights obtained from improved predictions of proteasomal cleavage. *Immunogenetics* (2005) 57:33–41. doi:10.1007/s00251-005-0781-7
71. Jørgensen KW, Rasmussen M, Buus S, Nielsen M. Net MHC stab - predicting stability of peptide-MHC-I complexes; impacts for cytotoxic T lymphocyte epitope discovery. *Immunology* (2014) 141:18–26. doi:10.1111/imm.12160
72. Sers C, Kuner R, Falk CS, Lund P, Sueltmann H, Braun M, Buness A, Ruschhaupt M, Conrad J, Mang-Fatehi S, et al. Down-regulation of HLA Class I and NKG2D ligands through a concerted action of MAPK and DNA methyltransferases in colorectal cancer cells. *Int J Cancer* (2009) 125:1626–1639. doi:10.1002/ijc.24557
73. Sconocchia G, Eppenberger-Castori S, Zlobec I, Karamitopoulou E, Arriga R, Coppola A, Caratelli S, Spagnoli GC, Lauro D, Lugli A, et al. HLA class II antigen expression in colorectal carcinoma tumors as a favorable prognostic marker. *Neoplasia* (2014) 16:31–42. doi:10.1593/neo.131568
74. Holloway G, Fleming FE, Coulson BS. MHC class I expression in intestinal cells is reduced by rotavirus infection and increased in bystander cells lacking rotavirus antigen. *Sci Rep* (2018) 8:67. doi:10.1038/s41598-017-18464-x
75. Fukuyo Y, Inoue M, Nakajima T, Higashikubo R, Horikoshi NT, Hunt C, Usheva A, Freeman ML, Horikoshi N. Oxidative Stress Plays a Critical Role in Inactivating Mutant BRAF by Geldanamycin Derivatives. *Cancer Res* (2008) 68:6324–6330. doi:10.1158/0008-5472.CAN-07-6602

76. Wang L, Cunningham JM, Winters JL, Guenther JC, French AJ, Boardman LA, Burgart LJ, McDonnell SK, Schaid DJ, Thibodeau SN. BRAF mutations in colon cancer are not likely attributable to defective DNA mismatch repair. *Cancer Res* (2003) 63:5209–12. doi:10.1158/0008-5472.can-08-1430
77. Lill JR, van Veelen PA, Tenzer S, Admon A, Caron E, Elias JE, Heck AJR, Marcilla M, Marino F, Müller M, et al. Minimal Information About an Immuno-Peptidomics Experiment (MIAIPE). *Proteomics* (2018) 18:1800110. doi:10.1002/pmic.201800110
78. Battistella M, Fraitag S, Teillac DH, Brousse N, de Prost Y, Bodemer C. Neonatal and Early Infantile Cutaneous Langerhans Cell Histiocytosis. *Arch Dermatol* (2010) 146:149–56. doi:10.1001/archdermatol.2009.360
79. Tong C, Jia X, Jia Y, He Y. Langerhans cell histiocytosis in Chinese adults: absence of BRAF mutations and increased FOXP3(+) regulatory T cells. *Int J Clin Exp Pathol* (2014) 7:3166–73.
80. Zeng K, Wang Z, Ohshima K, Liu Y, Zhang W, Wang L, Fan L, Li M, Li X, Wang Y, et al. BRAF V600E mutation correlates with suppressive tumor immune microenvironment and reduced disease-free survival in Langerhans cell histiocytosis. *Oncoimmunology* (2016) 5:e1185582. doi:10.1080/2162402X.2016.1185582
81. Mitchell JM, Berzins SP, Kannourakis G. A potentially important role for T cells and regulatory T cells in Langerhans cell histiocytosis. *Clin Immunol* (2018) 194:19–25. doi:10.1016/J.CLIM.2018.06.004
82. Ilieva KM, Correa I, Josephs DH, Karagiannis P, Egbuniwe IU, Cafferkey MJ, Spicer JF, Harries M, Nestle FO, Lacy KE, et al. Effects of BRAF Mutations and BRAF Inhibition on Immune Responses to Melanoma. *Mol Cancer Ther* (2014) 13:2769–2783. doi:10.1158/1535-7163.MCT-14-0290
83. Bradley SD, Chen Z, Melendez B, Talukder A, Khalili JS, Rodriguez-Cruz T, Liu S, Whittington M, Deng W, Li F, et al. BRAFV600E Co-opts a Conserved MHC Class I Internalization Pathway to Diminish Antigen Presentation and CD8+ T-cell Recognition of Melanoma. *Cancer Immunol Res* (2015) 3:602–9. doi:10.1158/2326-6066.CIR-15-0030
84. Beyrend G, van der Gracht E, Yilmaz A, van Duikeren S, Camps M, Höllt T, Vilanova A, van Unen V, Koning F, de Miranda NFCC, et al. PD-L1 blockade engages tumor-infiltrating lymphocytes to co-express targetable activating and inhibitory receptors. *J Immunother Cancer* (2019) 7:217. doi:10.1186/s40425-019-0700-3
85. Melief CJM. "License to Kill" Reflects Joint Action of CD4 and CD8 T Cells. *Clin Cancer Res* (2013) 19:4295–4296. doi:10.1158/1078-0432.CCR-13-1432
86. Saraiva DP, Jacinto A, Borralho P, Braga S, Cabral MG. HLA-DR in Cytotoxic T Lymphocytes Predicts Breast Cancer Patients' Response to Neoadjuvant Chemotherapy. *Front Immunol* (2018) 9:2605. doi:10.3389/fimmu.2018.02605
87. Kitamura T, Doughty-Shenton D, Pollard JW, Carragher NO. Real Time Detection of In Vitro Tumor Cell Apoptosis Induced by CD8+ T Cells to Study Immune Suppressive Functions of Tumor-infiltrating Myeloid Cells. *J Vis Exp* (2019) doi:10.3791/58841
88. Ghorani E, Rosenthal R, Mcgranahan N, Reading JL, Lynch M, Peggs KS, Swanton C, Quezada SA, Quezada SA, Swanton C. Differential binding affinity of mutated peptides for MHC class I is a predictor of survival in advanced lung cancer and melanoma. *Ann Oncol* (2018) 29:271–279. doi:10.1093/annonc/mdx687
89. Kessler JH, Khan S, Seifert U, Le Gall S, Chow KM, Paschen A, Bres-Vloemans SA, de Ru A, van Montfoort N, Franken KLMC, et al. Antigen processing by nardilysin and thimet oligopeptidase generates cytotoxic T cell epitopes. *Nat Immunol* (2011) 12:45–53. doi:10.1038/ni.1974
90. Tenzer S, Peters B, Bulik S, Schoor O, Lemmel C, Schatz MM, Kloetzel P-M, Rammensee H-G, Schild H, Holzhütter H-G. Modeling the MHC class I pathway by combining predictions of proteasomal

cleavage,TAP transport and MHC class I binding. *Cell Mol Life Sci* (2005) 62:1025–1037. doi:10.1007/s00018-005-4528-2

91. Trolle T, McMurtrey CP, Sidney J, Bardet W, Osborn SC, Kaever T, Sette A, Hildebrand WH, Nielsen M, Peters B. The Length Distribution of Class I-Restricted T Cell Epitopes Is Determined by Both Peptide Supply and MHC Allele-Specific Binding Preference. *J Immunol* (2016) 196:1480–7. doi:10.4049/jimmunol.1501721
92. Kreiter S, Vormehr M, van de Roemer N, Diken M, Löwer M, Diekmann J, Boegel S, Schrörs B, Vascotto F, Castle JC, et al. Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature* (2015) 520:692–6. doi:10.1038/nature14426
93. Spitzer MH, Carmi Y, Reticker-Flynn NE, Kwek SS, Madhireddy D, Martins MM, Gherardini PF, Prestwood TR, Chabon J, Bendall SC, et al. Systemic Immunity Is Required for Effective Cancer Immunotherapy. *Cell* (2017) 168:487-502.e15. doi:10.1016/j.cell.2016.12.022
94. Tran E, Turcotte S, Gros A, Robbins PF, Lu Y-C, Dudley ME, Wunderlich JR, Somerville RP, Hogan K, Hinrichs CS, et al. Cancer Immunotherapy Based on Mutation-Specific CD4+ T Cells in a Patient with Epithelial Cancer. *Science* (2014) 344:641–645. doi:10.1126/science.1251102
95. Linnemann C, van Buuren MM, Bies L, Verdegaal EME, Schotte R, Calis JJA, Behjati S, Velds A, Hilkmann H, Atmioui D el, et al. High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. *Nat Med* (2014) 21:81–85. doi:10.1038/nm.3773
96. Verdegaal EME, de Miranda NFCC, Visser M, Harryvan T, van Buuren MM, Andersen RS, Hadrup SR, van der Minne CE, Schotte R, Spits H, et al. Neoantigen landscape dynamics during human melanoma–T cell interactions. *Nature* (2016) 536:91–95. doi:10.1038/nature18945
97. Veatch JR, Lee SM, Fitzgibbon M, Chow I-T, Jesernig B, Schmitt T, Kong YY, Kargl J, Houghton AM, Thompson JA, et al. Tumor-infiltrating BRAFV600E-specific CD4+ T cells correlated with complete clinical response in melanoma. *J Clin Invest* (2018) 128:1563–1568. doi:10.1172/JCI98689
98. Chowell D, Morris LGT, Grigg CM, Weber JK, Samstein RM, Makarov V, Kuo F, Kendall SM, Requena D, Riaz N, et al. Patient HLA class I genotype influences cancer response to checkpoint blockade immunotherapy. *Science* (2018) 359:582–587. doi:10.1126/science.aao4572
99. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SAJR, Behjati S, Biankin A V, Bignell GR, Bolli N, Borg A, Børresen-Dale A-L, et al. Signatures of mutational processes in human cancer. *Nature* (2013) 500:415–21. doi:10.1038/nature12477
100. Chalmers ZR, Connelly CF, Fabrizio D, Gay L, Ali SM, Ennis R, Schrock A, Campbell B, Shlien A, Chmielecki J, et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. *Genome Med* (2017) 9:34. doi:10.1186/s13073-017-0424-2
101. Rollins BJ. Genomic Alterations in Langerhans Cell Histiocytosis. *Hematol Oncol Clin North Am* (2015) 29:839–851. doi:10.1016/j.hoc.2015.06.004
102. Goyal G, Lau D, Nagle AM, Vassallo R, Rech KL, Ryu JH, Davidge-Pitts CJ, Tobin WO, Koster MJ, Bennani NN, et al. Tumor mutational burden and other predictive immunotherapy markers in histiocytic neoplasms. *Blood* (2019) 133:1607–1610. doi:10.1182/blood-2018-12-893917
103. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJM, Robert L, Chmielowski B, Spasic M, Henry G, Ciobanu V, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* (2014) 515:568–571. doi:10.1038/nature13954
104. van Gool IC, Eggink FA, Freeman-Mills L, Stelloo E, Marchi E, de Bruyn M, Palles C, Nout RA, de Kroon CD, Osse EM, et al. POLE Proofreading Mutations Elicit an Antitumor Immune Response in Endometrial Cancer. *Clin Cancer Res* (2015) 21:3347–3355. doi:10.1158/1078-0432.CCR-15-0057



SECTION D

GENERAL DISCUSSION

XIII

Hemophagocytic lymphohistiocytosis

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

Hemophagocytic lymphohistiocytosis (HLH) is a potentially fatal hyperinflammatory syndrome caused by an over-activated immune response. If untreated, HLH may lead to irreversible multi- organ failure and death. There are numerous underlying conditions which can lead to HLH. These are generally grouped with HLH caused by inborn genetic alterations, named familial HLH (FHL) or HLH occurring secondary to a distinct trigger, referred to as secondary HLH (sHLH). This chapter focuses on biochemical, molecular, and clinical aspects of both FHL and sHLH.

DISEASE BIOMARKERS FOR HLH IN MCD PATIENTS

HLH is often difficult to distinguish from other conditions. Additional markers are needed to distinguish HLH from these other conditions. Sepsis is an example of a distinct condition that clinically overlaps with HLH.[1] Infections drive the pathogenesis of sepsis but can also drive the pathogenesis of HLH.[2] Therefore, HLH may easily be misdiagnosed as sepsis.[3] However, the etiology of the two diseases are markedly distinct. Sepsis is a justified immune response to an infection. The immune system can return to its resting state, provided that the pathogen is promptly and adequately cleared. In bacterial sepsis, this antigen clearance can be facilitated by antibiotics. On the contrary, HLH is characterized by a dysregulated and inadequate immune activation which is initiated once the immune system exceeds the HLH-threshold (Figure 5, introduction). Infections can drive the immune system toward this threshold and can trigger HLH (Figure 1). In HLH, the immune system cannot always restore its homeostatic balance. This different etiology of HLH and sepsis leads to differing clinical and biochemical profiles. Compared to sepsis, HLH is characterized by a markedly greater $CD8^+$ T-cell activation and clonal expansion.[4,5] Furthermore, numerous cytokine and chemokine markers differ between HLH and sepsis.[1,6,7] Recent and yet unpublished data by Jordan *et al*. demonstrates strikingly distinct protein patterns between FHL and sepsis employing broad spectrum proteomics. [unpublished data, presented at the annual meeting of the Histiocyte Society 2022 by M. Jordan]. As could be expected, these proteomics show that proteins related to IFN-γ and innate inflammatory response are more enriched in FHL compared to sepsis. In addition, proteins related to complement activation, apoptosis, and IL-6 signaling are upregulated to a larger extent in FHL patients compared to septic patients. In sepsis, INF-α shows evidently more upregulation, when compared to the levels in FHL.

Other clinical conditions besides sepsis involve proliferating lymphocytes and excessive cytokine production, and thereby show similarities to HLH. Multicentric Castleman's disease (MCD), such an inflammatory condition, with cytokine profiles

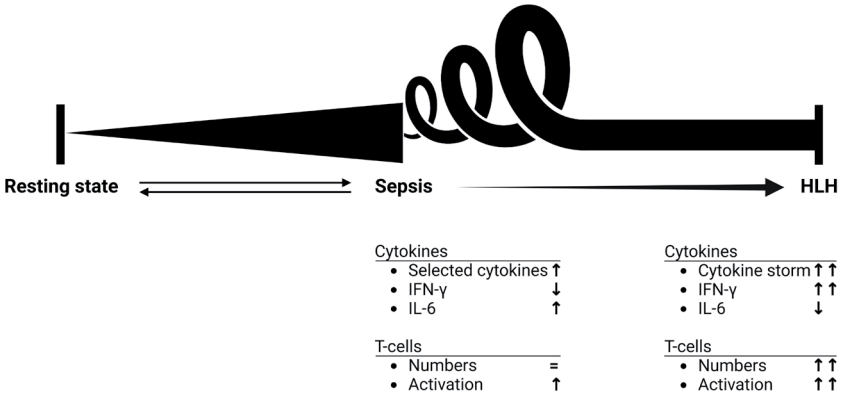


Figure 1 | HLH and sepsis are both triggered by an infection.
This schematic overview displays the differences between HLH and sepsis. In the resting state, there is no active immune response against a pathogen. Upon infection, the immune system is activated which can lead to a sepsis. If the pathogenic trigger is effectively cleared, the immune system can return to its resting state. However, once the HLH-threshold is exceeded, a hyperinflammatory immune response is initiated, leading up to fulminant HLH. A limited number of discriminating features between HLH and sepsis are presented.

similar to HLH.[8,9] Distinguishing between HLH, MCD, or sepsis remains a clinical challenge, especially in rare cases where all three conditions co-exist. Such a case is presented in Chapter V, where we focused on distinguishing HLH, sepsis, and MCD by presenting IL-10 as a new discriminative biomarker for HLH. This biomarker proves valuable for diagnosing and monitoring MCD-triggered HLH and remains discriminative for HLH in the case of additional sepsis. The presented case, an apparent immunocompetent patient who presented with HHV-8-triggered plasmacellular MCD, subsequently developed HLH. Later, with the progression of the disease, the patient developed sepsis caused by multiple bacterial pathogens including *Pseudomonas*, *Staphylococcus,* and *Enterococcus*. The patient eventually died of bacterial sepsis. Careful exploration of cytokine and viral load kinetics revealed that IL-10, INF-γ, sIL-2R, and HHV-8 viral load corresponded with the clinical course of the HLH, and not with the sepsis or MCD. On the contrary, C-reactive protein (CRP), ferritin, and IL-6 resembled the course of sepsis in our patient. Especially IL-6 corresponding with sepsis and not with HLH contradicts the recent results of proteomics that are discussed above. Although TNF-α is known to be elevated during sepsis, these levels remained low in our patient.[10,11] Although a definite explanation is not evident, low TNF-α may be caused by prolonged exposure to dexamethasone, which is known to inhibit TNF-α production by peripheral blood mononuclear cells.[12]

In the context of EBV-triggered HLH, it is widely excepted that the EBV viral load corresponds with HLH severity and therefore acts as a prognostic factor for disease outcome.[13] In this regard, it is unsurprising that HHV-8 corresponds to HLH severity in our case. However, a crucial difference between EBV-triggered HLH and our case, is

that the HHV-8 virus itself sets off the MCD, which in turn triggers the HLH. Thus, the HHV-8 viral load does not directly, but rather indirectly triggers HLH. This hypothesis is supported by the absence of reported cases of HLH triggered by HHV-8 outside the context of MCD.[2]

Cytokines, including IL-6, IFN-γ, and IL-10 drive the development of both MCD and HLH.[6,8,9,14-18] Moderate increase of IL-10 is predominantly associated with pleiotropic suppressive immune effects.[19-22] If the production of IL-10 further increases to high serum levels, $CD8^+$ T-cells are stimulated.[23-25] Moreover, IL-10 together with IL-18 are shown to drive macrophages to a proinflammatory state and induce an HLH-like phenotype in mice.[26] The importance of IL-10 in the pathophysiology is demonstrated by the protective effect of IL-10 blockade in HLH mouse models.[26] As MCD patients are known to have elevated levels of IL-10, it could therefore account for the stimulation of $CD8^+$ T-cells, a hallmark of HLH (Figure 3, introduction). Consequently, the upregulation of cytokines in MCD, especially IL-10, might form an additional element and together with the HHV-8 antigen, stress can buildup to reach the HLH-threshold (Figure 5, introduction).

Cytokines including IFN-γ and IL-10 are previously suggested to distinguish HLH from sepsis.[6] In Chapter V, we show that plasma cytokine profiling, specifically IFN-γ and IL-10, adds value in monitoring the course of HLH, even in the case of underlying MCD complicated by infections. HHV-8 viral load, IFN-γ, and IL-10 are relatively easy and inexpensive assays and thus have a great potential for clinical implementation. Additional research might reveal superior biomarkers that pinpoint specific clinical markers of HLH. This could be done by Luminex or mass spectrometry-based proteomics. With this technique, a large amount of proteins can be simultaneously quantified from small plasma volumes. These results might help identify specific cytokine patterns for HLH, MCD, or bacterial sepsis. Further research can also shed more light on the possibility of combining biomarkers to better classify and monitor the treatment response.

