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Immunohistochemical analysis of potentially targetable markers in histiocytic disorders

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Dedicated to my dear dad.
Thank you for being the firmest believer in me.

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LIST OF ABBREVIATIONS

AEC	3-Amino-9-Ethylcarbazole
AKT	Protein kinase B
ALK	Anaplastic lymphoma kinase
ASIR	Age-standardised incidence ratio
AXG	Adult XG
BCH	Benign cephalic histiocytosis
BRAF	B-rapidly accelerated fibrosarcoma
CD	Cluster of differentiation
CI	Confidence interval
CNS	Central nervous system
CRD	Cutaneous Rosai-Dorfman disease
CT	Computer tomography
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cells
ECD	Erdheim-Chester disease
ERK	Extracellular-signal regulated kinase
FDA	Food and drug administration (United States of America)
FFPE	Formalin-fixed paraffin-embedded
GEH	Generalized eruptive histiocytosis
HAM-56	Human alveolar macrophage-56
HE	Haematoxylin-eosin
HLH	Haemophagocytic lymphohistiocytosis
HRP	Horseradish peroxidase
ICH	Indeterminate cell histiocytosis
IHC	Immunohistochemistry, immunohistochemical
IL-1R	Interleukin-1 receptor
IFN-α	Interferon α
irAEs	Immune-related adverse events
IVIG	Intravenous immunoglobulins
JXG	Juvenile XG
LC	Langerhans cells
LCH	Langerhans cell histiocytosis

MAPK	Mitogen-activated protein kinase
MAP2K	Mitogen-activated protein kinase kinase
MEK	MAP2K
MGUS	Monoclonal gammopathy of undetermined significance
MH	Malignant histiocytosis
MM	Multiple myeloma
MoAb	Monoclonal antibody
MPS	Mononuclear phagocyte system
MRH	Multicentric reticulohistiocytosis
MRI	Magnetic resonance imaging
MS-LCH	Multisystemic LCH
mTOR	mechanistic/mammalian target of rapamycin
n.d.	Not determinable
NGS	Next-generation sequencing
NHL	Non-Hodgkin lymphoma
NLCH	Non-Langerhans cell histiocytosis
NOS	Not otherwise specified
NTRK	Neurotrophic tyrosine receptor kinase gene
NXG	Necrobiotic XG
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death-ligand 1
pERK/MEK	phosphorylated ERK/MEK
PI3K	Phosphoinositide 3-kinase
PNH	Progressive nodular histiocytosis
PUVA	Psoralen and ultraviolet A
RAS	Rat sarcoma
RAF	Rapidly accelerated fibrosarcoma
RDD	Rosai-Dorfman(-Destombes) disease
RNA	Ribonucleic acid
$RO^{+/-}$	Risk organ involved/ not involved
ROS1	Proto-oncogene tyrosine-protein kinase 1
SD	Standard deviation
SHML	Sinus histiocytosis with massive lymphadenopathy

SRH	Solitary reticulohistiocytoma
SS-LCH	Single-system-LCH
TBS	Tris buffered saline
TBST	TBS with Tween20
Th1	T helper cells type 1
TMA	Tissue microarray
TNF-α	Tumour necrosis factor α
TPM3	Tropomyosin 3
TRAF1	TNF receptor-associated factor 1
TRK	Tropomyosin receptor kinase
WHO	World health organization
XD	Xanthoma disseminatum
XG	Xanthogranuloma

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INTRODUCTION

1.1 The mononuclear phagocyte system

The mononuclear phagocyte system (MPS) is a group of cells that stem from progenitor cells of the bone marrow. MPS contains phagocytes of mononuclear morphology in contrast to polymorphonuclear phagocytes, such as neutrophil granulocytes (Hume, 2006). Macrophages, monocytes and dendritic cells (DC) belong to the MPS (Collin and Bigley, 2018). Both monocytes and macrophages are known for their function as phagocytic cells of apoptotic cells, whilst DC as a non-phagocytic member of the MPS have an immunologic importance as antigen-presenting cells that mostly reside in peripheral lymphoid organs and activate naïve T lymphocytes (Steinman and Witmer, 1978).

Macrophages and DC are believed to derive from haematopoietic stem cells of the lympho-myeloid lineage (Collin and Bigley, 2018). Monocytes were thought to be the precursor cells of the non-motile macrophages as a result of their capability to differentiate into various cells of the MPS, especially in inflammatory settings but recent studies observe a self-renewing nature of macrophages under steady-state conditions. DC are divided into myeloid and plasmacytoid DC with further subsets that express clusters of differentiation (CD) like CD141 and CD1c. (Emile et al., 2016). Histiocytes are macrophages of the extravascular tissue and are tissue-resident compared to the mostly motile monocytes which circulate in the peripheral blood, inter alia (van Furth and Cohn, 1968).

Langerhans cells (LC) are DC located in the epidermis, in mucous membranes and in the epithelium of the lungs (Emile et al., 2016). LC were presumed to solely be of bone marrow origin (Katz et al., 1979), although a self-renewing ability seems to exist (Merad et al., 2008). When activated by pathogens as an immune response, LC migrate to lymph nodes where they act as antigen-presenting cells to naïve T cells (Collin and Bigley, 2018). They appear as mononuclear cells with bean-shaped nuclei and contain Birbeck granules, which can be viewed electronmicroscopically. Immunohistologically, LC express cell surface markers such as CD1a and S100 (Schmieder et al., 2019). A marker that is almost primarily expressed in LC is CD207, it is the content of Birbeck granules and responsible for the induction of their synthesis (Valladeau et al., 2000).

Recent findings now challenge the concept of the MPS once created by van Furth and Cohn. Current literature has emerged that offers contradictory observations questioning the strictly myeloid origin of the MPS members (Radzun, 2015; Schulz et al., 2012) and suggesting a certain plasticity of the cells and a capability to transdifferentiate into other cells within and outside MPS family (Hume, 2006).

1.2 Histiocytoses

Histiocytosis describes a heterogenous group of rare disorders of the macrophage dendritic cell lineage(Stojkovic et al., 2000). These disorders can clinically present at any age, in any organ and with a varying severity. Their presentation can range from a single cutaneous lesion with spontaneous remission to a severe therapy-resistant multisystemic disease (Broadbent et al., 1989). In the past, histiocytoses were considered to be solely of reactive nature (Broadbent et al., 1989) but due to more recent findings showing cell clonality and mutations of tumour oncogenes, a neoplastic origin is also assumed (Badalian-Very et al., 2010; Willman et al., 1994; Yu et al., 1994). A recent review proposes a new classification redefining that created by the Working Group of the Histiocyte Society in 1987. It suggests a more detailed arrangement. Instead of the classic divide of histiocytoses into LC, non-LC related and malignant (Writing Group, 1987), Emile et al. offer a constellation in 2016 based on features, such as clinical presentation, histological, genetic and molecular similarity, creating the following groups: Langerhans-related histiocytoses ("L" group), cutaneous and mucocutaneous histiocytoses ("C" group), Rosai-Dorfman disease (RDD) ("R" group), malignant histiocytoses (MH) ("M" group) and haemophagocytic lymphohistiocytosis (HLH) and macrophage activation syndrome ("H" group) (Emile et al., 2016) *(Table 1.1).*

In the newly defined "L" group, Langerhans cell histiocytosis (LCH) and Erdheim-Chester disease (ECD) are observed to have more in common than previously believed due to their similar clonal mutations in the mitogen-activated protein kinase (MAPK) pathway (Badalian-Very et al., 2010; Haroche et al., 2012b) and clinical conditions that occur in both disorders like diabetes insipidus (Emile et al., 2016). The otherwise large group of non-Langerhans cell histiocytosis (NLCH) was attempted to be subclassified as the cutaneous group and others. Within the former, disorders are categorized into the xanthogranuloma (XG) family and non-XG family. Representatives of the XG family are, among others, juvenile XG (JXG), adult XG (AXG), generalized eruptive histiocytosis (GEH), progressive nodular histiocytosis (PNH) and xanthoma disseminatum (XD). The non-XG family is mainly composed of the cutaneous Rosai-Dorfman disease (CRD) and necrobiotic XG (NXG) (Emile et al., 2016) *(Table 1.1).*

In the same year as Emile et al, 2016, Swerdlow et al. updated their world health organization (WHO) classification of histiocytic and dendritic cell neoplasms from 2008 (Swerdlow et al., 2016). The WHO classification differs significantly from that of the Histiocyte Society by assuming a lymphoid origin of the neoplasms, while the Histiocyte Society view histiocytoses as myeloid disorders. Furthermore, only a few histiocytic disorders are featured in the WHO classification, leaving many NLCH uncategorized *(Table 1.1).*

In this study, histiocytoses will mainly be observed through the lens of the original classification by the Histiocyte Society from 1987, while being mindful of the more current classifications to have a better overview and understanding of the disorders discussed. The following table juxtaposes the most formative classifications of histiocytoses *(Table 1.1)*.

Histiocyte Society Classification (1987)	WHO Classification (2016)	Histiocyte Society Classification (2016)

LCH
- Histiocytosis X
  o Eosinophilic granuloma
  o Letterer-Siwe disease
  o Hand-Schüller-Christian syndrome
  o Hashimoto-Pritzker syndrome
- Self-healing histiocytosis
- Pure cutaneous histiocytosis
- Langerhans cell granulomatosis
- Type II histiocytosis
- Non-lipid reticuloendotheliosis
NLCH
- HLH
- Infection associated haemophagocytic syndrome
- RDD
- XG
- Reticulohistiocytoma
- Miscellaneous, other, unclassified
MH
- Acute monocytic leukaemia
- MH
- True histiocytic lymphomas (histiocytic sarcomas)
	LCH
	
	Disseminated JXG
	
	Histiocytic sarcoma
	
	Langerhans cell sarcoma
	
	Indeterminate dendritic cell tumour
	
	Follicular dendritic cell sarcoma
	
	Fibroblastic reticular cell tumour
	
	ECD
		L Group
		- LCH
		- ICH
		- ECD
		- Mixed LCH/ECD
		C Group
		- Cutaneous NLCH
		  o XG family:
		  ▪ JXG, AXG, SRH, BCH, GEH, PNH
		  o Non-XG family:
		  ▪ CRD, NXG, other NOS
		- Cutaneous NLCH with a major systemic component
		  o XG family:
		  ▪ XD
		  o Non-XG family:
		  ▪ MRH
		R Group
		- Familial RDD
		- Sporadic RDD:
		  o Classical RDD
		  o Extra-nodal RDD
		  o RDD with neoplasia or immune disease
		  o Unclassified
		M Group
		- MH (primary, secondary)
		H Group
		- HLH (primary, secondary, unknown/uncertain origin)

Table 1.1: Comparison of the most significant classifications of histiocytosis. (Emile et al., 2016; Swerdlow et al., 2016; Writing Group, 1987)

1.2.1 Langerhans cell histiocytosis

LCH is a neoplastic disorder that can affect all age groups and both sexes with varying severity(Gurnee and Lawley, 2018). Clinical presentation ranges from a single self-healing lesion to fatal multisystemic disease (Broadbent et al., 1989). A preponderance of young male patients has been observed with an approximate sex ratio (male: female) of 1.5:1 and a median age of 3 years. The occurrence of LCH depends on the age group, as its incidence reduces with age. (Guyot-Goubin et al., 2008; Ribeiro et al., 2015). The age-standardized incidence ratio (ASIR) in children is approximately at 4 to 6 per 1 million per year (Erdmann F, 2020; Guyot-Goubin et al., 2008; Kaatsch and Spix, 2006; Salotti et al., 2009). In adults, the incidence is estimated to be lower, at a minimum of 0.07 to 2 cases per million per year (Goyal et al., 2018; Schmieder et al., 2019).

The aetiology of LCH remains widely unknown. The findings of somatic mutations of MAPK pathway genes in most lesions revolutionized how LCH is viewed (Jouenne et al., 2020). Mutations such as BRAF V600E mutations occur in approximately 50% of all LCH lesions (Badalian-Very et al., 2010; Haroche et al., 2012b; Sahm et al., 2012; Satoh et al., 2012) and mitogen-activated protein kinase kinase (MAP2K)1, also known as MEK1, mutations are found in 50% of LCH cases with BRAF wild-type (Badalian-Very et al., 2010; Brown et al., 2014). In the past, Nezelof et al. suspected LCH lesions developed in various organs when pathological LC of the epidermis experienced a malignant or immunoproliferative activation (Nezelof and Basset, 2004). This concept is described in the “Activated-Immature” model. A more recent model is that of the “Misguided Myeloid DC Precursor” model proposing that LCH cells derive from pathological DC precursors that migrate and differentiate to CD207-positive DC. These cells then attract activated T-lymphocytes. This assumption is supported by studies that show the presence of myeloid DC precursors in LCH lesions (Allen et al., 2010; Rolland et al., 2005; Schmieder et al., 2019). Zinn et al support the idea of the misguided precursor myeloid cells, further hypothesizing that the recently described MAPK pathway mutations, which lead to various MEK and ERK hyperactivation, may be responsible for the differentiation of LCH cells. It is also suggested that the clinical presentation of LCH depends on the stage of differentiation at which ERK is activated. A mutation in the haematopoietic stem cell might lead to more severe LCH types, like multisystemic diseases or LCH with high-risk organ involvement, while mutations at more differentiated stages lead to milder LCH manifestations, such as unifocal lesions. (Berres et al., 2014; Zinn et al., 2016)

LCH clinically presents in various forms. In the past, clusters of clinical features were described as different LCH variations. Eosinophilic granuloma, Hand-Schüller-Christian disease, Letterer-Siwe disease and Hashimoto-Pritzker disease were defined as clinical subtypes of LCH. This categorization is now considered outdated due to the discovery of identical histopathological findings. Furthermore, the clinical presentation of LCH cases often matched that of several subtypes instead of only one, making a classification impossible. (Writing Group, 1987) Recent classifications sort clinical features into two groups based on their prognostic relevance. A differentiation is made between single-system and multisystemic disease. Single-system-LCH (SS-LCH) describes the involvement of only one organ system, while multisystemic LCH (MS-LCH) refers to multiple affected organs. Within MS-LCH, the distinction between high-risk (RO^+) and low-risk (RO^-) organ involvement is made. The liver, bone marrow/ haematopoietic system and spleen are considered high-risk organs (Emile et al., 2016; Zinn et al., 2016). The organs most affected by LCH are the bone (approximately 77%) and the skin (approximately 30%) (Salotti et al., 2009). Other often affected organs include lymph nodes, the central nervous system (CNS) and the lung. (Schmieder et al., 2019)

LCH lesions are cellularly heterogenous, containing LCH cells, lymphocytes, macrophages and eosinophils and possibly CD207-positive DC. Histopathological findings of LCH show oval cells with kidney shaped nuclei and eosinophilic cytoplasm with Birbeck granules within granulomatous lesions in the papillary dermis (Schmieder et al., 2019).

Immunohistochemically, they present as CD207-positive DC that have a similar phenotype to epidermal LC but are suspected to be of a different origin (Allen et al., 2010; Nelson et al., 2015). LCH possesses a characteristic immunostaining with expressions of S100, CD1a and CD207 (Chikwava and Jaffe, 2004).

Treatment of SS-LCH is not always required. Isolated forms in the bone or skin seldom profit from systemic therapy. Surveillance is mostly sufficient. In some cases, local therapy, such as surgical excision, phototherapy or topical treatment with ointments containing nitrogen mustard, for example, is needed. MS-LCH often profits from systemic therapy with vinblastine and prednisolone and/or methotrexate. (Emile et al., 2021; Schmieder et al., 2019) When patients no longer respond to first- or second-line treatment, salvage therapy can be used. Recent treatment options are BRAF inhibitors for patients with verified BRAF mutations (Diamond et al., 2018) and MEK inhibitors for patients with BRAF wild-type (Diamond et al., 2019).

Various factors predict a positive clinical outcome. Patients that respond positively to systemic therapy in the first six weeks have a better prognosis (Gadner et al., 2013), as well as the occurrence of unifocal LCH, SS-LCH or self-resolving lesions (Alston et al., 2007; Schmieder et al., 2019). Cigarette smoking poses as a risk factor for pulmonary LCH in adults as it possibly causes activation and migration of LCH cells to the lung (Colby and Lombard, 1983; Friedman et al., 1981; Vassallo et al., 2000). Long-term consequences of LCH can be neurodegeneration (Emile et al., 2021; Schmieder et al., 2019) and diabetes insipidus. The latter can either occur before LCH manifests as an indicator or LCH can contribute to its development in the future (Richards et al., 2011).

1.2.2 Non-Langerhans cell histiocytosis

NLCH encompasses a diverse group of uncommon disorders characterized by the abnormal proliferation of non-Langerhans histiocytes. Unlike LCH, which primarily affects LC, NLCH involves various subsets of histiocytes derived from the monocyte/macrophage lineage. (Fritsch and Schwarz, 2018) This review aims to provide a comprehensive overview of NLCH, including its clinical manifestations, pathogenesis, diagnostic approaches, treatment strategies and recent advances in understanding the disease *(Table 1.2).*

NLCH can affect multiple organ systems, including the skin, bones, lungs, liver and CNS. Clinical presentation varies depending on the specific subtype of NLCH. Cutaneous manifestations range from solitary or multiple nodules to diffuse skin involvement. Bone lesions, especially joint lesions, can lead to symptoms of arthritis as well as pain, fractures and skeletal deformities. Pulmonary involvement often presents as interstitial lung disease, leading to respiratory symptoms. (Fritsch and Schwarz, 2018) An idiopathic hepatosplenomegaly with secondary thrombocytopenia can occur which can lead to splenic sequestration (Sangüeza et al., 1995).

The exact aetiology and pathogenesis of NLCH remain largely unknown. However, emerging evidence suggests that dysregulated immune responses and genetic predisposition may play a role. Abnormalities in monocyte/macrophage differentiation and activation have been implicated in NLCH. Genetic alterations, including mutations in genes involved in cytokine signalling pathways, have also been identified in some NLCH cases, providing insights into disease mechanisms. (Weitzman and Jaffe, 2005)

Accurate diagnosis of NLCH requires a multidisciplinary approach, combining clinical evaluation, histopathological examination and immunohistochemical (IHC) analysis. Histological features often reveal the presence of histiocytic infiltrates with distinct immunophenotypic markers. Additional investigations, such as imaging studies (X-rays, CT scans, MRI) and laboratory tests (complete blood count, liver function tests), are crucial for evaluating the extent of organ involvement and ruling out other potential differential diagnoses.

- Xanthogranuloma

XG is a rare NLCH of benign nature. It often presents at a very young age, with male patients being more affected than their female counterparts and a tendency to self-regression. (Hernandez-Martin et al., 1997; Vahabi-Amlashi et al., 2020) A distinction between JXG and AXG can be made, however AXG is merely defined as the occurrence of JXG in adulthood (Gartmann and Tritsch, 1963). XG lesions are often heterogenous, however varying depending on the maturity of the lesions.

Disseminated XG is not as common as singular, especially cutaneous, XG. It has a predilection for mucosal sites such as the upper respiratory tract. Though mostly still of a benign character, its appearance in certain locations, like the CNS, can have a mass effect and cause severe symptoms by compressing neighbouring tissue and even be lethal. Systemic disseminated XG can be treated with the systemic therapy used for LCH. (Pileri et al., 2022)

JXG is a benign NLCH that predominantly affects infants and young children with a higher incidence than AXG. JXG typically manifests as solitary or multiple papules or nodules on the skin, with a predilection for the face, neck and upper trunk. While JXG primarily affects infants younger than one year of age, it can also occur in older children. The characteristic lesions exhibit a reddish-brown colour in their early stages and transition to a reddish-yellow appearance as they mature. In some cases, JXG may involve extracutaneous sites such as the lungs, bones, heart and gastrointestinal tract. (Fritsch and Schwarz, 2018)

Histopathologically, JXG presents with monomorphous histiocytic infiltrates in the dermis during its early stages. As the lesions progress, foam cells, Touton giant cells and foreign-body giant cells become prominent in the superficial dermis. (James et al., 2011) IHC analysis reveals positive staining for CD68, CD11, CD36, factor XIIIa and vimentin, while markers such as S100B and CD1a are not expressed. (Sangüeza et al., 1995) It is differentiated from LCH by the absence of CD1a and S100 expression and the presence of stabilin-1 (Traupe and Hamm, 2006). Other differential diagnoses include molluscum contagiosum, haemangioma and neurofibroma, among others.

AXG is a rare NLCH also characterized by benign proliferation of histiocytes derived from the monocyte/macrophage lineage. AXG predominantly affects adults with a higher incidence in males. It often presents as solitary or multiple papules or nodules on the skin, with a preference for the face, neck and upper trunk. (Fritsch and Schwarz, 2018) The lesions exhibit a wide range of coloration, reflecting their varying cellular composition and maturity. Early lesions appear as brownish-red nodules or papules, composed of histiocytes, fibroblasts and a few Touton cells. In contrast, mature lesions exhibit a yellowish hue due to the increased presence of lipid-rich foam cells and Touton giant cells, accompanied by a higher proportion of fibroblasts. (Fritsch and Schwarz, 2018)

IHC analysis plays a crucial role in confirming the diagnosis of AXG. The expression of stabilin-1 has been identified as a common marker for AXG. (Traupe and Hamm, 2006) This marker and others help differentiate AXG from other histiocytic disorders and contribute to accurate diagnosis. Furthermore, IHC staining can aid in assessing lesion maturity and guiding treatment decisions.

Treatment options for XG depend on various factors, including lesion characteristics, patient age and location. Surgical excision and CO^2 laser therapy are common approaches for localized lesions. (Classen et al., 2016) However, the treatment approach should be tailored to the individual patient, considering the potential for self-regression.

