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CHAPTER 1

GENERAL INTRODUCTION
CLASSIFICATION OF LANGERHANS CELL HISTIOCYTOSIS

The histiocytoses are a heterogeneous group of syndromes. From 1987, their classification was based on the type of histiocyte ‘tissue cell’ from the mononuclear phagocyte system which is dysregulated.\textsuperscript{1-3} Langerhans Cell Histiocytosis (LCH) is currently classified by the World Health Organisation as a dendritic cell disorder with a heterogeneous clinical presentation.\textsuperscript{1} In view of the recent findings in many of the histiocytoses, a new classification was recently proposed by physicians and scientist around the world who are members of the Histiocyte Society.\textsuperscript{4} In addition to the current classification based on histologic and phenotypic characteristics they suggest to classify histiocytoses based on molecular alterations, clinical and imaging characteristics. LCH is proposed to be included in the “L” (Langerhans) group.\textsuperscript{4} It is the most common histiocytic disorder in humans. Because about 3% of all children with oncologic disorders are diagnosed with LCH, this disease is much more studied in children. The exact number of new cases in children is about as high as in adults, but because of many more ‘adult’ diseases and many more adults than children, the true knowledge of LCH’s incidence in adults is still lacking. In the paediatric population the incidence rate is about two to ten children per one million each year, but like in adults, this may be underestimated also due to poor registration or misdiagnosis.\textsuperscript{5-10}

HISTOLOGICAL FEATURES AND DIAGNOSIS

The unifying feature of the presence of aberrant dendritic histiocytes in LCH lesions has been recognized since 1953. Then, Lichtenstein described a group of syndromes as Histiocytosis X which were characterized by similar clinical and histological features.\textsuperscript{11} These include eosinophilic granuloma (referring to a single osteolytic lesion), Hand-Schüller-Christian syndrome (describing multiple osteolytic lesions mostly located in the skull, exophthalmos and polyuria) and Abt-Letterer-Siwe disease (consisting of multiple osteolytic lesions skin involvement and multi-organ failure).\textsuperscript{11-13} In 1973, Nezelof demonstrated by electronic microscopy that the aberrant histiocytes in Histiocytosis X contained tennis-racket-shaped Birbeck granules which were previously been identified in normal skin- or mucosa-residing Langerhans Cells (LCs) by Birbeck et al.\textsuperscript{14,15} As the presence of these special organelles was considered as its pathology hallmark Histiocytosis X was called LCH, after its presumed cell of origin. The name LCH was accepted by the Histiocyte Society in 1987.\textsuperscript{2}

Another shared feature between normal LCs and aberrant LCH-cells is the expression the glycoprotein CD1a as shown in Figure 1D-f and 2. In addition, the type II Ca\textsuperscript{2+}-dependent lectin Langerin (CD207) is constitutively associated with Birbeck Granules and expressed by LCs and LCH-cells (Figure 1G-i and 2).\textsuperscript{16-18} Both
**Figure 1. Histology of a therapy-naïve LCH skin lesion obtained from a multi-system LCH patient (LCH344).**

1A) Hematoxylin and Eosin-staining demonstrating the presence of conventional, epidermal LCs and a dense dermal infiltration of rounded LCH-cells. A high power magnification of the indicated area (b) and a LCH-cell with a bean shaped nucleus present in the magnified area (c) as indicated by the box. 1D) Immunohistochemical staining with CD1a (brown) and hematoxylin (blue) demonstrating positive staining on round LCH-cells (e) and on conventional, epidermal LCs displaying dendrites (f). 1G) Immunofluorescent Langerin staining of a serial section demonstrating positive intracellular and membranous green staining of CD207 –expressing LCH-cells (h) and LCs (i) in the same areas.

markers are differential expressed on subpopulations of DCs, as discussed later and depicted in **Figure 2.** LCH diagnosis is based on histopathologic evaluation of a biopsy taken from the affected site(s) demonstrating the presence of CD1a- and/or Langerin expressing histiocytes (**Figure 1D-i**) in combination with the classical clinical symptoms. LCH-cells are easily recognized in the subsequently prepared Hematoxylin-Eosin-stained tissue sections as they clearly differ from conventional epidermal LCs found in skin. While epidermal LCs and conventional DCs are irregularly shaped cells and display long extensions called dendrites (**Figure 1f and 1i**), LCH-cells completely lack these dendrites and are more rounded (**Figure 1c, 1e and 1h**). In addition, LCH-cells have moderate amounts of pink cytoplasm and a folded to round 'coffee-bean' nucleus with several small nuclei (**Figure 1c**).

LCH lesions typically show the presence of granulomas, fibrosis, necrosis, haemorrhage and eosinophilic abscesses. Here the LCH-cells are intermixed with different types of immune cells, such as macrophages, eosinophils, multinucleated giant cells (MGC), stromal cells and certain T-cell subsets. The putative roles of these bystander cells are presented in a later section of this introduction wherein the previous hypothesis on LCH pathophysiology is discussed.
Figure 2. Simplified overview of human myelo-and lymphopoiesis and the presumed cells from which myeloid-derived LCH-cells may originate.