HLH CONCERNS A SPECTRUM RATHER THAN A DICHOTOMOUS CLASSIFICATION IN FHL OR SHLH

FHL and sHLH are often strictly distinguished as two different (dichotomous) entities. However, these two entities are not always well-demarcated. Instead, it is increasingly being embraced that FHL and sHLH should be classified as a disease spectrum instead of a dichotomous classification (Figure 2).[13] This was well illustrated by Miao *et al.* who showed that mutations in known HLH-associated genes, especially missense monoallelic mutations in *LYST, UNC13D, AP3B1, and STXBP2* are very common among adult sHLH patients.[27] These patients might have an increased susceptibility to HLH owing to their genetic background. However, the contribution of these genetic variations in reaching

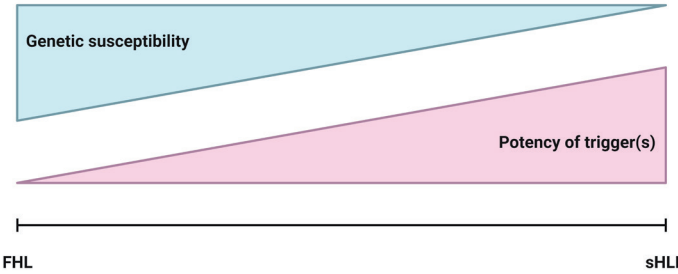


Figure 2 | HLH should be considered a disease spectrum rather than a dichotomous classification of FHL or sHLH.
The host defense is an interplay between the antigen that the host encounters and the functionality of the immune system. Individuals have varied ability to cope with antigens based on particular genetic aberrancies. If the potency of the trigger is too high for the immune system to cope with this trigger, inadequate termination of the immune response is the result, thus leading to HLH.

the HLH-threshold is limited, and not as large as that of the known mutations in FHL2-5 (Figure 5, introduction). Therefore these patients need to encounter a potent (or multiple) trigger(s) before developing HLH (such as triggers listed in Table 2 in the introduction). If such a trigger is not encountered during childhood, these patients present with HLH during adulthood. Besides, in definite FHL cases, a trigger of varying potency is mostly identified as the initiator of the HLH episode, which supports the idea of HLH being a spectrum of disorders.

Chapters VI and VII substantiate the notion of HLH being a spectrum of disease. In Chapter VI, we present a case in which the classification of FHL or sHLH is a matter of debate. At first presentation, this was a convincing case of sHLH due to the older age at presentation with EBV being the obvious trigger, and retained (although reduced) cytotoxic function. However, after genetic testing and careful clinical evaluation, we show that a mutation in the *RAB27A* gene underlays the patient's clinical picture including that of late-onset HLH. The patient was able to sufficiently clear pathogens throughout childhood and early adulthood due to sufficient residual function of the cytotoxic machinery. Later in life, the patient experienced difficulties in clearing endogenously present EBV-virus, which resulted in lymphadenopathy and persisting high EBV viral load. The patient's residual cytolytic machinery was insufficient to cope with this persisting and chronic pathogenic stress. Consequently, this genetically susceptible patient developed an HLH episode. This is not a case of classic FHL, in which the cytotoxic function is frequently completely abolished. Moreover, the pathogenesis in the presented case is assumed to be driven largely by the EBV-trigger, suggesting its classification as sHLH.[28] In favor of categorizing to FHL, a genetic variation is found to interfere with adequate cytotoxicity and is thereby crucial for the HLH episode to evolve. Thus, this case is best classified as HLH rather than strictly FHL or sHLH.

For the case presented in Chapter VII, one could also argue the HLH to be both FHL and sHLH. In this APDS patient, the cytotoxic machinery is intact and an acknowledged trigger of HLH was present, which are both classic features of sHLH. On the other hand, it could be regarded as FHL because a genetic mutation caused persistent antigen presentation with subsequent HLH. Similar to decisive FHL, a trigger preceded the development of HLH in this case.[13,29] It would therefore be appropriate to classify this patient somewhere between FHL and sHLH.

INCREASED GENETIC SUSCEPTIBILITY OF PID PATIENTS TO HLH

In FHL, a germline mutation accounts for a dysfunctional cytotoxic function by cytotoxic immune cells. FHL is classified as a primary immunodeficiency (PID) because an inborn error impairs a specific aspect of the immune system.[30] There is an increasing number of reports in which HLH occurs secondary to a PID other than FHL. These PID patients harbor mutations that do not necessarily affect the cytotoxic function of immune cells (and are thus not classified as FHL), but can still predispose a patient to developing HLH. This susceptibility is caused by a different mechanism, for instance, by hampered function of pathogen clearance.[13] For both FHL and sHLH, it is hypothesized that persisting antigen presentation is a key factor in HLH pathophysiology.[29,31] Thus, if persisting antigen load is the consequence of a PID, this can lead to excessive immune activation, which can further escalate into hyperinflammation, as typically seen in the context of HLH.[32,33] Thus, a PID can be regarded as a circumstance in which the potency of a trigger is increased. Therefore, the HLH-threshold is more easily reached in these PID patients (Figure 5, introduction). Indeed, PID patients with functionally impaired T-cells are reported to be more susceptible to HLH.[33] In Chapter VII, we present a patient with a gain-of-function mutation in the *PIK3CD* gene who developed HLH. This genomic aberration results in activated PI3K delta syndrome (APDS). In APDS the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway is over-activated. Especially effector T-cells of APDS patients are driven toward an activated and exhausted phenotype, which is typically associated with senescence.[34-36] The subsequent impairment of immunity in APDS patients is likely to result in persisting antigen load which could therefore contribute to HLH development.[37] When an APDS patient encounters a potent HLH-trigger (*e.g.,* the EBV virus), the persisting antigen could evoke an episode of sHLH.

The exact role of PIDs in the development of HLH is unknown. Research is scarce owing to the rarity of PIDs and the infrequent episodes of HLH developed by these patients.[32,33,38] However, these cases can shed light on the pathophysiology of HLH. For this research,

existing registries such as the ESID registry for primary immunodeficiencies could be used to detect HLH patients with an underlying PID.[39] It should be determined which types of PIDs underlie HLH and which pathophysiologic characteristics are displayed by these patients. There might be analogous features within the pathophysiology of these PIDs that simultaneously increase the susceptibility to HLH. As suggested by the case presented in Chapter VII, such specific pathophysiologic features could include senescence of CTLs, but might also include senescence of NK-cells, apoptosis of CTLs, or impaired phagocytosis by macrophages or granulocytes.

GRISCELLI SYNDROME TYPE 2 WITH LATE-ONSET HLH BUT NORMAL PIGMENTATION

Griscelli syndrome type 2 (GS2) is a disease caused by a dysfunctional RAB27A protein. RAB27A has a crucial role in the docking of cytotoxic granules (Figure 4, introduction) in CTLs and NK-cells. Additionally, in melanocytes, RAB27A is crucial for the transport of intracellular melanosomes to the periphery. Consequently, dysfunctional RAB27A can lead to both HLH and flawed dispersion of pigment, evident as hypopigmentation. [40,41] A few cases of GS2 have been reported in which the cytotoxic function is selectively impaired, without abnormal pigmentation.[42-45] These cases present opportunities to detail the role of RAB27A protein in the cytotoxic machinery and how its molecular interactions affect pigmentation. In Chapter VI, such an adult case is presented and thoroughly detailed. The patient initially presented with an EBV-driven lymphoproliferation and a few months later, the patient developed HLH. Upon molecular evaluation, a novel homozygous *RAB27A* c.551G>A p.(R184Q) variant was discovered. This mutation resulted in normal *in vivo* and *in vitro* expression levels of the mutant RAB27Ap.R184Q protein, suggesting that the mutant protein is not degraded by the proteasome. This is different from other known *RAB27A* mutations in which the translated protein is mostly degraded.[40] Furthermore, the patient had an atypical clinical presentation because he did not show clear signs of hypopigmentation.[40] Further *in vitro* studies of the RAB27Ap.R184Q variant revealed a retained function of melanosome distribution, but reduced cytotoxic function of NK-cells and CTLs, corresponding to the patient's clinical features. Next, the binding potentials to effector proteins of the RAB27Ap.R184Q variant were assessed. The binding of the RAB27Ap.R184Q variant to SLP2A (Figure 4, introduction) was reduced, compared to the wild type RAB27A protein. However, the increased binding of the RAB27Ap.R184Q variant to MUNC13-4 was remarkable. We hypothesize that this altered binding affinity of RAB27Ap.R184Q to several effector proteins will disturb adequate protein interactions and thereby lead to a malfunction of the cytolytic pathway and thus reduced target cell killing. Likely, the increased MUNC13-4 binding is not the only factor responsible for

reduced exocytosis of cytotoxic components. Rather a combination of altered binding properties to several other effectors such as SLP2A underlies dysfunctional cytotoxic function. This assumption is supported by the variant concerning an autosomal recessive variant. If it was only the increased MUNC13-4 binding causing the decreased exocytosis, then one would expect an autosomal dominant disease. After all, a gain-of-binding variant normally penetrates as an autosomal dominant disease. Therefore, it is likely that not only the increased MUNC13-4 binding, but also the altered/reduced binding of other effectors is necessary for this autosomal recessive disease to penetrate. As we have only tested the binding capacity of the RAB27Ap.R184Q variant to MUNC13-4 and SLP2A, we could only speculate that protein interactions of other effectors such as other SNARE-proteins will also be affected.[46,47]

Our finding that a constructed "guanosine triphosphate (GTP)-bound, active" RAB27Ap.Q78L variant bound MUNC13-4 repeatedly with less affinity than the constructed "guanosine diphosphate (GDP)-bound, inactive" RAB27Ap.T23N variant is in disagreement with published studies.[48,49] Moreover, when combining the two variants of the RAB27Ap.T23N (GDP bound, inactive) and RAB27Ap.R184Q (the variant found in our patient), this double mutant shows an even stronger binding to MUNC13-4. We could not explain these unexpected binding properties of proteins encoded by our plasmid constructs. A methodological error seems unlikely as the results of the parallel experiment with the RAB27A constructs to SLP2A and MELANOPHILIN are in line with previous literature. A fault in the plasmid constructs itself is also unlikely because all constructs were sequenced, yielding high-quality reads, confirming the desired sequences including the presence of the point mutations at the correct genomic positions. Thus, our findings suggest a true result and warrant further studies into the various protein interactions of RAB27A.

STEM CELL TRANSPLANT FOR ADULT HLH PATIENTS HARBORING AN HLH-DRIVING MUTATION

In the introduction and in Figure 5 of the introduction, we presented a schematic overview of how several etiologic elements build-up toward a certain threshold above which HLH is provoked.[13] The added value of each of these elements is highly variable. For example in the case of an inborn *PRF1* mutation, the genetic background comprises a large part in the development of FHL. A relatively small trigger can then evoke a detrimental HLH episode. For mutations in alternative immune function-effecting genes, the subsequent genetic background might be of less significance for reaching the HLH-threshold. In this case, a more potent trigger is (or even multiple triggers are) necessary to reach the HLH-threshold. In adults suffering from HLH, a genetic mutation can often

be found, as is demonstrated in Chapter VI and Chapter VII.[27,50,51] Mutations in adults comprise primarily missense variants, which occasionally occur biallelically.[27] These variants could be expected to contribute to the genetic background and attribute to the buildup toward the HLH-threshold. As these patients develop HLH during adulthood, this implies that the attribution of the genetic background is limited and small enough to prevent HLH from arising after common periods of pathogenic stress. Otherwise, HLH would have already developed during childhood. Only if a potent trigger such as EBV invades these patients, the HLH-threshold will be reached.

For the classic mutations occurring in FHL, the cytotoxic function is greatly abolished. As this concerns a genetic defect, patients typically present with HLH during childhood. Cure can only be achieved through allogenic SCT, after which functional donor T- and NK-cells restore adequate control of pathogenic episodes such as viral infections or malignancies.[52-54] Adults concealing a genetic mutation that does not lead to overt immune dysfunction require a different approach to allogenic SCT.[55,56] As allogenic SCT is associated with frequent and serious morbidity and mortality, the clinical benefits and risks of allogenic SCT should be weighed against the clinical benefit and risks associated with alternative treatment with immunosuppressant medication.[57-60] Although genetic variations or mutations in HLH-related genes can be found in nearly half of the adult HLH patients, a substantial proportion of HLH patients only display a single HLH episode.[27,61,62] Together with the high risk of morbidity and mortality of allogenic SCT, this justifies a reserved use of allogenic SCT in adult patients. To this extent, screening for a corresponding mutation in siblings of genetically susceptible HLH patients should not be performed routinely, because the detection of a corresponding mutation will not justify a preventive allogenic SCT. Accordingly, genetic screening of the siblings of the patient presented in Chapter VI will not attribute to the treatment strategy and will thus only advance research questions. Allogenic SCT might be considered for patients with recurrent HLH episodes. Enduring attributors to the HLH-threshold are likely present in these patients because they prove to reach the HLH-threshold easily. Such an enduring attributor is likely of genetic origin.[27] Therefore, these patients will likely benefit from allogenic SCT, which eliminates their genetic susceptibility. Future research on the genetic landscape of adult HLH is expected to pinpoint the subgroup(s) of patients with high risk of HLH relapse. This might then justify (preventive) allogenic SCT in this subgroup of patients and genetic screening of siblings or infants. Of note, the alternative of gene-corrected autologous SCT might not be a suitable alternative for adults with recurrent HLH episodes, because the genetic susceptibility for HLH is probably not determined by a single gene but rather a combination of genetic variations.[27,63,64] On the contrary, this approach could be of interest in all patients with confirmed FHL-associated mutations because a single mutation entirely accounts for dysfunctional cytotoxicity in these patients. Correcting this single mutational defect will normalize cytotoxic function

and thereby cure or prevent HLH. Cases such as that reported in Chapter VI and Chapter VII help to pinpoint causal mutations and specify their role in disease pathology, which will eventually lead to the identification of patients who will benefit from SCT.

ETOPOSIDE FOR ADULT HLH PATIENTS

The management of HLH remains a major clinical challenge. Most of our understanding of HLH is based on research in pediatric patients, which often concerns FHL.[2,52,65] HLH in adults is rarely associated with acknowledged FHL-related genes.[2] The pediatric treatment schedules designed primarily for FHL (Figure 6, introduction) can therefore not simply be implemented for the adult (secondary) HLH population.[55] The recommendations of the international working group on adult HLH of the Histiocyte Society include proposed treatment guidelines, which are adapted to adult HLH patients.[55,56] These guidelines comprise recommendations on the use of etoposide in an adapted HLH-94 schedule for specific clinical situations such as malignancy-associated HLH or refractory disease (Figure 7, introduction). However, there is little evidence that all adult HLH patients need prompt treatment with etoposide.

In Chapter VIII, we conducted a systematic review and meta-analysis on the effect of etoposide on survival in adult HLH patients. In this study, we could not demonstrate a beneficial effect of etoposide. An important consideration is that none of the retrieved studies was specifically designed to evaluate the clinical benefit of etoposide. Moreover, there is a huge heterogeneity among the different study populations. Adult HLH, comprising patients with limited genetic susceptibility, has a wide clinical spectrum and includes diverse underlying triggers. Analogous treatment with etoposide will thus certainly have different effects among patients. Moreover, the treatment regimen of a hematologic malignancy, as the underlying trigger of sHLH, often contains etoposide and thereby exerts an effect not only on the HLH but also on the underlying trigger. Consequently, patients included in our meta-analysis will have a variable (potential) benefit from etoposide. To overcome this limitation, our analysis should be stratified according to the type of HLH-trigger. However, most studies do not present enough data on causative triggers and patient numbers will consequently be too small to carry out a sensible analysis. Another limitation of our study is the bias by indication. As none of the included studies apply randomized treatment schedules, the consideration to start etoposide is primarily based on the clinicians' judgment and the centers' preferences. As etoposide is frequently withheld in less severe cases, the patients who are treated with etoposide likely overrepresented a subgroup of severe disease.[66] This subgroup has a worse prior survival compared to the group that is withheld from etoposide. Therefore, the results produced by this meta-analysis likely underestimate the clinical

benefits of etoposide. Although our study could not demonstrate a beneficial effect of etoposide, several individual studies did indicate beneficial effects.[66-70] Therefore, we do not recommend completely abandoning etoposide application in adult HLH, but rather reserve its use for individualized cases. Especially in refractory or severe disease with multi-organ failure, we underscore the recommendations of the international working group to consider etoposide as part of the treatment schedule.[55,56] With this meta-analysis and review of the literature, we provide the best available evidence on which clinical decision-making can be based regarding etoposide in the adult sHLH population. We also show that there are no conclusive studies on etoposide as a superior treatment modality in adults. A prospective and multicentric randomized clinical trial is necessary for definite conclusions on this topic.

Our review only focused on a single treatment modality. Newer treatment modalities might be preferable to etoposide. Since a cytokine storm is important in the pathogenesis and drives the clinical symptoms, new developments in therapeutics largely focus on cytokine inhibition.[71,72] Although several studies present promising results, cytokine inhibitors alone seem to be of limited value.[73-77] A recent study of tocilizumab (anti-IL-6) indicates that this drug might even be harmful.[73] Preliminary studies focusing on inhibiting INF-γ, which is known to be an important driver of HLH, also lack sufficient positive results.[74] [and additionally based on preliminary and unpublished data from running trails]. Anakinra (anti-IL-1) seems to have potentially beneficial effects, although strong evidence is lacking.[75-77] The explanation for these results from cytokine inhibition might lie within the large amount of different cytokines that are involved in the cytokine storm. Inhibiting just a single cytokine might have an insignificant effect on the total cytokine storm. Therefore, ruxolitinib forms a rational treatment for HLH, because it constrains the effect of the cytokine storm.[78,79] Ruxolitinib inhibits both JAK1 and JAK2 which function in numerous pathways downstream of HLH-associated cytokines such as IL-6, INF-γ, IL-2, and IL-10.[78] Although the cytokine storm itself is not reduced, the effect thereof is significantly dampened and results in improved survival of HLH patients.[80,81] Alemtuzumab is another alternative to anti-cytokine therapy. This anti-CD52 antibody depletes T- and B-cells and to a lesser extent monocytes, macrophages, and NK-cells through complement-directed cell-killing.[82] Therefore, it prevents these cells from producing large quantities of cytokines that cause HLH. Although the number of patients who received this treatment is rather small, alemtuzumab seems highly effective in both bridging FHL patients toward allogenic SCT and as salvage therapy.[83,84] Alemtuzumab depletes a wide range of immune cells that are involved in HLH, including macrophages, T- and NK-cells.[82] Since etoposide only depletes a limited number of immune cells (primarily activated $CD8^+$ T-cells), alemtuzumab can serve as a good alternative to etoposide.[85] A comparative study should ideally be performed to compare efficiency and side-effects.

HLH TRIGGERED BY HERPES SIMPLEX VIRUS

Numerous triggers are known to cause sHLH. The largest proportion of these triggers concern infectious triggers, more specifically viral.[2] Although EBV is the most prevalent viral trigger, other viruses like herpes simplex virus (HSV) are also reported to trigger sHLH.[86] In Table 1, we present data on HLH patients with an HSV (co)infection who have been treated in our academic center. Although HLH occurs mostly during the reactivation of HSV, we show that HLH can also occur during primary infection (Table 1).[87] As patient six demonstrates in our series, both HSV-specific IgM and IgG can still be negative at HLH presentation. In this patient, the Ig class switch was observed a few days later. Therefore the routine diagnostic workup of all cases suspected of sHLH should include evaluation of HSV by PCR-based technology as a positive HSV viral load could be missed by only performing serologic diagnostics.