- Rosai-Dorfman disease

Rosai-Dorfman(-Destombes) disease, otherwise known as sinus histiocytosis with massive lymphadenopathy (SHML), is a rare NLCH. RDD clinically presents as an indolent cervical lymphadenopathy that affects patients around the beginning of their third decade of life.(Mosheimer et al., 2017) Sometimes an extranodal organ manifestation, as well as isolated extranodal manifestation can occur. (Foucar et al., 1990; McClain et al., 2004) The disease course often varies but there are a few cases that report spontaneous regression. Disease surveillance appears difficult due to rare follow-up examinations. (Inoue and Onwuzurike, 2005; Mosheimer et al., 2017; Pagel et al., 2007)

RDD histopathology displays a typical histiocyte proliferation which can lead to sinus dilatation of the lymph node (Foucar et al., 1990). A pathognomonic feature of RDD histiocytes is that of emperipolesis, the non-destructive phagocytosis of lymphocytes and erythrocytes. Its presence is indispensable for diagnosis confirmation (Dalia et al., 2014; Rosai and Dorfman, 1969).

RDD seldom shows osseous involvement (10% of cases). Osseous manifestations can have a mass effect on its surrounding, like nearby nervous structures and thereby cause severe symptoms. Milder symptoms include pain and swelling. Mostly one or two bone regions are involved. The cranial bones and long bones are possibly the most predilected sites. Up to 75% of patients also have extraosseous involvement with sites in the soft tissue, lymph nodes and sinuses. In conclusion, the head and neck regions seem to be the most affected bone regions in RDD. (Foucar et al., 1990; McClain et al., 2004)

RDD treatment can consist of surgery, glucocorticoids or combination therapy, among others. Treatment intensity depends on the severity and manifestation of RDD. Due to the rarity of the disease, diagnosis and treatment are challenging. (Inoue and Onwuzurike, 2005; Pagel et al., 2007)

- Xanthoma disseminatum

XD is a rare benign, non-familial histiocytosis. It has a more xanthomatous histopathological presentation with lipid deposits than a histiocytic. (Ghorpade, 2009; Rupec and Schaller, 2002) The aetiology of this is unkown. (Yusuf et al., 2008) XD has been reported to be more of reactive than of neoplastic nature. (Eisendle et al., 2008) It clinically presents with xanthomatous lesions of the skin, typically affecting the flexural regions. XD lesions can also occur in ocular, oral, pharyngeal, visceral and skeletal sites. An involvement of the CNS can also be noticed. (Rupec and Schaller, 2002; Yağci et al., 2008) XD can manifest in any age. (Carpo et al., 1999) A male preference can be observed in XD, with a male to female ratio of 2:1. (Zak et al., 2006)

XD can be categorized in three clinical subtypes that also affect disease prognosis and vary in frequency of occurrence: persistent XD being the most prevalent, systemic progressive XD less prevalent and self-limited XD the least prevalent variant. (Zak et al., 2006) Differential diagnoses of XD are eruptive xanthomas, MH and LCH. Necessary diagnostic tools are clinicohistopathologic manifestation, serum lipoprotein levels and IHC. (Wayman and Margo, 2005)

XD was first described by Montgomery and Osterberg in 1938 (Montgomery and Osterberg, 1938). It remains one of the rarest histiocytic disorders with an estimated average incidence of 1.5 cases per annum (Pruvost et al., 2004). Although XD lesions are characterized by gradual self-regression, some lesions can at obstructive sites that may lead to high morbidity and/or mortality. Due to a frequent lack of response to therapy, the most efficient XD treatment is yet to be established. (Pruvost et al., 2004) However, a few cases presented a favourable response to therapy with cyclophosphamide (Seaton et al., 2004) and 2-chlorodeoxyadenosine (Khezri et al., 2011).

- Necrobiotic xanthogranuloma

Kossard and Winkelmann were the first to describe NXG in 1980(Kossard and Winkelmann, 1980). It is a rare form of NLCH with no known gender predilection. Age of onset is mostly in the sixth decade of life. An association with cell proliferative disorders, such as multiple myeloma (MM) or monoclonal gammopathy of undetermined significance (MGUS) is known in the medical literature.(Steinhelfer et al., 2022) NXG has an unknown aetiopathogenesis. It is assumed that paraproteins are of significance and play a part in the formation of granuloma (Chave et al., 2001; Dholaria et al., 2016; Kossard and Winkelmann, 1980; Silapunt and Chon, 2010). NXG clinically presents as asymptomatic lesions with yellow or brown macules or nodules. Mature lesions often develop telangiectasias, ulcerations, atrophies and/or scars (Gün et al., 2004). The most common site is in the periorbital area. Though rare, systemic involvement has also been described in the literature (Frank and Weidman, 1952; Hunter and Burry, 1985; Umbert and Winkelmann, 1995). The most common extracutaneous sites are located in the oropharyngeal tract, bronchi of the lungs, liver, spleen and heart (Mehregan and Winkelmann, 1992; Novak et al., 1992; Rose et al., 2012; Spicknall and Mehregan, 2009; Winkelmann et al., 1997). The histopathology of NXG often shows granulomas that can be found in the dermis and subcutaneous fat. Touton giant cells can also appear (Wood et al., 2009). Cholesterol clefts are pathognomonic (Hallermann et al., 2010). As NXG is rare, like many other histiocytoses, literature mostly contains of case reports and series. Many patients with NXG undergo prior therapy before finally receiving adequate treatment (Steinhelfer et al., 2022).

In a systematic review of March 2022, several papers from the literature were screened for systemic therapy of NXG. 79 papers and 175 cases were reviewed and analysed to detect the most effective therapy for NXG which was: intravenous immunoglobulins (IVIG), corticosteroids and combination treatments including corticosteroids. (Steinhelfer et al., 2022) Due to the lack of NXG therapy guidelines, prospective, and randomized controlled studies are needed to establish the best therapeutic approach, though, this is hard to execute due to the rarity of NXG.

- Erdheim-Chester disease

ECD is a type of NLCH that histopathologically presents with lesions infiltrated by CD68-positive, CD1a-/S100-negative foamy histiocytes (Haroche et al., 2012a). Their proliferation can be associated with mutations of MAPK components (Wright et al., 1999). It is a rare disease with a preponderance of older male patients, with an average age of onset between the fifth and seventh decade of life (Haroche et al., 2014). In the WHO classification, ECD is described as a histiocytic neoplasm (Swerdlow et al., 2016). Though, its origin is unknown, in the literature, it has been discussed whether ECD is of a malignant or reactive nature (Mazor et al., 2013).

ECD has a varying clinical presentation. Symptoms may present themselves in form of neurological deficits, bone pain and diabetes insipidus. Predilected sites of ECD are the retroperitoneum, skin, heart and lungs. (Cavalli et al., 2013; Diamond et al., 2014) Neurological involvement plays a crucial role in the onset of severe functional disabilities in most patients. This can lead to a higher mortality of ECD (Lachenal et al., 2006). Typical diagnostic tools, such as MRIs, tissue biopsies and immunohistochemistry (IHC) are vital in detecting the extent of ECD manifestation. ECD treatment often includes a combination of surgery and pharmacological therapy (Boyd et al., 2020).

Due to various studies that describe activating mutations of BRAF in 54% of ECD patients, ECD therapy has been revolutionised. Targeted therapy with BRAF inhibitors have vastly improved treatment options. (Haroche et al., 2012b; Munoz et al., 2014; Rizzo et al., 2014). Therapy particularly used are BRAF inhibitor vemurafenib (Haroche et al., 2013), the anti-TNF-α monoclonal antibody (moAb) infliximab (Dagna et al., 2012) and the interleukin-1 receptor (IL-1R) antagonist anakinra (Aouba et al., 2010). The overall clinical response is favourable, yet inconsistent.

- Generalized eruptive histiocytosis

GEH is often an asymptomatic self-healing NLCH. It mostly affects young adults. GEH lesions appear as symmetrical papular, non-lipidic eruptions of several blue/red papules in successive crops.on face, trunk and proximal extremities, usually vanishing spontaneously. (Aso et al., 1982; Chu, 2010)

Clinicohistopathological and IHC studies play an significant part in diagnosing and differentiating GEH from other infiltrative diseases, like leprosy. (Sharath Kumar et al., 2011) It stains positively for CD68, factor XIIIa and HAM-56, however no positivity is displayed with S100 (Salsberg, 2019).

A case report of Y-J. Deng et al. in the British Journal of Dermatology suggest a possible therapeutic assessment with a combination of glucocorticoids and hydroxychloroquine or thalidomide to improve GEH treatment. Furthermore, follow-up examinations are necessary for further evaluation of adequate therapy. (Deng et al., 2004)

Management of NLCH depends on the subtype, extent of disease and organ involvement. Therapeutic options include observation, surgery, systemic therapies (corticosteroids, chemotherapy, immunomodulatory agents) and targeted therapies (Classen et al., 2016). The choice of treatment modality should be individualized, considering disease severity, patient age, organ dysfunction and potential adverse effects. Multidisciplinary collaboration involving dermatologists, haematologists/oncologists, rheumatologists and other specialists is crucial for optimal patient care.

Recent advancements in the understanding of NLCH have shed light on its molecular pathogenesis and potential therapeutic targets. Genetic studies have revealed recurrent mutations and gene fusions in certain NLCH subtypes, providing opportunities for targeted therapies. (Wu et al., 2022) Moreover, the identification of specific cytokine dysregulation and immune cell interactions has opened avenues for immune-modulating approaches. Clinical trials investigating novel agents and immune-based therapies are underway, aiming to improve outcomes for patients with NLCH.

Histiocytosis	Age, sex	Typical locations	Appearance, symptoms, clinical features	Immunophenotype	Therapy
(Juvenile) xanthogranuloma	Children more commonly than young adults; male more often than female	Head, neck, upper trunk; buccal possible; any site possible	Single nodule (>2cm) or multiple papules (red to yellow brown)	$CD68^+$, $CD163^+$, $FXIIIa^+$, $CD1a^-$	Chemotherapy, surgery, radiotherapy, haematopoietic stem cell transplantation depending on the involvement
Rosai-Dorfman(-Destombes) disease	Mean age, 20.6 years, mostly males affected	Cervical localization	Lymphadenopathy; cutaneous nodules; obstruction of upper respiratory tract, nosebleed, deformity of the nasal dorsum; dyspnoea, dry cough; signs of CNS involvement; testicular enlargement	$S100^+$, $CD68^+$ and $CD1a^-$	Corticosteroids, sirolimus, methotrexate, azathioprine
Xanthoma disseminatum	Young adults; male more often than female	Any skin, eyelid, skin folds; mucosae involved in 50% of patients, sometimes life-threatening	Hundreds of lesions: symmetrical, coalescing, round to oval, orange to yellow-brown papules and nodules	$CD68^+$, $CD163^+$, $FXIIIa^+$, $CD1a^-$	Spontaneous resolution, conservative surgery
Necrobiotic xanthogranuloma	Median age 55 years; male and female equally often	Face, mostly periocular; frequent eye involvement; trunk	Xanthelasmas on upper eyelids, confluent nodules forming firm yellowish plaques; telangiectasias; ulceration; atrophy	$CD68^+$, $CD163^+$, $FXIIIa^-$, $CD1a^-$	IVIG, corticosteroids and combination therapies including corticosteroids
Erdheim-Chester disease	Between 40-70 years; men are more affected than women	CNS, osseous, cutaneous, retrobulbary and peritoneal involvement	Lower limb pain; general symptoms (fatigue, weight loss, fever); xanthelasma; diabetes insipidus; exophthalmos; dyspnoea, dry cough; signs of cardiac involvement (for example, tamponade); signs of CNS involvement (degenerative or tumoural)	$CD68^+$, CD207, S100 weak positive	IFN α-2a or pegylated IFN-α-2a; other potential options are anakinra, infliximab, sirolimus plus corticosteroids; BRAF or MEK inhibitors in life-threatening cases (for example, CNS or heart involvement)
Generalized eruptive histiocytosis	Young adults more commonly than children; male more often than female	Trunk and proximal limbs; spares skin folds and mucosae	Innumerable macules and papules that vary from flesh-coloured to red	$CD68^+$, $CD163^+$, $FXIIIa^+$, $CD1a^-$	Corticosteroids, PUVA

Table 1.2: Overview of non-Langerhans histiocytoses. (Abla et al., 2018; Alexander et al., 2005; Diamond et al., 2014; Emile et al., 2021; Goyal et al., 2020; Lan Ma et al., 2007; Papo et al., 2019; Ratzinger and Zelger, 2018; Steinhelfer et al., 2022; Zou et al., 2023)

1.3 Diagnostic markers

Diagnostic markers play a crucial role in accurately identifying and distinguishing various diseases and conditions. In this subchapter, I will focus on five specific diagnostic markers: S100, CD1a, CD207, stabilin-1 and CD68 in order to provide an in-depth exploration of these markers, their significance in different pathological contexts and their utility in clinical practice.

S100 is a calcium-binding protein that is widely expressed in various cell types, including melanocytes, glial cells and antigen-presenting cells. The clinical presentation of LCH may vary from mild chronic forms with a good prognosis to multisystemic forms with therapy-resistance and high mortality. LCH cells have Birbeck granules containing CD207and high concentrations of S100B. Additionally, serum S100B appears to be of prognostic and predictive value regarding the therapeutic outcome of metastatic melanoma. (Porter and Scully, 2000) The sensitivity of S100 protein for LCH is high, reported by various authors to be in the range of 90% to 100%. (Hage et al., 1993; Helm et al., 1993; Kahn and Thorner, 1990; Kanitakis et al., 1991; Mierau and Favara, 1986) S100 protein is not specific for LCH cells (Kanitakis et al., 1991; Mierau and Favara, 1986).

Despite the CD1 molecule being the first described in the CD nomenclature system in 1982(1984), it still belongs to one of the least understood CDs in terms of its functional roles. The density of CD1a-positive DC within human tumours has been associated with survival. (Coventry and Heinzel, 2004) In contrast to NLCH, LCH lesions often contain CD1a/CD207-positive mononuclear phagocytes (Berres et al., 2015). In a Study of Piotr Dziêgiel et al, co-expression of CD1a with CD207 was noted in 11 cases, as well as the presence of Birbeck granules. Furthermore, in all examined biopsies the expression of S100 protein on inflammatory cells was found. The results corroborate the usefulness of IHC studies on CD1a and CD207 expression in diagnosis of LCH. (Dziegiel et al., 2007)

Langerin (CD207) is a marker that owes its name to its appearance in the Birbeck granules of LC. It is almost solely expressed in LC and plays an important part in the induction of their synthesis. (Valladeau et al., 2000)

Stabilin-1 (STAB1, FEEL-1, CLEVER-1, KIAA0246, MS-1) is a scavenger receptor protein that can be found in splenic sinusoidal endothelial cells. Furthermore, it is expressed by tissue macrophages and other sinusoidal endothelial cells. Its expression is generated when chronic inflammation and tumourigenesis occur. (Kzhyshkowska et al., 2006) Five cases of JXG and one case of NXG were stabilin-1-positive, while granulomatous diseases that expressed Th1-cytokines, like sarcoidosis were stabilin-1-negative. Stabilin-1-positive vessels were found in NLCH and melanocytic lesions. (Schönhaar et al., 2014)

CD68 is a glycoprotein expressed on macrophages and monocytes, serving as a pan-macrophage marker. It plays a vital role in IHC evaluation of various disorders. CD68 staining is widely utilized to identify and quantify macrophages in inflammatory conditions as well as in tumours (for example, invasive ductal carcinoma and melanoma (Pernick et al., 1999)). (Emile et al., 1994)

Diagnostic markers such as S100, CD1a, CD207, stabilin-1 and CD68 play indispensable roles in differentiating and characterizing various diseases. Their IHC detection aids in accurate diagnosis, differential diagnosis and monitoring disease progression. Understanding these markers' expression patterns and significance enhances our diagnostic capabilities and improves patient care and management.

1.4 Experimental markers

Histiocytic disorders encompass a diverse group of diseases characterized by abnormal proliferation and accumulation of histiocytes (Desai et al., 2013). While several diagnostic markers have been established for histiocytosis, the evaluation of additional markers, such as markers of the MAPK pathway, neurotrophic tyrosine receptor kinase gene (NTRK) family proteins and programmed cell death-ligand 1 (PD-L1), can possibly provide further insights into disease pathogenesis and potential therapeutic targets. This subchapter aims to investigate the origin of these markers and their potential relevance to histiocytoses.

1.4.1 MAPK pathway

MAPK signalling pathway dysregulation has been implicated in various malignancies, including some histiocytic disorders. Mutations in genes encoding proteins involved in this pathway, such as BRAF and MAP2K1, have been identified in specific histiocytic subtypes. The activation of MAPK pathway components suggests their potential role in promoting histiocytic proliferation and survival *(Figure 1.1).* Therefore, exploring the expression and activation status of MAPK pathway markers could aid in understanding the underlying molecular mechanisms driving histiocytoses. (Chakraborty et al., 2014; Nelson et al., 2014; Nelson et al., 2015)

Over 90% of LCH and ECD cases share MAPK mutations. An (over)activation of the pathway is responsible for ERK phosphorylation and nuclear translocation, in LCH without detected mutations. (Chakraborty et al., 2014) Various proportions of cases of other histiocytoses (Diamond et al., 2016; Go et al., 2014) , including the “R” group histiocytosis (Fatobene et al., 2018; Richardson et al., 2018) , also show MAPK pathway mutations. The aetiopathogenesis of RDD, however, is varies across its phenotypic spectrum. (Abla et al., 2018) In a study of 55 cases of JXG, 73% had a mutation in the MAPK pathway. (Durham et al., 2019) Furthermore, mutations activating the MAPK pathway could be found in a series of 21 MH cases. (Egan et al., 2020) Understanding the relationship between MAPK and histiocytes is critical to unravelling the pathogenesis of histiocytic disorders and developing targeted therapies to restore normal MAPK signalling and halt disease progression.

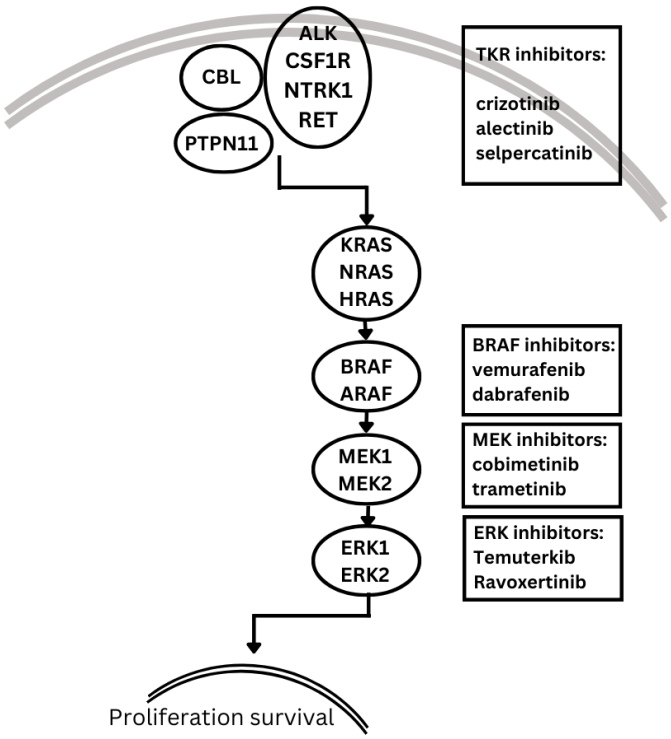

Figure 1.1: Proteins of the MAPK pathway with clinically approved inhibitors*.
This figure shows all involved proteins, from the tyrosine kinases in the cell membrane to the activation of the RAS-RAF-MEK-ERK pathway. At every point a mutation or fusion can take place, inhibitors are described. Figure adapted and modified from Emile et al., 2021 (Emile et al., 2021; PubChem, 2023; Yang et al., 2023)
**except ERK inhibitors: currently in clinical trials*

1.4.2 TRK receptors

Tropomyosin receptor kinase (TRK) receptors immunohistochemically serve as stable diagnostic markers. They can present in non-NTRK-rearranged mesenchymal malignancies (Brčić et al., 2021). While TRK receptor alterations have not been extensively studied in histiocytoses, their potential presence suggests a possible role in disease development. They can be used as a cost-efficient alternative to mutation analyses. As surrogate markers, they can help detect NTRK fusions. However, mutation analysis, more specifically RNA-based next-generation sequencing (NGS), is indispensable for identification and confirmation of specific NTRK fusions before affected patients can receive targeted therapy (Brčić et al., 2021). Evaluating the expression and fusion status of TRK receptors in histiocytic lesions may shed light on novel diagnostic and therapeutic avenues for targeted therapy.

The relationship between TRK receptors and histiocytes is of utmost importance in understanding the molecular mechanisms underlying histiocytic disorders. TRK receptors are a family of receptor tyrosine kinases that are involved in regulating cellular growth, differentiation and survival.

Recent studies have identified activating mutations and fusions involving TRK genes in certain histiocytic disorders, such as ECD (Huang and Feng, 2019) and RDD (Dabbs, 2017). These genetic alterations lead to constitutive activation of TRK receptor signalling, resulting in dysregulated histiocyte proliferation and survival. Targeting TRK receptor aberrations with specific inhibitors has shown promising therapeutic effects in histiocytic disorders, highlighting the critical role of TRK receptors in histiocyte biology and their potential as therapeutic targets (Brčić et al., 2021).