Human DCs are heterogeneous of phenotype and can originate from hematopoietic stem cells (HSC) that give rise to granulocyte-macrophage progenitors (GMP) or multi-lymphoid progenitors (MLP) in bone marrow. All lymphoid cell types and myeloid cell types such as monocytes, macrophages and DCs originate from MLP. In peripheral blood (middle row), classical monocytes and blood myeloid DC (both classical type 1 (cDC1) and type 2 (cDC2) are putative precursors of tissue DC and macrophages (bottom row). Transcriptional profiles suggest that CD14+ DC (interstitial DC) arise from monocytes and dermal CD141+ and CD1c+ DC arise from their counterparts in blood. In vitro data and flowcytometry based expression profiles suggest that LC most likely repopulate in situ and may arise from cDC1 cells in blood. In addition, monocytes are able to differentiate in vitro into CD1a and Langerin expressing cells. As gene expression profiles are different between both cells, it is nowadays questionable whether LC origin from monocytes in vivo. Broken arrows indicate relationships that have found in vitro studies and in mice but require further confirmation in humans. Stars indicate the phenotype of cells wherein BRAFV600E was detected in LCH-patients; this was detected either in bone marrow, peripheral blood or LCH-affected tissues. Adapted from 34,35.
CLINICAL SYMPTOMS AND DISEASE STAGING

LCH lesions can manifest as a single or multiple lesions in various organs such as bone, the gastro-intestinal tract, the hypothalamic-pituitary axis, lymph nodes (LN), lungs, central nervous system (CNS), thyroid, skin and the so called ‘risk-organs’: hematopoietic system, spleen and liver (Table 1). Birbeck granules seldom detected in histiocytes present in liver samples.\(^{17}\) The extent of the disease is classified according to the tissue types involved (Table 1).\(^{23,24}\) Single-system disease comprises LCH lesions in one tissue type. These include uni-or multifocal lesions in bone, referred to as mono-ostotic or poly-ostotic disease, or in other tissue types. Multi-system disease is defined as LCH manifestation in different organs or tissue types. Those patients are further classified according to the involvement of the above described risk-organs. ‘High-risk’ patients show signs of risk-organ dysfunction while ‘low-risk’ patients lack these signs.\(^{23-25}\)

The most prevalent LCH manifestations are skeletal and skin involvement. Depending on other organs involved, around 28 to 80% of the LCH cases presents with osteolytic lesions in bone. These lesions are frequently painful with adjacent soft tissue swelling.\(^{24}\) Skin involvement is seen in 10 to 48% of the patients where nearly all patients display skin lesions when multiple organs are involved. Cutaneous LCH manifests as a red papular eczematous-like rash, with or without ulcerations and vesicles mostly located in the groins, diaper areas and on the scalp.\(^{8,25}\) Gross abnormalities in lungs result in clinical symptoms like dyspnoea, coughing, haemoptysis, recurrent pneumonias or thoracic pain. Enlarged and often painful LN may point to LCH localisation and/or involvement. CNS-involvement may lead to various symptoms which depends on the affected site.

Due to hypothalamic or pituitary dysfunction for example, several endocrine deficiencies may occur which result in symptoms associated with Diabetes Insipidus (DI), hypothyroid disease and growth hormone deficiency or lead to sleep dysfunction.\(^{36,37}\) In contrast, gross cerebral abnormalities may lead to difficulties in balance and coordination. Neurodegenerative abnormalities from cerebrum associate with a wide spectrum of clinical signs ranging from subtle tremor or reflex abnormalities to severe psychiatric disease.\(^{36}\) When liver, spleen or hematopoietic system are involved, their functions are affected which can be measured by routine blood testing. Liver and spleen are then enlarged and patients may become icteric and may suffer from symptoms related to reduced haemostasis capacity and lower leukocyte numbers, such as epistaxis and recurrent infections.\(^{24}\) Liver involvement can lead to a sclerosing cholangitis and may result in biliary cirrhosis.\(^{38}\) In case of BM involvement, common findings are either lineage cytopenias or hyperactivation of lymphocytes and macrophages. When lymphocytes and macrophages produce high amounts of inflammatory cytokines it becomes a life-threatening disorder called hemophagocytic lymphohistiocytosis (HLH).\(^{39}\) Due to this heterogeneous clinical
Table 1. Staging LCH patients according to the extent and anatomic location of the LCH lesion(s).