In line with observations from the literature, we noticed that all our HSV-HLH patients presented with hepatitis.[86,88,89] Since HSV-associated hepatitis is a very rare phenomenon, it might seem remarkable that all our HLH patients presented with hepatitis.[90] This association between HSV-HLH and hepatitis is probably due to the viral load of the HSV. Previous studies show that patients with HSV-hepatitis have a markedly higher HSV viral load compared to HSV patients without hepatitis.[91] Moreover, similar to observations in EBV-driven HLH, the HSV viral load could be expected to correspond with the HLH disease activity.[13,92,93] Thus, having a high HSV viral load drives both the HSV-hepatitis and the HLH. Alternatively, the hepatitis (which is diagnosed by elevated transaminases) might simply be secondary to the HLH rather than caused by a HSV-hepatitis.

Patient three presented with HLH that was caused by a primo HSV-2 infection. The treatment consisted of corticosteroids and acyclovir; the patient survived the HLH episode but was unable to control her HSV-2 infection. Thus far, she still has recurring HSV-2 episodes in the skin with persisting viremia. Such a persistent and life-threatening infection could be indicative of an underlying primary immunodeficiency, especially because numerous genes affect successful immune defense against the herpes virus.[94] Indeed, whole exome sequencing (WES) uncovered a potentially interesting genetic variation in the *Ring Finger Protein (RNF) 213* gene. The *RNF213*p.R4215X variant has a population frequency of less than 0.00001 (GnomAD v3.1.2).[95] In addition, the mutation is predicted to be damaging, which is illustrated by a high CADD score of 35.[96,97] However, the genetic constraint of *RNF213* for loss-of-function variants is only 0.57 (90% CI 0.49–0.66) according to GnomAD's *observed/expected* metric, indicating that the gene is fairly tolerant to loss-of-function mutations.[95] *RNF213* mutations are drivers of Moyamoya disease (MMD).[98,99] MMD is characterized by specific vascular anomalies. The arteries around the circle of Willis show stenosis or occlusion and collateral vessels are formed.[100] MMD patients often present with an ischemic stroke or a transient ischemic attack

Table 1 | HLH triggered by HSV (co-)infection.

Pt nr	Age	HSV type	Primary or reactivation	HSV IgM	HSV IgG	Ct value	Peak ALAT	WES	Potential alternative HLH trigger	Outcome
	Years			*Presentation*	*Presentation*	*Lowest*	*U/L*			
1	17	Unknown	Primary	Positive	Positive	Negative	3421	No potential pathogenic variant	Still's disease	Deceased. Multi-organ failure due to HLH
2	58	HSV-1	Reactivation	Negative	Positive	31.8	2632	Not done	None	Deceased. Multi-organ failure due to HLH
3	63	HSV-2	Primary	Positive	Negative	9.4	5128	RNF213 variant	None	Alive with recurrent HSV infections
4	45	HSV-1	Reactivation	Unknown	Positive	31.5	473	No potential pathogenic variant	Lymphoma, EBV	Deceased. Multi-organ failure due to HLH + bacterial sepsis
5	30	HSV-2	Primary	Unknown	Negative	9.4	1139	Not done	Pregnancy	Deceased. Multi-organ failure due to HLH
6	62	HSV-1	Primary	Negative	Negative	15.2	2690	Not done	None	Deceased. Multi-organ failure due to HLH

(TIA).[100,101] Indeed, patient three had a history of TIA and other neurological symptoms such as headache, cognitive impairment, and seizures. However, the MRI of this patient did not display gross vascular anomalies fitting MMD. Thus this *RNF213*p.R4215X variant probably does not penetrate as MMD in this patient. However, the RNF213 protein also plays a crucial role in antimicrobial defense including HSV defense.[102] A dysfunctional RNF213 could hinder HSV clearance and thereby explain the patient's persisting HSV infection. A high viral load is the probable driver of the HLH and hepatitis episode in this patient.[91-93] Protein expression and *in vitro* functional assays of this specific RNF213 variant are needed to confirm its contribution to this patient's clinical features of HSV-HLH and persistent HSV infection.[102] In this stage of research, definitive conclusions cannot be drawn, but the described observations may lead to more insights into herpes-driven HLH.

XIV

Langerhans cell histiocytosis

LANGERHANS CELL HISTIOCYTOSIS

Langerhans cell histiocytosis (LCH) is a neoplastic condition in which pathologic histiocytes accumulate in a varying number of lesions at various anatomical sites. For a long time, LCH was considered an inflammatory disease with lesions consisting of apparently normal, reactive immune cells in which cytokines play a key role.[103,104] However, modern technology helped discover that the LCH-cells in the lesions are clonal and LCH should be categorized as a neoplastic disorder. Somatic activating mutations in the MAPK-pathway drive the pathogenesis by promoting the cell's proliferation and survival.[105] The same applies to Erdheim–Chester disease (ECD), where lesion-accumulating macrophage-like histiocytes express similar MAPK pathway-activating somatic driver mutations.

THE FREQUENCY OF MAPK-PATHWAY MUTATIONS IN LCH

As of 2010, we know that somatic activating MAPK-pathway mutations drive LCH. Clinical research is primarily performed in children, showing the *BRAF*p.V600E mutations accounts for approximately 60% of cases.[105-109] Since 2010, numerous additional mutations in the MAPK-pathway have been found to drive LCH pathogenesis, with MAP2K1 hotspot mutations being the second most prevalent type of mutation.[110-116] In pediatric LCH patients, a MAPK-pathway mutation is found in approximately 80% of cases.[117] In Chapter IX, we confirm these observations by collaborating in an international multicenter study resulting in the largest cohort of pediatric patients analyzed to date. Fewer studies report on the frequency of LCH-associated mutations among the adult population. In Chapter X, we report the *BRAF*p.V600E frequency among adult LCH cases. There is a slightly lower frequency of *BRAF*p.V600E in the adult population compared to the pediatric population (46% versus 51%, respectively). Since not all patients from our pediatric and adult cohorts have been evaluated for mutations other than *BRAF*p.V600E, we are not able to present exact frequencies of the other frequently occurring somatic mutations.

THE ROLE OF MAPK-PATHWAY MUTATIONS ON DISEASE EXTENT

LCH is a heterogeneous disease regarding its clinical manifestation and disease course.[118] The factors that determine the extent of the disease remain largely unknown. Several pediatric studies show a correlation between mutational status and disease extent,

indicating that MAPK-pathway mutations have the potential to influence the extent of the disease.[106,119-121] In Chapter IX, we confirm correlations from previous literature by showing that lesional *BRAF*p.V600E is associated with significantly more MS disease, multifocal SS-skin disease, risk-organ involvement, skin involvement, and lower age of disease presentation.[106] In addition to previous literature, we show a new correlation between *BRAF*p.V600E and involvement of bones of the upper extremity and CNS risk bone lesions. This is the first study to show a correlation between *MAP2K1* mutations and SS-bone disease. Taken together, these results indicate that genetic alterations play a part in the development of specific disease extent in the pediatric population. *BRAF*p.V600E seems to drive LCH toward a more extensive disease, whereas *MAP2K1* hotspot mutations limit disease extent to a confined bone disease. Intriguingly, correlations between *BRAF* mutational status and disease extent that are apparent in children, seem to be absent as per several reports on adults.[122-124] To confirm these observations made by small case series, we analyzed a large international cohort of 156 adult LCH patients in Chapter X. In line with previous literature, this cohort did not show a correlation between *BRAF*p.V600E status and disease extent, and neither did we observe a trend toward a difference. Moreover, this lack of correlation remained when our cohort was combined with cases reported in the literature in a meta-analysis. We can, therefore, conclude that the *BRAF*p.V600E mutation and disease extent correlate in children, but not in adults. This finding highlights that we should be cautious when extrapolating genetic insights from pediatric studies to the adult population.

THE ROLE OF MAPK-PATHWAY MUTATIONS ON PROGNOSIS

Since somatic activating MAPK-pathway mutations underlie LCH, it would be reasonable to hypothesize that these mutations affect a patient's prognosis. Indeed, a large previous study by Héritier *et al.* demonstrated the correlation between mutational status and prognosis in pediatric patients.[106] They showed a higher reactivation rate, increased permanent consequences, and higher treatment failure in *BRAF*p.V600E positive patients. These findings are widely supported by other publications.[107,118,120,125-128] In Chapter IX, we show a reduced event-free survival in *BRAF*p.V600E positive pediatric LCH patients in the univariate analysis. In contrast with earlier studies, *BRAF*p.V600E, did not affect the overall survival in our cohort.[119] Additionally, when the patients are stratified by disease extent in the univariate analysis, the correlation between *BRAF*p.V600E and event-free survival disappeared. This finding was confirmed by our multivariate analysis, in which *BRAF*p.V600E did not correlate with event-free survival but did correlate with disease extent. As discussed before, *BRAF*p.V600E drives LCH toward a more extensive

disease including MS-LCH and risk organ lesions.[106] This extensive disease accounts for a worse prognosis, and thus, *BRAF*p.V600E exerts its (indirect) effects on prognosis only by driving the disease extension.[129-131]

Taking these observations into account, the prognosis of the adult LCH population should not be affected by a *BRAF*p.V600E mutation, as *BRAF*p.V600E does not correlate with disease extent in the adult population. Indeed, in Chapter X, the univariate analysis shows that the *BRAF*p.V600E mutation does not correlate with event-free survival in adults with LCH. From our data, we can conclude that *BRAF*p.V600E does not correlate directly with prognosis, but rather exerts an effect on prognosis by altering the disease extent. In the future, these findings should be confirmed within other cohorts of LCH.

THE CONTRIBUTION OF MAPK-PATHWAY MUTATIONS TO LCH DEVELOPMENT

The somatic activation of MAPK-pathway mutations is presumed to drive most of the pathogenesis of LCH. An almost universal phosphorylation of ERK in LCH indicates that MAPK-pathway activation has an essential role in the pathogenesis.[105,111] Moreover, MAPK-pathway activation is known to stimulate proliferation and survival and thereby presents a rationale for the role of its activation in the pathophysiology.[132,133] Although it is evident that MAPK-pathway activation is necessary for LCH development, other contributors to the pathophysiology might currently be underexposed. As mouse models demonstrate, overexpression of the *BRAF*p.V600E mutation alone, induce an LCH-like phenotype instead of an accurate representation of the divers clinical features of LCH disease.[107] When a *BRAF*p.V600E mutation is introduced in a CD11c-positive DC progenitor, these mice develop histiocytic infiltrates in the skin, liver, spleen, and lungs.[107] These mice generally lack bone involvement and only display severe and multisystem disease. This deviates from human LCH in which bone involvement is very common, particularly presenting as single-system disease.[134] Moreover, in humans harboring *BRAF*p.V600E in hematopoietic progenitor cells, the disease extent is often limited to SS-LCH and does not always present as MS-LCH as is the case for these mouse models.[135] It is questionable to what extent transgenic murine LCH models represent human LCH pathogenesis. Nonetheless, it could be regarded as an indication that the sole introduction of a *BRAF*p.V600E mutation is not enough to initiate LCH disease and that other features contribute to the pathophysiology of LCH. Moreover, there is no report of patients with an activating germline MAPK-pathway mutation (which causes the cardio-facio-cutaneous syndrome in humans) that has been reported to develop LCH.[136] Patients harboring an activating germline MAPK-pathway mutation are not even evidently susceptible to developing a neoplasm.[137] In this regard, it should be noted that

there is no report of a patient with the cardio-facio-cutaneous syndrome that harbored the specific germline *BRAF*p.V600E mutation that, as a somatic mutation, is the most prevalent driver of MAPK-pathway activation in LCH patients. If an activating MAPK-pathway mutation would be the sole driver behind LCH, it could be expected to give rise to a single disease instead of both LCH and ECD. Moreover, if the MAPK-pathway mutation was the sole disease driver, identical correlations between this MAPK-pathway mutation and prognosis would be expected in both children and adults. As shown in Chapter IX and Chapter X, the effect of *BRAF*p.V600E in children is different from that in adults. Taken together, there is enough rationale to state that MAPK-pathway mutations are crucial for the disease pathophysiology, but that they do not account for the full LCH pathophysiology. Further research is needed to clarify the contributing role of additional elements in the pathogenesis of LCH. This research should focus on the genetic landscape of LCH-cells to find other genetic alterations contributing to LCH pathogenesis. It is also likely that alterations in various pathways are important in the disease pathology. Several papers have already assessed the activity of clonality-associated pathways in LCH, such as the NOTCH pathway, transforming growth factor (TGF)-β pathway, and p53 pathway.[138-140] It remains unknown if these pathways are altered concurrently and if they have a role in the pathogenesis of LCH. The next step could be to assess the activity of such pathways simultaneously within an LCH-cell. Additionally, forced overactivation or suppression of these pathways in LCH mouse-models could shed light on their role in the disease pathology.

ANTI-TUMOR DEFENSE IN THE LCH-LESION

The results presented in Chapter XI demonstrate several mechanisms for tumor escape by the LCH-cell. Immunological tumor defense is a long-known phenomenon in neoplastic disorders.[141-143] Both the innate and adaptive immune systems contribute to anti-tumor activity.[144-146] The adaptive anti-tumor response is primarily the result of $CD8^+$ T-cell killing.[146] Intracellular proteins encoded by cancer-associated, somatic mutations called neoantigens are cut into smaller peptide fragments during protein degradation. These tumor-derived peptides are loaded onto HLA class I molecules and subsequently transported to the cell membrane. There, they form a crucial activating stimulus for the adaptive anti-tumor response.[142] When an antigen-presenting cell (APC) presents a tumor-derived peptide to a naïve $CD8^+$ T-cell, it is stimulated and will differentiate into cytotoxic T-cells (CTLs).[142] When these CTLs re-encounter their neopeptide, which is presented by a HLA class I molecule on tumor cells, the CTLs will eliminate these tumor cells presumed that the cancer cells do not additionally express inhibitory ligands that dampen the cytolytic T-cell function.[142]

In Chapter XI, we explore the immunological anti-tumor response in LCH-lesions and study if LCH-cells are capable of escaping from the immunological tumor defense. We used publicly available bioinformatic tools to demonstrate that neopeptides are predicted to be formed from the mutant BRAFp.V600E protein.[147] If this neopeptide would be potent enough to initiate an anti-tumor response, then neopeptide-guided cell killing could potentially play part in LCH-cell homeostasis.[148] In this context, studies on melanoma patients indicate that BRAFp.V600E-derived neopeptides seem to be capable of initiating an anti-tumor immune response.[149,150] Neopeptide presentation by HLA class I is crucial for this anti-tumor response to be effective. In Chapter XI, we analyze the ability of BRAFp.V600E-derived neopeptides to be presented by HLA class I molecules on the cell surface. We show that the potential for neopeptide presentation is present, indicated by normal expression of HLA class I by LCH-cells and stable *in vitro* HLA binding capacity of a BRAFp.V600E-derived neopeptide (KIGDFGLAT**E**K) to particular HLA molecules. Although there is a potential for neopeptide presentation, evaluation of several BRAFp.V600E overexpressing cell lines showed that the BRAFp.V600E-derived neopeptide (KIGDFGLAT**E**K) was not detected in the peptidome extracted from HLA class I molecules stripped from the surface of these cells. It could be speculated that the BRAFp.V600E protein is not effectively degraded by the proteasome. This immunological 'escape' mechanism would then be comparable to the RAB27Ap.R184Q variant of Chapter VI, where the mutated protein is expressed at normal levels but not (quickly) degraded by the proteasome. The consequence in LCH-cells seems to be insignificant neopeptide presentation by LCH-cells *in vivo*. This lack of neoantigen presentation will consequently prevent the immune system to detect the presence of neoplastic LCH-cells and will result in escape from the immunological anti-tumor response. In line with this observation is the lack of correlation between $CD8^+$ T-cell density and event-free survival that we show in Chapter XI. The $CD8^+$ T-cell density of a tumor lesion is crucial for tumor defense and is known to correlate with disease prognosis in several neoplasias.[151] A higher density of $CD8^+$ T-cells implicates a higher rate of tumor-directed immune response and thereby better tumor control and better prognosis.[151] Therefore, it could be expected that an increased $CD8^+$ T-cell density in LCH-lesions improves the patients prognosis. However, because LCH-cells do not present neopeptide on their surface, an anti-tumor response will not be initiated. Higher numbers of $CD8^+$ T-cells within a lesion will not enhance the anti-tumor effect and will thus not affect a patient's prognosis. Alternatively, the cellular anti-tumor response in LCH-lesions is likely to be dampened by the observed presence of regulatory T-cells (Tregs) within LCH-lesions.[152,153] Moreover, LCH-cells are believed to induce Treg expansion and thereby, further enhance the anti-tumor escape.[153] A conclusive explanation of the heterogeneous composition of LCH-lesions and how it affects disease features such as disease extent and prognosis remains unavailable.[107,154]

The escape from anti-tumor immunosurveillance that is reported in Chapter XI is important with regard to the rationale for immunotherapies in LCH patients. Immunotherapy with immune checkpoint inhibitors is quickly expanding in the oncology field, with impressive results in numerous neoplasms.[155] It is therefore not surprising that immunotherapy has been suggested for patients with refractory LCH.[156,157] However, in Chapter XI, we show that BRAFp.V600E protein- derived neopeptides are not presented on the cell's surface. This is a strong indication that immunotherapy will not be effective for patients that exclusively harbor this particular mutation. Moreover, the potency of immunotherapy is highly dependent on the mutational load of the neoplasia.[155] As the mutational load of LCH is very low with approximately 0.03 mutations per megabase (Mb) (compared to approximately 10 mutations per Mb in melanoma), it is unlikely that immunotherapy will benefit LCH patients.[111,158,159]

Anti-tumor control is additionally operated by NK-cells. NK-cells take part in tumor defense by detecting a lack of major histocompatibility complex (MHC) class I molecules on the cell surface.[160] Healthy cells present enough MHC class I molecules on their cell surface and thereby inhibit targeting by NK-cells, whereas tumor or virus-infected cells frequently display decreased levels or lack MCH class I and are thus eliminated by the NK-cell.[160] In Chapter XI, we show that LCH-cells express normal levels of MHC class I molecules at their cell surface, thus these cells could also escape from elimination by NK-cells. Therefore, LCH-cells are also not susceptible to this additional, NK-driven mechanism of elimination. This further explains the observed escape of LCH-lesions from the immunological anti-tumor response.