1.4.3 PD-L1

PD-L1 (CD274) is a ligand expressed by tumour cells. It prevents the activation of cytokine production and cytotoxic activity by binding with its receptor, programmed cell death protein 1 (PD-1 or CD279). PD-1 is a T-cell receptor of inhibitive nature. PD-1-positive tumour-infiltrating T lymphocytes promote tumour progression. (Gatalica et al., 2015) PD-L1/PD-1 interaction can be inhibited by using PD-L1 specific immune checkpoint inhibitors to treat various malignancies, such as melanoma, renal cell carcinoma and non-small cell lung cancer. (Herbst et al., 2014; Taube et al., 2014; Topalian et al., 2012)

PD-L1 and histiocytes are crucial in the context of immune regulation and tumour microenvironment. PD-L1 is a cell surface protein that interacts with its receptor PD-1 on immune cells, including histiocytes. This interaction plays a significant role in suppressing immune responses and promoting immune tolerance. (Herbst et al., 2014; Taube et al., 2014; Topalian et al., 2012) In the tumour microenvironment, histiocytes can express PD-L1, enabling them to engage with PD-1 on T cells and inhibit their function (Xu et al., 2016). This immune evasion mechanism is exploited by tumours to escape immune surveillance. Understanding the interplay between PD-L1 expression on histiocytes and the immune response is essential for developing targeted immunotherapies that can effectively modulate the tumour microenvironment and increase anti-tumour immune reaction (Proietti et al., 2020).

1.5 Aim of the project

Current literature shows an association of MAPK pathway activation with severity and hereby prognosis of histiocytosis development. To confirm this observation, I focused on a potential correlation between the expression intensity of members of the MAPK pathway and the occurrence of high-risk cases, like cases with involvement of risk organs, with disseminated, multisystemic or multifocal clinical presentation in histiocytosis. Due to their targetable nature, the expression intensity of NTRK family proteins and PD-L1 were also observed in order to detect correlation between these markers and high-risk histiocytic disorders. My focus will be on LCH and XG (AXG and JXG), as their cohort size allowed a thorough examination within and between both groups regarding the experimental marker expressions and patient data. Due to the insufficient sample sizes of the other histiocytoses, solely a descriptive analysis of results was possible.

This project is composed of two phases: In the first phase, clinicohistopathological data and tissue samples were acquired in a multicentric setting in which six German dermatology clinics participated. In the second phase, LCH and NLCH tissue samples were analysed immunohistochemically with their known diagnostic markers and additionally with experimental markers. The examined histiocytic disorders were LCH, AXG and JXG, NXG, XD, RDD, ECD, GEH.

The aim of this project was to analyse clinical data of histiocytosis patients and identify clinical parameters to better understand the demographics of my cohort and draw more specific conclusions when targeting the MAPK pathway, NTRK family and PD-L1. Additionally, potentially targetable markers of patients diagnosed with either LCH or NLCH should be identified. The intent was to analyse druggable key signalling pathways and molecules, as to increase the therapy options of patients with severe or therapy resistant histiocytosis forms, like cases with involvement of risk organs, with disseminated, multisystemic or multifocal clinical presentation and recognize the prognostic value of the analysed parameters according to their expression. The analysed components were members of the MAPK pathway, proteins of the NTRK family and the immunorelevant PD-L1.

2 MATERIAL AND METHODS

2.1 Materials

Table 2.1: Primary antibodies

antibody	company	catalogue number	dilution in blocking buffer	pH	host
BRAF V600E Clone VE1	SpringBioScience	E19290	1:100	9.0	mouse
p44/42 MAPK (ERK1/2) (137F5)	Cell Signaling Technology	4695S	1:250	6.0	rabbit
ERK2 (E460)	abcam	ab32081	1:300	6.0	rabbit
MEK1/2 (L38C12)	Cell Signaling Technology	4694S	1:40	9.0	mouse
MEK2 (Y78)	abcam	ab32517	1:500	6.0	rabbit
CD68 Clone PG-M1	Dako	M0876	1:50	9.0	mouse
PD-L1 (E1L3N)	Cell Signaling Technology	13684S	1:10	9.0	rabbit
pan-TRK (EPR17341)	abcam	ab181560	1:100	9.0	rabbit
S100 (B)	Dako	Z0311	1:500	6.0	rabbit
CD1a clone 10	Dako	M3571	1:100	9.0	mouse
stabilin-1 clone RS1	Peptide Specialty Laboratories	Box 4,22	1:500	6.0	rabbit
CD207 (Langerin) 12D6	Medac	392M-15	1:40	9.0	mouse
phospho-MEK1/2 (Ser221) (166F8)	Cell Signaling Technology	2338S	1:10	9.0	rabbit
phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (20G11)	Cell Signaling Technology	4376	1:40	9.0	rabbit

Table 2.2: Chemicals (including reagents and kits / solutions and buffers)

product	**company**	**catalogue number**
aqua dest.	Fresenius Kabi	13KBP211
Dako Antibody Diluents	Dako	S0809
Dako Faramount Aqueous Mounting Medium	Dako	S3025
Dako Pen	Dako	S2002
ethanol	Roth	K928.4
Mayer's hemalum solution	Merck	1.09249-2500
Eosin Y-solution 0.5%, aqueous	Merck	1.09844.1000
HIER Citrate Buffer	Zytomed	ZUCO028-500
HIER T-EDTA Buffer	Zytomed	ZUCO029-500
PBS Buffer 1×	PanReac Applichem	A0964
Tris-Wash Buffer, TBS 20×	Zytomed	ZUC52-500
Tween20 1%	Sigma Life Science	SLBx6047
Xylol	Roth	K928.4
Dako Kit Rabbit: - Peroxidase-Blocking Solution - Dako EnVision+ System- HRP Labelled Polymer Anti-Rabbit - AEC Substrate Chromogen	Dako	K4009
Dako Kit Mouse: - Peroxidase-Blocking Solution - Dako EnVision+ System- HRP Labelled Polymer Anti-Mouse - AEC Substrate Chromogen	Dako	K4005

Table 2.3: Laboratory equipment and routine laboratory material

product	company	catalogue number
laboratory incubator	Heraeus Instruments	T6060
humidity box 4 °C	Simport	M920-2
refrigerator 4 °C	Liebherr	042.KÜS.207
microscopes	Leitz Olympus	Laborlux K BX51
Autostainer XL	Leica	ST5010
water bath	Memmert	Type WNB 10
rotary microtome	Leica	RM2065
tissue arrayer	3DHistech	TMA Grand Master
slide scanner	Leica	Aperio AT2
cover glasses 24×60 mm	Engelbrecht	KN00010044452
slides for HE 76×26 mm	R. Langenbrinck	03-0004
slides for IHC 25×75×1 mm	R. Langenbrinck	03-0060

Table 2.4: Software tools

software	version	company
QuPath	0.3	developed at University of Edinburgh, UK
Endnote	20.4	Clarivate Analytics, London, UK
SAS	9.4	SAS Institute, Cary, USA

2.2 Methods

2.2.1 Study concept

In the present study, tissue microarray (TMA) data from patients with a diagnosed form of histiocytosis were analysed. Data and tissue samples of these patients were collected within the scope of a multicentric study in which six German dermatology clinics participated. These teaching hospitals were located in Mannheim, Tübingen, Augsburg, Würzburg, Dresden and Frankfurt am Main. The tissue punches were taken from infiltrate-rich regions of LCH, AXG and JXG, NXG, XD, RDD, ECD and GEH.

Only tissue samples with histopathologically confirmed diagnoses were collected from the tissue archives and included in the study. Additionally, specific IHC stainings were performed against diagnostic markers, such as S100, CD1a, CD207 for LCH and stabilin-1 and CD68 for NLCH, to verify each diagnosis.

Three TMAs were generated using collected tissue samples. Proteins of the RAS-RAF-MEK-ERK pathway, NTRK family proteins and PD-L1 were immunohistochemically stained with specific antibodies. After digitalization, IHC analyses were performed using an open-source software for digital pathology, QuPath 0.3. Analysis was based on a semiquantitative IHC scoring method, which incorporates staining intensity and percentage of positive cells. Following the IHC analysis, results were statistically evaluated with the aim of detecting possible correlations between experimental marker expressions and clinical and/or histopathological data.

2.2.2 Patient collective

The patient collective of this nationwide multicentric study includes patients from six German dermatology clinics. After contacting 54 centres nationwide with 17 responses, of which 11 were in the affirmative, 6 teaching hospitals ultimately participated. These centres contributed a total of 124 cases. Data collection happened between September 2018 and March 2020. Only 69 out of 124 cases had histological samples, thereby excluding the other 55 from the study. The collected samples dated from 1998 to 2019.

A positive vote on ethics (2018-816R-MA) was issued for this study with the conditions to solely evaluate pre-existing patient data retrospectively without contacting, interviewing, or examining patients. Evaluation of the data had to be performed in an anonymized or pseudonymized manner.

After undergoing pre-selection, the cohort contained 11 patients with diagnosed LCH cases, 25 patients with diagnosed XG, 3 patients with diagnosed NXG cases, 1 patient with a diagnosed XD, 4 patients with diagnosed RDD cases, 1 patient with a diagnosed ECD, 2 patients with diagnosed cases of GEH and 1 patient with a diagnosed NLCH, NOS (not otherwise specified). Cases with no, low and medium expression of diagnostic markers (S100, CD1a, CD207, stabilin-1 and CD68) were excluded.

In summary, this study presents data from 3 TMAs containing 192 tissue punches from 69 patients with a diagnosed form of histiocytosis. 74 TMA punches were taken from LCH, 82 punches from XG, 9 punches from NXG, 3 punches from XD, 12 punches from RDD, 3 punches from ECD, 6 punches from GEH and 3 punches from NLCH, NOS. IHC stainings against experimental markers of LCH and XG were statistically analysed, determining cut-off values for groups with clinical high-risk factors, such as involvement of risk organs, disseminated, multisystemic or multifocal clinical presentation. The small sample size of the other histiocytoses only allowed to describe and discuss the immunostaining results without further statistical analysis.

2.2.3 TMA preparation

Prior to the TMA production, collected formalin-fixed paraffin-embedded (FFPE) tissue blocks were cut into thin slices, affixed on microscope slides and haematoxylin-eosin (HE) stained. HE staining was performed with the Autostainer XL from Leica. Initially, tissue sections were deparaffinized using a xylene bath 4 times for a duration of 1 minute each. This was followed by the removal of xylene and hydration of the samples with a series of ethanol baths with gradually reduced ethanol concentrations (99%, 96% and 70%) for 1 minute each. Prior to and after haematoxylin staining for 4 minutes, slices were rinsed in water for 1 and 10 minutes. Afterwards, the samples were counterstained with eosin 0.5% for 2 minutes. This was followed by a short water bath of 30 seconds. Once rinsed with water, the slices were differentiated with 80% ethanol for 30 seconds and dehydrated by incubating 2 times in 96% ethanol for 30 seconds each and 3 times in 99% ethanol for 1 minute each. The staining procedure was concluded with clearing the samples by incubating 2 times in a xylene bath for 1 minute each. This was followed by mounting coverslips with mounting medium (Eukitt) *(Table 2.3)*.

The slides were inspected under the microscope before and after staining for aspects such as quantity and quality of tissue, visibility of infiltration by histiocytosis typical cells, abnormal cells or inflammatory cells. Three regions of interest of a size of 1.0 mm were marked for punching on each slide and an array plan of the planned TMAs was generated, grouping the tissue punches by diagnosis: TMA 1 for LCH, TMA 2 for XG and TMA 3 for other histiocytoses. Based on the generated TMA array plans, TMAs were created before proceeding to the IHC stainings

2.2.4 TMA production

The concept behind TMAs is that of a high-throughput method. It enables a rapid and simultaneous analysis of large amounts of tissue samples whilst preserving the rest of the specimen by specifically excerpting regions of interest. Other methods would require the use of the complete sample. Representative tissues from FFPE samples are retrieved as donors and inserted into an acceptor paraffin block in a predetermined array. With the TMA method, analyses can be performed with an increased reliability of measures and less subjection to experimental variability. Once all punched cylindric cores of a predefined size are amalgamated into one TMA, sections of varying sizes are cut from the block and stained, for example immunohistochemically in order to identify certain protein expressions in tissues with similar pathologies. (Kampf et al., 2012; Kononen et al., 1998)

For this study, a core size with a diameter of 1 mm was selected. A maximum of three core biopsies was extracted from all 71 samples from 69 patients. This amounted to a total of 192 tissue punches. These cores were embedded in 3 TMA recipient blocks using the automated tissue arrayer, TMA Grand Master *(Table 2.3)*. Although each recipient block can incorporate up to 96 tissue cores, the blocks were filled according to diagnosis: LCH, XG and other histiocytoses. Due to this categorization, the blocks were only filled with 78 cores in TMA1, 86 cores in TMA2 and 38 cores in TMA3. Each block contained a total of 4 muscle punches, 2 samples per array. Muscle samples at the start of each array served as orientation cores.

After the production of the TMA samples, IHC stainings were performed within the framework of my study using various antibodies in order to stain against diagnostic markers that were used to confirm the diagnoses in the TMA cores before proceeding with IHC stainings against nine experimental markers.

2.2.5 TMA processing

Prior to IHC staining, the TMA blocks were cut into 1 µm thin sections and affixed on microscope slides, similar to the procedure used to prepare the FFPE tissue blocks for HE staining. IHC staining with any of the 14 used antibodies began with the deparaffinization of samples in a xylene bath 3 times for 5 minutes. Afterwards, xylene was removed and the slices were hydrated by incubating them, first in absolute ethanol twice for 3 minutes and then in a series of solutions with gradually reduced ethanol concentration (90%, 80% and 70%) for another 3 minutes each. Afterwards, the samples were rinsed with distilled water for 15 seconds *(Table 2.2).*

For staining with the primary antibodies (anti-BRAF V600E, anti-MEK1/2, anti-CD68, anti-PD-L1, anti-pan-TRK, anti-CD1a, anti-CD207, anti-phospho-MEK1/2 (anti-pMEK1/2) and anti-phospho-ERK1/2 (anti-pERK1/2)), the TMA samples were washed with Tris-buffered saline (TBS) for 5 to 10 minutes *(Table 2.1)*. For antigen retrieval, slides were cooked in HIER T-EDTA Buffer (pH 9.0) at 95°C for 30 minutes using a water bath. After a 20–30-minute cool down phase, the samples were encircled with a hydrophobic circle using a Dako pen to prevent reagents that were to be applied in the following steps from running down the slide. This was followed by further rinsing with distilled water for 15 seconds and a repeated treatment with TBS, this time performed twice for 5 minutes each. Afterwards, samples were hydrated with a combination of TBS and Tween20 (1%) (TBST) for 5 minutes and incubated in the humidity box at room temperature (RT) for 10 minutes using peroxidase-blocking solution. Upon being washed twice in TBS for 5 minutes and in TBST for additional 5 minutes, the primary antibodies were diluted with a 1:100 ratio for BRAF V600E, a 1:40 ratio for MEK1/2, a 1:50 ratio for CD68, a 1:10 ratio for PD-L1, a 1:100 ratio for pan-TRK, a 1:100 ratio for CD1a, a 1:40 ratio for CD207 and a 1:10 ratio for pMEK1/2 and a 1:40 ratio for pERK1/2 using Dako Antibody Diluent and applied to the respective samples. The samples incubated overnight in the humidity box at 4 °C. Samples that were not incubated with primary antibody served as negative controls. The next day, prior to and after the 1-hour incubation with secondary antibody Dako EnVision+ System- HRP Labelled Polymer Anti-Rabbit in the humidity box at RT, the TMA slides were washed in TBS twice for 5 minutes and in TBST for another 5 minutes. Thereafter, a drop of 3-Amino-9-Ethylcarbazole (AEC) Substrate Chromogen was applied for a duration of 10 to 30 minutes. Staining was stopped by rinsing the samples with aqua dest. and counterstaining with Mayer's hemalum solution (1:10) for 7 minutes. This was followed by double rinsing with tap water for 3 to 5 minutes and distilled water. The immunostaining procedure was concluded with the stabilization of samples with Dako Faramount Aqueous Mounting medium before mounting the cover slip *(Table 2.3)*.

For the other primary antibodies, anti-ERK1/2, anti-ERK2, anti-MEK2, anti-S100 and anti-stabilin-1, a nearly identical staining procedure was implemented except for the following details: Immunostaining with these antibodies involved the use of HIER Citrate Buffer (pH 6.0) as a substitute for T-EDTA Buffer, with the samples incubating in a water bath at 95°C for 1 hour instead of 40 minutes. Incubation of these primary antibodies with Dako Antibody Diluent was performed in a dilution of 1:250 for ERK1/2, 1:300 for ERK2 and 1:500 for MEK2, for S100 and for stabilin-1. The washing buffer for all TMA slides could easily be replaced with phosphate-buffered saline (PBS) without further consequences *(Table 2.2)*.

2.2.6 Digitalization and immunohistochemical analysis

After successful completion of all stains, the stained TMAs were scanned digitally at 400x magnification using a slide scanner (Aperio AT2), thereby making a digital evaluation and analysis of the slides and performed immunostainings possible. The scans were viewed with the software tool QuPath *(Table 2.4).* Each scan was viewed and reviewed to safeguard its quality. With the function of *TMA dearraying*, the scans were sorted according to their array plan location. TMA cores were also viewed and reviewed according to evaluability, excluding those that lacked tissue or were not evaluable due to artefacts. The evaluation was performed by 2 independent investigators, one of which is an experienced dermatohistopathologist.

Analysis of the stained slides was based on the semiquantitative multiplicative quickscore method (Brazão-Silva et al., 2013; Detre et al., 1995), which allows the evaluation of expression intensity and proportion of immunopositive cells by estimation. In this study, I used a modified approach. The staining intensity was categorized into three groups (1-3), lowest intensity = 1, medium intensity = 2 and highest intensity = 3. Depending on the location of the antigen, the staining was categorized as nuclear and/or cytoplasmic. In addition to the intensity score, the quantity of positive cells in percentage of the whole punch was estimated and grouped accordingly (0-4): 0% = 0, 1-25% = 1, 26-50% = 2, 51-75% = 3, 76-100% = 4. Multiplication of both evaluated scores yielded the IHC score ranging from 0-12. (Thanan et al., 2016)

Prior to the analysis of the experimental markers, a pre-selection of all TMA samples took place as a plausibility check to verify the presence of LCH or NLCH cells in each punch. The scores of the diagnostic markers (anti-S100, anti-CD1a and anti-CD207 for LCH and anti-stabilin-1 and anti-CD68 for NLCH) were used to confirm the known diagnosis of each case. Each patient was represented by a maximum of three TMA punches, each receiving individual scores, which were summarized by determining the mean value after excluding category outliers. Solely for the purpose of pre-selection, the scores ranging from 0-12 were categorized in no expression (0), low expression (1-3), medium expression (4-6), high expression (7-9), very high expression (10-12) (Thanan et al., 2016). After allocating each punch to one of the categories, the mean score value of each patient was determined. Samples that had mean values showing a high to very high marker expression counted as confirmed cases. With this method, 11 out of 28 LCH cases, 25 out of 29 XG cases and all other histiocytoses cases were confirmed, thereby excluding cases with no to medium marker expression from further IHC analysis. The evaluation of the experimental markers proceeded with the confirmed cases.

2.2.7 Statistical analysis

Data management was performed using Excel 2010 *(Table 2.4).* Statistical analysis was performed using SAS software (version 9.4) *(Table 2.4).* LCH and XG were the focus of the statistical analysis as their respective cohort size allowed analyses and comparisons within and between both groups regarding the experimental marker expressions and the clinicohistopathological data. Due to the insufficient cohort size of the other histiocytoses, their score results can solely be of descriptive nature.

For the statistical analysis, the median of all TMA punches belonging to one tissue sample was calculated and assigned as the main score value. Due to the asymmetrical distribution of data, the Mann-Whitney U test was used to determine if there was a difference between the groups formed, setting the significance level at 5%. Following the U test, logistic regressions were performed to determine if an effect of each marker could be found using standard regression coefficients. Additionally, logistic regression was used to establish the cut-off value of significant markers. The cut-off value defined the mark at which a patient in all probability belonged to LCH or XG. Finally, multiple regression analysis was performed to find out if the combination of markers increased the effect on the analysed group.

3 RESULTS

Histiocytosis describes a heterogenous group of rare disorders of the MPS. These disorders can clinically present in any organ with varying severity and age of onset. They can manifest in form of a single cutaneous lesion with spontaneous regression or as a severe therapy-resistant disseminated disease. Salvage therapy is needed in, for example, therapy resistant histiocytoses. Recent targeted therapy options are BRAF inhibitors for patients with verified BRAF mutations and MEK inhibitors for patients with BRAF wild-type.(Emile et al., 2016)

The aim of this study is to find more targetable markers that increase the range of possible treatment for LCH and NLCH patients. There is emerging evidence suggesting that dysregulated immune responses and genetic predisposition appear to play a role in the pathogenesis of histiocytoses. For this reason, the analysed parameters in this study were MAPK pathway components, proteins of the NTRK family and immunorelevant PD-L1.

In this work, clinicohistopathological and TMA data from patients with a diagnosed form of histiocytosis were analysed. Data and tissue samples of patients with LCH, AXG and JXG, NXG, XD, RDD, ECD and GEH were collected within the scope of a multicentric study. Only tissue samples with confirmed diagnoses were stained immunohistochemically. IHC staining was firstly performed against histiocytosis specific diagnostic markers (S100, CD1a, CD207, stabilin-1, CD68), then against proteins of the MAPK pathway, NTRK family proteins and PD-L1.

XG and LCH were each grouped in high- and low-risk subtypes. AXG and JXG were combined to form XG, which was then divided into two groups: disseminated XG and XG with singular lesions, while LCH was also divided into two groups: high-risk LCH (containing MS-LCH, RO^{+}LCH and multifocal LCH) and low-risk LCH (containing SS-LCH, RO^{-}LCH and unifocal LCH). They were analysed based on their IHC scores. As previously described, the IHC score is a product of the marker expression intensity and proportion of immunopositive cells. The higher the marker expression, the higher the IHC score ranging from 0 to 12. Antibodies of significant value were the following: anti-PD-L1, anti-ERK2, anti-MEK2, anti-pMEK1/2, anti-MEK1/2 and anti-ERK1/2 *(Figure 3.1).*

The first statistical analysis using the U-test revealed some significant differences between the examined groups. For this reason, the U-test was followed up by logistic regressions. These were performed to determine if an effect of each marker could be found using standard regression coefficients. Furthermore, cut-off points were determined to better help the distinction between LCH and NLCH, more specifically XG. Additionally, multiple regression analysis was performed with the intent to detect potentially significant marker combinations that surpassed the effect of individual markers on the analysed group. Lastly, the other histiocytoses were analysed by measures of central tendency and statistical dispersion with the aim to find high experimental marker expressions among them.