| Single-system LCH: single lesion or multiple lesions in one organ or tissue type |
|---|---|
| Mono-ostotic | single lesion in bone |
| Poly-ostotic | multiple lesions in bone* |
| CNS-risk bone lesions | lesion(s) in mastoid, orbital and temporal bones with an increased risk for DI* |
| Special site bone lesions | lesions located in functionally critical anatomical sites*, ** |
| Lymph nodes (LN) | Draining LN from LCH-affected sites are classified as single system LCH like thyroid, or thymus |
| Other | |
| Skin | |
| Lung | |

| Multi-system LCH: single lesion or multiple lesions in two or more tissue types/organ systems* |
|---|---|
| Low-risk | without risk-organ (RO-) dysfunction |
| High risk | with risk-organ (RO+) dysfunction (hematopoietic system, spleen, liver) |

* These lesions justify systemic therapy; **such as odontoid peg and vertebral lesions with intraspinal soft tissue extension. Adapted from LCH-III and LCH-IV (Eudract number 2011-001699-20).

manifestation, LCH can easily be missed or delayed. Painful ostotic LCH can be difficult to distinguish clinically and radiologically from osteomyelitis while skin LCH is unfortunately frequently misdiagnosed as eczema or cradle cap.

**THERAPY**

Different types of LCH treatment are available and either applied as standard therapy or tested in clinical trials. Patients receive a particular type of treatment guided by the extent and location of the disease as shown in Table 1. The Histiocyte Society has given guidelines and has developed treatment regimens with incorporation of the results from the different clinical trials.

Single-system patients are often conservatively treated by ‘watchful waiting’ or receive mild therapy after histology has confirmed LCH diagnosis. While mono-ostotic lesions often regress after taking a biopsy from the affected site, the biopsy procedure of these lesions can be followed by curettage or intralesional corticosteroid infiltration. The latter treatment seems especially useful for painful, non-regressing mono-ostotic lesions. Topical steroids or systemic methotrexate are used to successfully treat single-system skin disease. Only single-system patients diagnosed with poly-ostotic LCH, CNS-risk or special-site lesions are treated with vincristine and systemic prednisone or similar regimens. All multi-system patients receive aggressive therapies consisting of a combination of chemotherapy and systemic corticosteroids. How many agents are administered and for which period they should be given is discussed below. Patients’ characteristics, including the risk for collapse or fractures, guides
whether radiation therapy or the administration of bisphosphonates or non-steroidal anti-inflammatory medications (NSAIDS) are justified.

**PROGNOSIS**

LCH outcome is variable and may range from a sporadic spontaneously self-regressing single skin, bone or lung lesion to life-threatening multi-organ failure.\(^{43,44,46-48}\) While patients below the age of two are more prone to develop multi-system LCH than elder patients, the involvement of risk-organs is more predictive for unfavorable outcome than age.\(^{23}\) To date, the extent of the disease, the involvement of risk-organs and the appearance of new lesions are the best documented prognostic factors for outcome.\(^{43,49-52}\) Especially the appearance of new LCH lesions during active disease or during therapy, so called ‘LCH-progression’, is associated with increased risk of permanent late effects ‘sequelea’.\(^{49,53}\) In addition, a study from Jubran et al. described that all six high-risk multi-system patients who had LCH progression died.\(^{51}\) Moreover, the results from LCH-I and DAL-HX 83 and 90 trials demonstrate that multi-system patients who did not respond to therapy had a higher mortality risk.\(^{52}\) These results indicated that the lack of initial response to therapy and disease progression strongly correlates with increased mortality rates in multi-system LCH patients.\(^{51,52}\) For those multi-system patients who do not respond well or even show disease progression on therapy, it is currently recommended to intensify LCH-therapy.\(^{42,43,46,52}\) This so called salvage therapy includes a combination of two aggressive chemotherapeutic agents. If needed, this is followed by allogeneic hematopoietic stem cell transplantation of which the optimal choice of conditioning remains uncertain.\(^{54}\) Aside to reduce mortality, these intensified treatment may further reduce sequelae which may affect the quality of life.\(^{55}\) The most common sequelae include DI due to hypothalamic/pituitary dysfunction, cognitive dysfunction, cerebellar involvement, lung fibrosis and skeletal abnormalities.\(^{37,51,53,55}\) Importantly, DI may become manifest prior to the clinical and histologic diagnosis, during therapy or (like most sequelae) years after therapy-induced non-active status has been achieved.

The vast majority of LCH patients responds well to first or second line treatment. However, the appearance of new lesions after a period of non-active disease, generally referred to as ‘LCH-reactivation’, still remains a critical factor affecting long term outcome as it also seems related with an increased risk for sequelae.\(^{43,49,50}\) Dependent on the affected tissue site, three to 25% of the single-system patients reactivates, albeit with excellent survival rates.\(^{24}\) In contrast, multi-system patients and particular ‘high-risk’ patients have poor prognosis with respect to mortality rates (∼14% with and ∼2% without risk-organ involvement) while reactivation rates are comparable (∼35% with and ∼26% without risk-organ involvement) (personal communication and analysis of randomized and non-randomized patients by M. Minkov and U. Pötschger,
Biological prognostic markers that identify patients at risk for unfavorable outcome before treatment initiation are currently lacking.