Notably, other mechanisms of immune tolerance might also play part in LCH and should be taken into consideration when concluding on the anti-tumor response. These mechanisms include the upregulation of ligands for inhibitory receptors, and the immunosuppressive microenvironment.[150,156,157,161]

SECOND PRIMARY MALIGNANCIES IN LCH PATIENTS

Recent data show that LCH patients are at a higher risk of developing a second primary malignancy (SPM).[162] This is especially pronounced in adults with estimates of an SPM ranging from 6% to 32%.[129,162,163] Indeed, in Chapter X we show a remarkable incidence of SPMs in our adult LCH cohort. Since these additional malignancies often occur prior to, or concurrent with the LCH, they probably represent a true correlation rather than a therapy-related comorbidity. No association with SPMs was discovered in the pediatric cohort that is described in Chapter IX. SPMs are to be expected in our adult cohort due to the general risk among aging individuals to develop any kind of neoplasm.[164] However, in our cohort there is a higher frequency and a deviating distribution of types

of malignancies compared to the overall population. In line with the literature, we primarily report second hematologic malignancies (SHM) in our adult LCH cohort.[162,165,166] Notably, this finding may shed more light on the pathogenesis of LCH. As hematologic malignancies and LCH-cells are thought to share common hematopoietic progenitors, it is valid to hypothesize that both LCH and the SHM arise from the same progenitor cell and therefore share clonality. Indeed, a case has previously been demonstrated in which both LCH-cells and concurrent SHM from T-cell origin share the same genetic T-cell receptor rearrangement (although not expressed by the LCH-cell), suggesting clonality.[167,168] In Chapter X, we add a case in which identical immunoglobulin gene rearrangements were found in an LCH biopsy and a lymph node displaying a diffuse large B-cell lymphoma (DLBCL), which occurred a few years later. In this patient, the LCH-lesion harbored the *BRAF*p.V600E mutation, whereas the lymph node in which DLBCL presented did not. The identical immunoglobulin gene rearrangement is an indication that a common hematopoietic progenitor cell may acquire different genomic alterations over time, turning it into a pathological clone heading toward a distinct type of hematologic malignancy. A subsequent mutation in *BRAF*p.V600E then deflects part of the descendant cells toward LCH. Both LCH and an SHM can then sequentially arise (Figure 3). This hypothesis is supported by the observation that identical proteins are mutated in patients with hematologic malignancies and patients with LCH. Examples

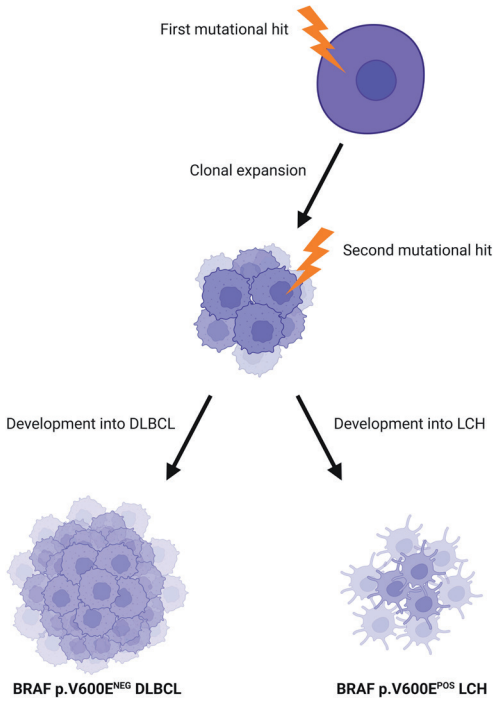


Figure 3 | Clonal relationship between DLBCL and LCH.
Identical immunoglobulin gene rearrangements in a patient with LCH and the subsequent diffuse large B-cell lymphoma (DLBCL) indicates a shared precursor of both diseases.

include JAK2 mutations in myeloproliferative neoplasms and NOTCH pathway alterations in B-cell malignancies, which are both found to be mutated in selected LCH patients.[138,169,170] Future studies need to clarify how two distinct, but clonally related neoplasms can arise in the same patient. The first step in this process should be a thorough prospective registration of these SPMs to determine the frequency and types of malignancies. All SPMs in this cohort should be evaluated for a clonal relationship with the LCH, for example with immunoglobulin or T-cell receptor gene rearrangement assays. Furthermore, studies are needed to make the clinical implications of these SPMs clear. There might be a rationale for intensified and long-term follow-up after LCH diagnosis and treatment in the adult patient.

LCH AS A DIFFERENT DISEASE ENTITY IN CHILDREN COMPARED TO ADULTS

Accumulating evidence suggests that LCH in children and adults forms a distinct entity.[122-124] As discussed before, Chapter IX and Chapter X highlight these differences concerning the disease extent and prognosis in relation to the somatic *BRAF*p.V600E mutation. Although both pediatric and adult LCH are generally driven by MAPK-pathway mutations, other factors will result in a different effect of mutations on clinical parameters. Additionally, Chapters IX and X demonstrate that SPMs are common in adult LCH but not evident in pediatric LCH.[162] Adults presenting with pulmonary LCH are distinct from the pediatric population because this disease is highly related to smoking and often presents as single-system lung disease, which is an infrequent finding in children.[124] Taken together, these observations indicate a distinct disease biology and thus, a distinct LCH entity between children and adults. Whether the origin of these differences lies within genetic factors, (micro)environmental factors, or within a difference in the cell that is hit by the somatic mutation, remains unknown.

SOMATOSTATIN RECEPTOR EXPRESSION IN ECD

Somatostatin receptor (SSTR) expression is a common finding in a large number of malignancies, most notably neuroendocrine tumors.[171,172] ECD, which is often considered the macrophage counterpart of LCH, could also be expected to express the SSTR. It has repeatedly been demonstrated that monocytes and monocyte-derived macrophages produce SSTR2 messenger ribonucleic acid (RNA) with subsequent protein expression on the cell's surface.[173] The ECD-cell might therefore have the potency of expressing SSTR because the hematopoietic origin of ECD is assumed to be of the (pre) monocytic

Figure 4 | Expression of SSTR2.
Data obtained from single-cell transcriptomics produced by RNA-sequencing are pooled and compared by their normalized transcripts per million (nTPM). Several immune and blood cells show the expression of SSTR2, including Langerhans cells, dendritic cells, and macrophages. As the ECD-cell is thought to descend from the monocytic lineage, the ECD-cell may express SSTR2. In addition, other lesional cells can also express SSTR2 contributing to the observed lesional expression of SSTR2 in ECD-lesions. All the data presented in this figure were obtained from Human Protein Atlas proteinatlas.org[186]

lineage.[174-178] Moreover, other lesional cells in ECD could also express SSTRs, including lymphocytes and mononuclear phagocytes (Figure 4).[173,179-186] Several cells in the ECD-lesion could thus express SSTR. Indeed, in Chapter XII, SSTR scintigraphy demonstrates the expression of SSTR2 in ECD-lesions. So far, there is only one previous report showing a positive SSTR scintigraphy in an ECD patient.[187] It would be relevant to determine whether MAPK-pathway activation can result in the upregulation of the SSTR, which could account for a possible enhancement of the ECD-lesion on the SSTR scintigraphy.

The results in Chapter XII present a discrepancy in SSTR expression obtained by SSTR scintigraphy and the staining of the biopsy. The SSTR scintigraphy demonstrates positivity for the ECD-lesions. Indium-111-pentetreotide (the type of SSTR scintigraphy used in our patient) binds primarily to SSTR2 and a lesser extent to SSTR 3 and 5. Therefore, one could conclude that this ECD-lesion harbors SSTRs.[188-190] However, the immunohistochemical staining of one of the LCH-lesions was negative for SSTR2, 3 and 5. A possible explanation could be the processing of the biopsy which had taken place more than 20 years prior to *in vivo* staining and the subsequent prolonged exposure of the tissue to formalin fixatives. This might have impacted the quality of SSTR immunostaining.[191] Alternatively, the fragment of the biopsy that was used for staining could have lacked the SSTR-positive cells. Also, a low expression of SSTR could be physiologically relevant but insufficient for staining. It would be of great interest to

prospectively stain a series of ECD biopsies for SSTR to conclude on SSTR expression in ECD-lesions.

THE POTENTIAL OF SOMATOSTATIN RECEPTOR RADIONUCLIDE THERAPY IN ECD

Immunosuppression and chemotherapy have long been the main treatment modalities for ECD patients.[131,192,193] Since the discovery of MAPK-pathway activation, targeted therapy is increasingly being applied in ECD patients.[192,194] Although these MAPK-pathway inhibitors are promising, several downsides warrant careful application. As demonstrated for vemurafenib, the ECD-lesions quickly regress, but either ECD-cells or its progenitor cells seem to be incompletely eliminated, as evident from the high recurrence of disease after treatment discontinuation.[195] Therefore, prolonged treatment with MAPK-pathway inhibitors is necessary. This chronic treatment is often hindered by the frequent side effects associated with targeted therapy and commonly results in treatment discontinuation.[196] Although targeted therapy has markedly improved disease outcomes over time, new treatment modalities with limited toxicity might impose a definitive cure.

In Chapter XII we present a case of refractory ECD in which SSTR radionuclide therapy proved effective. Over the 17 years following the SSTR radionuclide therapy, the patient displayed slow regression of the various ECD-lesions, although the lesions did not entirely disappear as evidenced by the persisting lesion upon several repeated radiographic evaluations. The positive SSTR scintigraphy could explain the response to the peptide-targeted radionuclide ([(177)Lu-DOTA(0), Tyr(3)]octreotate) therapy. However, this finding should be interpreted with great caution. Further research is needed to confirm SSTR expression in ECD-lesions and to specify which type(s) of cells express these receptors. An additional consideration before concluding on the efficiency of radiolabeled SST analog therapy is the patient's simultaneous and long-term exposure to immunosuppressive therapy. The patient was on prednisone and azathioprine during and after radiolabeled SST analog therapy. Thus, we could not exclude a potential synergistic effect of these agents on long-term disease remission. However, it should be noted that these (and other) agents did not induce remission prior to the radiolabeled SST analog therapy. Therefore, it is unlikely that prednisone or azathioprine alone induced remission, but it is plausible that these agents contributed to long-term remission of the disease. Taken together, these results warrant extensive further research before considering radiolabeled SST analog therapy as a treatment option for ECD.

XV

Histiocytic overlap

HISTIOCYTIC OVERLAP

Diseases can be classified according to numerous classification systems. When classifying diseases by hematopoietic cell lineage, diseases involving (elements of) the histiocytic lineage can be grouped and are termed histiocytic disorders. Therefore, the common feature of the different histiocytic disorders is the involvement of histiocytes. When grouped according to this nomenclature, the diseases involved are highly heterogeneous. The current classification of HLH as histiocytic disorder might not be correct. HLH is historically classified as a histiocytic disorder because the disease phenotype presents active and abundant macrophages.[197] Indeed, macrophages are involved in HLH, which is illustrated by their evident contribution to the fundamental cytokine storm and the presence of macrophages showing hemophagocytosis. However, modern insights into FHL show that the driving force behind FHL is the defective cytotoxicity of CTLs and NK-cells. The activation of macrophages is reactive/secondary to this process and is mediated by IFN-γ production by the cytotoxic cells.[198] In the case of other histiocytic disorders such as LCH, a histiocyte (*e.g.,* the LCH-cell) has a primary and not reactive role in disease development. Being only reactively involved in the disease pathogenesis, HLH might not be regarded as histiocytic disorder but rather as an immunodeficiency.

Both LCH and ECD are typically driven by a MAPK-pathway mutation, leading to a gain-of-function of the mutated protein.[199] It is unknown why both LCH and ECD can result from identical MAPK-pathway mutations and what determines the differentiation into either of these two diseases. As there is no report of an LCH-lesion evolving into an ECD-lesion, or vice versa, it is probable that LCH and ECD both concern cells in an end-stage differentiation without the ability to transdifferentiate. The time point at which the differentiation trajectory toward LCH- or ECD-cells is set, is a matter of debate. This differentiation could either be established by the microenvironment in which the LCH or ECD precursor cell is seeded, or during the hematopoietic development. If the differentiation is established by the microenvironment, a circulating precursor has the sustained ability to differentiate into either an LCH- or ECD-cell. Upon entering the designated tissue, local stimuli such as cytokines, chemokines, or cell–cell interaction steer the precursor toward either an LCH- or ECD-cell.[176] This hypothesis is supported by the observation that LCH and ECD tend to develop in distinct tissues, in which the different micro-environment leads to either LCH or ECD.[193,200] However, both LCH and ECD patients have MAPK-pathway mutated, blood-borne (precursor) cells.[135,201] As these circulating MAPK-pathway mutated cells will circulate through both LCH- and ECD-associated tissue, one would expect that these cells seed in both ECD- and LCH-associated tissue. In turn, this should lead to frequent co-occurrence of LCH and ECD. However, this concurrent presentation of LCH and ECD is only observed sporadically.[199] Moreover, this scarcely reported co-occurrence of LCH and ECD is even reported within a

single lesion.[199] If the trajectory toward either LCH or ECD is set by the microenvironment, the specific microenvironment of the future lesion will not allow the development of both LCH and ECD in a single lesion. Therefore, it is most probable that the precursor is already primed before it enters the designated tissue. Future research should unravel the essential stimuli for the differentiation into either LCH or ECD, in order to explain how identical driver mutations are able to cause these two different disease entities.

A link between HLH and LCH has been reported by a few studies. In these reports, HLH seems to be triggered by the presence of LCH-cells.[202-205] Among MS-LCH with the involvement of risk organs, the development of HLH is fairly common with some studies reporting an incidence as high as 10%.[202] This co-occurrence is more common in patients aged <2 years and results in an inferior 5-year overall survival.[202] The underlying mechanism of HLH development in LCH patients is unknown. As we show in Chapter XI, the *BRAF*p.V600E mutated cell lines do not present BRAFp.V600E protein-derived neoantigens on the cell surface and could, therefore, not evoke an anti-tumor response. Due to the lack of neoantigen exposure to the immune system, the BRAFp.V600E mutant protein is not likely to be a contributing trigger to the HLH. Other (concurrent) mutated proteins might be degraded into (neo) antigens which, can effectively be presented at the cell surface. This could provide the persisting antigen exposure needed for sHLH to develop. Alternatively, the development of HLH in LCH patients could be explained by excessive cytokine production. In both diseases, a cytokine upregulation (or cytokine storm) is thought to play a crucial role in the pathophysiology.[71,72,103,204] In LCH-lesions, there is a high production of pro-inflammatory cytokines, chemokines, and growth factors. These cytokines include, but are not limited to, IFN-γ, IL-6, IL-10, IL-18, and CXCL9, which are both present in the LCH-lesion and in the patient's serum.[8,9,14,103,104,206] These signal molecules account for an inflammatory component in otherwise neoplastic myeloid disease. HLH on the other hand, is characterized by a pathologic over-activated immune response driven by a cytokine storm, with a large overlap in the aforementioned cytokines and chemokines. It could be speculated that the cytokine upregulation observed in LCH contributes to a chain of events resulting in an uncontrolled upregulation of the immune system, and thus a clinical picture of HLH. The cytokines produced by the LCH-lesion are thereby an independent element, or trigger, leading up to the threshold for HLH induction (Figure 5, introduction). This could also explain the observation that HLH is primarily seen in MS-LCH with risk organ involvement.[202] In the case of unifocal LCH, there is just one lesion producing cytokines, resulting in relatively low systemic quantities thereof. In the case of MS-LCH, there are more (and often larger) lesions which together produce larger quantities of cytokines. These larger quantities of cytokines will facilitate the patient in reaching the HLH-threshold. Besides, LCH in itself can cause features that are characteristic of HLH. As Galluzzo *et al.* demonstrated, more than 40% of MS-LCH patients show hemophagocytosis in their bone marrow and could

therefore ease constructing an HLH diagnosis.[205] Furthermore, additional HLH triggers, such as an infection or chemotherapy (prescribed in the context of LCH treatment) are often present in LCH patients, and could contribute to reaching the HLH-threshold and developing sHLH.[204]

XVI

Future perspectives

FUTURE PERSPECTIVES ON HLH

The current understanding of HLH has significantly increased in the last decade. This is due to the increasing scientific interest in HLH, together with state-of-the-art technologies such as whole genome sequencing, proteomics, and sophisticated murine models. However, a lot remains to be uncovered on a wide range of HLH aspects. While the pathophysiology of FHL has been unraveled to a great extent, the pathophysiology of sHLH remains largely unexplained, and future research should focus thereon. Several hypotheses have been proposed, but none of these could sufficiently explain the process underlying and driving sHLH. The first step to attaining such insights probably lies within extensive genetic profiling. As discussed in this thesis, there are numerous clues to expect FHL and sHLH to be more of a spectrum rather than a dichotomous classification. Whole genome sequencing of a large international cohort of adult HLH patients could further clarify the role of genetics and show its clinical implications.[27] In addition to the pathophysiology, future research should focus on the diagnostic process of HLH. Although the HLH04 criteria and HScore are acceptably sensitive and specific, recognizing HLH clinically and making a swift diagnosis remains challenging. It is particularly challenging to adequately distinguish HLH from severe sepsis. Moreover, several diagnostic criteria are laborious, time-consuming, and/or not commonly available. Additional disease-specific (bio) markers could facilitate a more timely and smooth diagnosis, distinguishing HLH from sepsis. Numerous proteomic techniques could aid in finding novel biomarkers. Mass spectrometry-based proteomics is of particular great value due to its ability to detect and quantify huge numbers of proteins and also aid further functional analysis by mapping protein interactions and modifications.[207] This technique might help detect HLH-specific biomarkers such as cytokines or proteins related to complement activation and detail the function of these proteins in the pathophysiology. Additional research is also needed to optimize disease management. Currently, there are no prospective trials published on disease management for the adult population. Treatment recommendations are based on small case series and expert opinions. Trials are currently running on the treatment of HLH in adults.[208,209] These and other multicenter trials are needed to find an optimal treatment strategy.

HLH triggered by immune-stimulatory therapies, such as chimeric antigen receptor T-cell (CART) therapy and immune checkpoint inhibitors including ipilimumab (blocking cytotoxic T-lymphocyte-associated protein 4; CTLA4) and nivolumab/pembrolizumab (blocking programmed cell death protein 1 PD1) are relatively new to the field.[210,211] These immune system-activating therapies are increasingly applied in the context of solid and hematologic malignancies but occasionally provoke acute toxicities like cytokine-release syndrome and even fulminant HLH. In the case of CART therapy, the

infused malignancy-specific T-cell encounters high quantities of its (mostly leukemic) target cells. This results in the expansion of CARTs which produce high levels of cytokines, facilitating the buildup toward the HLH-threshold (Figure 5, introduction).[212] For immune checkpoint inhibitors, an inhibitory receptor (*i.e.,* CTLA4 or PD1) on T-cells is blocked. Thereby, the T-cell response is enhanced by the lack of target cell-induced downregulation. Activated T-cells will produce cytokines that further accelerate the patient toward the HLH-threshold. Further research should confirm whether these therapies give rise to true HLH or rather to an HLH-mimicking clinical condition. In addition, specific diagnostic criteria should be assessed and possibly altered to fit this specific situation. Treatment strategies for these patients are still poorly defined and require further research as performed by, amongst others, the CARTOX working group.[213]

FUTURE PERSPECTIVES ON LCH

This thesis supplements the existing knowledge regarding several aspects of LCH. Future work needs to explore disease pathophysiology, which could ultimately lead to new prognostic insights and therapeutic strategies such as novel targeted therapies. To this end, uncovering the cell of origin will greatly enhance our understanding of LCH and the likelihood that patients will develop a second primary malignancy. Numerous studies indicate the hematopoietic developmental chain as origin of the LCH-cell. Specifically, a somatic mutation (MAPK-pathway mutation in the majority of cases) is thought to initiate the pathophysiology. The developmental stage in which the mutation occurs will then contribute to the disease characteristics such as disease presentation, extent, and prognosis. Future research should confirm this hypothesis and specify the contributing role of the cell of origin to the disease characteristics.