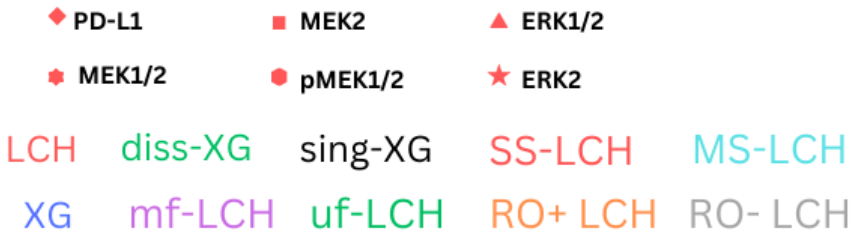

Figure 3.1: Legend of following charts
Diss-XG: disseminated XG; sing-XG: singular XG; mf-LCH: multifocal LCH; uf-LCH: unifocal LCH

3.1 XG versus LCH

As LCH and XG can both be the differential diagnosis of the other, I compared the average IHC scores of both groups to determine if and which antibodies detected significant differences in expression based on the disorder and created cut-off values that can be used as clinical tools to assist distinction between differential diagnoses *(Table 3.1).*

Table 3.1: Immunohistochemical scores of experimental markers for LCH and XG

Pat-ID	BRAF	MEK1/2	MEK2	pMEK1/2	ERK1/2	ERK2	pERK1/2	pan-TRK	PD-L1
LCH									
1	4.0	2.0	4.0	4.0	4.0	8.0	0.0	8.0	0.0
2	4.0	2.0	8.0	4.0	8.0	6.0	6.0	4.0	4.0
3	12.0	3.0	12.0	6.0	12.0	12.0	8.0	4.0	0.0
4	4.0	6.0	10.0	3.5	7.0	4.0	8.0	1.0	0.0
5	8.0	6.0	9.0	6.0	10.0	8.0	9.0	8.0	0.0
6	8.0	6.0	8.0	n.d.	12.0	8.0	4.0	6.0	0.0
7	9.0	9.0	8.0	4.0	12.0	4.0	9.0	0.0	3.0
8	8.0	12.0	12.0	4.0	12.0	8.0	2.0	12.0	0.0
9	10.0	12.0	7.5	2.5	12.0	8.0	5.0	4.0	4.0
10	n.d.	8.0	n.d.	n.d.	n.d.	n.d.	4.0	12.0	0.0
11	n.d.	6.0	8.0	6.0	n.d.	n.d.	4.0	0.0	3.0
Sample size	9	11	10	9	9	9	11	11	11
Median	8.0	6.0	8.0	4.0	12.0	8.0	5.0	4.0	0.0
Average	7.4	6.5	8.7	4.4	9.9	7.3	5.4	5.4	1.3
Standard deviation	2.71	3.34	2.21	1.19	2.77	2.31	2.80	4.10	1.71
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	1.77	1.97	1.37	0.78	1.81	1.51	1.66	2.42	1.01
XG									
1	0.0	8.0	12.0	8.0	4.0	12.0	12.0	0.0	4.0
2	0.0	3.0	12.0	12.0	0.0	12.0	12.0	4.0	4.0
3	0.0	8.0	12.0	10.5	8.0	12.0	5.0	5.0	8.0
4	0.0	8.0	9.0	12.0	4.0	12.0	8.0	12.0	8.0
5	0.0	8.0	10.0	4.0	4.0	12.0	8.0	8.0	8.0
6	6.0	12.0	12.0	12.0	12.0	12.0	10.5	12.0	5.0
7	5.0	8.0	10.0	12.0	8.0	12.0	12.0	4.0	8.0
8	12.0	8.0	10.0	12.0	12.0	8.0	8.0	0.0	8.0
9	8.0	8.0	8.0	n.d.	8.0	12.0	n.d.	0.0	8.0
10	8.0	8.0	n.d.	n.d.	12.0	n.d.	n.d.	12.0	n.d.
11	12.0	n.d.	n.d.	8.0	2.0	12.0	12.0	8.0	12.0
12	0.0	10.5	12.0	6.0	12.0	8.0	8.0	4.0	2.0
13	0.0	4.0	8.0	8.0	8.0	12.0	4.0	9.0	3.0
14	0.0	8.0	8.0	8.0	8.0	9.0	0.0	12.0	2.0
15	4.5	8.0	8.0	8.0	12.0	12.0	0.0	12.0	8.0
16	10.0	8.0	12.0	n.d.	9.0	8.0	n.d.	8.0	0.0
17	9.0	8.0	9.0	10.0	12.0	12.0	10.0	12.0	8.0
18	10.5	10.0	8.0	n.d.	12.0	10.0	n.d.	n.d.	2.0
19	9.0	8.0	12.0	n.d.	8.0	8.0	n.d.	n.d.	n.d.
20	12.0	8.0	10.0	8.0	6.0	12.0	6.0	12.0	8.0
21	12.0	7.0	12.0	8.0	12.0	8.0	5.5	12.0	8.0
22	4.0	12.0	12.0	12.0	9.0	12.0	12.0	4.0	8.0
23	12.0	2.0	12.0	n.d.	6.0	12.0	n.d.	n.d.	n.d.
24	n.d.	n.d.	n.d.	12.0	8.0	8.0	0.0	4.0	5.0
25	n.d.	n.d.	n.d.	4.0	8.0	n.d.	12.0	0.0	0.0
Sample size	23	22	21	19	25	23	19	22	22
Median	6.0	8.0	10.0	8.0	8.0	12.0	8.0	8.0	8.0
Average	5.8	7.8	10.4	9.2	8.2	10.7	7.6	7.0	5.8
Standard deviation	4.83	2.33	1.68	2.63	3.38	1.77	4.17	4.53	3.15
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	1.97	0.97	0.72	1.18	1.32	0.73	1.88	1.89	1.31

3.1.1 Anti-MEK2

The median IHC score for anti-MEK2 in the LCH group was 8 with a mean value of 8.7 (7.33-10.07; CI 95%), while the XG group had a median IHC score of 10 and an average score of 10.4 (9.68-11.12; CI 95%). Due to the asymmetric distribution of the data, the Mann-Whitney U test was chosen to test whether there was a difference between LCH and XG regarding anti-MEK2. The difference found here was significant with a p-value of 0.0338. Furthermore, logistic regression was used to then determine the difference in marker expression level between both groups. This resulted in a significant p-value of 0.0444 *(Figure 3.6)*. This indicates that there was a significant positive correlation between high anti-MEK2 IHC scores and XG. The cut-off value determined for XG was at 10 *(Figure 3.7)*. An odds ratio (OR) of 1.6 means that patients with marginal lesions that showed a high MEK expression had a 1.6 times higher probability of having XG lesions rather than LCH lesions. The model had 73% validity *(Figure 3.2)*.

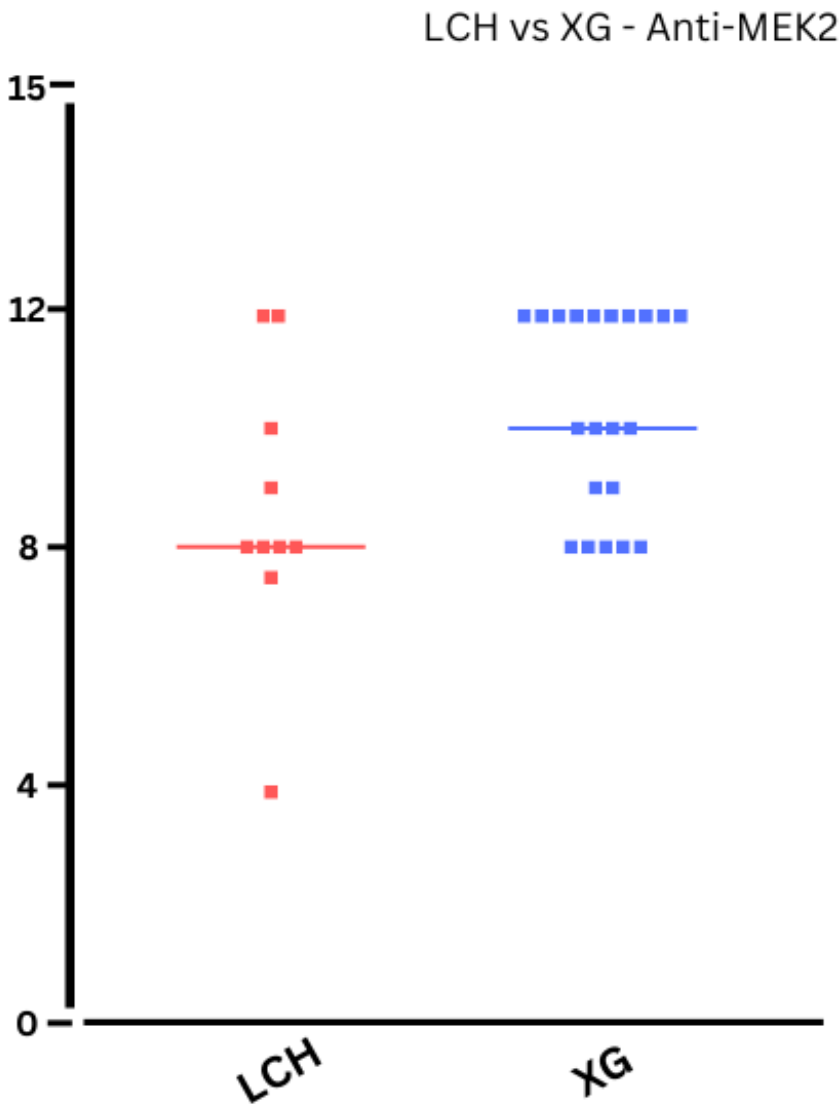

Figure 3.2: Graphical presentation of the distribution of the IHC scores of LCH versus XG for anti-MEK2, including their median IHC scores.

3.1.2 Anti-pMEK1/2

The median IHC score for anti-pMEK1/2 in the LCH group was 4 with a mean value of 4.4 (3.62-5.18; CI 95%), while the XG group had a median IHC score of 8 and an average score of 9.2 (8.02-10.38; CI 95%). The U test was chosen to test whether there was a difference between LCH and XG regarding anti-pMEK1/2. The difference found here was highly significant with a p-value of 0.0002. Furthermore, logistic regression was then used to determine the difference in marker expression level between both groups. This resulted in a very significant p-value of 0.0078 *(Figure 3.6)*. This means that high anti-pMEK1/2 IHC scores could be associated with XG. The cut-off value determined for XG was at 8 *(Figure 3.7)*. The odds ratio (OR) of 2.6 means that patients with marginal lesions that highly expressed pMEK1/2 had a 2.6 times higher probability of having XG lesions rather than LCH lesions. The model had 93% validity *(Figure 3.3).*

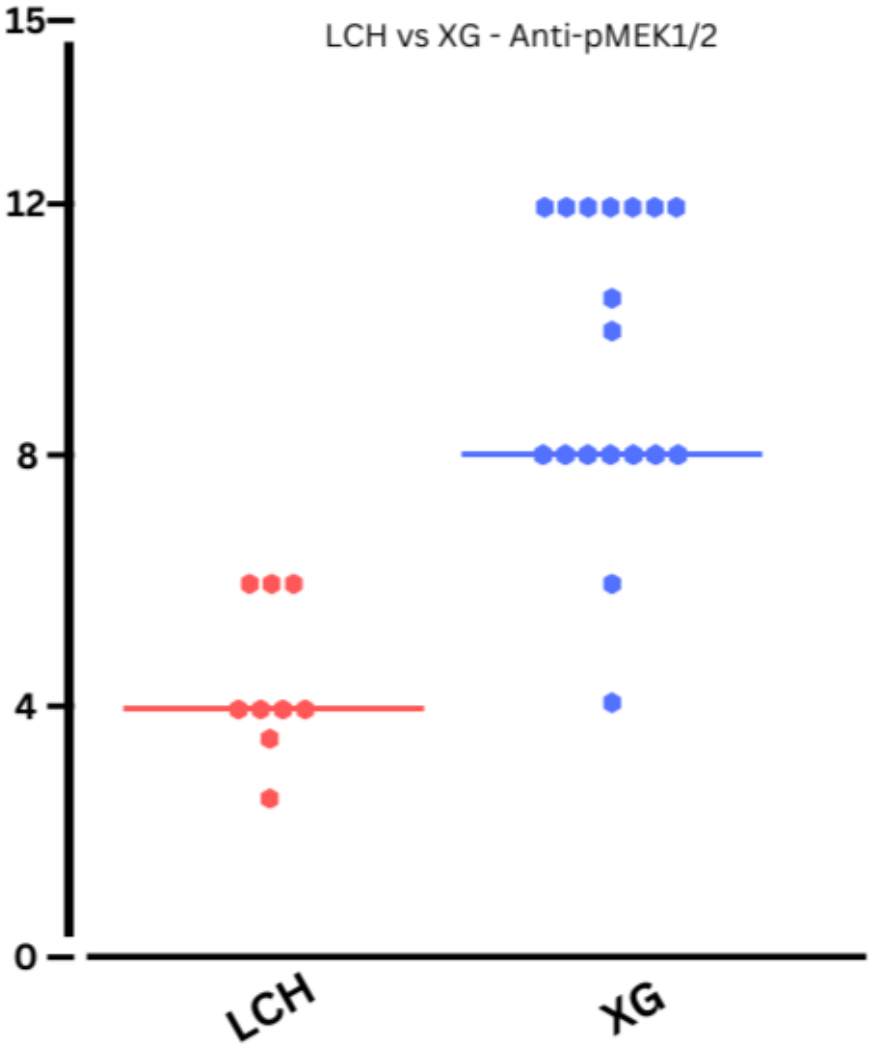

Figure 3.3: Graphical presentation of the distribution of the IHC scores of LCH versus XG for anti-pMEK1/2, including their median IHC scores.

3.1.3 Anti-ERK2

The median IHC score for anti-ERK2 in the LCH group was 8 with a mean value of 7.3 (5.79-8.81; CI 95%), while the XG group had a median IHC score of 12 and an average score of 10.7 (9.97-11.43; CI 95%). The U test was used to test whether there was a difference between LCH and XG regarding anti-ERK2 expression. The difference found here was highly significant with a p-value of 0.0008. Furthermore, logistic regression was then used to determine the difference in marker expression level between both groups. This resulted in a very significant p-value of 0.0095 *(Figure 3.6)*. This shows that there was a significant positive correlation between high anti-ERK2 IHC scores and XG. The cut-off value determined for XG was at 9 *(Figure 3.7)*. The odds ratio (OR) of 2.1 means that patients with marginal lesions that showed a high expression of ERK2 had a 2.1 times higher probability of having XG lesions rather than LCH lesions. The model had 85% validity *(Figure 3.4).*

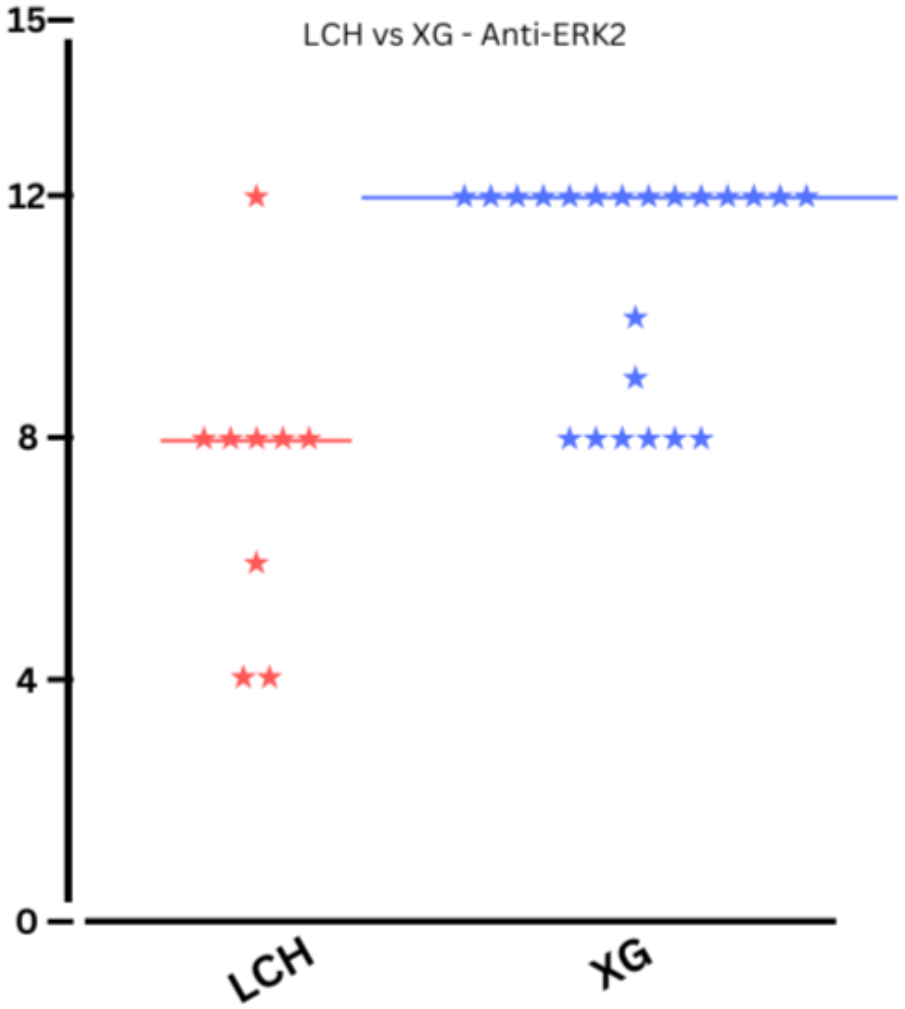

Figure 3.4: Graphical presentation of the distribution of the IHC scores of LCH versus XG for anti-ERK2, including their median IHC scores.

3.1.4 Anti-PD-L1

The median IHC score for anti-PD-L1 in the LCH group was 0 with a mean value of 1.3 (0.29-2.31; CI 95%), while the XG group had a median IHC score of 8 and an average score of 5.8 (4.49-7.11; CI 95%). The Mann-Whitney U test was used to discover a possible difference between LCH and XG regarding anti-PD-L1. The difference found here was highly significant with a p-value of 0.0005. Furthermore, logistic regression was then used to determine the difference in marker expression level between both groups. This resulted in a very significant p-value of 0.0048 *(Figure 3.6)*. This suggests that high anti-PD-L1 IHC scores could be associated with XG. The cut-off value determined for XG was at 5 *(Figure 3.7)*. The odds ratio (OR) of 1.8 means that patients with marginal lesions highly expressed PD-L1 had a 1.8 times higher probability of having XG lesions rather than LCH lesions. The model had 87% validity.

In addition to simple logistic regression, I aimed to determine if a combination of antibodies would lead to an increased effect on LCH or XG. For this, I used backward multiple logistic regression of all experimental markers with exemption of pMEK1/2, ERK1/2 and pERK1/2 due to model incompatibility. The following result was obtained: A clear benefit of combining experimental markers could not be identified in this analysis. Only anti-PD-L1 showed a significant p-value of 0.0313. Comparing the two results from both the simple and the backward multiple logistic regression, it can be seen that there was a significantly positive correlation between high anti-PD-L1 IHC scores and XG. The odds ratio of the backward multiple regression (OR) of 2.5 further emphasises that patients with marginal lesions that exhibited high PD-L1 expression were more likely to suffer from an XG lesion, more precisely a 2.5 times higher probability than suffering from an LCH lesion. The model was valid by 92% *(Figure 3.5).*

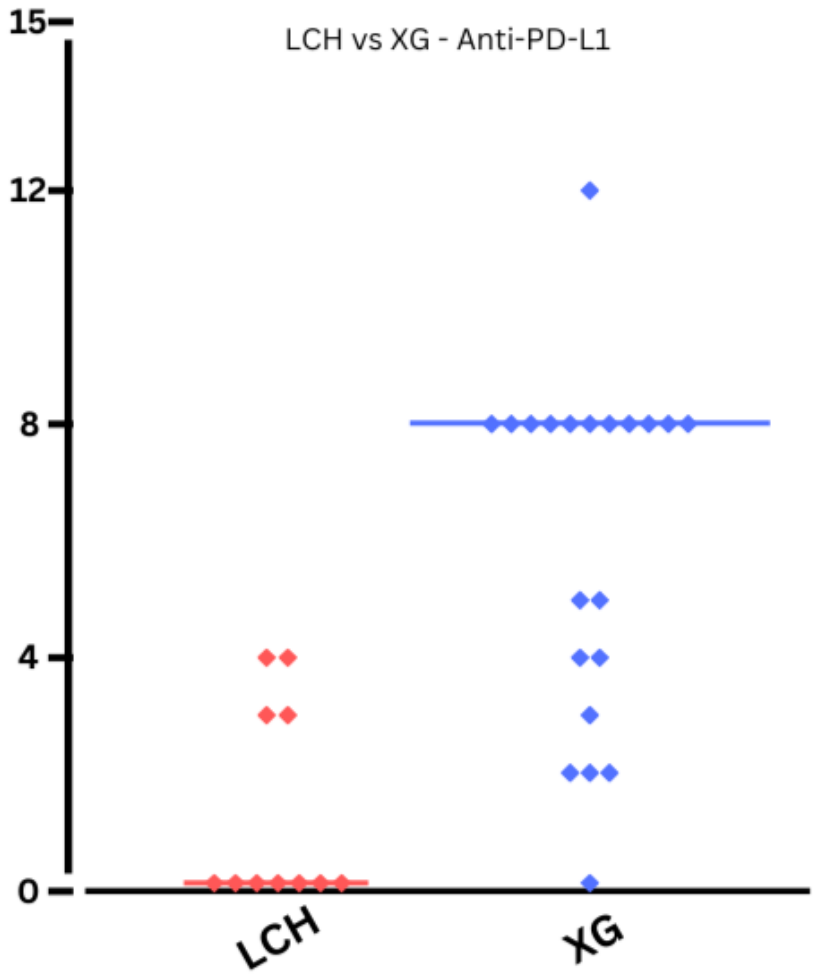

Figure 3.5: Graphical presentation of the distribution of the IHC scores of LCH versus XG for anti-PD-L1, including their median IHC scores.