Figure 3. Improvement on survival and reactivations of randomized and non-randomized multi-system patients with (‘high risk’) and without (‘low-risk’) risk-organ involvement. Kaplan Meier curves showing that reactivation rates (a) of low-risk and (b) high-risk multi-system patients in LCH-III were decreased as compared to the reactivation rates in LCH-I and LCH-II. Survival rates (c) of high-risk multi-system patients were increased in LCH-III as compared to LCH-I and LCH-II. Kindly provided by M. Minkov and U. Pötschger.

Early identification of non-responsive patients and subsequent treatment intensification administration may thus decrease the risk for reactivations, the development of sequelae and further improve outcome and survival. The Histiocyte Society and other study-groups, such as the DAL-HX group in Europe, conducted various international clinical trials in order to improve outcome for patients at risk of poor prognosis. Indeed, due to treatment intensification (DAL-HX83/90 and LCH-II) and prolongation (LCH-III), the reactivation and mortality rates have improved significantly. Results from LCH-II and the DAL studies showed that therapy intensification with two or more chemotherapeutic agents led to faster disease resolution and increased survival rates in ‘high-risk’ patients compared to a single drug. In addition, an early response to first-line therapy was a positive prognostic value for survival. When a second course of initial treatment was introduced in LCH-III, it resulted in improved survival of 84% in ‘high-risk’ patients as compared to LCH-I (62%) and LCH-II (69%) and lower reactivation rates of 27% than in LCH-I (55%) and LCH-II (44%) (Figure 3). Importantly, reactivations were significantly reduced to 37% in low-risk multi-system patients when they were treated for 12 months instead of 6 months in the same trial (54%) or as compared to reactivation rates in the past trials LCH-I (52%) and LCH-II (48%). Given these promising results, the recently opened LCH-IV trial investigates whether 1. therapy intensification and prolongation from 12 to 24 months even further reduces the incidence of reactivations, sequelae and mortality in multi-system patients 2. the effect of therapy prolongation in single-system
patients where systemic therapy is justified (Table 1) and 3. the effects from a switch to intensified treatment in patients who fail first-line therapy response at early evaluation (Eudract number 2011-001699-20).

Although prolongation of chemotherapy seems beneficial, it is potentially associated with long-term side effects, such as liver failure, bone marrow depression, increased risk for infections, infertility and burden for the patient and for the health care system. LCH research over the last decade has focused on unravelling its pathogenesis to find new rationales for improved therapeutic options. In addition, it aimed to find biological risk markers that identify patients at the time of LCH onset who are at risk for sequelae or LCH reactivation. Despite this effort, only few candidate biological risk markers are known that predict reactivation and progression risk. These risk markers involve genes and proteins that are associated with cell-to-cell adhesion and cellular motility (E-cadherin, matrix metalloproteinase 12 (MMP12) and Gelsolin) or with cell proliferation and anti-apoptosis (Gelsolin, Caspase-3, Bcl-2, p16, and SHP-1). Most of these risk markers are associated with multi-system LCH which might explain their association with increased risk for progression; none predict early response to treatment. Such (bio)markers may help to prevent unnecessary overtreatment of patients with a favourable expected outcome and to prevent undertreatment of patients at risk by not administrating initial intensive treatment. None of the above-mentioned biological risk markers have been integrated as yet in international guidelines for therapy decisions.

In conclusion, the extent of the disease is clearly correlated to outcome where the poorest prognosis is seen in ‘high risk’ multi-system patients. These patients receive intense therapy for a long period. Current trials address whether even a longer treatment is beneficial for outcome.

PATHOPHYSIOLOGY HYPOTHESES

Due to development of new laboratory techniques and international collaborations between expert centres, the last two decades have shed much more light in to the etiology and pathophysiology of LCH.

LCH as a reactive disease

The hypothesis saying that LCH is the consequence of a dysregulated immune response has long been considered as the reason underlying the accumulation of LCH-cells. As mentioned above, LCH lesions are histological reminiscent of chronic inflammation and LCH-cells are intermixed with various immune cells. Together they produce a ‘cytokine-storm’ consisting of a variety of pro-and anti-inflammatory cytokines, interleukins, growth-factors and chemokines. This ‘cytokine-storm’ may promote the cellular activation, differentiation, expansion and attraction of different types of immune cells from peripheral blood to the lesion.
Several observations suggest that LCH-cell-T-cell ‘cross-talk’ occurs in LCH lesions. These cells are indeed in close proximity and LCH-cells express costimulatory molecules and ligands important for T-cell activation.\textsuperscript{20,21,65,69} Lesional polyclonal CD4\textsuperscript{+} T-cells have an activated memory phenotype and are a major source of locally produced cytokines.\textsuperscript{20,22,69} In addition to these conventional T-cells (T-CONV), locally proliferating regulatory T-cells (T-REG) are frequently observed in LCH-lesions.\textsuperscript{21} In analogy to their peripheral-blood counterparts in healthy individuals, T-REG isolated from LCH patients’ blood cells were fully functional in inhibiting T-cell proliferation.\textsuperscript{21}