LCH patients are assumed to be at an increased risk of developing an SPM, mostly concerning hematologic malignancies.[162,165,166,214,215] Recently, this is gaining attention from researchers. Focusing on this phenomenon could shed more light on the cell of origin of LCH and its development into LCH. Future research should map clonal relationships between the SPM and LCH or ECD. It is of particular interest to stratify this mapping by LCH disease extent. Our preliminary findings, together with previously reported findings suggest that SPMs occur in both SS- and MS-LCH. However, it remains unknown if the shared clonality of these entities is common. If LCH patients with a clonally-related SPM concern MS-LCH, this will further strengthen the hypothesis that high-risk disseminated disease originates from a cell of origin higher up in the hematopoietic developmental chain (Figure 9, introduction). Further exploring SPM occurrence will likely change the way adult patients are monitored after their LCH has resolved. Close monitoring for

the development of an SPM could be desirable for specific subgroups, if not all, of LCH patients.

Future research should also focus on other contributing factors to disease characteristics. The disease characteristics will most certainly not only be determined by genetics, but numerous other factors such as immune-homeostasis and escape from anti-tumor surveillance. The role of these factors in this multifactorial disease is still largely unknown.

The studies described in this thesis did not specifically focus on LCH treatment. Considering the fast-developing field of targeted therapy, this should be in the scope of future directives. Especially the potential for targeted therapy and when to initiate it should be clarified. Studies reveal the substantial efficacy of the current MAPK-pathway inhibiting agents, but also present downsides. As disease presentation seems only actively suppressed and not cured, chronic treatment is necessary. It is largely unknown what the long-term consequences of these agents are and how the disease activity develops over time. Moreover, it is not clear which clinical features warrant the initiation of effective disease-suppressive therapy (*i.e.,* BRAF inhibition) instead of curative options (*e.g.,* chemotherapy). From melanoma patients, who are treated with BRAF inhibitors, it is known that the disease will eventually progress under dual BRAF/MEK inhibition and that secondary malignancies sporadically occur due to monotherapy with a BRAF inhibitor. It remains unknown if similar effects occur in LCH patients. Thus, long-term studies should clarify these and other aspects of BRAF inhibitors for LCH patients. Until then, targeted therapy should be applied cautiously in LCH patients.

XVII

Final conclusions

FINAL CONCLUSIONS

This thesis contributes to our understanding of clinical, immunological, and genetic features of histiocytic disorders. We show that the development of HLH is a sum of genetic and non-genetic determinants that accumulate toward a physiological threshold that sets off HLH (Figure 5, introduction). Once this threshold is exceeded, an uncontrolled immune reaction will become evident as HLH. To this extent, HLH should not be classified as either FHL or sHLH, but rather a spectrum of diseases. The genetic component that helps to reach the HLH-threshold is variable and always needs an additional trigger to initiate full-blown HLH. We show that the genetic component does not only comprise known germline mutations in FHL genes but potentially include all mutations that result in persistent or excessive (neo)antigen presentation. Furthermore, cytokine production by other disease entities such as LCH and MCD could contribute to prolonged activation of effector immune cells and therefore contribute to reaching the HLH-threshold. Diseases associated with excessive cytokine production should therefore be regarded as a potential risk of triggering HLH. These insights should be taken into account and should change the way we think of HLH. Future research should appreciate the concept of HLH being a spectrum rather than having a sole focus on either genetic or secondary HLH. Studies should focus on the interplay and contributing role of these diverse elements in the HLH pathophysiology. In this regard, the treatment of HLH should not be guided by guidelines adapted to FHL or sHLH, but should acknowledge HLH to be a heterogeneous disorder requiring tailored treatment.

LCH pathophysiology is multifactorial and is not merely dependent on the MAPK-pathway mutation. In this thesis, we show how LCH-cells can escape from immune surveillance by the lack of neoantigen presentation at the cells' surface. In addition, we underscore the heterogeneity of LCH and show differences between children and adults with respect to genetic implications and the presentation of secondary primary malignancies, which is found to have a clonal relationship to the LCH in selected cases. These findings emphasize the lack of knowledge and justify further research into the pathophysiologic and clinical characteristics of LCH in adults. This research should reach beyond studies on the MAPK-pathway because these mutations do not exclusively explain the pathophysiology and clinical features. Additional determinants such as alternative intracellular signaling pathways, additional genetic alterations, and cytokine exposure should be explored to find additional drivers of disease characteristics.

XVIII

Summary

SUMMARY

Histiocytes are a group of hematopoietic cells consisting of dendritic cells, Langerhans cells (LCs), and macrophages. When (a subset of) these cells acquire pathogenic features, the subsequent disease is classified as a histiocytic disorder. This thesis mainly focused on two of the most prevalent histiocytic disorders, HLH and LCH. The specific objective regarding HLH is to understand the contributing factors in setting of an HLH-episode, including the role of genetic alterations in the pathogenesis. Furthermore, the added value of etoposide in HLH patients is studied. For LCH, we aim to understand the effect of lesional composition, anti-tumor (immune) surveillance, and genetic alterations on disease characteristics and outcomes.

HLH is characterized by a pathological and hyperinflammatory state with high mortality rates. Genetic predisposition and environmental triggers such as viruses and malignancies collectively add to reach a certain threshold. Above this threshold, the immune response spins out of control, leading to a severe clinical state called HLH. In contrast, LCH is a neoplastic disease where lesions are formed which consist of pathognomonic LCH-cells in a background of immune cells, and sometimes fibrosis. The pathophysiology is largely driven by functionally aberrant histiocytes expressing somatic, activating MAPK-pathway mutations.

In Chapter VI, we describe a novel *RAB27A* variant in an adult patient with fatal HLH. We show that the RAB27A protein variant is stably expressed and that it impairs the cytotoxic function of CTLs and NK-cells but leaves melanosome distribution (and pigmentation) intact. At the molecular level, this mutant RAB27A protein affects the binding properties of its effector proteins that are necessary for cytotoxic function. Together with the case in Chapter VII, this case emphasizes the concept that HLH is a spectrum of disease rather than a dichotomous classification in secondary HLH or familial HLH.

Next, we present a general mechanism by which primary immunodeficiency patients are susceptible to developing HLH. According to this mechanism, patients with a congenital impaired immune defense have higher and persisting antigen stress. This pushes the patient toward the HLH-threshold and can contribute to the development of HLH. This concept is illustrated by a patient with activated PI3KD syndrome (APDS) which is described in Chapter VII. The leucocytes of APDS patients are driven toward activated and exhausted states, inducing senescence. The subsequently reduced capability of antigen clearance could have resulted in the development of HLH in this patient.

Adequate treatment of adult patients with HLH cannot be presented as a one-protocol-fits-all principle. Although etoposide is an established modality in pediatric HLH, it is not clear what place it should take in the treatment of adult HLH. In Chapter VIII, we present a systematic review and meta-analysis of the literature. We conclude that

there is no substantial scientific evidence demonstrating that etoposide has a beneficial effect on adult HLH patients. However, because several studies do show results that support a beneficial effect, we recommend to consider etoposide for the treatment of refractory or severe cases (*i.e.,* multi-organ failure) of HLH.

In Chapter IX, we report the frequency of LCH driver mutations that are found in pediatric patients. Moreover, we report the association of these mutations with clinical parameters. We confirm the results of published studies by showing a correlation between lesional *BRAF*p.V600E and multisystem disease, risk-organ involvement, skin involvement, and lower age at the time of LCH diagnosis. In addition, we show that lesional *MAP2K1* is associated with single-system bone disease. *BRAF*p.V600E is known as an unfavorable prognostic parameter. We show that *BRAF*p.V600E affects disease extension and thereby prognosis, rather than having an independent effect on the prognosis.

The majority of clinical cohort studies on LCH are performed in children. In Chapter X, we show that the clinicogenomic associations of *BRAF*p.V600E as found in the pediatric population seem not to be present in the adult population. A remarkably high incidence of second primary malignancies (SPMs) is noted in our cohort of LCH patients, particularly in patients with *BRAF*p.V600E positive LCH. A substantial proportion of these SPMs concern hematologic malignancies. This could be an indication that the two diseases share a common precursor or at least pathophysiological elements. In one of these patients, the LCH-cell and subsequent diffuse large B-cell lymphoma presented identical immunoglobulin rearrangements. This is a strong indication that the two diseases are clonally related.

$CD8^+$ T-cell mediated anti-tumor response is a known immunologic tumor-defense mechanism in neoplasias. In Chapter XI, we show that, although the HLA class I molecules are theoretically capable of binding BRAFp.V600E-derived neoantigens, this neopeptide could not be detected at the surface of *BRAF*p.V600E transduced B-cell lines. This likely results in the escape of LCH-cells from the immunologic tumor-defense and explains why the $CD8^+$ T-cell density in LCH-lesions does not impact disease outcome. Extending this observation, LCH patients are not expected to benefit from immune checkpoint inhibitors, which aim to stimulate anti-tumor-directed T-cells.

Erdheim–Chester disease (ECD) is a disease that is closely related to LCH with positive staining for CD68 and CD163 but negative staining for CD1a and CD207. Accordingly, ECD is often regarded as the macrophage counterpart of LCH. Somatostatin receptors (SSTRs) are expressed by a wide range of cells including monocytes and macrophages. In Chapter XII, we confirm that ECD-lesions appear positive upon SSTR scintigraphy. In this patient with refractory ECD, peptide-targeted radionuclide therapy preceded long-term remission of ECD activity. Although these results suggest a therapeutic potential, careful and further exploration is needed to draw definite conclusions.

XIX

Samenvatting

SAMENVATTING

Histiocyten vormen een groep van hematopoëtische cellen die bestaan uit dendritische cellen, Langerhans cellen (LC) en macrofagen. De groep van ziektes die uitgaan van deze histiocyten worden 'histiocytose' genoemd. Dit proefschrift beschrijft onderzoek naar de twee meest voorkomende vormen van histiocytose, te weten HLH en LCH. Er wordt onderzocht wat de bijdragende componenten zijn die tot een HLH-episode leiden, waarbij er speciale aandacht is voor genetische afwijkingen. Daarnaast wordt onderzocht of etoposide een effectieve therapie is voor HLH patiënten. Betreffende LCH ligt de focus van dit proefschrift op de invloed van lesionale samenstelling, de anti-tumor (immuun)response en genetische afwijkingen op de ziekte karakteristieken en uitkomst.

Bij HLH is er sprake van een overactief immuunsysteem. Genetische aanleg en omgevingsfactoren zoals een infectie of maligniteit stapelen zich op totdat een specifieke drempel is bereikt. Boven deze drempel slaat het immuunsysteem op hol, wat tot het klinisch beeld van HLH leidt. LCH is een neoplastische ziekte waarbij laesies gevormd worden in één of meerdere weefsels, die bestaan uit pathognomonische LCH-cellen, omringd door een scala aan immuun-cellen en fibrose. De pathofysiologie wordt grotendeels gedreven door activerende mutaties in de MAPK-cascade.

In Chapter VI wordt een volwassen HLH patiënt beschreven die een variatie laat zien in het *RAB27A* gen met een nog onbekende functionele betekenis. Het corresponderende eiwit laat een stabiele expressie zien en veroorzaakt een verminderde cytotoxische functie van cytotoxische T-cellen en NK-cellen. Opvallend is de behouden melanosoom distributie en hiermee de normale pigmentatie. Op eiwit niveau zorgt dit gemuteerde RAB27A eiwit ervoor dat de binding met andere eiwitten die nodig zijn voor de cytotoxische functie verstoord wordt. In combinatie met de data gepresenteerd in Chapter VII laat deze casus zien dat HLH gezien moet worden als een breed spectrum van aandoeningen. De klassieke indeling in secundaire HLH of familiaire HLH is hiermee achterhaald.

We beschrijven een algemeen mechanisme dat verklaart waarom patiënten met een primaire immuundeficiëntie gemakkelijker vatbaar zijn voor het ontwikkelen van HLH. Patiënten met een verzwakt immuunsysteem ten gevolge van congenitale veranderingen in hun genoom hebben vaak langdurig circulerend antigeen in grotere hoeveelheden vergeleken met gezonde personen. Hierdoor bereiken deze patiënten sneller de HLH-drempel. In Chapter VII wordt dit principe beschreven in een patiënt met APDS. De leukocyten in patiënten met APDS hebben een geactiveerd en uitgeput fenotype. Hierdoor kan antigeen minder goed geklaard worden, een situatie die in deze patiënt lijkt te hebben bijgedragen aan het ontwikkelen van HLH.

Tot nu toe is er geen sprake van een eenduidig en universeel behandelprotocol voor volwassen HLH patiënten. Hoewel de meerwaarde van etoposide in zekere mate

is aangetoond bij kinderen met aangeboren defecten in de cytolytische machinerie, is de effectiviteit hiervan bij volwassenen nog onduidelijk. In Chapter VIII worden de resultaten van een systematisch review met meta-analyse beschreven naar de klinische effectiviteit van etoposide. Hieruit wordt duidelijk dat er geen solide bewijs is dat etoposide een toegevoegde waarde heeft bij de behandeling van volwassen HLH patiënten. Wel benadrukken we dat afzonderlijke artikelen een potentieel voordelig effect laten zien van etoposide. Derhalve adviseren we om etoposide te gebruiken in refractaire patiënten of patiënten met ernstige HLH met multi-orgaanfalen.

In Chapter IX rapporteren we de frequentie van mutaties die geassocieerd zijn met LCH onder kinderen met LCH. In dit hoofdstuk tonen we aan dat zowel de *BRAF*p.V600E mutatie, als de *MAP2K1* mutatie een associatie laten zien met specifieke ziekte lokalisaties. *BRAF*p.V600E kennen we vanuit eerder onderzoek als een mutatie met een slechte prognostische waarde. We laten echter zien dat *BRAF*p.V600E de prognose niet onafhankelijk bepaalt, maar dat deze mutatie geassocieerd is met de uitgebreidheid van de ziekte en op die wijze indirect een effect uitoefent op de prognose.

Het overgrote deel van gerapporteerde klinische studies naar de uitkomst van LCH in relatie tot de aanwezigheid van somatische mutaties zijn gedaan onder kinderen. In Chapter X laten we zien dat de resultaten van deze onderzoeken niet zondermeer vertaald kunnen worden naar de volwassen populatie. Associaties van *BRAF*p.V600E bij kinderen (zoals beschreven in Chapter IX) worden immers niet gevonden bij volwassenen. Opvallend is dat we veel additionele, tweede primaire maligniteiten (TPM) vinden onder onze groep van volwassen LCH patiënten, in het bijzonder in de patiënten met *BRAF*p.V600E positieve LCH laesies. Met name hematologische TPMs komen frequent voor. Dit vormt een indicatie dat de twee ziektes mogelijk een gezamenlijke voorloper cel hebben. Dit wordt bevestigd door een patiënt waar we identieke immuunglobuline gen herschikkingen vinden in zowel zijn LCH als in zijn diffuus grootcellig B-cel lymfoom dat zich enkele jaren later ontwikkelde.

$CD8^+$ T-cellen spelen ook een belangrijke rol bij de immunologische afweer tegen genetisch ontspoorde cellen. In Chapter XI laten we zien dat bepaalde HLA klasse I moleculen neopeptides die afkomstig zijn van het BRAFp.V600E eiwit zouden moeten kunnen binden. In werkelijkheid wordt dit eiwit echter niet gepresenteerd op het celmembraan van verschillende cellijnen die de *BRAF*p.V600E mutatie met zich meedragen. Het gevolg hiervan is dat de LCH-cel lijkt te kunnen ontsnappen aan de immunologische tumor afweer. Hiermee wordt ook verklaard dat de $CD8^+$ T-cel dichtheid niet van invloed lijkt te zijn op de uitkomst van LCH patiënten. Deze resultaten vormen tevens een rationale om terughoudend te zijn met het gebruik van immuun checkpoint remmers in refractaire LCH patiënten, waarin de *BRAF*p.V600E mutatie hoog prevalent is.

ECD is een ziekte die sterk gelieerd is aan LCH met positieve aankleuring voor CD68 en CD163, maar negatieve aankleuring voor CD1a en CD207 op het celmembraan.

Het wordt regelmatig de macrofaag-tegenhanger van LCH genoemd. Somatostatine receptoren (SSTR) worden tot expressie gebracht op een veelheid van lichaamscellen, waaronder monocyten en macrofagen. In Chapter XII tonen wij aan dat ECD laesies aan kunnen kleuren op een SSTR scintigrafie. Bij deze patiënt werd na het geven van radioactief lutetium-gekoppeld somatostatine analoge therapie langdurige remissie van zijn refractaire ECD bereikt. Ondanks deze bemoedigende observatie moet er nog veel opgehelderd worden alvorens deze therapie geschikt is voor de dagelijkse praktijk.

REFERENCES

1. Machowicz R, Janka G, Wiktor-Jedrzejczak W. Similar but not the same: Differential diagnosis of HLH and sepsis. *Crit Rev Oncol Hematol*. 2017;114:1-12.
2. Ramos-Casals M, Brito-Zeron P, Lopez-Guillermo A, Khamashta MA, Bosch X. Adult haemophagocytic syndrome. *Lancet*. 2014;383(9927):1503-1516.
3. Lachmann G, Spies C, Schenk T, Brunkhorst FM, Balzer F, La Rosee P. Hemophagocytic Lymphohistiocytosis: Potentially Underdiagnosed in Intensive Care Units. *Shock*. 2018;50(2):149-155.
4. Chaturvedi V, Marsh RA, Zoref-Lorenz A, et al. T-cell activation profiles distinguish hemophagocytic lymphohistiocytosis and early sepsis. *Blood*. 2021;137(17):2337-2346.
5. De Matteis A, Colucci M, Rossi MN, et al. Expansion of CD4dimCD8+ T cells characterizes macrophage activation syndrome and other secondary HLH. *Blood*. 2022;140(3):262-273.
6. Tang Y, Xu X, Song H, et al. Early diagnostic and prognostic significance of a specific Th1/Th2 cytokine pattern in children with haemophagocytic syndrome. *Br J Haematol*. 2008;143(1):84-91.
7. Han XC, Ye Q, Zhang WY, Tang YM, Xu XJ, Zhang T. Cytokine profiles as novel diagnostic markers of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in children. *J Crit Care*. 2017;39:72-77.
8. Bower M, Veraitch O, Szydlo R, et al. Cytokine changes during rituximab therapy in HIV-associated multicentric Castleman disease. *Blood*. 2009;113(19):4521-4524.
9. Dispenzieri A, Fajgenbaum DC. Overview of Castleman disease. *Blood*. 2020;135(16):1353-1364.
10. Spooner CE, Markowitz NP, Saravolatz LD. The role of tumor necrosis factor in sepsis. *Clin Immunol Immunopathol*. 1992;62(1 Pt 2):S11-17.
11. Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. Into the eye of the cytokine storm. *Microbiol Mol Biol Rev*. 2012;76(1):16-32.
12. Bessler H, Mendel C, Straussberg R, Gurary N, Aloni D, Sirota L. Effects of dexamethasone on IL-1beta, IL-6, and TNF-alpha production by mononuclear cells of newborns and adults. *Biol Neonate*. 1999;75(4):225-233.
13. Brisse E, Wouters CH, Matthys P. Advances in the pathogenesis of primary and secondary haemophagocytic lymphohistiocytosis: differences and similarities. *Br J Haematol*. 2016;174(2):203-217.
14. Dossier A, Meignin V, Fieschi C, Boutboul D, Oksenhendler E, Galicier L. Human herpesvirus 8-related Castleman disease in the absence of HIV infection. *Clin Infect Dis*. 2013;56(6):833-842.
15. Wing A, Xu J, Meng W, et al. Transcriptome and unique cytokine microenvironment of Castleman disease. *Mod Pathol*. 2022;35(4):451-461.
16. Pai RL, Japp AS, Gonzalez M, et al. Type I IFN response associated with mTOR activation in the TAFRO subtype of idiopathic multicentric Castleman disease. *JCI Insight*. 2020;5(9).
17. Polizzotto MN, Uldrick TS, Wang V, et al. Human and viral interleukin-6 and other cytokines in Kaposi sarcoma herpesvirus-associated multicentric Castleman disease. *Blood*. 2013;122(26):4189-4198.
18. Oksenhendler E, Carcelain G, Aoki Y, et al. High levels of human herpesvirus 8 viral load, human interleukin-6, interleukin-10, and C reactive protein correlate with exacerbation of multicentric castleman disease in HIV-infected patients. *Blood*. 2000;96(6):2069-2073.
19. Bedke T, Muscate F, Soukou S, Gagliani N, Huber S. Title: IL-10-producing T cells and their dual functions. *Semin Immunol*. 2019;44:101335.