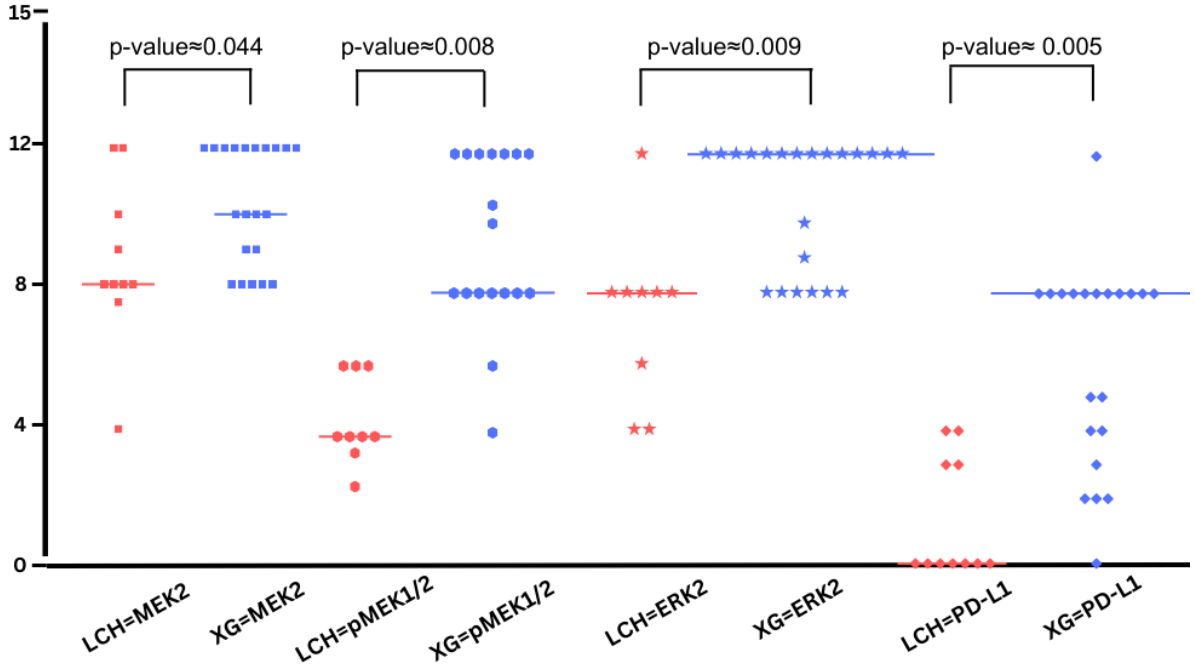

Figure 3.6: Summary of the distribution of the IHC scores of LCH versus XG for significant experimental markers, including their median IHC scores and p-values.

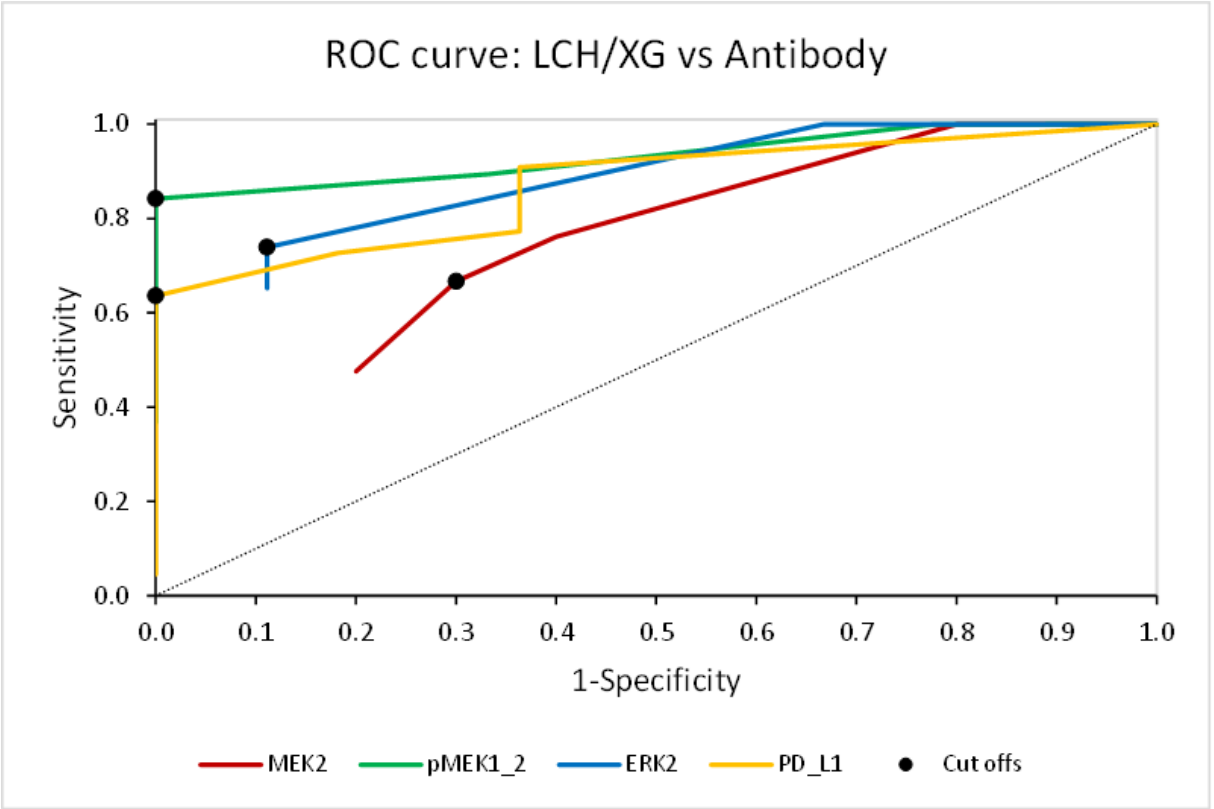

Figure 3.7: ROC (Receiver-Operating-Characteristics) curve of LCH versus XG for significant experimental markers, including cut-off values. X-axis: 1-specificity, also known as false-positive rate. Y-axis: sensitivity.

3.1.5 Non-significant markers

The following antibodies showed a higher mean IHC value in XG than in LCH without significance: For anti-MEK1/2 the median IHC score of the XG group was 8 with an average score of 7.8 (6.83-8.77; CI 95%), while the LCH group had a median IHC score of 6 and an average score of 6.5 (4.53-8.47; CI 95%). The p-value was 0.1535. For anti-pERK1/2 the median IHC score of the XG group was 8 with an average score of 7.6 (5.72-9.48; CI 95%), while the LCH group had a median IHC score of 5 and an average score of 5.4 (3.74-7.06; CI 95%). The p-value was 0.0941. For anti-pan-TRK the median IHC score of the XG group was 8 with an average score of 7 (5.11-8.89; CI 95%), while the LCH group had a median IHC score of 4 and an average score of 5.4 (2.98-7.82; CI 95%). The p-value was 0.3369.

The following antibodies showed a higher mean IHC value in LCH than in XG without significance: For anti-BRAF V600E the median IHC score of the LCH group was 8 with an average score of 7.4 (5.63-9.17; CI 95%), while the XG group had a median IHC score of 6 and an average score of 5.8 (3.83-7.77; CI 95%). The p-value was 0.5805. For anti-ERK1/2 the median IHC score of the LCH group was 12 with an average score of 9.9 (8.09-11.71; CI 95%), while the XG group had a median IHC score of 8 and an average score of 8.2 (6.88-9.52; CI 95%). The p-value was 0.2081.

3.2 Disseminated XG versus singular XG

XG can clinically appear as disseminated or singular, thereby having disparate severity and prognosis. I compared the average IHC scores of both groups as a measure of the expression of specific experimental markers to ascertain if and which markers showed significant differences in expression based on the clinical manifestation, with hope to determine prognostic markers with which a disseminated outcome could possibly be predicted at initial manifestation *(Table 3.2).*

Table 3.2: Immunohistochemical scores of experimental markers for disseminated and singular XG

Pat-ID	BRAF	MEK1/2	MEK2	pMEK1/2	ERK1/2	ERK2	pERK1/2	pan-TRK	PD-L1
disseminated XG									
10	8.0	8.0	n.d.	n.d.	12.0	n.d.	n.d.	12.0	n.d.
11	12.0	n.d.	n.d.	8.0	2.0	12.0	12.0	8.0	12.0
Sample size	2	1	0	1	2	1	1	2	1
Median	10.0	8.0	n.d.	8.0	7.0	12.0	12.0	10.0	12.0
Average	10.0	8.0	n.d.	8.0	7.0	12.0	12.0	10.0	12.0
Standard deviation	2.00	0.00	n.d.	0.00	5.00	0.00	0.00	2.00	0.00
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	2.77	n.d.	n.d.	n.d.	6.93	n.d.	n.d.	2.77	n.d.
singular XG									
1	0.0	8.0	12.0	8.0	4.0	12.0	12.0	0.0	4.0
2	0.0	3.0	12.0	12.0	0.0	12.0	12.0	4.0	4.0
3	0.0	8.0	12.0	10.5	8.0	12.0	5.0	5.0	8.0
4	0.0	8.0	9.0	12.0	4.0	12.0	8.0	12.0	8.0
5	0.0	8.0	10.0	4.0	4.0	12.0	8.0	8.0	8.0
6	6.0	12.0	12.0	12.0	12.0	12.0	10.5	12.0	5.0
7	5.0	8.0	10.0	12.0	8.0	12.0	12.0	4.0	8.0
8	12.0	8.0	10.0	12.0	12.0	8.0	8.0	0.0	8.0
9	8.0	8.0	8.0	n.d.	8.0	12.0	n.d.	0.0	8.0
12	0.0	10.5	12.0	6.0	12.0	8.0	8.0	4.0	2.0
13	0.0	4.0	8.0	8.0	8.0	12.0	4.0	9.0	3.0
14	0.0	8.0	8.0	8.0	8.0	9.0	0.0	12.0	2.0
15	4.5	8.0	8.0	8.0	12.0	12.0	0.0	12.0	8.0
16	10.0	8.0	12.0	n.d.	9.0	8.0	n.d.	8.0	0.0
17	9.0	8.0	9.0	10.0	12.0	12.0	10.0	12.0	8.0
18	10.5	10.0	8.0	n.d.	12.0	10.0	n.d.	n.d.	2.0
19	9.0	8.0	12.0	n.d.	8.0	8.0	n.d.	n.d.	n.d.
20	12.0	8.0	10.0	8.0	6.0	12.0	6.0	12.0	8.0
21	12.0	7.0	12.0	8.0	12.0	8.0	5.5	12.0	8.0
22	4.0	12.0	12.0	12.0	9.0	12.0	12.0	4.0	8.0
Sample size	20	20	20	16	20	20	16	18	19
Median	4.8	8.0	10.0	9.0	8.0	12.0	8.0	8.0	8.0
Average	5.1	8.1	10.3	9.4	8.4	10.8	7.6	7.2	5.8
Standard deviation	4.72	2.05	1.68	2.43	3.37	1.76	3.83	4.52	2.76
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	2.07	0.90	0.73	1.19	1.48	0.77	1.88	2.09	1.24

3.2.1 Anti-PD-L1

For anti-PD-L1 the median and average IHC score of the group with disseminated XG was 12 (SD± 0), while the singular XG group had a median IHC score of 8 and an average score of 5.8 (4.56-7.04; CI 95%). Due to the asymmetric distribution of the data, the U test was chosen to test whether there was a difference between disseminated and singular XG regarding PD-L1 expression. The difference found showed a non-significant p-value of 0.0707. However, further investigation with simple regression analysis was used to predict how much the marker expression changed depending on the group. This resulted in a significant p-value of 0.0471 and a standardised regression coefficient for disseminated XG of +6.21, which states that patients with a disseminated XG had a significantly higher IHC score by +6.21 than patients with singular XG. This means that high anti-PD-L1 IHC scores could have a poor prognostic value, being more predominant in analysed disseminated XG. The coefficient of determination (R^2) of 0.20 indicates that 20% of the influence on anti-PD-L1 expression could be explained by the presence of the disseminated XG group. These results must be interpreted with caution due to the small sample size and disproportionate distribution with solely one disseminated case showing a limited generalizability *(Figure 3.8).*

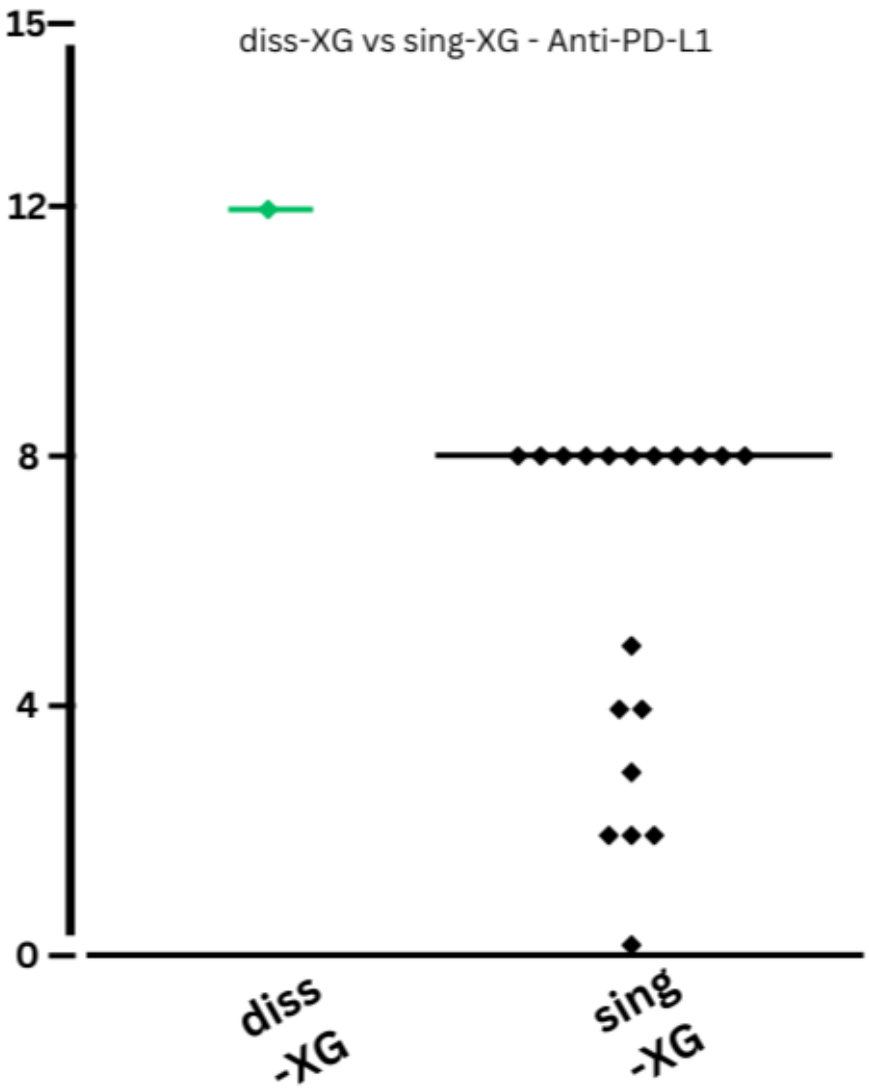

Figure 3.8: Graphical presentation of the distribution of the IHC scores of disseminated XG versus singular XG for anti-PD-L1, including their median IHC scores.

3.2.2 Non-significant markers

The following antibodies showed a higher mean IHC value in disseminated XG than in singular XG without significance: For anti-BRAF V600E the median and average IHC score of the group with disseminated XG was 10 (7.23-12; CI 95%), while the singular XG group had a median IHC score of 4.8 and an average score of 5.1 (3.03-7.17; CI 95%). The p-value was 0.1964. For anti-ERK2 the median and average IHC score of the group with disseminated XG was 12 (SD± 0), while the singular XG group had a median IHC score of 12 and an average score of 10.8 (10.03-11.57; CI 95%). The p-value was 0.4870. For anti-pERK1/2 the median and average IHC score of the group with disseminated XG was 12 (SD± 0), while the singular XG group had a median IHC score of 8 and an average score of 7.6 (5.72-9.48; CI 95%). The p-value was 0.2118. For anti-pan-TRK the median and average IHC score of the group with disseminated XG was 10 (7.23-12; CI 95%), while the singular XG group had a median IHC score of 8 and an average score of 7.2 (5.11-9.29; CI 95%). The p-value was 0.4708.

3.3 High-risk versus low-risk LCH

LCH can be classified as high-risk and low-risk LCH. MS-LCH, LCH with risk organ involvement (RO^+) and multifocal LCH are all clinical appearances of LCH that are associated with a severity and poor prognosis, while their counterparts SS-LCH, LCH with low-risk organ involvement (RO^-) and LCH with unifocal lesions mostly have a better outcome. I compared the median IHC scores of both groups to ascertain if and which antibodies showed significant differences in expression based on the clinical manifestation, with hope to determine prognostic markers with which a high-risk outcome could possibly be predicted at initial manifestation. By comparing both groups, I wanted to find out if a high expression of the experimental markers could be correlated with one of the groups. For this purpose, I used the Mann-Whitney U test to determine if a difference between both groups exist and multiple regression analysis, in which all six variables (MS-LCH versus SS-LCH, RO^+ -LCH versus RO^- -LCH and multifocal LCH versus unifocal LCH) were correlated with experimental marker expressions *(Table 3.3).*

Table 3.3: Immunohistochemical scores of experimental markers for high-risk versus low-risk LCH (MS-LCH versus SS-LCH, multifocal LCH versus unifocal LCH and RO^+-LCH versus RO^--LCH)

Pat-ID	BRAF	MEK1/2	MEK2	pMEK1/2	ERK1/2	ERK2	pERK1/2	pan-TRK	PD-L1
MS-LCH									
3	12.0	3.0	12.0	6.0	12.0	12.0	8.0	4.0	0.0
6	8.0	6.0	8.0	n.d.	12.0	8.0	4.0	6.0	0.0
8	8.0	12.0	12.0	4.0	12.0	8.0	2.0	12.0	0.0
9	10.0	12.0	7.5	2.5	12.0	8.0	5.0	4.0	4.0
11	n.d.	6.0	8.0	6.0	n.d.	n.d.	4.0	0.0	3.0
Sample size	4	5	5	4	4	4	5	5	5
Median	9.0	6.0	8.0	4.0	12.0	8.0	4.0	4.0	0.0
Average	9.5	7.8	9.5	4.6	12.0	9.0	4.6	5.2	1.4
Standard deviation	1.66	3.60	2.05	1.47	0.00	1.73	1.96	3.92	1.74
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	1.63	3.16	1.80	1.44	n.d.	1.70	1.72	3.44	1.53
SS-LCH									
1	4.0	2.0	4.0	4.0	4.0	8.0	0.0	8.0	0.0
2	4.0	2.0	8.0	4.0	8.0	6.0	6.0	4.0	4.0
4	4.0	6.0	10.0	3.5	7.0	4.0	8.0	1.0	0.0
5	8.0	6.0	9.0	6.0	10.0	8.0	9.0	8.0	0.0
7	9.0	9.0	8.0	4.0	12.0	4.0	9.0	0.0	3.0
10	n.d.	8.0	n.d.	n.d.	n.d.	n.d.	4.0	12.0	0.0
Sample size	5	6	5	5	5	5	6	6	6
Median	4.0	6.0	8.0	4.0	8.0	6.0	7.0	6.0	0.0
Average	5.8	5.5	7.8	4.3	8.2	6.0	6.0	5.5	1.2
Standard deviation	2.23	2.69	2.04	0.87	2.71	1.79	3.21	4.23	1.67
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	1.95	2.15	1.79	0.76	2.38	1.57	2.57	3.39	1.34

Pat-ID Multifocal LCH	BRAF	MEK1/2	MEK2	pMEK1/2	ERK1/2	ERK2	pERK1/2	pan-TRK	PD-L1
6	8.0	6.0	8.0	n.d.	12.0	8.0	4.0	6.0	0.0
7	9.0	9.0	8.0	4.0	12.0	4.0	9.0	0.0	3.0
8	8.0	12.0	12.0	4.0	12.0	8.0	2.0	12.0	0.0
9	10.0	12.0	7.5	2.5	12.0	8.0	5.0	4.0	4.0
10	n.d.	8.0	n.d.	n.d.	n.d.	n.d.	4.0	12.0	0.0
11	n.d.	6.0	8.0	6.0	n.d.	n.d.	4.0	0.0	3.0
Sample size	4	6	5	4	4	4	6	6	6
Median	8.5	8.5	8.0	4.0	12.0	8.0	4.0	5.0	1.5
Average	8.8	8.8	8.7	4.1	12.0	7.0	4.7	5.7	1.7
Standard deviation	0.83	2.48	1.66	1.24	0.00	1.73	2.13	4.96	1.70
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	0.81	1.98	1.46	1.22	n.d.	1.70	1.71	3.97	1.36
Unifocal LCH									
1	4.0	2.0	4.0	4.0	4.0	8.0	0.0	8.0	0.0
2	4.0	2.0	8.0	4.0	8.0	6.0	6.0	4.0	4.0
3	12.0	3.0	12.0	6.0	12.0	12.0	8.0	4.0	0.0
4	4.0	6.0	10.0	3.5	7.0	4.0	8.0	1.0	0.0
5	8.0	6.0	9.0	6.0	10.0	8.0	9.0	8.0	0.0
Sample size	5	5	5	5	5	5	5	5	5
Median	4.0	3.0	9.0	4.0	8.0	8.0	8.0	4.0	0.0
Average	6.4	3.8	8.6	4.7	8.2	7.6	6.2	5.0	0.8
Standard deviation	3.20	1.83	2.65	1.08	2.71	2.65	3.25	2.68	1.60
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	2.80	1.61	2.33	0.94	2.38	2.33	2.85	2.35	1.40

Pat-ID RO+ LCH	BRAF	MEK1/2	MEK2	pMEK1/2	ERK1/2	ERK2	pERK1/2	pan-TRK	PD-L1
8	8.0	12.0	12.0	4.0	12.0	8.0	2.0	12.0	0.0
9	10.0	12.0	7.5	2.5	12.0	8.0	5.0	4.0	4.0
11	n.d.	6.0	8.0	6.0	n.d.	n.d.	4.0	0.0	3.0
Sample size	2	3	3	3	2	2	3	3	3
Median	9.0	12.0	8.0	4.0	12.0	8.0	4.0	4.0	3.0
Average	9.0	10.0	9.2	4.2	12.0	8.0	3.7	5.3	2.3
Standard deviation	1.00	2.83	2.01	1.43	0.00	0.00	1.25	4.99	1.70
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	1.39	3.20	2.28	1.62	n.d.	n.d.	1.41	5.65	1.92
RO- LCH									
1	4.0	2.0	4.0	4.0	4.0	8.0	0.0	8.0	0.0
2	4.0	2.0	8.0	4.0	8.0	6.0	6.0	4.0	4.0
3	12.0	3.0	12.0	6.0	12.0	12.0	8.0	4.0	0.0
4	4.0	6.0	10.0	3.5	7.0	4.0	8.0	1.0	0.0
5	8.0	6.0	9.0	6.0	10.0	8.0	9.0	8.0	0.0
6	8.0	6.0	8.0	n.d.	12.0	8.0	4.0	6.0	0.0
7	9.0	9.0	8.0	4.0	12.0	4.0	9.0	0.0	3.0
10	n.d.	8.0	n.d.	n.d.	n.d.	n.d.	4.0	12.0	0.0
Sample size	7	8	7	6	7	7	8	8	8
Median	8.0	6.0	8.0	4.0	10.0	8.0	7.0	5.0	0.0
Average	7.0	5.3	8.4	4.6	9.3	7.1	6.0	5.4	0.9
Standard deviation	2.88	2.49	2.26	1.02	2.86	2.59	2.96	3.71	1.54
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	2.13	1.72	1.67	0.81	2.12	1.92	2.05	2.57	1.06

3.3.1 Anti-MEK1/2

The following scores are the IHC values of the individual groups.