Conventional LCs and DCs are essential cells with respect to initiate an optimal response of the innate immune and adaptive immune system against invading pathogens. Key steps in this process are antigen uptake and processing, up and down-regulation of a certain set of chemokine receptors upon activation and maturation, migration to lymph nodes and presentation to and activation of T-cells (Figure 4). Stimuli for classical LC activation and maturation are thus present in LCH lesions and local T-cells seem activated.\textsuperscript{20,22,65,66,69,70} However, lesional LCH-cells in all paediatric cases, seem immature as evidenced by data showing that they express CCR6, but mostly lacked CCR7 and other maturation markers such as CD83 or CD86 (Figure 4).\textsuperscript{58,65-67,71,72} In line with the mostly intracellular expression of Major Histocompatibility Complex (MHC) Class II, LCH-cells poorly stimulated allogeneic T-cell proliferation in vitro.\textsuperscript{67} In addition, LCH-cells do express markers of immature differentiation like the pattern recognition receptor CD14 and the monocyte antigen CD68, while LCs and dermal Langerin\textsuperscript{+} clearly lack these receptors (Figure 2).\textsuperscript{67} It has therefore been postulated that tissue accumulation of LCH-cells is the result of an undefined process leading to ‘maturation-arrest’ and, consequently, to their inability to migrate to regional LN.

Other arguments that advocate for a frustrated, local immune response are the following. First, the spontaneously self-regressing nature of some biopsied lesions suggests that exogenous triggering of the local immune response either leads to the elimination of LCH-cells or induces inflammation-induced migration towards the LN.\textsuperscript{21,44,47,48} Second, in contrast to malignancies, LCH-cells display low frequencies of mitotic figures and few stain positive for the proliferation marker Ki67, which suggests that they are not highly proliferative.\textsuperscript{21,73}

**The 21\textsuperscript{st} century: LCH as a neoplastic disorder**

The hypothesis saying that LCH is the consequence of a malignant transformation of LCs has been long investigated. Although the authors of a previous study clearly stated that point mutations could not be excluded, no genomic aberrations on flowsorted lesional LCH-cells could be detected up to a few years ago.\textsuperscript{74}

Arguments that advocate in favour of this hypothesis are the familial clustering of a few LCH cases.\textsuperscript{88} LCH-cells in non-pulmonary lesions are clonal, and some display DNA aneuploidy with genetically instable tumor-suppressor genes.\textsuperscript{74,89-91} LCH-cells
Figure 4. Key steps of the innate immune response initiated by LCs and dermal and interstitial DCs in relation to the phenotype of LCH-cells.

DCs and LCs both take-up antigens via (macro)-pinocytosis and phagocytosis; antigens bound to Langerin expressed on LCs are also internalized via endocytosis. Once activated and matured, specific receptors are down-regulated (such as the chemokine receptor CCR6) while the chemokine receptors CXCR4 and CCR7 are up-regulated. After this alteration, these antigen-presenting cells (APC) subsequently migrate to lymph nodes (LN). In addition to the extracellular expression of MHC class II, Constitutive expression CD1a expression, facilitates antigen presentation in the LN to T-cells. Cross-talk between professional APC and T-cells provide essential signals for full activation T-cells and, together with cytokines present in the LN, directs their differentiation into particular subtypes. Besides T-Cell-Receptor ligation, the activation of naïve T-cells involves a second signal delivered by the interaction of co-stimulatory molecules. Well known co-stimulatory molecules are CD80 (B7.1) and CD86 (B7.2), inducible costimulatory molecule Ligand (ICOSL) and CD40 expressed on APC which interact with respectively CD28, ICOS and CD40L (CD154) on T-cells. After T-cells have been fully activated they up-regulate activation markers, clonally expand and differentiate into effector cells. Then, they may induce cytotoxicity (CD8-cytotoxic T-cells) and/or secrete cytokines (both cytotoxic T-cells as CD4+ T-helper T cells) which are instrumental for different types of immune responses. Please note that the phenotype of LCH-cells is most similar to the expression seen immature LCs, with the exception of the molecules in bold. Adapted from: 78,80
additionally exhibit activation of several pathways which promote survival, proliferation and resistance to apoptosis. These include NF-κB activation, upregulation of the oncogene product MDM2 and overexpression of cell-cycle related proteins that control proliferation such as p16, p21, p53, Rb, Bcl-2, FADD, FLICE and FLIP. \(^{59,92,98}\) Especially LCH-cells from multi-system patients display low levels of the apoptosis-initiating factor caspase-3, \(^{93}\) high expression levels of the anti-apoptotic protein Bcl-2, \(^{93,97}\) and overexpression of Gelsolin which blocks apoptosis. \(^{61}\) Despite these malignant-pleading arguments, no genomic aberrations in lesional LCH-cells could be found in biopsied tissues for decades.\(^{74,90}\)