20. Varma TK, Toliver-Kinsky TE, Lin CY, Koutrouvelis AP, Nichols JE, Sherwood ER. Cellular mechanisms that cause suppressed gamma interferon secretion in endotoxin-tolerant mice. *Infect Immun*. 2001;69(9):5249-5263.
21. Aste-Amezaga M, Ma X, Sartori A, Trinchieri G. Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. *J Immunol*. 1998;160(12):5936-5944.
22. Noble A, Giorgini A, Leggat JA. Cytokine-induced IL-10-secreting CD8 T cells represent a phenotypically distinct suppressor T-cell lineage. *Blood*. 2006;107(11):4475-4483.
23. Emmerich J, Mumm JB, Chan IH, et al. IL-10 directly activates and expands tumor-resident CD8(+) T cells without de novo infiltration from secondary lymphoid organs. *Cancer Res*. 2012;72(14):3570-3581.
24. Fujii S, Shimizu K, Shimizu T, Lotze MT. Interleukin-10 promotes the maintenance of antitumor CD8(+) T-cell effector function in situ. *Blood*. 2001;98(7):2143-2151.
25. Naing A, Infante JR, Papadopoulos KP, et al. PEGylated IL-10 (Pegilodecakin) Induces Systemic Immune Activation, CD8(+) T Cell Invigoration and Polyclonal T Cell Expansion in Cancer Patients. *Cancer Cell*. 2018;34(5):775-791 e773.
26. Tang Y, Xu Q, Luo H, et al. Excessive IL-10 and IL-18 trigger hemophagocytic lymphohistiocytosis-like hyperinflammation and enhanced myelopoiesis. *J Allergy Clin Immunol*. 2022;150(5):1154-1167.
27. Miao Y, Zhu HY, Qiao C, et al. Pathogenic Gene Mutations or Variants Identified by Targeted Gene Sequencing in Adults With Hemophagocytic Lymphohistiocytosis. *Front Immunol*. 2019;10:395.
28. Bryceson YT, Pende D, Maul-Pavicic A, et al. A prospective evaluation of degranulation assays in the rapid diagnosis of familial hemophagocytic syndromes. *Blood*. 2012;119(12):2754-2763.
29. Brisse E, Wouters CH, Matthys P. Hemophagocytic lymphohistiocytosis (HLH): A heterogeneous spectrum of cytokine-driven immune disorders. *Cytokine Growth Factor Rev*. 2015;26(3):263-280.
30. Picard C, Al-Herz W, Bousfiha A, et al. Primary Immunodeficiency Diseases: an Update on the Classification from the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency 2015. *J Clin Immunol*. 2015;35(8):696-726.
31. Brisse E, Imbrechts M, Put K, et al. Mouse Cytomegalovirus Infection in BALB/c Mice Resembles Virus-Associated Secondary Hemophagocytic Lymphohistiocytosis and Shows a Pathogenesis Distinct from Primary Hemophagocytic Lymphohistiocytosis. *J Immunol*. 2016;196(7):3124-3134.
32. Bode SF, Ammann S, Al-Herz W, et al. The syndrome of hemophagocytic lymphohistiocytosis in primary immunodeficiencies: implications for differential diagnosis and pathogenesis. *Haematologica*. 2015;100(7):978-988.
33. Faitelson Y, Grunebaum E. Hemophagocytic lymphohistiocytosis and primary immune deficiency disorders. *Clin Immunol*. 2014;155(1):118-125.
34. Lucas CL, Kuehn HS, Zhao F, et al. Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110delta result in T cell senescence and human immunodeficiency. *Nat Immunol*. 2014;15(1):88-97.
35. Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. *Nat Rev Immunol*. 2003;3(4):317-330.
36. Cannons JL, Preite S, Kapnick SM, Uzel G, Schwartzberg PL. Genetic Defects in Phosphoinositide 3-Kinase delta Influence CD8(+) T Cell Survival, Differentiation, and Function. *Front Immunol*. 2018;9:1758.
37. Bloomfield M, Klocperk A, Zachova R, Milota T, Kanderova V, Sediva A. Natural Course of Activated Phosphoinositide 3-Kinase Delta Syndrome in Childhood and Adolescence. *Front Pediatr*. 2021;9:697706.

38. Cetinkaya PG, Cagdas D, Gumruk F, Tezcan I. Hemophagocytic Lymphohistiocytosis in Patients With Primary Immunodeficiency. *J Pediatr Hematol Oncol*. 2020;42(6):e434-e439.
39. Seidel MG, Kindle G, Gathmann B, et al. The European Society for Immunodeficiencies (ESID) Registry Working Definitions for the Clinical Diagnosis of Inborn Errors of Immunity. *J Allergy Clin Immunol Pract*. 2019;7(6):1763-1770.
40. Menasche G, Pastural E, Feldmann J, et al. Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. *Nat Genet*. 2000;25(2):173-176.
41. Van Gele M, Dynoodt P, Lambert J. Griscelli syndrome: a model system to study vesicular trafficking. *Pigment Cell Melanoma Res*. 2009;22(3):268-282.
42. Cetica V, Hackmann Y, Grieve S, et al. Patients with Griscelli syndrome and normal pigmentation identify RAB27A mutations that selectively disrupt MUNC13-4 binding. *J Allergy Clin Immunol*. 2015;135(5):1310-1318 e1311.
43. Netter P, Chan SK, Banerjee PP, et al. A novel Rab27a mutation binds melanophilin, but not Munc13-4, causing immunodeficiency without albinism. *J Allergy Clin Immunol*. 2016;138(2):599-601 e593.
44. Ohishi Y, Ammann S, Ziaee V, et al. Griscelli Syndrome Type 2 Sine Albinism: Unraveling Differential RAB27A Effector Engagement. *Front Immunol*. 2020;11:612977.
45. Tesi B, Rascon J, Chiang SCC, et al. A RAB27A 5' untranslated region structural variant associated with late-onset hemophagocytic lymphohistiocytosis and normal pigmentation. *J Allergy Clin Immunol*. 2018;142(1):317-321 e318.
46. Kuroda TS, Fukuda M, Ariga H, Mikoshiba K. The Slp homology domain of synaptotagmin-like proteins 1-4 and Slac2 functions as a novel Rab27A binding domain. *J Biol Chem*. 2002;277(11):9212-9218.
47. Izumi T. In vivo Roles of Rab27 and Its Effectors in Exocytosis. *Cell Struct Funct*. 2021;46(2):79-94.
48. Menager MM, Menasche G, Romao M, et al. Secretory cytotoxic granule maturation and exocytosis require the effector protein hMunc13-4. *Nat Immunol*. 2007;8(3):257-267.
49. Shirakawa R, Higashi T, Tabuchi A, et al. Munc13-4 is a GTP-Rab27-binding protein regulating dense core granule secretion in platelets. *J Biol Chem*. 2004;279(11):10730-10737.
50. Wang Y, Wang Z, Zhang J, et al. Genetic features of late onset primary hemophagocytic lymphohistiocytosis in adolescence or adulthood. *PLoS One*. 2014;9(9):e107386.
51. Jin Z, Wang Y, Wang J, et al. Primary hemophagocytic lymphohistiocytosis in adults: the utility of family surveys in a single-center study from China. *Orphanet J Rare Dis*. 2018;13(1):17.
52. Bergsten E, Horne A, Arico M, et al. Confirmed efficacy of etoposide and dexamethasone in HLH treatment: long-term results of the cooperative HLH-2004 study. *Blood*. 2017;130(25):2728-2738.
53. Henter JI, Elinder G, Soder O, Ost A. Incidence in Sweden and clinical features of familial hemophagocytic lymphohistiocytosis. *Acta Paediatr Scand*. 1991;80(4):428-435.
54. Janka GE. Familial hemophagocytic lymphohistiocytosis. *Eur J Pediatr*. 1983;140(3):221-230.
55. La Rosee P, Horne A, Hines M, et al. Recommendations for the management of hemophagocytic lymphohistiocytosis in adults. *Blood*. 2019;133(23):2465-2477.
56. Hines MR, von Bahr Greenwood T, Beutel G, et al. Consensus-Based Guidelines for the Recognition, Diagnosis, and Management of Hemophagocytic Lymphohistiocytosis in Critically Ill Children and Adults. *Crit Care Med*. 2022;50(5):860-872.
57. Machowicz R, Suarez F, Wiktor-Jedrzejczak W, et al. Allogeneic hematopoietic stem cell transplantation for adult HLH: a retrospective study by the chronic malignancies and inborn errors working parties of EBMT. *Bone Marrow Transplant*. 2022;57(5):817-823.

58. Arnaout K, Patel N, Jain M, El-Amm J, Amro F, Tabbara IA. Complications of allogeneic hematopoietic stem cell transplantation. *Cancer Invest*. 2014;32(7):349-362.
59. Akhmedov M. Infectious complications in allogeneic hematopoietic cell transplant recipients: Review of transplant-related risk factors and current state of prophylaxis. *Clin Transplant*. 2021;35(2):e14172.
60. Mohty B, Mohty M. Long-term complications and side effects after allogeneic hematopoietic stem cell transplantation: an update. *Blood Cancer J*. 2011;1(4):e16.
61. Yildiz H, Bailly S, Van Den Neste E, Yombi JC. Clinical Management of Relapsed/Refractory Hemophagocytic Lymphohistiocytosis in Adult Patients: A Review of Current Strategies and Emerging Therapies. *Ther Clin Risk Manag*. 2021;17:293-304.
62. Park HS, Kim DY, Lee JH, et al. Clinical features of adult patients with secondary hemophagocytic lymphohistiocytosis from causes other than lymphoma: an analysis of treatment outcome and prognostic factors. *Ann Hematol*. 2012;91(6):897-904.
63. Sepulveda FE, Garrigue A, Maschalidi S, et al. Polygenic mutations in the cytotoxicity pathway increase susceptibility to develop HLH immunopathology in mice. *Blood*. 2016;127(17):2113-2121.
64. Staal FJT, Aiuti A, Cavazzana M. Autologous Stem-Cell-Based Gene Therapy for Inherited Disorders: State of the Art and Perspectives. *Front Pediatr*. 2019;7:443.
65. Henter JI, Arico M, Egeler RM, et al. HLH-94: a treatment protocol for hemophagocytic lymphohistiocytosis. HLH study Group of the Histiocyte Society. *Med Pediatr Oncol*. 1997;28(5):342-347.
66. Song Y, Wang Y, Wang Z. Requirement for etoposide in the initial treatment of Epstein-Barr virus-associated haemophagocytic lymphohistiocytosis. *Br J Haematol*. 2019;186(5):717-723.
67. Arca M, Fardet L, Galicier L, et al. Prognostic factors of early death in a cohort of 162 adult haemophagocytic syndrome: impact of triggering disease and early treatment with etoposide. *Br J Haematol*. 2015;168(1):63-68.
68. Bigenwald C, Fardet L, Coppo P, et al. A comprehensive analysis of Lymphoma-associated haemophagocytic syndrome in a large French multicentre cohort detects some clues to improve prognosis. *Br J Haematol*. 2018;183(1):68-75.
69. Bubik RJ, Barth DM, Hook C, et al. Clinical outcomes of adults with hemophagocytic lymphohistiocytosis treated with the HLH-04 protocol: a retrospective analysis. *Leuk Lymphoma*. 2020;61(7):1592-1600.
70. Li B, Guo J, Li T, et al. Clinical Characteristics of Hemophagocytic Lymphohistiocytosis Associated with Non-Hodgkin B-Cell Lymphoma: A Multicenter Retrospective Study. *Clin Lymphoma Myeloma Leuk*. 2021;21(2):e198-e205.
71. Osugi Y, Hara J, Tagawa S, et al. Cytokine production regulating Th1 and Th2 cytokines in hemophagocytic lymphohistiocytosis. *Blood*. 1997;89(11):4100-4103.
72. Henter JI, Elinder G, Soder O, Hansson M, Andersson B, Andersson U. Hypercytokinemia in familial hemophagocytic lymphohistiocytosis. *Blood*. 1991;78(11):2918-2922.
73. Kim JY, Kim M, Park JK, Lee EB, Park JW, Hong J. Limited efficacy of tocilizumab in adult patients with secondary hemophagocytic lymphohistiocytosis: a retrospective cohort study. *Orphanet J Rare Dis*. 2022;17(1):363.
74. Locatelli F, Jordan MB, Allen C, et al. Emapalumab in Children with Primary Hemophagocytic Lymphohistiocytosis. *N Engl J Med*. 2020;382(19):1811-1822.
75. Bami S, Vagrecha A, Soberman D, et al. The use of anakinra in the treatment of secondary hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer*. 2020;67(11):e28581.

76. Baverez C, Grall M, Gerfaud-Valentin M, et al. Anakinra for the Treatment of Hemophagocytic Lymphohistiocytosis: 21 Cases. *J Clin Med*. 2022;11(19).
77. Naymagon L. Anakinra for the treatment of adult secondary HLH: a retrospective experience. *Int J Hematol*. 2022.
78. Albeituni S, Verbist KC, Tedrick PE, et al. Mechanisms of action of ruxolitinib in murine models of hemophagocytic lymphohistiocytosis. *Blood*. 2019;134(2):147-159.
79. Keenan C, Nichols KE, Albeituni S. Use of the JAK Inhibitor Ruxolitinib in the Treatment of Hemophagocytic Lymphohistiocytosis. *Front Immunol*. 2021;12:614704.
80. Zhang Q, Zhao YZ, Ma HH, et al. A study of ruxolitinib response-based stratified treatment for pediatric hemophagocytic lymphohistiocytosis. *Blood*. 2022;139(24):3493-3504.
81. Ahmed A, Merrill SA, Alsawah F, et al. Ruxolitinib in adult patients with secondary haemophagocytic lymphohistiocytosis: an open-label, single-centre, pilot trial. *Lancet Haematol*. 2019;6(12):e630-e637.
82. Corporation G. Vol. 2022; 2014.
83. Marsh RA, Allen CE, McClain KL, et al. Salvage therapy of refractory hemophagocytic lymphohistiocytosis with alemtuzumab. *Pediatr Blood Cancer*. 2013;60(1):101-109.
84. F. R. Achini-Gutzwiller CLV, J. M. van Montfrans, D. Moshous, A. M. Jansen-Hoogendijk, M. M. van Ostaijen-ten Dam, M. W. Schilham, D. J. A. Moes, A. C. Lankester, E. P. Buddingh. The 48th Annual Meeting of the European Society for Blood and Marrow Transplantation: Physicians - Oral Sessions (O009-O155). *Bone Marrow Transplantation*. 2022;57(1):16-99.
85. Johnson TS, Terrell CE, Millen SH, Katz JD, Hildeman DA, Jordan MB. Etoposide selectively ablates activated T cells to control the immunoregulatory disorder hemophagocytic lymphohistiocytosis. *J Immunol*. 2014;192(1):84-91.
86. Alidjinou EK, Dewilde A, Terriou L, Lazrek M, Engelmann I, Hober D. Persistent viral DNA detection in blood after primary herpes simplex 1 infection revealed by hepatitis with hemophagocytic syndrome. *J Clin Virol*. 2015;69:101-103.
87. Cusini A, Gunthard HF, Stussi G, et al. Hemophagocytic syndrome caused by primary herpes simplex virus 1 infection: report of a first case. *Infection*. 2010;38(5):423-426.
88. Norvell JP, Blei AT, Jovanovic BD, Levitsky J. Herpes simplex virus hepatitis: an analysis of the published literature and institutional cases. *Liver Transpl*. 2007;13(10):1428-1434.
89. Lasserre M, Huguet C, Terno O. Acute severe herpes simplex hepatitis with virus-associated hemophagocytic syndrome in an immunocompetent adult. *J Hepatol*. 1993;18(2):256-257.
90. Schiodt FV, Davern TJ, Shakil AO, McGuire B, Samuel G, Lee WM. Viral hepatitis-related acute liver failure. *Am J Gastroenterol*. 2003;98(2):448-453.
91. Beersma MF, Verjans GM, Metselaar HJ, Osterhaus AD, Berrington WR, van Doornum GJ. Quantification of viral DNA and liver enzymes in plasma improves early diagnosis and management of herpes simplex virus hepatitis. *J Viral Hepat*. 2011;18(4):e160-166.
92. van Laar JA, Buysse CM, Vossen AC, et al. Epstein-Barr viral load assessment in immunocompetent patients with fulminant infectious mononucleosis. *Arch Intern Med*. 2002;162(7):837-839.
93. Liu P, Pan X, Chen C, et al. Nivolumab treatment of relapsed/refractory Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in adults. *Blood*. 2020;135(11):826-833.
94. Jouanguy E, Beziat V, Mogensen TH, Casanova JL, Tangye SG, Zhang SY. Human inborn errors of immunity to herpes viruses. *Curr Opin Immunol*. 2020;62:106-122.
95. Karczewski KJ, Francioli LC, Tiao G, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature*. 2020;581(7809):434-443.