MS-LCH versus SS-LCH: The median IHC score for anti-MEK1/2 of the MS-LCH group was 6 with a mean value of 7.8 (4.64-10.96; CI 95%), while the SS-LCH group also had a median IHC score of 6 but an average score of 5.5 (3.35-7.65; CI 95%).

Multifocal LCH versus unifocal LCH: The median IHC score for anti-MEK1/2 of the multifocal LCH group was 8.5 with an average score of 8.8 (6.82-10.78; CI 95%), the unifocal LCH group, however, had a median IHC score of 3 and an average score of 3.8 (2.19-5.41; CI 95%).

RO^+-LCH versus RO^--LCH: The median IHC score for anti-MEK1/2 of the RO^+-LCH group was 12 with a mean value of 10 (6.8-12; CI 95%), while the RO^--LCH group had a median IHC score of 6 and an average score of 5.3 (3.58-7.02; CI 95%).

Due to the asymmetric distribution of the data, the Mann-Whitney U test was chosen to test whether there was a difference between high-risk LCH and low-risk LCH regarding MEK1/2 expression. The difference found in multifocal LCH versus unifocal LCH was significant with a p-value of 0.0146. While there was no significant difference in MS-LCH versus SS-LCH (p-value 0.3478) and in RO^+ -LCH versus RO^--LCH (p-value 0.0744).

Furthermore, multiple regression analysis was used to then predict how much the marker expression changes depending on the group (high-risk/low-risk) containing all three subgroups (MS-LCH versus SS-LCH, RO^+ -LCH versus RO^--LCH and multifocal LCH versus unifocal LCH). This resulted in a significant cumulative p-value of 0.0443, stating that MEK1/2 expression significantly correlated with one or more of the three analysed groups. Further analysis of the data revealed that only the multifocal LCH group with a standardised regression coefficient of +4.09 had a marginally significant individual p–value of 0.0566 due to its notable MEK1/2 expression. The RO^+-LCH group also had considerable MEK1/2 expression resulting in a standardised regression coefficient of +3.45 and an individual p-value of 0.1920. The opposite was true for the MS-LCH group with low MEK1/2 expression and therefore a standardised regression coefficient of -1.68 and an individual p-value of 0.4295. Overall, these results state that the combination of all three subgroups showed a significant correlation between high-risk LCH and anti-MEK1/2. However, no significant correlation within individual groups could be observed. Due to the small sample size of the multifocal group, a significance can, nevertheless, be assumed. In summary, this means that patients with high-risk LCH have a higher probability of expressing high MEK1/2 compared to their low-risk counterparts. Additionally, patients with multifocal LCH lesions had a higher IHC score by +4.09 than patients with single LCH lesions. This means that high anti-MEK1/2 IHC scores could have a poor prognostic value, being more predominant in the analysed high-risk, more specifically, in multifocal LCH. The model had 66% validity (R^2= 0.66) *(Figure 3.9).*

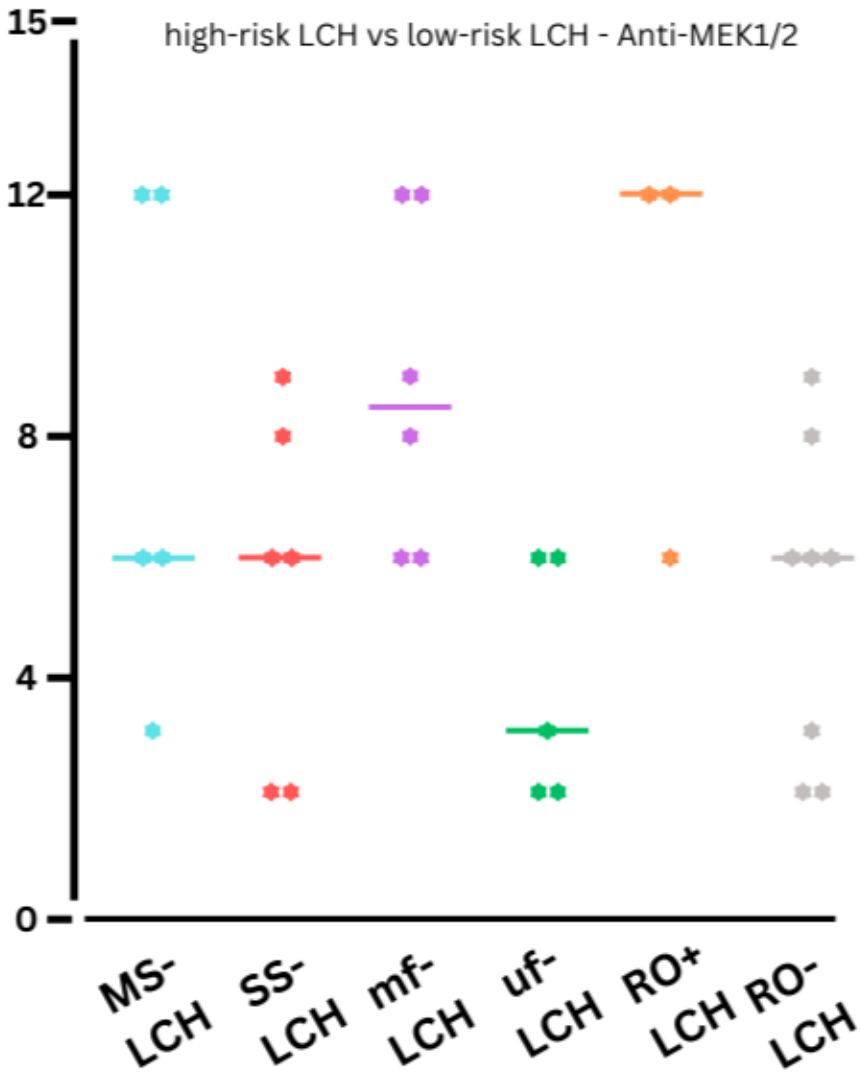

Figure 3.9: Graphical presentation of the distribution of the IHC scores of high-risk LCH versus low-risk LCH (MS-LCH versus SS-LCH, multifocal LCH versus unifocal LCH and RO^+-LCH versus RO^--LCH) for anti-MEK1/2, including their median IHC scores.

3.3.2 Anti-ERK2

The following scores are the IHC values of the individual groups.

MS-LCH versus SS-LCH: The median IHC score for anti-ERK2 of the MS-LCH group was 8 with a mean value of 9 (7.3-10.7; CI 95%), while the SS-LCH group had both a median and an average IHC score of 6 (4.43-7.57; CI 95%).

Multifocal LCH versus unifocal LCH: The median IHC score for anti-ERK2 of the multifocal LCH group was 8 with an average score of 7 (5.3-8.7; CI 95%), while the unifocal LCH group had a median IHC score of 8 and an average score of 7.6 (5.27-9.93; CI 95%).

RO^+-LCH versus RO^--LCH: The median and average IHC score for anti-ERK2 of the RO^+-LCH group were both 8 (SD± 0), while the RO^--LCH group had a median IHC score of 8 and an average score of 7.1 (5.18-9.02; CI 95%).

The U test (suitable for asymmetrically distributed data) was used to determine whether there was a difference between high-risk LCH and low-risk LCH regarding anti-ERK2. The difference found in MS-LCH versus SS-LCH was marginally significant with a p-value of 0.0591. While there was no significant difference in multifocal LCH versus unifocal LCH (p-value 0.8927) and in RO^+ -LCH versus RO^--LCH (p-value 0.5191).

Furthermore, multiple regression analysis was used to predict how much the marker expression changes depend on the group (high-risk versus low-risk) containing all three subgroups (MS-LCH versus SS-LCH, RO^+ -LCH versus RO^--LCH and multifocal LCH versus unifocal LCH). This resulted in a marginally significant cumulative p-value of 0.0542, stating that one or more of the three analysed groups had a slightly significant effect on anti-ERK2. Further analysis of the data showed that the MS-LCH group with a standardised regression coefficient of +4.92 and a significant individual p–value of 0.0144 positively influences anti-ERK2 expression. The RO^+-LCH group had a standardised regression coefficient of -0.46 and an individual p-value of 0.7933, negatively influencing anti-MEK1/2 expression. While the multifocal LCH group with a standardised regression coefficient of -3.08 had an individual p-value of 0.0703, also negatively influencing anti-MEK1/2 expression. Overall, these results state that the combination of all three subgroups showed a slightly significant correlation between high-risk LCH and ERK2 expression. The results also show that patients with MS-LCH had a higher IHC score by +4.92 than patients with SS-LCH. This means that high anti-ERK2 IHC scores could have a poor prognostic value, being more predominant in MS-LCH. The model had a validity of 76% (R^2= 0.76) *(Figure 3.10).*

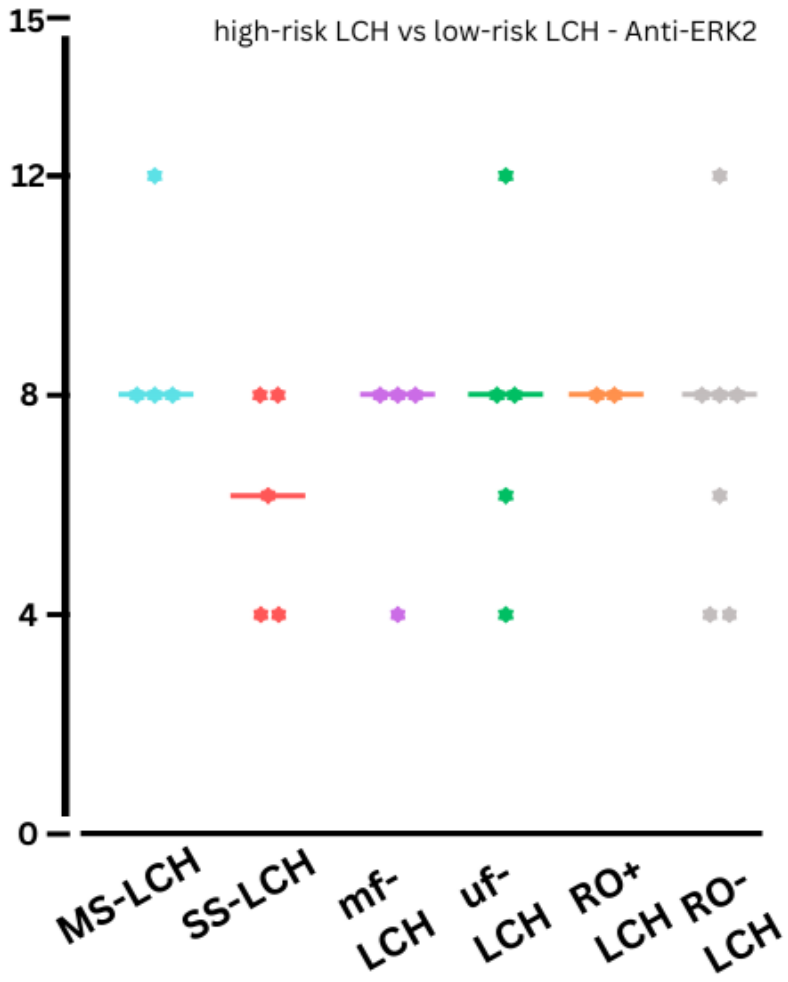

Figure 3.10: Graphical presentation of the distribution of the IHC scores of high-risk LCH versus low-risk LCH (MS-LCH versus SS-LCH, multifocal LCH versus unifocal LCH and RO^+-LCH versus RO^--LCH) for anti-ERK2, including their median IHC scores.

3.3.3 Anti-ERK1/2

The following scores are the IHC values of the individual groups.

MS-LCH versus SS-LCH: The median and average IHC score for anti-ERK1/2 of the MS-LCH group was 12 (SD± 0), the SS-LCH group had a median IHC score of 8 and an average score of 8.2 (5.82-10.58; CI 95%).

Multifocal LCH versus unifocal LCH: The median and average IHC score for anti-ERK1/2 of the multifocal LCH group was 12 (SD± 0), while the unifocal LCH group had a median IHC score of 8 and an average score of 8.2 (5.82-10.58; CI 95%).

RO^+-LCH versus RO^--LCH: The median and average IHC score for anti-ERK1/2 of the RO^+-LCH group were both 12 (SD± 0), while the RO^--LCH group had a median IHC score of 10 and an average score of 9.3 (7.18-11.42; CI 95%).

Due to the asymmetric distribution of data, the Mann-Whitney U test was used to determine whether there was a difference between high-risk LCH and low-risk LCH regarding anti-ERK1/2. The difference found between not only multifocal LCH and unifocal LCH, but also between MS-LCH and SS-LCH, was significant with a p-value of 0.0318 respectively, while there was no significant difference in RO^+ -LCH versus RO^--LCH (p-value 0.1995). These results show that a high ERK1/2 expression is present in MS- and multifocal LCH compared to their low-risk counterparts, which suggests a correlation that could be of prognostic value. Due to these results, multiple regression analysis was then performed with the hope to predict how much the marker expression changes depending on the group (high-risk versus low-risk). However, these results were of no significant value.

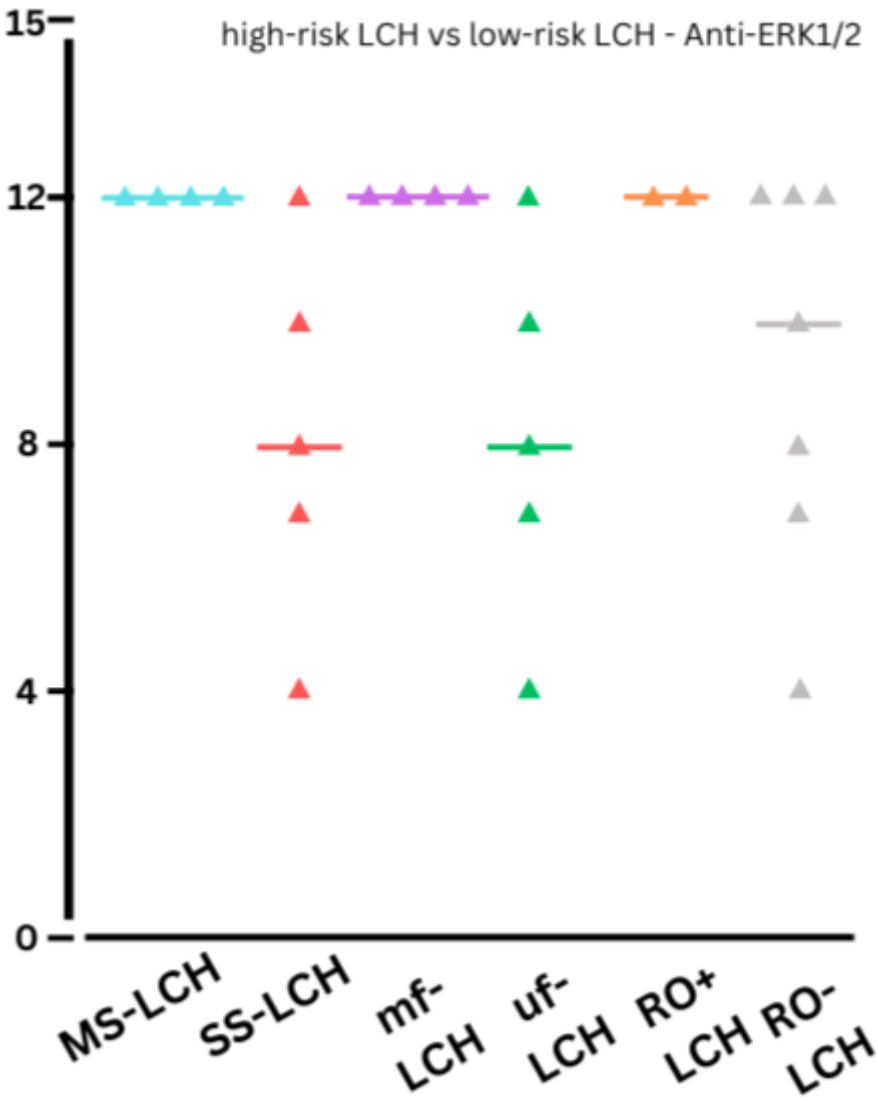

Figure 3.11: Graphical presentation of the distribution of the IHC scores of high-risk LCH versus low-risk LCH (MS-LCH versus SS-LCH, multifocal LCH versus unifocal LCH and RO^+-LCH versus RO^--LCH) for anti-ERK1/2, including their median IHC scores.

3.3.4 Non-significant markers

The following antibodies showed a higher mean IHC value in MS-LCH than in SS-LCH without significance: For anti-BRAF V600E, the median IHC score of the group with MS-LCH was 9 and the average score was 9.5 (7.87-11.13; CI 95%), while the SS-LCH group had a median IHC score of 4 and an average score of 5.8 (3.85-7.75; CI 95%). The p-value was 0.0759. For anti-MEK2, the median IHC score of the group with MS-LCH was 8 and the average score was 9.5 (7.77-11.3; CI 95%), while the SS-LCH group had a median IHC score of 8 and an average score of 7.8 (6.01-9.59; CI 95%). The p-value was 0.5888. For anti-pMEK1/2, the median IHC score of the group with MS-LCH was 4 and the average score was 4.6 (3.16-6.04; CI 95%), while the SS-LCH group had a median IHC score of 4 and an average score of 4.3 (3.54-5.06; CI 95%). The p-value was 0.6958. For anti-pERK1/2, the median IHC score of the group with MS-LCH was 4 and the average score was 4.6 (2.88-6.32; CI 95%), while the SS-LCH group had a median IHC score of 7 and an average score of 6 (3.43-8.57; CI 95%). The p-value was 0.3086.

The following antibodies showed a higher mean IHC value in multifocal LCH than in unifocal LCH without significance: For anti-BRAF V600E, the median IHC score of the group with multifocal LCH was 8.5 with an average score of 8.8 (7.99-9.61; CI 95%), while the unifocal LCH group had a median IHC score of 4.0 and an average score of 6.4 (3.6-9.2; CI 95%). The p-value was 0.2049. For anti-pan-TRK, the median IHC score of the group with multifocal LCH was 5 with an average score of 5.7 (1.73-9.67; CI 95%), while the unifocal LCH group had a median IHC score of 4 and an average score of 5 (2.65-7.35; CI 95%). The p-value was 1.0. For anti-PD-L1 the median IHC score of the group with multifocal LCH was 1.5 with an average score of 1.7 (0.34-3.06; CI 95%), while the unifocal LCH group had a median IHC score of 0 and an average score of 0.8 (0-2.2; CI 95%). The p-value was 0.4565.

The following antibodies showed a higher mean IHC value in RO^{+}-LCH than in RO^{-}-LCH without significance: For anti-PD-L1 the median IHC score of the RO^{+}-LCH group was 3 with an average score of 2.3 (0.38-4.22; CI 95%), the RO^{-}-LCH group had a median IHC score of 0 and an average score of 0.9 (0-1.96; CI 95%). The p-value was 0.2343.