The Ras-Raf-MEK-ERK pathway, also known as the Mitogen-Activated Protein kinases (MAPK) pathway, is normally activated by receptor-ligand interaction. This leads to transiently induced survival and proliferation of activated cells (Figure 5). Most cancer-related mutations occurring in key elements of the MAPK-pathway, like mutations in Braf, lead to constitutive activation of MAPK-pathway.\(^{99-101}\) In 2010, the group of Rollins published a hallmark paper wherein they described that laser-captured LCH-cells carried the cancer-related point mutation Braf\(^{V600E}\) in approximately 55% of the LCH cases examined in their study.\(^{31}\) While the Braf\(^{V600E}\) mutation is the most common mutation found in LCH-cells, LCH-cells may also display other Braf mutations such as Braf \(^{600DLAT},\) Braf \(^{599A},\) Braf \(^{V600D}\) and Braf \(^{V600K}.\)\(^{31,102,103}\) Similar to those described in cancer,\(^{100,101}\) the LCH-related mutations in Braf lead to constitutive activation of MAPK-pathway.\(^{31}\) Indeed, the key components which indicate constitutive MAPK-pathway activation (phosphorylated MEK and ERK kinases) were detected by immunohistochemistry in Braf\(^{V600E}\) mutated LCH-cells.\(^{31,104}\) Unexpectedly, Rollins et al. showed that these elements were also phosphorylated in Braf\(^{wild}\) type LCH-cells.\(^{31}\) They postulated that LCH-cells may carry somatic mutations in other components of the MAPK pathway. In collaboration with the group of Rollins, we indeed demonstrated that LCH-cells carry mutations in MAP2K1 and MAP3K1, which are kinases of the MAPK-pathway.\(^{105}\) Additional mutations in these genes were also found by others.\(^{104,106}\)

Based on these data, LCH might be classified as a neoplastic disease. However, some clinicians and scientists still question the true malignant character of LCH and prefer to call it a neoplastic disorder.

**LCH as a myeloid neoplastic disease with a clear inflammatory component**

The latest hypothesis is proposed recently and says that LCH is the consequence of a dysregulated, neoplastic differentiation of cells belonging to the myeloid cell lineage. This is supported by the finding that peripheral blood from LCH patients contains increased CD34\(^{+}\) precursors and lin\(^{-}\) HLA-DR\(^{+}\)CD11c\(^{+}\)myeloid DCs as compared with age-matched controls.\(^{32,107,108}\)
Figure 5. The MAPK pathway.
Under normal conditions, only a stimulus as delivered for instance by growth factors, chemokines, cytokines and other stress factors activate the MAKP pathway. Either activated RAS leads to the activation of RAF proteins ARAF, BRAF and CRAF. Consequently, a phosphorylation (P) cascade occurs of downstream Mitogen-Extracellular signal-regulated Kinases (MEK) MEK1 (MAP2K1) and MEK2 (MAP2K2) and subsequently phosphorylation of the Extracellular signal-Regulated Kinases (ERK) ERK1 (MAPK3) and ERK2 (MAPK1). The latter MAPKs activate transcription factors, such as c-myc, which leads to cellular proliferation and differentiation. MEKK1 (MAP3K1) is under normal conditions activated by mobilized G-proteins and promotes proliferation and survival via phosphorylation of MAP2K1 which then activates ERK signalling. When BRAF, ARAF, MAP2K1 or MAP3K1 is mutated, MEK1/2 and ERK1/2 are continuously phosphorylated whereby the mutated cells have acquired capacity to inhibited proliferation, increased survival, differentiation of transformation and increased proliferation. Stars indicate the elements wherein kinase activating mutations have been detected in LCH-cells. Adapted from.\textsuperscript{101,112}
**Figure 2** gives a schematic overview of of human myelo-and lymphopoiesis and the presumed cells from which myeloid-derived LCH-cells may originate. This is based immunohistochemical, gene expression data and BRAF analysis in LCH-cells and other cells of the myeloid lineage.\textsuperscript{16,29,32-34,60,109-111} In addition to the earlier described shared expression of Langerin and CD1a by LCs and LCH-cells, they also express another family member CD1c. CD1a and CD1c are also expressed on dermal and interstitial DCs (Figure 2).\textsuperscript{28,29,73,111} A subpopulation of dermal DCs additionally expresses Langerin but its expression has not been detected on interstitial DCs or DC subsets in peripheral blood Langerin (Figure 2).\textsuperscript{29,34}