96. Rentzsch P, Schubach M, Shendure J, Kircher M. CADD-Splice-improving genome-wide variant effect prediction using deep learning-derived splice scores. *Genome Med*. 2021;13(1):31.
97. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet*. 2014;46(3):310-315.
98. Kamada F, Aoki Y, Narisawa A, et al. A genome-wide association study identifies RNF213 as the first Moyamoya disease gene. *J Hum Genet*. 2011;56(1):34-40.
99. Bang OY, Chung JW, Kim DH, et al. Moyamoya Disease and Spectrums of RNF213 Vasculopathy. *Transl Stroke Res*. 2020;11(4):580-589.
100. Scott RM, Smith ER. Moyamoya disease and moyamoya syndrome. *N Engl J Med*. 2009;360(12):1226-1237.
101. Lee S, Rivkin MJ, Kirton A, deVeber G, Elbers J, International Pediatric Stroke S. Moyamoya Disease in Children: Results From the International Pediatric Stroke Study. *J Child Neurol*. 2017;32(11):924-929.
102. Thery F, Martina L, Asselman C, et al. Ring finger protein 213 assembles into a sensor for ISGylated proteins with antimicrobial activity. *Nat Commun*. 2021;12(1):5772.
103. Laman JD, Leenen PJ, Annels NE, Hogendoorn PC, Egeler RM. Langerhans-cell histiocytosis 'insight into DC biology'. *Trends Immunol*. 2003;24(4):190-196.
104. Egeler RM, Favara BE, van Meurs M, Laman JD, Claassen E. Differential In situ cytokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expression of cytokines relevant to disease and treatment. *Blood*. 1999;94(12):4195-4201.
105. Badalian-Very G, Vergilio JA, Degar BA, et al. Recurrent BRAF mutations in Langerhans cell histiocytosis. *Blood*. 2010;116(11):1919-1923.
106. Heritier S, Emile JF, Barkaoui MA, et al. BRAF Mutation Correlates With High-Risk Langerhans Cell Histiocytosis and Increased Resistance to First-Line Therapy. *J Clin Oncol*. 2016;34(25):3023-3030.
107. Berres ML, Lim KP, Peters T, et al. BRAF-V600E expression in precursor versus differentiated dendritic cells defines clinically distinct LCH risk groups. *J Exp Med*. 2014;211(4):669-683.
108. Hayase T, Saito S, Shioda Y, et al. Analysis of the BRAF and MAP2K1 mutations in patients with Langerhans cell histiocytosis in Japan. *Int J Hematol*. 2020;112(4):560-567.
109. Feng S, Han L, Yue M, et al. Frequency detection of BRAF V600E mutation in a cohort of pediatric langerhans cell histiocytosis patients by next-generation sequencing. *Orphanet J Rare Dis*. 2021;16(1):272.
110. Brown NA, Furtado LV, Betz BL, et al. High prevalence of somatic MAP2K1 mutations in BRAF V600E-negative Langerhans cell histiocytosis. *Blood*. 2014;124(10):1655-1658.
111. Chakraborty R, Hampton OA, Shen X, et al. Mutually exclusive recurrent somatic mutations in MAP2K1 and BRAF support a central role for ERK activation in LCH pathogenesis. *Blood*. 2014;124(19):3007-3015.
112. Diamond EL, Durham BH, Haroche J, et al. Diverse and Targetable Kinase Alterations Drive Histiocytic Neoplasms. *Cancer Discov*. 2016;6(2):154-165.
113. Nelson DS, van Halteren A, Quispel WT, et al. MAP2K1 and MAP3K1 mutations in Langerhans cell histiocytosis. *Genes Chromosomes Cancer*. 2015;54(6):361-368.
114. Chakraborty R, Burke TM, Hampton OA, et al. Alternative genetic mechanisms of BRAF activation in Langerhans cell histiocytosis. *Blood*. 2016;128(21):2533-2537.
115. Heritier S, Helias-Rodzewicz Z, Chakraborty R, et al. New somatic BRAF splicing mutation in Langerhans cell histiocytosis. *Mol Cancer*. 2017;16(1):115.
116. Nelson DS, Quispel W, Badalian-Very G, et al. Somatic activating ARAF mutations in Langerhans cell histiocytosis. *Blood*. 2014;123(20):3152-3155.

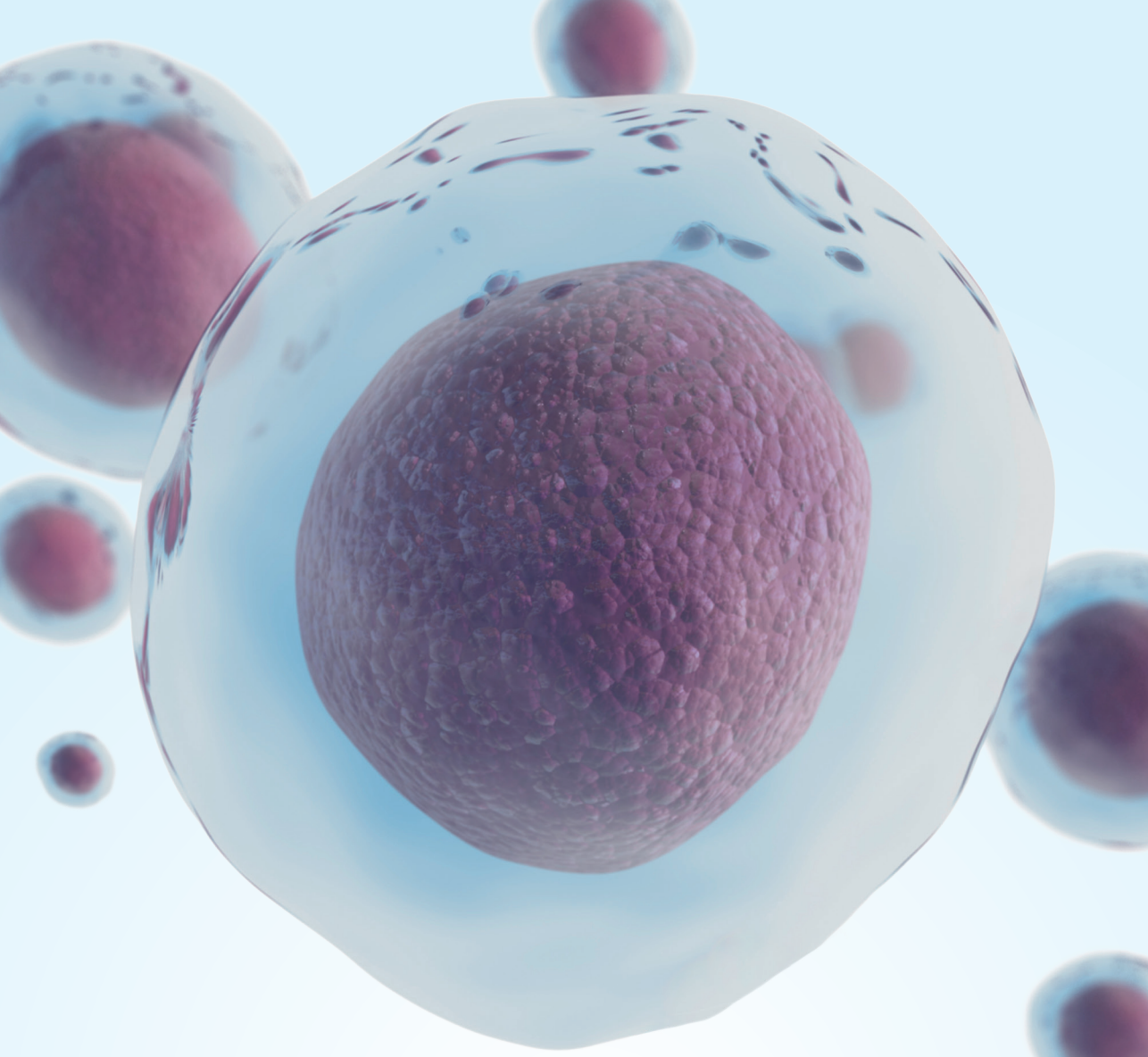
117. Heritier S, Emile JF, Helias-Rodzewicz Z, Donadieu J. Progress towards molecular-based management of childhood Langerhans cell histiocytosis. *Arch Pediatr*. 2019;26(5):301-307.
118. Rodriguez-Galindo C, Allen CE. Langerhans cell histiocytosis. *Blood*. 2020;135(16):1319-1331.
119. Bhatia P, Singh M, Sharma M, et al. BRAF V600E mutation in childhood Langerhans cell histiocytosis correlates with multisystem disease and poor survival. *Blood Cells Mol Dis*. 2020;82:102356.
120. Ozer E, Sevinc A, Ince D, Yuzuguldu R, Olgun N. BRAF V600E Mutation: A Significant Biomarker for Prediction of Disease Relapse in Pediatric Langerhans Cell Histiocytosis. *Pediatr Dev Pathol*. 2019;22(5):449-455.
121. Phan TDA, Phung BG, Duong TT, et al. A study of pathological characteristics and BRAF V600E status in Langerhans cell histiocytosis of Vietnamese children. *J Pathol Transl Med*. 2021;55(2):112-117.
122. Gaurav G, Aldo AA-M, Marie H, et al. Phenotypes and prognostic factors in adults with Langerhans cell histiocytosis. *Journal of Clinical Oncology*. 2021;39(15_suppl):7049-7049.
123. Cao XX, Duan MH, Zhao AL, et al. Treatment outcomes and prognostic factors of patients with adult Langerhans cell histiocytosis. *Am J Hematol*. 2022;97(2):203-208.
124. Jouenne F, Chevret S, Bugnet E, et al. Genetic landscape of adult Langerhans cell histiocytosis with lung involvement. *Eur Respir J*. 2020;55(2).
125. Heritier S, Barkaoui MA, Miron J, et al. Incidence and risk factors for clinical neurodegenerative Langerhans cell histiocytosis: a longitudinal cohort study. *Br J Haematol*. 2018;183(4):608-617.
126. McClain KL, Picarsic J, Chakraborty R, et al. CNS Langerhans cell histiocytosis: Common hematopoietic origin for LCH-associated neurodegeneration and mass lesions. *Cancer*. 2018;124(12):2607-2620.
127. Lahey ME. Prognostic factors in histiocytosis X. *Am J Pediatr Hematol Oncol*. 1981;3(1):57-60.
128. Ladisch S, Gadner H. Treatment of Langerhans cell histiocytosis--evolution and current approaches. *Br J Cancer Suppl*. 1994;23:S41-46.
129. Arico M, Girschikofsky M, Genereau T, et al. Langerhans cell histiocytosis in adults. Report from the International Registry of the Histiocyte Society. *Eur J Cancer*. 2003;39(16):2341-2348.
130. Rigaud C, Barkaoui MA, Thomas C, et al. Langerhans cell histiocytosis: therapeutic strategy and outcome in a 30-year nationwide cohort of 1478 patients under 18 years of age. *Br J Haematol*. 2016;174(6):887-898.
131. Gadner H, Minkov M, Grois N, et al. Therapy prolongation improves outcome in multisystem Langerhans cell histiocytosis. *Blood*. 2013;121(25):5006-5014.
132. Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science*. 1999;286(5443):1358-1362.
133. Zhang W, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res*. 2002;12(1):9-18.
134. Kemps PG, Zondag TC, Arnardottir HB, et al. Clinicogenomic associations in childhood Langerhans cell histiocytosis: an international cohort study. *Blood Adv*. 2022.
135. Xiao Y, van Halteren AGS, Lei X, et al. Bone marrow-derived myeloid progenitors as driver mutation carriers in high- and low-risk Langerhans cell histiocytosis. *Blood*. 2020;136(19):2188-2199.
136. Riller Q, Rieux-Laucat F. RASopathies: From germline mutations to somatic and multigenic diseases. *Biomed J*. 2021;44(4):422-432.
137. Kratz CP, Rapisuwon S, Reed H, Hasle H, Rosenberg PS. Cancer in Noonan, Costello, cardiofaciocutaneous and LEOPARD syndromes. *Am J Med Genet C Semin Med Genet*. 2011;157C(2):83-89.

138. Hutter C, Kauer M, Simonitsch-Klupp I, et al. Notch is active in Langerhans cell histiocytosis and confers pathognomonic features on dendritic cells. *Blood*. 2012;120(26):5199-5208.
139. Alexandrescu S, Tatevian N, Czerniak BA, Covinsky MH, Burns NK, Brown RE. Morphoproteomics provides support for TGF-beta pathway signaling in the osteoclastogenesis and immune dysregulation of osteolytic Langerhans cell histiocytosis. *Int J Clin Exp Pathol*. 2012;5(6):503-511.
140. Mehrazma M, Mahjoob F, Rafsanjani KA, Jafari D, Farahani SJ, Lahouti HA. VEGF and p53 biomarkers in pediatric patients with Langerhans cell histiocytosis. *J Pediatr Hematol Oncol*. 2013;35(7):e292-295.
141. Gonzalez H, Hagerling C, Werb Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes Dev*. 2018;32(19-20):1267-1284.
142. Foss FM. Immunologic mechanisms of antitumor activity. *Semin Oncol*. 2002;29(3 Suppl 7):5-11.
143. Jakobisiak M, Lasek W, Golab J. Natural mechanisms protecting against cancer. *Immunol Lett*. 2003;90(2-3):103-122.
144. Berraondo P, Minute L, Ajona D, Corrales L, Melero I, Pio R. Innate immune mediators in cancer: between defense and resistance. *Immunol Rev*. 2016;274(1):290-306.
145. Candeias SM, Gaipl US. The Immune System in Cancer Prevention, Development and Therapy. *Anticancer Agents Med Chem*. 2016;16(1):101-107.
146. Hanson HL, Donermeyer DL, Ikeda H, et al. Eradication of established tumors by CD8+ T cell adoptive immunotherapy. *Immunity*. 2000;13(2):265-276.
147. Nielsen M, Lundegaard C, Lund O, Kesmir C. The role of the proteasome in generating cytotoxic T-cell epitopes: insights obtained from improved predictions of proteasomal cleavage. *Immunogenetics*. 2005;57(1-2):33-41.
148. Rizzo FM, Cives M, Simone V, Silvestris F. New insights into the molecular pathogenesis of langerhans cell histiocytosis. *Oncologist*. 2014;19(2):151-163.
149. Somasundaram R, Swoboda R, Caputo L, et al. Human leukocyte antigen-A2-restricted CTL responses to mutated BRAF peptides in melanoma patients. *Cancer Res*. 2006;66(6):3287-3293.
150. Ilieva KM, Correa I, Josephs DH, et al. Effects of BRAF mutations and BRAF inhibition on immune responses to melanoma. *Mol Cancer Ther*. 2014;13(12):2769-2783.
151. Fridman WH, Zitvogel L, Sautes-Fridman C, Kroemer G. The immune contexture in cancer prognosis and treatment. *Nat Rev Clin Oncol*. 2017;14(12):717-734.
152. Mitchell J, Kelly J, Kvedaraite E, et al. Foxp3(+) Tregs from Langerhans cell histiocytosis lesions co-express CD56 and have a definitively regulatory capacity. *Clin Immunol*. 2020;215:108418.
153. Senechal B, Elain G, Jeziorski E, et al. Expansion of regulatory T cells in patients with Langerhans cell histiocytosis. *PLoS Med*. 2007;4(8):e253.
154. Egeler RM, van Halteren AG, Hogendoorn PC, Laman JD, Leenen PJ. Langerhans cell histiocytosis: fascinating dynamics of the dendritic cell-macrophage lineage. *Immunol Rev*. 2010;234(1):213-232.
155. Yarchoan M, Johnson BA, 3rd, Lutz ER, Laheru DA, Jaffee EM. Targeting neoantigens to augment antitumour immunity. *Nat Rev Cancer*. 2017;17(4):209-222.
156. Gatalica Z, Bilalovic N, Palazzo JP, et al. Disseminated histiocytoses biomarkers beyond BRAFV600E: frequent expression of PD-L1. *Oncotarget*. 2015;6(23):19819-19825.
157. Zeng K, Wang Z, Ohshima K, et al. BRAF V600E mutation correlates with suppressive tumor immune microenvironment and reduced disease-free survival in Langerhans cell histiocytosis. *Oncoimmunology*. 2016;5(7):e1185582.
158. Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. *Nature*. 2013;500(7463):415-421.

159. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Science*. 2015;348(6230):69-74.
160. Marcus A, Gowen BG, Thompson TW, et al. Recognition of tumors by the innate immune system and natural killer cells. *Adv Immunol*. 2014;122:91-128.
161. Hogstad B, Berres ML, Chakraborty R, et al. RAF/MEK/extracellular signal-related kinase pathway suppresses dendritic cell migration and traps dendritic cells in Langerhans cell histiocytosis lesions. *J Exp Med*. 2018;215(1):319-336.
162. Ma J, Laird JH, Chau KW, Chelius MR, Lok BH, Yahalom J. Langerhans cell histiocytosis in adults is associated with a high prevalence of hematologic and solid malignancies. *Cancer Med*. 2019;8(1):58-66.
163. Haupt R, Nanduri V, Calevo MG, et al. Permanent consequences in Langerhans cell histiocytosis patients: a pilot study from the Histiocyte Society-Late Effects Study Group. *Pediatr Blood Cancer*. 2004;42(5):438-444.
164. Mattiuzzi C, Lippi G. Current Cancer Epidemiology. *J Epidemiol Glob Health*. 2019;9(4):217-222.
165. Bagnasco F, Zimmermann SY, Egeler RM, et al. Langerhans cell histiocytosis and associated malignancies: A retrospective analysis of 270 patients. *Eur J Cancer*. 2022;172:138-145.
166. Egeler RM, Neglia JP, Arico M, et al. The relation of Langerhans cell histiocytosis to acute leukemia, lymphomas, and other solid tumors. The LCH-Malignancy Study Group of the Histiocyte Society. *Hematol Oncol Clin North Am*. 1998;12(2):369-378.
167. Feldman AL, Berthold F, Arceci RJ, et al. Clonal relationship between precursor T-lymphoblastic leukaemia/lymphoma and Langerhans-cell histiocytosis. *Lancet Oncol*. 2005;6(6):435-437.
168. Yokokawa Y, Taki T, Chinen Y, et al. Unique clonal relationship between T-cell acute lymphoblastic leukemia and subsequent Langerhans cell histiocytosis with TCR rearrangement and NOTCH1 mutation. *Genes Chromosomes Cancer*. 2015;54(7):409-417.
169. Bonometti A, Bagnoli F, Fanoni D, et al. JAK2-mutated Langerhans cell histiocytosis associated with primary myelofibrosis treated with ruxolitinib. *Hum Pathol*. 2018;73:171-175.
170. Arruga F, Vaisitti T, Deaglio S. The NOTCH Pathway and Its Mutations in Mature B Cell Malignancies. *Front Oncol*. 2018;8:550.
171. Reubi JC, Laissue J, Krenning E, Lamberts SW. Somatostatin receptors in human cancer: incidence, characteristics, functional correlates and clinical implications. *J Steroid Biochem Mol Biol*. 1992;43(1-3):27-35.
172. Reubi JC, Krenning E, Lamberts SW, Kvols L. Somatostatin receptors in malignant tissues. *J Steroid Biochem Mol Biol*. 1990;37(6):1073-1077.
173. Dalm VA, van Hagen PM, van Koetsveld PM, et al. Expression of somatostatin, cortistatin, and somatostatin receptors in human monocytes, macrophages, and dendritic cells. *Am J Physiol Endocrinol Metab*. 2003;285(2):E344-353.
174. Emile JF, Abla O, Fraitag S, et al. Revised classification of histiocytoses and neoplasms of the macrophage-dendritic cell lineages. *Blood*. 2016;127(22):2672-2681.
175. Aubart FC, Poupel L, Saint-Charles F, et al. Profound systemic alteration of the immune phenotype and an immunoglobulin switch in Erdheim-Chester disease in 78 patients from a single center. *Haematologica*. 2022;107(6):1347-1357.
176. Milne P, Bigley V, Bacon CM, et al. Hematopoietic origin of Langerhans cell histiocytosis and Erdheim-Chester disease in adults. *Blood*. 2017;130(2):167-175.
177. Durham BH, Roos-Weil D, Baillou C, et al. Functional evidence for derivation of systemic histiocytic neoplasms from hematopoietic stem/progenitor cells. *Blood*. 2017;130(2):176-180.
178. Haroche J, Cohen-Aubart F, Amoura Z. Erdheim-Chester disease. *Blood*. 2020;135(16):1311-1318.