3.4 Other histiocytoses

Due to the small sample size, prognostic statistical analyses could not be performed on the other histiocytoses. Therefore, I solely undertook a descriptive statistical analysis for NXG, XD, RDD, ECD and GEH. The analysis contained measures of central tendency and statistical dispersion with the aim to detect which markers was highly expressed (median/average IHC score ≥ 8) in the analysed histiocytoses *(Table 3.4).*

Table 3.4: Immunohistochemical scores of all experimental markers for other histiocytoses (NXG, XD, RDD, ECD and GEH)

Pat-ID	BRAF	MEK1/2	MEK2	pMEK1/2	ERK1/2	ERK2	pERK1/2	Pan-TRK	PD-L1
NXG									
58	4.0	4.0	8.0	4.0	4.0	8.0	0.0	2.0	0.0
59	8.0	4.0	6.0	3.0	3.5	10.0	0.0	8.0	0.0
60	12.0	10.0	8.0	4.0	8.0	8.0	0.0	8.0	4.0
Sample size	3	3	3	3	3	3	3	3	3
Median	8.0	4.0	8.0	4.0	4.0	8.0	0.0	8.0	0.0
Average	8.0	6.0	7.3	3.7	5.2	8.7	0.0	6.0	1.3
Standard deviation	3.3	2.8	0.9	0.5	2.0	0.9	0.0	2.8	1.9
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	3.70	3.20	1.07	0.53	2.28	1.07	n.d.	3.20	2.13
XD									
61	8.0	8.0	4.0	4.0	8.0	8.0	0.0	8.5	1.5
Sample size	1	1	1	1	1	1	1	1	1
Median	8.0	8.0	4.0	4.0	8.0	8.0	0.0	8.5	1.5
Average	8.0	8.0	4.0	4.0	8.0	8.0	0.0	8.5	1.5
Standard deviation	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
RDD									
62	12.0	9.0	12.0	4.0	12.0	12.0	0.0	12.0	4.0
63	5.0	4.5	4.0	4.0	9.0	8.0	1.5	6.0	n.d.
64	12.0	12.0	9.0	8.0	4.0	8.0	3.0	9.0	4.0
65	7.0	8.0	8.5	0.0	4.0	10.0	0.0	8.0	4.0
Sample size	4	4	4	4	4	4	4	4	3
Median	9.5	8.5	8.8	4.0	6.5	9.0	0.8	8.5	4.0
Average	9.0	8.4	8.4	4.0	7.3	9.5	1.1	8.8	4.0
Standard deviation	3.1	2.7	2.9	2.8	3.4	1.7	1.2	2.2	0.0
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	3.02	2.62	2.80	2.77	3.35	1.63	1.22	2.12	n.d.
ECD									
66	8.0	9.0	8.0	4.0	12.0	12.0	3.0	8.0	4.0
Sample size	1	1	1	1	1	1	1	1	1
Median	8.0	9.0	8.0	4.0	12.0	12.0	3.0	8.0	4.0
Average	8.0	9.0	8.0	4.0	12.0	12.0	3.0	8.0	4.0
Standard deviation	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GEH									
67	12.0	12.0	8.0	0.0	12.0	8.0	0.0	6.0	0.0
69	12.0	9.0	12.0	4.0	n.d.	12.0	2.0	6.0	4.0
Sample size	2	2	2	2	1	2	2	2	2
Median	12.0	10.5	10.0	2.0	12.0	10.0	1.0	6.0	2.0
Average	12.0	10.5	10.0	2.0	12.0	10.0	1.0	6.0	2.0
Standard deviation	0.0	1.5	2.0	2.0	0.0	2.0	1.0	0.0	2.0
Alpha value	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Confidence interval	n.d.	2.08	2.77	2.77	n.d.	2.77	1.39	n.d.	2.77

3.4.1 NXG

The following antibodies detected a high marker expression in NXG: anti-ERK2 with a median IHC score of 8 and an average score of 8.7 (7.63-9.77; CI 95%), anti-BRAF V600E with a median and average IHC score of 8 (4.3-11.7; CI 95%), anti-MEK2 with a median IHC score of 8 and an average score of 7.3 (6.23-8.37; CI 95%) and anti-pan-TRK with a median IHC score of 8 and an average score of 6 (2.8-9.2; CI 95%). This suggests an increased expression of the proteins of the MAPK pathway in NXG, as well as an increased expression of TRK.

3.4.2 XD

The following antibodies showed a high marker expression in XD: anti-pan-TRK with a median and average IHC score of 8.5 (SD± 0) and anti-BRAF V600E, anti-MEK1/2, anti-ERK1/2 and anti-ERK2 with a median and average IHC score of 8 (SD± 0). This also suggests an increased expression of the proteins of the MAPK pathway in XD, as well as an increased expression of TRK.

3.4.3 RDD

The following antibodies detected a high marker expression in RDD: anti-ERK2 with a median IHC score of 9 and an average score of 9.5 (7.87-11.13; CI 95%), anti-BRAF V600E with a median IHC score of 9.5 and an average score of 9 (5.98-12; CI 95%), anti-pan-TRK with a median value of 8.5 and an average score of 8.8 (6.68-10.92; CI 95%), anti-MEK2 with a median IHC value of 8.8 and an average score of 8.4 (5.6-11.2; CI 95%) and anti-MEK1/2 with a median IHC of 8.5 and an average score of 8.4 (5.78-11.02; CI 95%). An increased expression of the proteins of the MAPK pathway, as well as of TRK, in RDD could be assumed.

3.4.4 ECD

The following antibodies showed a high marker expression in ECD: anti-ERK1/2 and anti-ERK2 with a median and average IHC score of 12 (SD± 0), anti-MEK1/2 with a median and average IHC score of 9 (SD± 0) and anti-BRAF V600E, anti-MEK2 and anti-pan-TRK with a median and average IHC score of 8 (SD± 0).Like the aforementioned histiocytoses, the findings here suggest an increased expression of the proteins of the MAPK pathway in ECD, as well as an increased expression of TRK.

3.4.5 GEH

The following antibodies detected a high marker expression in GEH: anti-ERK1/2 and anti-BRAF V600E with a median and average IHC score of 12 (SD± 0), anti-MEK1/2 with a median and average IHC score of 10.5 (8.42-12; CI 95%) and anti-MEK2 and anti-ERK2 with a median and mean IHC value of 10 (7.23-12; CI 95 This also suggests an increased expression of the proteins of the MAPK pathway in GEH.

4 DISCUSSION

Histiocytic disorders encompass a group of rare diseases characterized by the abnormal proliferation and accumulation of histiocytes, immune cells derived from the monocyte-macrophage lineage. (Adam et al., 2022) Among histiocytic disorders, LCH and NLCH represent distinct entities with varying clinical presentations, prognoses and treatment approaches. This discussion aims to explore the IHC analysis of prognostic markers in these histiocytic disorders, shedding light on the diagnostic challenges and potential therapeutic implications based on my findings.

4.1 State of research

4.1.1 MAPK signalling pathway

The impact of MAPK signalling on LCH and NLCH has been a significant area of research in recent years. The prsence of the BRAF V600E mutation in approximately 50-60% of LCH cases has provided crucial insights into the pathogenesis of LCH (Badalian-Very et al., 2010). This activating mutation leads to an (over)activation of the downstream effectors MEK and ERK, promoting uncontrolled cell growth and survival of LCH cells (Classen et al., 2016).

In LCH, the presence of the BRAF V600E mutation has been associated with specific clinical features, such as a higher severity and more systemic infestation. This mutation has also emerged as a potential therapeutic target, with BRAF and MEK inhibitors showing promising results in clinical trials, leading to improved outcomes and durable remissions in some patients. (Badalian-Very et al., 2012; Haroche and Abla, 2015)

In NLCH, MAPK pathway alterations have been identified in certain subtypes, further emphasising the importance of MAPK signalling in these disorders. For instance, the BRAF V600E mutation has been identified in a subset of cases with ECD, particularly those involving bone and other organ systems. Additionally, mutations in other MAPK pathway components, such as MAP2K1, have been found in other NLCH entities like JXG and RDD suggesting a potential role of MAPK dysregulation in these disorders as well as in a broader category of histiocytic and dendritic cell neoplasms. (Diamond et al., 2016; Emile et al., 2016; Haroche et al., 2012b; Hyman et al., 2015)

BRAF V600E and MEK1 can be considered as prognostic markers in certain histiocytic disorders, including LCH, JXG, ECD and RDD. Prognostic markers are factors that provide valuable information about the likely clinical outcome and disease progression in patients with a specific condition. In the context of LCH and NLCH, the presence or absence of certain protein overexpression, such as BRAF V600E and MAP2K1, can influence the disease course and treatment response. (Hyman et al., 2015) An influence of the disease course can be their impact on disease severity: Studies have shown that LCH patients with the BRAF V600E mutation often present with a more severe and multisystem disease compared to those without the mutation. The presence of this mutation is associated with a higher risk of disease relapse and recurrence. This also impacts treatment response; patients with the BRAF V600E mutation in LCH have shown more favourable responses to targeted therapy with BRAF inhibitors (for example vemurafenib) compared to patients without this mutation. The outcome is also affected by this mutation. The BRAF V600E mutation status has been associated with differences in overall survival and progression-free survival in LCH patients. On the other side, MEK1 mutations have been identified as an alternative driver mutation in LCH patients without the BRAF V600E mutation. The presence of MAP2K1 mutations is associated with specific disease characteristics and can influence the course of the disease.

MAP2K1 is a component of the MAPK signalling pathway. Mutations in this gene can lead to uncontrolled activation of the pathway, promoting cell proliferation and survival. In the context of histiocytosis, mutations in the MAP2K1 gene have been identified and can influence disease characteristics and prognosis. The main disease characteristics are related to the histopathology, the disease specific symptoms and the diseases site. Some reports suggest that histiocytosis lesions with MAP2K1 mutations might have distinct histopathological features, such as less frequent eosinophilic infiltration compared to BRAF V600E-mutated lesions. The presence of MAP2K1 mutations might correlate with certain systemic symptoms, but this aspect needs further research to establish definitive associations.

MAP2K1 mutations might be associated with certain sites of disease involvement. For example, some studies suggest a higher frequency of CNS and bone involvement in ECD patients with MAP2K1 mutations compared to those with BRAF V600E mutations. (Brown et al., 2014; Diamond et al., 2016) These mutations also seem to have an influence on disease course, such as on prognosis and therapy. Some studies have indicated that MAP2K1 mutations might be associated with a more aggressive disease course or poorer prognosis in certain histiocytic disorders. However, the data can be somewhat inconsistent and larger studies are needed to confirm these associations. The presence of a MAP2K1 mutation could have implications for targeted therapy. MEK inhibitors, which target the protein encoded by MAP2K1, might be effective for patients with these mutations. (Brown et al., 2014; Diamond et al., 2016) Furthermore, MAP2K1 mutations can be found in many types of histiocytosis but some types have a stronger correlation with this mutation, such as ECD. While BRAF V600E mutations are the most common in ECD, a subset of BRAF V600E-negative ECD patients harbour MAP2K1 mutations. In LCH, MAP2K1 mutations are less common than BRAF V600E mutations but have been identified in some patients, especially those without the BRAF mutation. (Haroche et al., 2012b) It also has implications for treatment response and may influence the choice of targeted therapies in LCH patients without the BRAF V600E mutation. (Allen et al., 2018; Diamond et al., 2016)

It is important to note that while these mutations can serve as valuable prognostic markers in LCH and NLCH, disease prognosis is a complex interplay of various factors. Other clinical and genetic factors, along with the specific histiocytic disorder and its extent, should also be taken into consideration when determining the overall prognosis and tailoring treatment strategies for individual patients. BRAF V600E and MEK1 mutants can be targeted with specific drugs. Several targeted therapies have been developed to inhibit their signalling pathways in various disorders, including histiocytosis. The following drugs target the BRAF and MEK pathways: vemurafenib and dabrafenib (selective BRAF inhibitors) and trametinib (MEK inhibitor). All three inhibitors are approved for BRAF V600E-positive melanoma (Flaherty et al., 2011) but should also be considered for the therapy of histiocytosis. Off-label use of the aforementioned drugs, case reports as well as prospective experimental studies will help us improve treatment of LCH as well as of NLCH. When considering off-label use, side effects should be considered throughout therapy. Knowledge on side effects stem from melanoma therapy and range from skin rash, fatigue to peripheral oedema and photosensitivity. These side effects should be taken into consideration. (Allen and Parsons, 2015; Diamond et al., 2019)

In histiocytoses, clinical trials have shown promising results with the use of targeted therapies such as BRAF and MEK inhibitors, especially in LCH patients with BRAF V600E mutations. These inhibitors can effectively suppress the downstream signalling of BRAF and MEK, leading to regression and improved clinical outcomes in some cases. Regarding other drugs that can target the same pathway but have not been extensively tested in histiocytoses, some potential candidates include other MAPK pathway inhibitors such as ERK inhibitors. ERK is a downstream effector of the MAPK pathway. Targeting ERK may also hold therapeutic potential, although specific inhibitors are still under investigation. Combination of BRAF and MEK inhibitors have been shown to have synergistic effects in melanoma treatment (Flaherty et al., 2012). These combinations may also be explored in histiocytosis.

PI3K/AKT/mTOR pathway inhibitors: the PI3K/AKT/mTOR pathway interacts with the MAPK pathway and plays a role in cell growth and survival. Inhibitors targeting this pathway might also have potential in certain histiocytosis cases and should therefore be considered in future studies. (Allen and Parsons, 2015; Diamond et al., 2019; Senechal et al., 2007)

It is essential to emphasise that the use of targeted therapies in histiocytoses is still an area of active research. While some patients have shown favourable responses to these drugs, not all individuals with histiocytosis will benefit equally. Treatment decisions should be individualized based on a patient's specific genetic and clinical profile.

4.1.2 PD-L1

PD-L1 is an immune checkpoint molecule that plays a significant part in regulating immune reaction by binding to PD-1 (PD-L1 receptor) on T cells (Patsoukis et al., 2020). In LCH and NLCH, PD-L1 expression has been studied as a potential biomarker for disease prognosis and treatment response. In LCH, PD-L1 expression has been detected in lesional tissues, suggesting a possible role in immune evasion and tumour progression. Some studies have shown that higher PD-L1 expression in LCH lesions is associated with a more aggressive disease course and poor clinical outcomes. The presence of PD-L1 on LCH cells may inhibit the immune response and promote tumour survival by suppressing T cell activity. (Allen and Parsons, 2015; Senechal et al., 2007) In NLCH, which includes various rare forms of histiocytoses, the role of PD-L1 expression is less understood. Studies on specific NLCH subtypes, such as ECD and histiocytic sarcoma, have shown varying levels of PD-L1 expression. In this work, I could prove the overexpression of PD-L1 in XG, especially in disseminated XG. However, the impact of PD-L1 expression on clinical outcomes in these NLCH entities remains unknown and needs to be further elucidated. Targeting the PD-1/PD-L1 axis with immune checkpoint inhibitors has shown promise in various cancers, including some histiocytosis cases. Clinical trials exploring the effectiveness of PD-1/PD-L1 blockade in histiocytic disorders are ongoing and preliminary results suggest potential benefits for certain patients. (Diamond et al., 2019; Haroche et al., 2013; Satoh et al., 2012) In summary, PD-L1 expression is of interest in LCH and NLCH due to its potential as a prognostic marker and therapeutic target. However, more research is needed to fully understand the impact of PD-L1 on the pathogenesis and clinical behaviour of these histiocytic disorders.

PD-L1 is being investigated as a potential prognostic marker in various cancers and inflammatory diseases, including histiocytic disorders like LCH and NLCH. In this work, I investigated it as a prognostic marker for LCH and NLCH like XG. Higher levels of PD-L1 expression may indicate a stronger ability of the disease to suppress the immune response and evade immune surveillance, leading to disease progression and resistance to treatment. Therefore, PD-L1 expression in histiocytic lesions has been suggested as a potential negative prognostic marker, indicating a higher risk of disease relapse or progression. In NLCH, the role of PD-L1 as a prognostic marker is less established, as NLCH encompasses a heterogeneous group of rare histiocytic disorders. Studies exploring the association between PD-L1 expression and clinical outcomes in specific NLCH subtypes are limited. However, there have been reports of PD-L1 expression in some NLCH cases, raising the possibility of its potential as a prognostic marker in certain entities like XG. (Diamond et al., 2019; Haroche et al., 2013)

It is important to note that the prognostic value of PD-L1 as a marker in histiocytic disorders needs further investigation and validation. Additional research with larger patient cohorts and longer follow-up periods is necessary to fully understand the relationship between PD-L1 expression and disease prognosis in both LCH and NLCH. Moreover, PD-L1 expression is also considered a predictive marker for response to immune checkpoint inhibitors, such as PD-1/PD-L1 blockade therapy. Patients with higher levels of PD-L1 expression in their histiocytic disorder are more likely to respond to these immunotherapies.

This further emphasises the importance of exploring PD-L1 expression as a potential prognostic marker and as a predictor of treatment response in histiocytic disorders. (Diamond et al., 2019) Its expression has shown promise as a prognostic marker in disseminated and singular XG, indicating a potential association with disease aggressiveness and clinical outcomes. However, more comprehensive studies are required to establish its prognostic significance in histiocytic disorders and to explore its predictive value for response to targeted therapies, including immune checkpoint inhibitors.

Targeting PD-L1, which was shown to be a significant marker for XG in my study, should be taken into consideration. This marker is a target for immune checkpoint inhibitors, particularly PD-1/PD-L1 blockade therapies. These therapies aim to prevent the interaction between PD-1 receptors on immune cells and PD-L1 on tumour cells, thus preventing suppression of the immune system and allowing it to build a stronger anti-tumour response (Guha, 2014). Several of these checkpoint inhibitors have been approved for various cancers and are being investigated in clinical trials for potential use in other diseases, including histiocytic disorders. Some of the FDA-approved drugs are pembrolizumab (Keytruda), nivolumab (Opdivo), atezolizumab (Tecentriq), avelumab (Bavencio). (Ansell et al., 2015; Herbst et al., 2014; Li et al., 2019)

These drugs have shown promising results in various cancers by enabling the immune system's ability to recognize and attack tumour cells. However, their effectiveness and safety in histiocytic disorders, including LCH and NLCH, require further investigation in clinical trials. Side effects of PD-1/PD-L1 inhibitors, known as immune-related adverse events (irAEs), can affect different body parts and cause varying symptoms. Typical irAEs are, fatigue, diarrhoea, colitis and endocrine dysfunctions, rash, among others. (Diagnostics, 2023) While most irAEs are manageable with immunosuppressive drugs, they can sometimes be severe and require discontinuation of treatment. Other drugs that target the same pathway but have not been specifically tested in histiocytic disorders include cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibitors, like ipilimumab (yervoy). CTLA-4 is another immune checkpoint protein that downregulates T cell activation (Yun et al., 2015). Combined PD-1/PD-L1 and CTLA-4 blockade has shown synergistic effects in some cancers, leading to enhanced anti-tumour responses. (Ansell et al., 2015; Herbst et al., 2014; Li et al., 2019)

It is worth noting that the use of immune checkpoint inhibitors in histiocytic disorders is an active area of research and ongoing clinical trials are evaluating their safety and efficacy. Additionally, preclinical studies and case reports have provided some evidence of PD-1/PD-L1 expression in histiocytic lesions, suggesting the potential rationale for using immune checkpoint inhibitors in these diseases. (Fayiga et al., 2023; Haberecker et al., 2023; Haroun et al., 2017; Pileri et al., 2022) However, as with any investigational therapy, the use of immune checkpoint inhibitors in histiocytosis requires careful evaluation in clinical trials to establish their optimal dosing, safety and effectiveness. Furthermore, criteria for patient selection and potential biomarkers that could predict response to these therapies need to be identified to ensure their effective and appropriate use in histiocytic disorders. (Li et al., 2019)

4.1.3 TRK

The impact of TRK receptors on LCH and NLCH has not been extensively studied so far. Literature on the role of TRK receptors in histiocytic disorders is relatively limited and further research is needed to fully understand its significance in these diseases. In this study, I tried to determine if it could be used as a biomarker for LCH as well as for specific types of NCLH like XG, et cetera. This work detected a high IHC score (>8) of pan-TRK in XG but without significance (p-value >0.05), as well as high IHC scores in NXG, XD, RDD, ECD.

TRK receptors, including TRK A, TRK B and TRK C, are encoded by the NTRK1, NTRK2 and NTRK3 genes, respectively (Drilon et al., 2018). They are involved in various cellular processes, including cell survival, differentiation and proliferation. Abnormalities in the NTRK genes, such as gene fusions, result in constitutive activation of the TRK receptors, leading to oncogenic signalling. While the TRK pathway has been extensively studied in certain cancers, such as rare paediatric solid tumours and some adult malignancies, its specific role in histiocytic disorders is not well-defined. A few case reports and studies have described the presence of NTRK gene fusions, such as TPM3-NTRK1 and TRAF1-NTRK1, in some cases of LCH and NLCH. These fusions can lead to TRK activation, suggesting a potential role in disease pathogenesis. However, the overall frequency of these fusions and their clinical significance in histiocytosis are yet to be determined. (Cocco et al., 2018)

It is worth noting that there are targeted therapies that aim at inhibiting the TRK pathway, such as TRK inhibitors. They have shown remarkable efficacy in tumours harbouring NTRK fusions in other cancer types. Therefore, there is growing interest in exploring the potential therapeutic relevance of pan-TRK inhibitors in histiocytic disorders with NTRK fusions. Given the limited data available, further research is needed to explore the prevalence and clinical impact of TRK pathway alterations in LCH and NLCH. Large-scale genomic profiling studies, as well as functional and preclinical investigations, will be crucial to better understand the significance of TRK receptors in histiocytic disorders and to explore their potential as therapeutic targets.

There is limited evidence to consider TRK receptor expression as a prognostic marker in histiocytosis, including LCH and NLCH. Most of the available research on TRK receptors has focused on their role as potential therapeutic targets rather than a prognostic marker. The results from my work also show that they could not be trusted as prognostic markers. To determine whether pan-TRK can be considered a prognostic marker in histiocytosis, further research is needed. Large-scale studies with well-characterized patient cohorts and long-term follow-up are necessary to assess the relationship between TRK pathway activation and clinical outcomes in histiocytosis patients. Additionally, efforts to identify and validate other potential prognostic markers in histiocytosis are ongoing.