In addition, the integrin CD11c is expressed on LCH-cells and both dermal CD1a\textsuperscript{+}Langerin\textsuperscript{+} and CD1a\textsuperscript{+}Langerin\textsuperscript{−} DCs but not on epidermal LCs.\textsuperscript{29} While LCH was called after its presumed cell of origin, this data shows that LCH-cell most likely not originate from epidermal tissue residing LCs or plasmacytoid DC but from immature CD1c\textsuperscript{+} myeloid DCs.\textsuperscript{29,60,109,110} In order to study the pathophysiology of LCH in vivo, multiple research groups have tried to induce LCH in mice. Lacking knowledge of the exact LCH-precursor phenotype and differences in the expression profiles of myeloid cells in mice and human hamper, however, the establishment of murine LCH models. The most closely resembled multi-system LCH models are created when constructs are placed under the CD11c promotor as expressed by DC precursors. Mice with the expression of SV40, a polyomavirus that supresses retinoblastoma and p53 tumor-suppressor proteins, under CD11c developed multi-system-like LCH with accumulated Langerin-expressing CD8α DCs in bone, bone marrow, spleen liver, thymus and mesenteric lymph nodes.\textsuperscript{113,114} When BRAF\textsuperscript{V600E} was constructed under CD11c (CD11c_BRAF), it induced a disseminated, aggressive LCH-like phenotype with organ dysfunction. In both systems, CD11c-expressing committed DC progenitors that maintain their ability to differentiate into Langerin-expressing cells are transformed and not only differentiated tissue-resident Langerin-expressing DCs or LCs. Although both constructs induce murine histiocytosis, it is notable that BRAF\textsuperscript{V600E} mutations occur at high frequency in LCH lesions,\textsuperscript{31,102,103} while SV40 has not been detected in LCH cases yet. In contrast to the above systems, BRAF\textsuperscript{V600E} expression under the Langerin promotor (Langerin_BRAF), representing differentiated DCs, induced an mild LCH-like phenotype with mainly lung and liver involvement.\textsuperscript{32}

These observations collectively suggest that if the BRAF mutation occurs in a myelopoietic progenitor cell that expresses CD11c, it will further differentiate into a Langerin-expressing LCH-cell and spread throughout the body resulting in an aggressive type of LCH. In contrast, if the BRAF mutation occurs in a Langerin-expressing tissue-restricted precursor LCH-cell, it retains in this peripheral tissue site and thus manifests as a mild variant.\textsuperscript{32} The authors also suggest that if the mutation occurs in a tissue-restricted DC progenitor, with or without tissue tropism, could result
in poly-ostotic or single LCH lesions. This model does not explain the occurrence of circulating BRAF\textsuperscript{V600E} cells in a self-regressing skin lesion.\textsuperscript{32}

In summary, increasing data is supporting the hypothesis that LCH is a neoplastic myeloid disease, originating from either hematopoietic progenitor cells, from monocytes, DCs or LCs or from tissue-restricted DCs where the cellular differentiation status at the time of the mutational hit presumably determines the extent of the disease.

**OUTLINE OF THIS THESIS**

The pathology of LCH has been studied for decades but is not fully elucidated yet. While recent advances have provided more insight in the genetic and phenotypic make-up of the LCH-cells, the origin is LCH cells is still unknown and the clinical implication of the mutational status of the LCH-cells is far from understood. Also, the composition of the inflammatory microenvironment has scarcely been studied in the context of the clinical features of LCH. This is remarkable since its manifestation form and outcome are variable and the reason for LCHs' heterogeneity is not well understood.

Currently, LCH treatment and its duration is solely guided by clinical features and, if applicable, subsequent randomisation within the clinical trials. However, a substantial proportion of patients historically designated ‘at risk’ for unfavourable outcome never reactivates. Prolonged treatment given to these patients ‘at risk’ may, however, lead to an unnecessary burden for both the patient and the health care system. Thus, biological prognostic markers, for example the lesional microenvironment or the mutation status of the LCH-cells, identifying this subcategory of patients at the time of diagnosis are urgently needed.

The aim of this thesis was to provide more insight in LCH pathophysiology by genetic and immunologic “finger-printing” of LCH lesions in relation to clinical features. We therefore categorize LCH patients according to their 1. Genetic abnormalities in their lesional LCH-cells or genomic DNA 2. Functional characteristics of peripheral blood cells 3. Phenotype of lesional LCH cells and 4. Inflammatory composition of lesions. In addition, we aimed to find new (bio)markers which can be used, in addition to already defined clinical parameters, to identify future patients at risk for unfavourable outcome and to guide clinical decisions.

**Genetic fingerprinting of LCH-cells**

Cancer patients who have proven mutations in MAPK-pathway are currently enrolled in trials which test the efficacy of agents that block individual elements of the MAPK-pathway. As explained above, a significant proportion of LCH-cells display malignancy-associated somatic BRAF, MAP2K1 and MAP3K1 mutations that constitutive activates the MAPK pathway. Because this signalling pathway is activated in LCH-cells lacking
activating BRAF mutations, it is plausible that additional MAPK-pathway activating mutations are present in these LCH-cells. Recent technological advances in molecular analysis on genomic DNA derived from either unfixed, i.e. live, cells or formalin fixed paraffin embedded (FFPE) tissue samples have recently opened new possibilities to address additional mutations in LCH-cells. To increase treatment options, also for patients without proven BRAF mutations in their LCH-cells, we searched for additional mutations that could lead to MAPK-pathway activation. The results of this collaborative effort between one of the worlds centre of excellence that is dedicated to immunological profiling of LCH (LUMC) with one of the centres of excellence in genomic profiling (Dana Farber Cancer Institute) are described in Chapter 2 of this thesis.