179. Armani C, Catalani E, Balbarini A, Bagnoli P, Cervia D. Expression, pharmacology, and functional role of somatostatin receptor subtypes 1 and 2 in human macrophages. *J Leukoc Biol*. 2007;81(3):845-855.
180. Ozturk E, Gunalp B, Ozguven M, et al. The visualization of granulomatous disease with somatostatin receptor scintigraphy. *Clin Nucl Med*. 1994;19(2):129-132.
181. Ferone D, Pivonello R, Van Hagen PM, et al. Quantitative and functional expression of somatostatin receptor subtypes in human thymocytes. *Am J Physiol Endocrinol Metab*. 2002;283(5):E1056-1066.
182. Krantic S. Peptides as regulators of the immune system: emphasis on somatostatin. *Peptides*. 2000;21(12):1941-1964.
183. Bhathena SJ, Louie J, Schechter GP, Redman RS, Wahl L, Recant L. Identification of human mononuclear leukocytes bearing receptors for somatostatin and glucagon. *Diabetes*. 1981;30(2):127-131.
184. Nakamura H, Koike T, Hiruma K, Sato T, Tomioka H, Yoshida S. Identification of lymphoid cell lines bearing receptors for somatostatin. *Immunology*. 1987;62(4):655-658.
185. Juntikka T, Vaittinen S, Vahlberg T, Jyrkkio S, Minn H. Somatostatin Receptors and Chemokine Receptor CXCR4 in Lymphomas: A Histopathological Review of Six Lymphoma Subtypes. *Front Oncol*. 2021;11:710900.
186. Karlsson M, Zhang C, Mear L, et al. A single-cell type transcriptomics map of human tissues. *Sci Adv*. 2021;7(31).
187. Abdulrezzak U, Doğan S, Kula M, KocaaĞAoĞLu E, Köse Kurt Y, Tutuş A. Heterogeneous Manifestations of Four Cases With Erdheim-Chester Disease By Nuclear Medicine Imaging Modalities. *Turkiye Klinikleri Journal of Medical Sciences*. 2016;36.
188. Balon HR, Goldsmith SJ, Siegel BA, et al. Procedure guideline for somatostatin receptor scintigraphy with (111)In-pentetreotide. *J Nucl Med*. 2001;42(7):1134-1138.
189. Buscail L, Esteve JP, Saint-Laurent N, et al. Inhibition of cell proliferation by the somatostatin analogue RC-160 is mediated by somatostatin receptor subtypes SSTR2 and SSTR5 through different mechanisms. *Proc Natl Acad Sci U S A*. 1995;92(5):1580-1584.
190. Asnacios A, Courbon F, Rochaix P, et al. Indium-111-pentetreotide scintigraphy and somatostatin receptor subtype 2 expression: new prognostic factors for malignant well-differentiated endocrine tumors. *J Clin Oncol*. 2008;26(6):963-970.
191. Karlsson C, Karlsson MG. Effects of long-term storage on the detection of proteins, DNA, and mRNA in tissue microarray slides. *J Histochem Cytochem*. 2011;59(12):1113-1121.
192. Goyal G, Heaney ML, Collin M, et al. Erdheim-Chester disease: consensus recommendations for evaluation, diagnosis, and treatment in the molecular era. *Blood*. 2020;135(22):1929-1945.
193. Grois N, Potschger U, Prosch H, et al. Risk factors for diabetes insipidus in langerhans cell histiocytosis. *Pediatr Blood Cancer*. 2006;46(2):228-233.
194. Haroche J, Cohen-Aubart F, Emile JF, et al. Dramatic efficacy of vemurafenib in both multisystemic and refractory Erdheim-Chester disease and Langerhans cell histiocytosis harboring the BRAF V600E mutation. *Blood*. 2013;121(9):1495-1500.
195. Cohen Aubart F, Emile JF, Carrat F, et al. Targeted therapies in 54 patients with Erdheim-Chester disease, including follow-up after interruption (the LOVE study). *Blood*. 2017;130(11):1377-1380.
196. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. 2011;364(26):2507-2516.
197. Histiocytosis syndromes in children. Writing Group of the Histiocyte Society. *Lancet*. 1987;1(8526):208-209.

198. de Saint Basile G, Menasche G, Fischer A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nat Rev Immunol*. 2010;10(8):568-579.
199. Hervier B, Haroche J, Arnaud L, et al. Association of both Langerhans cell histiocytosis and Erdheim-Chester disease linked to the BRAFV600E mutation. *Blood*. 2014;124(7):1119-1126.
200. Diamond EL, Dagna L, Hyman DM, et al. Consensus guidelines for the diagnosis and clinical management of Erdheim-Chester disease. *Blood*. 2014;124(4):483-492.
201. Eder SK, Schwentner R, Ben Soussia P, et al. Vemurafenib acts as a molecular on-off switch governing systemic inflammation in Langerhans cell histiocytosis. *Blood Adv*. 2022;6(3):970-975.
202. Chellapandian D, Hines MR, Zhang R, et al. A multicenter study of patients with multisystem Langerhans cell histiocytosis who develop secondary hemophagocytic lymphohistiocytosis. *Cancer*. 2019;125(6):963-971.
203. Favara BE, Jaffe R, Egeler RM. Macrophage activation and hemophagocytic syndrome in langerhans cell histiocytosis: report of 30 cases. *Pediatr Dev Pathol*. 2002;5(2):130-140.
204. Hinson ARP, Patel N, Kaplan J. Hemophagocytic Lymphohistiocytosis in Langerhans Cell Histiocytosis: A Case Report and Review of the Literature. *J Pediatr Hematol Oncol*. 2021;43(1):24-27.
205. Galluzzo ML, Braier J, Rosenzweig SD, Garcia de Davila MT, Rosso D. Bone marrow findings at diagnosis in patients with multisystem langerhans cell histiocytosis. *Pediatr Dev Pathol*. 2010;13(2):101-106.
206. Morimoto A, Oh Y, Nakamura S, et al. Inflammatory serum cytokines and chemokines increase associated with the disease extent in pediatric Langerhans cell histiocytosis. *Cytokine*. 2017;97:73-79.
207. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature*. 2003;422(6928):198-207.
208. ClinicalTrails.gov. Etoposide in the First-line Treatment of Adult EBV-HLH. Vol. 2022; 2022.
209. ClinicalTrails.gov. Evaluate Efficacy, Safety and Tolerability, PK and PD of Emapalumab in Children and Adults With MAS in Still's or SLE (EMERALD). Vol. 2022; 2022.
210. Rajapakse P, Andanamala H. Hemophagocytic Lymphohistiocytosis Secondary to Immune Checkpoint Inhibitor Therapy. *World J Oncol*. 2022;13(2):49-52.
211. Lichtenstein DA, Schischlik F, Shao L, et al. Characterization of HLH-like manifestations as a CRS variant in patients receiving CD22 CAR T cells. *Blood*. 2021;138(24):2469-2484.
212. Sandler RD, Tattersall RS, Schoemans H, et al. Diagnosis and Management of Secondary HLH/MAS Following HSCT and CAR-T Cell Therapy in Adults; A Review of the Literature and a Survey of Practice Within EBMT Centres on Behalf of the Autoimmune Diseases Working Party (ADWP) and Transplant Complications Working Party (TCWP). *Front Immunol*. 2020;11:524.
213. Neelapu SS, Tummala S, Kebriaei P, et al. Chimeric antigen receptor T-cell therapy - assessment and management of toxicities. *Nat Rev Clin Oncol*. 2018;15(1):47-62.
214. Egeler RM, Neglia JP, Puccetti DM, Brennan CA, Nesbit ME. Association of Langerhans cell histiocytosis with malignant neoplasms. *Cancer*. 1993;71(3):865-873.
215. Egeler RM, Neglia JP, Arico M, Favara BE, Heitger A, Nesbit ME. Acute leukemia in association with Langerhans cell histiocytosis. *Med Pediatr Oncol*. 1994;23(2):81-85.



APPENDIX

List of abbreviations
List of publications
About the author
PhD portfolio
Dankwoord

LIST OF ABBREVIATIONS

AKT	Protein kinase B
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
APDS	Activated PI3K delta syndrome
BCL2L1	B-cell lymphoma-2 like protein 1
BiTE	Bispecific T-cell engager
CAR	Chimeric antigen receptor
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CD	Castleman's disease
CHOP	Cyclophosphamide, hydroxyduanorubicine, vincristine and prednisone
CMV	Cytomegalovirus
CNS	Central nervous system
CRP	C-reactive protein
CsA	Cyclosporine A
CTL	Cytotoxic T-cells
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	Chemokine CXC-motif ligand
DC	Dendritic cell
DLBCL	Diffuse large B-cell lymphoma
EBV	Epstein-Barr virus
ECD	Erdheim-Chester disease
EMP	Erythro-myeloid progenitor
FHL	Familial hemophagocytic lymphohistiocytosis
GDP	Guanosine diphosphate
GS2	Griscelli syndrome type 2
GTP	Guanosine triphosphate
HHV8	Human herpesvirus 8
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HLH	Hemophagocytic lymphohistiocytosis
HSV	Herpes simplex virus
ICH	Indeterminate cell histiocytosis
IFN-γ	Interferon gamma

IL	Interleukin
iv	Intravenous
IVIG	Intravenous immunoglobulin
JAK2	Janus kinase 2
LC	Langerhans cell
LCH	Langerhans cell histiocytosis
MAHS	Malignancy associated hemophagocytic syndrome
MAPK	Mitogen-activated protein kinase
MAS	Macrophage activating syndrome
Mb	Megabase
MCD	Multicentric Castleman's disease
MHC	Major histocompatibility complex
MMD	Moyamoya disease
MRI	Magnetic resonance imaging
MS	Multisystem
mTOR	Mammalian target of rapamycin
ND-LCH	Neuro-degenerative LCH
NK	Natural killer
PD1	Programmed cell death protein 1
PET	Positron emission tomography
PI3K	Phosphoinositide 3-kinase
PID	Primary immunodeficiency
pLCH	Pulmonary Langerhans cell histiocytosis
PRRT	Peptide receptor radionuclide therapy
RDD	Rosai-Dorfman disease
RNA	Ribonucleic acid
RNF	Ring finger protein
SA-beta-Gal	Senescence-associated acidic beta-galactosidase
SCT	Stem cell transplantation
sHLH	Secondary hemophagocytic lymphohistiocytosis
SHM	Second hematologic malignancy
sIL-2R	Soluble interleukin-2 receptor
SIRS	Systemic inflamatory response syndrome
SLE	Systemic lupus erythematosus
SNARE	Soluble N-ethyl-maleimide-sensitive-factor attachment protein receptor
SPM	Second primary malignancy

STX	Syntaxin
STXBP	Syntaxin binding protein
TGF	Transforming growth factor
TIA	Transient ischemic attack
TNF	Tumor necrosis factor
TPM	Tweede primaire maligniteit
Treg	Regulatory T-cells
WES	Whole exome sequencing
WHO	World Health Organization

LIST OF PUBLICATIONS

T.C.E. Zondag, C. Rokx, K van Lom, A.R. van den Berg, P. Sonneveld, W.A. Dik, G.J.J. van Doornum, K.H. Lam, J.A.M. van Laar. Cytokine and viral load kinetics in human herpesvirus 8-associated multicentric Castleman's disease complicated by hemophagocytic lymphohistiocytosis: a case report.
Int J Hematol. 2016 Apr;103(4):469-72

P.G. Kemps, **T.C.E. Zondag**, E.C. Steenwijk, Q. Andriessen, J. Borst, S. Vloemans, D.L. Roelen, L.M. Voortman, R.M. Verdijk, C.J.M. van Noesel, A.H.G. Cleven, C. Hawkins, V. Lang, A.H. de Ru, G.M.C. Janssen, G.W. Haasnoot, K.L.M.C. Franken, R. van Eijk, N. Solleveld-Westerink, T. van Wezel, R.M. Egeler, A. Beishuizen, J.A.M. van Laar, O. Abla, C. van den Bos, P.A. van Veelen, A.G.S. van Halteren. Apparent Lack of BRAF V600E Derived HLA Class I Presented Neoantigens Hampers Neoplastic Cell Targeting by CD8+ T Cells in Langerhans Cell Histiocytosis.
Front Immunol. 2020 Jan 10;10:3045

T.C.E. Zondag*, Z. Zhou*, M.A.W. Hermans, P.M. van Hagen, J.A.M. van Laar. Haemophagocytic lymphohistiocytosis in activated PI3K delta syndrome, an illustrative case report.
J Clin Immunol. 2021 Oct;41(7):1656-1659

A. Muleviciene, T. Sekine, **T.C.E. Zondag**, Y.T. Bryceson, B. Tesi, J. Rascon. Childhood Kaposi sarcoma related to hypomorphic severe combined immunodeficiency caused by a novel CORO1A mutation.
Pediatr Blood Cancer. 2022 May;69(5):e29487

T.C.E. Zondag*, L. Torralba-Raga*, J.A.M. van Laar, M.A.W. Hermans, A. Bouman, I.H.I.M. Hollink, P.M. van Hagen, D.A. Briggs, A.N. Hume, Y.T. Bryceson. Novel Rab27a variant associated with late-onset hemophagocytic lymphohistiocytosis alters effector protein binding.
J Clin Immunol. 2022 Nov;42(8):1685-1695

P.G. Kemps, **T.C.E. Zondag**, H.B. Arnardóttir, N. Solleveld-Westerink, J. Borst, E.C. Steenwijk, D. van Egmond, J.F. Swennenhuis, E.Stelloo, I. Trambusti, R.M. Verdijk, C.J.M. van Noesel, A.H.G. Cleven, M. Scheijde-Vermeulen, M.J. Koudijs, L. Krsková, C. Hawkins, R.M. Egeler, J. Brok, T. von Bahr Greenwood, K. Svojgr, A. Beishuizen, J.A.M. van Laar, U. Poetschger, C. Hutter, E. Sieni, M. Minkov, O. Abla, T. van Wezel, C. van den Bos, A.G.S. van Halteren. Clinicogenomic associations in childhood Langerhans cell histiocytosis: an international cohort study
Blood Adv. 2023 Feb 28;7(4):664-679

T.C.E. Zondag, A. Lika, J.A.M van Laar. The role of etoposide in the treatment of adult patients with hemophagocytic lymphohistiocytosis.
Exp Hematol Oncol. 2023 Jan 9;12(1):2

T.C.E. Zondag*, A.A. Acosta-Medina*, P.G. Kemps*, J.P. Abeykoon, J. Borst, E.C. Steenwijk, E.A.M. Feijen, J.C. Teepen, N.N. Bennani, S.M. Schram, M.V. Shah, C. Davidge-Pitts, M.J. Koster, J.H. Ryu, R. Vassallo, W.O. Tobin, J.R. Young, S. Dasari, K. Rech, A. Ravindran, A.H.G. Cleven, R.M. Verdijk, C.J.M. van Noesel, B.V. Balgobind, G.J. Bouma, P. Saeed, J.A.M. Bramer, R.A.L. de Groen, J.S.P. Vermaat, M.A.J. van de Sande, E.F. Smit, A.W. Langerak, T. van Wezel, S.H. Tonino, C. van den Bos, J.A.M. van Laar, R.S. Go, G. Goyal, A.G.S. van Halteren. *BRAF*^{V600E} is associated with higher incidence of second cancers in adults with Langerhans cell histiocytosis.
Accepted for publication in Blood

T.C.E. Zondag, S.E. Detiger, E. Stelloo, R.M. Verdijk, A.G.S. van Halteren, A.D.A. Paridaens, P.M. van Hagen, J.A.M. van Laar. Successful radiolabeled somatostatin analog therapy in Erdheim-Chester disease
In preparation

*authors contributed equally

ABOUT THE AUTHOR

Timo Zondag was born on the 23rd of June in 1993 in Amstelveen in the Netherlands. He grew up in Baarn with his brother and parents. During his primary education he spent two years in Bangkok, Thailand. Timo graduated secondary school at Griftland College in Soest where he developed an interest in the biology of the human body. In 2011 he was selected to attended medical school at the Erasmus University in Rotterdam, where he obtained his medical degree in 2018. During the second year of his medical study, Timo developed an interest in immunology and started a research project at the department of internal medicine, section of Allergy & Clinical Immunology. This would later evolve into a PhD-program on histiocytic diseases. Timo successfully applied for a *Ter Meulen* fund to finance his six-months working visit in 2019 to the Karolinska Institute in Stockholm, Sweden. He started his clinical career as resident at the Internal Medicine in the Franciscus Gasthuis & Vlietland hospital where he worked for over two years. In 2022 he was allowed to start his specialty training in Internal Medicine at the Erasmus Medical Center in Rotterdam.

Erasmus MC
Universitair Medisch Centrum Rotterdam

PHD PORTFOLIO

Institution	Erasmus Medical Center
Faculty	Faculteit der Geneeskunde en Gezondheidswetenschappen
Department	Internal medicine
Section	Clinical immunology
Graduate school	Erasmus MC Graduate School
PhD period	2015 until 2022
Name PhD student	Timo Carl Emile Zondag
Promotor	Prof.dr. P.M. van Hagen
Co-promotors	Dr. J.A.M. van Laar
	Dr. A.G.S. van Halteren

Courses and lectures

2022	Gezondheidsrecht
2022	Basiscursus Regelgeving en Organisatie voor Klinisch onderzoekers (BROK)
2021	Scientific integrity
2021	Systematic Literature Retrieval (in PubMed) 1 and 2
2019	Flowcytometry
2018 – 2019	Various laboratory-oriented trainings

Conferences and lectures

2019 – 2022	Regionale refereeravonden Allergologie & Immunologie
2019	Attendance of International primary immunodeficiencies congress (IPIC)
2019	Weekly HERM seminars
2015 – 2022	Attendance of several annual meetings of the histiocyte society
2015 – 2022	Regular seminars and trainings of the dept. of Internal Medicine/ Clinical Immunology
2015 – 2022	Occasional seminars and trainings of dept. of immunology

2015	Attendance of the European workshop on immune-mediated inflammatory diseases

Presentations

2021	Research meeting Laboratory of Medical Immunology. "*HLH; The immune system running wild*"
2021	37th annual meeting of the Histiocyte Society. Poster presentation: *A rare RAB27A variant associated with a case of hemophagocytic lymphohistiocytosis alters effector protein binding affinities*"
2019	4th international primary immunodeficiencies congress (IPIC). Poster presentation: "*A rare RAB27A variant associated with a case of hemophagocytic lymphohistiocytosis alters effector protein binding affinities*"
2016	32nd annual meeting of the histiocyte society. Poster presentation: "*Etoposide in adults with idiopathic HLH: is it necessary?*"
2016	Science days internal medicine. Poster presentation: "*Etoposide containing treatment in hemophagocytic lymphohistiocytosis, a systematic review*"
2015	31st annual meeting of the histiocyte society. Poster presentation: "*Etoposide containing treatment in hemophagocytic lymphohistiocytosis, a systematic review*"
2015	9th European Workshop on Immune-Mediated Inflammatory Diseases. Speed data presentation: "*Treating hemophagocytosis with etoposide, a case series and review of literature*"

Teaching

2022	Educatieve sessie NIV dagen: "*HLH; een op hol geslagen immuunsysteem*"
2020 – 2022	Various occasions at dept. of Internal Medicine/Clinical Immunology
2019 – 2022	Supervision of clinical interns in internal medicine
2017	Nieuwe ontwikkelingen in de diagnostiek en behandeling van immuungemedieerde aandoeningen. "*Langerhans cellen op oorlogspad*".

Other academic activities

2022	Peer review of manuscript considered for publication in Diagnostic Pathology
2016	Patient meeting Histiocytose Nederland
2015 – 2022	Co-developer of local treatment protocols of HLH and LCH
2015 – 2022	Participant in grand rounds immunology