While some studies have reported the presence of NTRK gene fusions, which activate the TRK pathway, in the case of histiocytosis, the impact of these fusions on disease prognosis remains unclear. The rarity of histiocytosis and the limited number of cases with confirmed TRK pathway activation make it challenging to draw definitive conclusions on its prognostic significance. As the field of histiocytosis research advances and more data become available, there may be a better understanding of the prognostic implications of TRK pathway activation in histiocytosis. For now, the focus remains on characterizing the molecular landscape of these diseases and exploring the potential for targeted therapies, including TRK inhibitors, to improve patient outcome.

TRK is druggable and there are compounds known as TRK inhibitors that have shown potency in cancers with NTRK gene fusions, which activate the TRK pathway. These inhibitors can block the activity of the TRK proteins and potentially suppress tumour growth driven by NTRK fusions. Some of the TRK inhibitors include larotrectinib (Vitrakvi), as well as entrectinib (Rozlytrek).

Larotrectinib targets TRK A, TRK B and TRK C. It has shown remarkable efficacy in treating solid tumours harbouring NTRK gene fusions. The most common side effects include fatigue, dizziness, nausea, vomiting and constipation. Entrectinib targets TRK A, TRK B and TRK C as well as other kinases like proto-oncogene tyrosine-protein kinase 1 (ROS1) and anaplastic lymphoma kinase (ALK). Typical side effects include fatigue, constipation, parageusia and oedema.(DailyMed, 2020)

Regarding histiocytosis, the relationship between TRK and its potential as a therapeutic target is an emerging area of research. There are limited data on the use of TRK inhibitors, specifically for the treatment of histiocytosis. The clinical experience with TRK inhibitors in histiocytosis is relatively limited compared to other neoplasms. Since histiocytosis is a rare and heterogeneous group of disorders, research efforts are ongoing to identify specific biomarkers, including NTRK gene fusions, that could be targeted by TRK inhibitors. It is essential to conduct clinical trials and gather more evidence to determine the efficacy and safety of TRK inhibitors in histiocytosis patients with NTRK gene fusions. Furthermore, there might be other drugs or investigational compounds that can target the same pathway but are yet to be thoroughly tested in histiocytosis. As research progresses, new treatment options may emerge that target the TRK pathway or other related pathways, potentially providing more therapeutic options for patients with histiocytosis.

4.2 Critical reflections on the results

4.2.1 PD-L1

In this work, the most unexpected finding was that of the high expression of PD-L1 in XG lesions. It implies the involvement of PD-L1 in XG pathobiology, suggesting a possible role in immune evasion and tumour progression, especially in disseminated XG. It can possibly explain the varying clinical presentation, clinical outcome and treatment response of XG. It can therefore be assumed that XG cells might be able to inhibit the immune response and promote tumour survival by suppressing T cell activity due to the presence of PD-L1, especially in disseminated lesions. This can be associated with a more aggressive disease progression and a poor clinical outcome, as is already known to occur in disseminated forms of adult and JXG (Dehner, 2003). These findings also indicate that patients with XG, particularly with disseminated XG, might benefit from treatment with immune checkpoint inhibitors, such as PD-1/PD-L1-blockade therapy (for example pembrolizumab). This study is the first to report a high expression of PD-L1 in XG. My results show high PD-L1 expression in most analysed XG cases, especially in disseminated XG. These findings contribute to Fayiga et al.'s recent case study which describes the presence of increased PD-L1 mRNA in a patient with disseminated XG (Fayiga et al., 2023). However, due to the small sample size and unequal distribution, caution must be applied when interpreting these results. In future investigations, it might be possible to acquire a larger cohort with more detailed clinical patient data to confirm and validate my findings. In a different manner, LCH and ECD surprisingly showed a very low expression of PD-L1, contrary to results reported by Gatalica, among others (Gatalica et al., 2015). This inconsistency may be caused by the difficulty to reproduce semi-quantitative IHC scoring results due to its subjective nature. In future studies, it might be beneficial to use an artificial intelligence-assisted quantitative method based on immunopositive cell count with hope of increasing reproducibility in IHC evaluation (Bencze et al., 2021). Future work may also benefit from prospective studies in which fresh tissue samples can be generated. Although recent literature asserts that older FFPE tissue has a similar quality and can reliably be used up to 30 years later (Ono et al., 2018), I propose the generation of fresh FFPE samples to better avoid artefacts and weaker stainability. Furthermore, mutational analysis, such as NGS in further research is required to confirm whether an actual genetic mutation is on hand or solely a high protein expression.

4.2.2 Proteins of the MAPK pathway

- BRAF V600E

Several reports have identified activating mutations in the MAPK pathway, including somatic BRAF V600E mutations, in LCH and NLCH. The results of my study further confirm the association between BRAF V600E expression and histiocytoses. Histiocytic disorders with high, but statistically insignificant, BRAF V600E expression were LCH, predominantly MS-LCH and multifocal LCH, disseminated XG, NXG, XD, RDD, ECD and GEH. The overexpression of BRAF V600E in MS-LCH and disseminated XG is consistent with the literature that describes a disease severity when BRAF V600E is highly expressed. However, a strong expression of MEK and ERK in LCH, which can be interpreted as a missing activation of downstream effectors, could not be observed. It is difficult to explain this result, but it might be related to the subjective analysis of histiocytes among non-histiocytic cells, such as lymphocytes and eosinophiles. Mutational analysis, such as cell free DNA (cfDNA) analysis, is needed in future work to confirm the presence of the BRAF V600E mutation (Hyman et al., 2015). These results further encourage the use of selective BRAF inhibitors (vemurafenib and dabrafenib) in LCH and NLCH with BRAF V600E overexpression or in severe LCH and NLCH cases in order to improve clinical outcome. In practice, a combination of BRAF and MEK inhibitors can be applied to profit from the synergistic effect (Flaherty et al., 2012).

- MEK

Some reports have identified activating mutations in MEK1 in LCH and NLCH. The results of my study further confirm the association between MEK expression and histiocytoses. Histiocytic disorders with high MEK (MEK1/2, MEK2 or pMEK1/2) expression of significant value were high-risk LCH, predominantly multifocal LCH and XG. The overexpression of MEK can be interpreted as a possible MEK mutation. Such mutations are often present in the absence of BRAF V600E mutations and can activate ERK1/2 (Allen et al., 2018; Diamond et al., 2016). MEK1 mutations of BRAF V600E-negative histiocytoses, such as XG have been reported in the literature, however my study indicates a MEK2-mutation in XG. Similar to BRAF V600E mutations, MEK mutations are associated with disease severity and poor prognosis. Mutational analysis is advised in future studies to confirm the presence of the MEK1 and/or MEK2 mutations (Hyman et al., 2015). My results further encourage the use of MEK inhibitors (for example trametinib) in MEK-positive LCH and NLCH without BRAF V600E overexpression or in severe LCH and NLCH cases in order to improve clinical outcome.

- ERK

In the literature, very little is known about ERK mutations in histiocytoses. The results of this study show an association between ERK overexpression and histiocytoses. Histiocytic disorders with significantly high ERK (ERK1/2 or ERK2) expression were LCH and XG. The overexpression of ERK in multifocal LCH, MS-LCH and XG is an intriguing finding and may be related to the upstream activation of MAPK pathway proteins. Although little is known as to how high ERK expressions can influence LCH and NLCH, a prognostic impact can be assumed, as is the case of the overexpression of other MAPK pathway components. Additional studies will be needed to confirm and validate these findings using a larger cohort. Future research with focus on mutational analysis is necessary to identify the specific ERK mutations. My findings indicate that patients with histiocytoses, especially LCH and XG with ERK overexpression might benefit from treatment with ERK inhibitors in the future, although these are not yet clinically established (Morris et al., 2013; PubChem, 2023; Yang et al., 2023).

Generally, the other NLCH (NXG, XD, RDD, ECD and GEH) showed a high expression of all MAPK pathway proteins, indicating a probable BRAF mutation that led to a general activation of downstream MAPK pathway proteins. Mutations of components of the MAPK pathway play a significant part in tumourigenesis by stimulating cell proliferation and migration (Kim and Choi, 2015). These findings are consistent with the literature, encouraging the use of BRAF and MEK inhibitors in NLCH cases with mutations of MAPK pathway components, especially BRAF.

4.2.3 TRK

Very little was found in the literature regarding TRK expression in histiocytoses. Haberecker et al. described a few NTRK fusions which also resulted in increased TRK expression confirming the presence of NTRK rearrangements in histiocytosis (Haberecker et al., 2023). My results are in line with those of Haberecker et al. and thereby further support the idea of NTRK fusions in histiocytic disorders. In my study, there were several NLCH with high TRK expression levels, though none of statistical significance. These results may not be reproducible on a wide scale due to the small sample size. Additional studies will be needed to confirm and validate these findings using a larger cohort, although acquiring a large number of histiocytosis cases might be challenging as most disorders are very rare. Histiocytic disorders with notable expression of TRK were multifocal LCH, XG, specifically disseminated XG, NXG, XD, RDD and ECD. High TRK protein expression detected with IHC stainings suggests the presence of NTRK fusions. NTRK gene fusions play a role in malignant tumours by enabling (over)expression of tyrosine kinases that contribute to tumour cell proliferation (Amatu et al., 2016). Further studies on this topic are therefore necessary and recommended. Future research with focus on mutational analysis, such as NGS is required to identify the specific NTRK rearrangements in disorders with high TRK protein expression, which will make targeted therapy aiming at the right TRK protein possible. My findings indicate that patients with histiocytoses, especially NLCH, might benefit from treatment with TRK inhibitors, such as entrectinib and larotrectinib, that both target TRK A, B and C.

4.3 Outlook

A mutation is a change or alteration in the DNA sequence of an organism's genome. It can occur due to various factors, including external environmental influences (for example radiation, chemicals) or internal cellular processes (for example errors during DNA replication or repair). Mutations can range in size, from small changes in a single nucleotide (point mutations) to large-scale structural alterations involving whole genes or chromosomal segments. Mutations in specific genes or regulatory regions can lead to aberrant protein expression, which may play a critical part in the development and progression of histiocytic disorders. One significant signalling pathway implicated in histiocytosis is the MAPK pathway, which regulates cell growth, differentiation and survival. Mutations in genes within this pathway, such as BRAF and MAP2K1/2 (encoding MEK1/2), are known to be associated with various histiocytic disorders.

For example, in LCH, activating mutations in the BRAF gene have been detected in a significant proportion of cases. These mutations lead to the constitutive activation of the BRAF protein, which is a key component of the MAPK pathway. As a result, the downstream signalling cascade is continuously activated, promoting uncontrolled cell proliferation and survival of pathological LC.

Similarly, in ECD, another type of histiocytosis, mutations in the MAP2K1 gene have been reported. These mutations also result in the hyperactivation of the MEK1 protein, leading to dysregulated cell growth and the formation of infiltrative lesions.

In both LCH and ECD, the presence of these mutations leads to an upregulation of key proteins in the MAPK pathway, such as phosphorylated MEK and ERK. These elevated protein levels can be detected through IHC analysis, indicating a high expression of the respective markers in affected tissues.

These high protein marker expressions are not only diagnostic indicators but also hold potential as prognostic markers. They can help identify patients with aggressive or treatment-resistant forms of histiocytosis, allowing for more targeted and personalized therapeutic approaches. By understanding the molecular basis of these disorders, including the impact of mutations on protein expression, researchers and clinicians can establish new treatments that specifically target the dysregulated pathways, ultimately improving patient outcome.

In the context of histiocytosis, the question of whether every biopsy should be analysed immunohistochemically requires careful consideration. IHC is a valuable tool that allows for the detection and characterization of specific proteins and their expression patterns within tissue samples. However, performing IHC on every biopsy may not always be necessary or practical due to various factors. The decision of when to conduct IHC analysis should be guided by several key considerations:

- Diagnostic uncertainty

In cases where the diagnosis of histiocytosis is uncertain based on routine histopathology alone, IHC can play a crucial role in confirming or further characterizing the specific type of histiocytosis. It can help to distinguish between different subtypes of histiocytoses or differentiate histiocytic disorders from other similar conditions, leading to a more accurate diagnosis.

- Clinical presentation

IHC analysis may be more warranted in patients with atypical clinical presentations or those who exhibit high-risk factors, such as involvement of risk organs, disseminated, multisystemic or multifocal clinical presentation. In such cases, IHC can provide additional insights into the disease's aggressiveness and prognosis, which may guide treatment decisions.

- Availability of resources

Conducting IHC analysis on every biopsy can be resource-intensive in terms of time, costs and skilled personnel. Therefore, it may be more feasible to prioritise IHC analysis for cases where it is most likely to impact clinical management or where there is diagnostic uncertainty.

- Confirmation of marker expression

The analysis of specific IHC markers associated with histiocytosis, such as S100, CD1a, CD207, stabilin-1 and CD68 have been the focus of histiocytosis studies in the past. In future studies or cases in which these markers have already been confirmed through pre-selection or previous analyses, additional IHC analysis with newly significant markers may not be necessary.

- Research Studies

In the context of research studies aimed at understanding the molecular mechanisms and prognostic markers of histiocytosis, comprehensive IHC analysis may be more appropriate. However, even in research settings, prioritisation based on clinical relevance and available resources remains essential.

Furthermore, while IHC is a valuable technique for characterizing histiocytosis and identifying prognostic markers, performing IHC analysis on every biopsy may not always be necessary or practical. Decisions regarding IHC analysis should be guided by factors such as diagnostic uncertainty, high-risk clinical features, available resources and research objectives. A thoughtful and strategic approach to IHC analysis can help ensure that it is utilized effectively to enhance the understanding and management of histiocytic disorders.

While off-label use of the abovementioned therapeutic targets could spark hope for some patients, it is crucial to approach this option with caution. Physicians must thoroughly evaluate the risks and potential benefits for each individual case. Close monitoring of patients undergoing off-label treatments is necessary to track their responses, potential side effects and overall safety. Despite the possibilities of off-label use, it is essential to emphasise the importance of ongoing research to identify and develop targeted and validated therapies for histiocytic disorders. Clinical trials focusing on specific genetic mutations and signalling pathways, such as the MAPK pathway, NTRK family and PD-L1, are fundamental to better understand the underlying mechanisms of the diseases. Research endeavours should also focus on exploring the factors contributing to disease severity, response to treatment and long-term outcomes. Comprehensive studies involving larger patient cohorts, standardised data collection and rigorous analysis will enhance our understanding of the disease and enable the development of more effective therapies.

To further advance the field, comprehensive mutation analysis can significantly contribute to understanding histiocytic disorders better. Exploring the genetic landscape of histiocytosis patients can help identify specific mutations associated with disease susceptibility, severity or response to treatment. Whole exome sequencing or targeted gene panels can be employed to identify novel genetic alterations and potential therapeutic targets. Additionally, the study of familiar cases of histiocytosis can shed light on the transmission of the disease to younger generations. Investigating possible genetic predispositions and heredity patterns may uncover critical insights regarding disease risk factors and facilitate early detection and intervention in at-risk families.

In conclusion, while off-label use may be considered in certain cases of histiocytic disorders, it should only be pursued cautiously when no approved therapies are available or when all other options have been exhausted. Prioritising targeted and validated therapies through rigorous research remains paramount. Comprehensive mutation analysis and exploration of transmission patterns will provide a deeper understanding of the disorders and guide the development of more effective, personalized treatments. The ultimate goal is to improve patient outcomes, enhance disease management and offer hope to patients and families affected by histiocytic disorders.

In the course of this work, biological markers that have been reported in the literature (MAPK pathway proteins), as well as markers that have not yet been tested (PD-L1, TRK) were identified and their expression in LCH and NLCH was analysed. I was able to demonstrate the significant representation of two of my marker groups (MAPK components and PD-L1) and the positive correlation with the prognosis of the associated histiocytosis.

In further studies, the limitations of this work should be considered and if need be, adjusted as possible. The analysis of some samples and markers was only possible to a limited extent. A larger cohort should be recruited for this purpose in order to expand the limited number of samples per histiocytosis type. Furthermore, my study could only generate retrospective data. A prospective cohort study with mutation analysis, targeted therapy and regular inspections would improve data quality and advance research into optimal diagnosis and therapy.

5 SUMMARY

This work aimed at unravelling crucial clinical parameters and prognostic markers for patients diagnosed with Langerhans cell histiocytosis (LCH) and non-Langerhans cell histiocytosis (NLCH). The objective was to enhance our understanding of the demographics of the cohort and derive specific conclusions related to the mitogen-activated protein kinase (MAPK) pathway, proteins of the neurotrophic tyrosine receptor kinase (NTRK) family and programmed cell death-ligand 1 (PD-L1). This in-depth analysis of druggable key signalling pathways and molecules sought to expand therapeutic options for patients facing severe or therapy-resistant forms of histiocytosis, particularly those with involvement of risk organs and disseminated, multisystemic or multifocal clinical presentations. I focused on members of the MAPK pathway, NTRK family proteins and on the immunorelevant PD-L1.

The study material encompassed tissue microarray (TMA) data obtained from patients diagnosed with histiocytosis, collected through a multicentric effort involving six renowned German dermatology clinics. Only tissue samples with histopathologically confirmed diagnoses were utilized, further validated through specific immunohistochemical (IHC) stainings against diagnostic markers after generating three TMAs. Further IHC stainings of the TMAs were conducted against members of the MAPK pathway, NTRK family proteins and PD-L1.

The nationwide multicentric study included 124 cases contributed by six teaching hospitals. After rigorous data collection and pre-selection, 69 patients with histiocytosis diagnoses were included in the final cohort. The breakdown of histiocytosis subtypes allowed for meaningful statistical analyses, with 11 LCH cases, 25 xanthogranuloma (XG) cases and a limited number of other histiocytosis. Other anlaysed NLCH were necrobiotic xanthogranuloma (NXG), xanthoma disseminatum (XD), Rosai-Dorfman disease (RDD), Erdheim-Chester disease (ECD) and generalized eruptive histiocytosis (GEH). Ethical guidelines were strictly adhered to and the study was conducted retrospectively in an anonymised manner.

The TMAs were created, focusing on LCH, XG and a few other histiocytoses. IHC stainings were performed on the TMA samples using specific antibodies (anti-S100, anti-CD1a, anti-CD207, anti-stabilin-1, anti-CD68, anti-BRAF, anti-MEK1/2, anti-MEK2, anti-pMEK1/2, anti-ERK1/2, anti-ERK2, anti-pERK1/2, anti-pan-TRK and anti-PD-L1).

Digital scanning of the stained TMAs enabled efficient evaluation and analysis. The semiquantitative multiplicative quickscore method was employed, considering staining intensity and the percentage of immunopositive cells. The experimental markers were statistically analysed to identify potential therapeutical targets and correlations with clinicohistopathological data.

The study's findings shed light on the clinical demographics of histiocytosis patients and provided valuable insights into the involvement of the MAPK pathway in histiocytosis pathology. By analysing druggable key signalling pathways and molecules, I have identified potential prognostic markers for patients with LCH and NLCH, particularly those with severe or therapy-resistant forms. Notably, I have explored the prognostic value of MAPK pathway members, NTRK family proteins and PD-L1 expression.

This study appears to be the first to detect significant PD-L1 overexpression in XG, especially in disseminated XG. It implies the involvement of PD-L1 in XG pathobiology and indicates a potential role of XG cells in immune evasion and tumour progression. Patients with higher levels of PD-L1 expression are more likely to benefit from therapy with immune checkpoint inhibitors.

Further results of my study confirm the known association between MAPK pathway proteins and histiocytoses. Firstly, several histiocytic disorders with BRAF V600E overexpression, though statistically insignificant, were found. High BRAF V600E expression could be detected in LCH, particularly multisystemic and multifocal LCH, as well as disseminated XG, NXG, XD, RDD, ECD and GEH. Secondly, there were findings of significant MEK (MEK1/2, MEK2 or pMEK1/2) overexpression in high-risk LCH, predominantly multifocal LCH and XG. High MEK expression can be synonymous with a possible MEK mutation which, in the literature, is often present in the absence of BRAF V600E mutations and can (over)activate ERK1/2. Although MEK1 mutations are known to appear in histiocytosis, my study sheds new light on the possible presence of MEK2-mutation in XG. Lastly, the analysis of ERK undertaken here showed a significant ERK (ERK1/2 or ERK2) overexpression in LCH, especially in multifocal and multisystemic LCH and in XG. This is an interesting result and is most likely due to (over)activation of upstream MAPK pathway components.

Finally, in most histiocytoses (multifocal LCH, XG, specifically disseminated XG, NXG, XD, RDD and ECD) high TRK expression levels could be observed, though none of statistical significance. These findings suggest the presence of NTRK fusions and indicates a possible benefit from treatment with TRK inhibitors.

In conclusion, this doctoral thesis represents a significant step forward in understanding histiocytic disorders and identifying potentially targetable markers for LCH and NLCH patients. The comprehensive IHC analysis has illuminated the importance of the MAPK pathway and other key molecules in these diseases. By expanding therapeutic options and recognizing crucial prognostic indicators, this research holds promise for improving patient outcomes and advancing our knowledge of histiocytosis pathogenesis. Further studies and clinical trials could build upon these findings, ultimately contributing to the benefit of patients with histiocytic disorders worldwide.

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7 CURRICULUM VITAE

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2006 – 2007	Luise-Hensel-Realschule, Aachen
2007 – 2013	Kaiser-Karls-Gymnasium, Aachen
29.06.2013	General qualification for university entrance (Abitur)
2013 – 2015	School of medical laboratory scientists (MTLA), University Hospital Cologne

ACADEMIC CAREER

2015 - 2021	University of Heidelberg (Medical Faculty Mannheim) Medicine
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Igbo	Basics

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