**Immunologic fingerprinting of LCH lesions**

Several cytokine and chemokine receptors make use of the MAPK-signalling pathway to temporarily induce transcription factors that lead to enhanced survival, proliferation and increased chemotactic properties. The chemokine receptor CXCR4 plays an important role in facilitating an appropriate immune response while it has also been reported to be key in more than 23 types of malignancies. In cancer, its expression is strongly associated with the extent and location of metastases as well as with poor prognosis. In Chapter 3, we hypothesized that CXCR4 first directs the migration of aberrant myeloid (LCH-like) cells to tissues where its ligand CXCL12 is expressed. Second, it orchestrates through MAPK-pathway activation their local accumulation in the LCH-affected tissues. By studying the individual components of the CXCR4-CXCL12 axis in relation to LCH manifestation, outcome and mutation status, we thus addressed the unsolved issue why LCH-cells also end up in other tissues than the typical LC homing sites being the epidermal layers of the skin, mucosa, LN and lungs.

Besides aberrant LCH-cells, many different types of immune cells end up in LCH lesions. Lymphocyte recruitment into normal secondary lymphoid organs, chronically inflamed tissues and tumor sites is mediated via specialized blood vessels called High Endothelial Venules (HEVs) and through the release of various chemoattractant molecules, mostly being chemokines. HEVS and organized lymphocyte aggregates are frequently detected in non-lymphoid tissues where they are called tertiary lymphoid structures (TLS) provided that they have similar characteristics as classic LN. Their presence along with the presence of certain T-cell subsets have been found in sites of chronic inflammation and seem positive prognostic factors in several types of cancer. Although CD3^+^ T-cells have been visualized in LCH lesions, the mechanisms that lead to their influx and tissue accumulation have barely been studied nor related to the extent of the disease or outcome. Because pathology reports often describe concurrent lymphocytic aggregates in LCH lesions, we studied in Chapter 4 whether
similar T-cell attractive elements are present in LCH lesions, if the aggregates represent TLS and if they predict LCH outcome.

Non-naïve T-cells obviously accumulate in LCH-affected tissues. However, this does not necessarily mean that the cells function as activated T-cells. Mechanisms that facilitate the distinct steps of the presumed T-cell activation inside LCH lesions are unknown. Costimulation via ICOS-ICOSL interaction (Figure 3) provides a second signal necessary for full activation of T-cells and might, depending on the activated T-cell subset, thus be relevant for both immune surveillance and immune evasion. When T-cells are functional, their interaction with tumor cells may lead to anti-tumor responses by elimination of tumor cells and thereby preventing tumor outgrowth. In contrast, tumor cells or other cellular ‘partners in crime’ such as T-REG efficiently orchestrate immune evasion through the interference with the influx of tumor-associated T-cells or altering local dendritic cell-T-cell interactions. In fact, tumor-cell derived immunosuppressive cytokines, such as Interleukin (IL)-10 and Transforming Growth Factor–beta (TGF-β), alter their microenvironment in such a way that tumor-reactive T-cells are rendered non-functional or skewed into immunosuppressive T-cells. Thus, in contrast to the classic differentiation and commitment of T-cell differentiation as depicted in Figure 3, the T-helper phenotype is more plastic as T-cells are highly flexible in cytokine production. In Chapter 5, we hypothesized that LCH-cells drive immune evasion in a similar fashion. Here, we describe the presence of immune suppressive cytokines in relation to the phenotype and activation status of lesion-infiltrating T-cells.

One of the cytokines which may be key to proper effector T-cell function in the lesion is Interferon-gamma (IFN-γ). As IFN-γ receptor (IFN-γR) are classically expressed by several myeloid cells including monocytes and macrophages, we questioned in Chapter 6 if defective IFN-γ signalling contributes to the pathogenesis of LCH. In fact, poly-ostotic LCH can be difficult to distinguish clinically and histologically from (recurrent) disseminated Mycobacterial infections in patients who display a mutation in exon 6 of the gene encoding the IFN-γR1 (ADIFNGR1 patients). ADIFNGR1 patients present with LCH-mimicking lesions which may appear in skin, bone, LN and/or visceral organs. Following misdiagnosis as LCH patients, they may receive harmful treatment regiments that may lead to disease progression. In Chapter 6, LCH patients and one ADIFNGR1 patient were both analysed for mutations in genes encoding for cytokine receptors belonging to the type 1 cytokine pathway and we assessed IFN-γ signalling in functional assays. We describe a simple new laboratory method which can used to properly differentiate between ADIFNGR1 patients and LCH patients before treatment is started.

In the final Chapters 7 and 8, the results of the studies presented in this thesis are summarized and discussed.