Received: 6 January 2023 | Revised: 5 April 2023 | Accepted: 10 April 2023

DOI: 10.1002/ajh.26938

RESEARCH ARTICLE

Molecular and clinicopathologic characterization of pediatric histiocytoses

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Funding information
Conquer Cancer Foundation ASCO Young Investigator Award funded by the Strike 3 Foundation, Grant/Award Number: 11413; Programme de Recherche Translationnelle en Cancérologie, Grant/Award Number: 19-143

Abstract

The spectrum of somatic mutations in pediatric histiocytoses and their clinical implications are not fully characterized, especially for non-Langerhans cell histiocytosis (-LCH) subtypes. A cohort of 415 children with histiocytosis from the French histiocytosis registry was reviewed and analyzed for *BRAF*^{V600E}. Most *BRAF*^{WT} samples were analyzed by next-generation sequencing (NGS) with a custom panel of genes for histiocytosis and myeloid neoplasia. Of 415 case samples, there were 366 LCH, 1 Erdheim-Chester disease, 21 Rosai-Dorfman disease (RDD), 21 juvenile xanthogranuloma (JXG, mostly with severe presentation), and 6 malignant histiocytosis (MH). *BRAF*^{V600E} was the most common mutation found in LCH (50.3%, $n = 184$). Among 105 non-*BRAF*^{V600E}-mutated LCH case samples, NGS revealed mutations as follows: *MAP2K1* ($n = 44$), *BRAF* exon 12 deletions ($n = 26$), and duplications ($n = 8$), other *BRAF* V600 codon mutation ($n = 4$), and non-MAP-kinase pathway genes ($n = 5$). Wild-type sequences were identified in 17.1% of samples. *BRAF*^{V600E} was the only variant significantly correlated with critical presentations: organ-risk involvement and

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neurodegeneration. MAP-kinase pathway mutations were identified in seven RDD (mostly *MAP2K1*) and three JXG samples, but most samples were wild-type on NGS. Finally, two MH samples had *KRAS* mutations, and one had a novel *BRAF*^{G469R} mutation. Rarely, we identified mutations unrelated to MAP-kinase pathway genes. In conclusion, we characterized the mutational spectrum of childhood LCH and clinical correlations of variants and subtypes. Variants responsible for JXG and RDD were not elucidated in more than half of the cases, calling for other sequencing approaches.

1 | INTRODUCTION

Histiocytosis constitutes a group of rare disorders arising from tissue infiltration by myeloid cells with diverse macrophage or dendritic cell phenotypes.[1] Oncogenic somatic mutations of the MAP-kinase pathway have been reported in varying frequencies depending on histiocytosis subgroup, age of the studied population, and sensitivity of the molecular method used.

Langerhans cell histiocytosis (LCH) and Erdheim-Chester disease (ECD) are included among the L group histiocytoses in the 2016 revised classification[2] because of their shared clinical and molecular characteristics. LCH is by far the most common histiocytosis with a pediatric predominance, whereas ECD is very rare in children. For this L group, the mutational spectrum is the best described, and molecular alterations involving the MAP-kinase pathway are assumed to be identifiable in about three of every four cases. In childhood LCH, *BRAF*^{V600E} is the most frequently identified mutation (about 50%), followed by alterations in exons 2 and 3 of *MAP2K1*, which are found in ~11%–30% of pediatric cases.[3–8] Recently, small deletions and duplications of *BRAF* exon 12 have been reported, but their prevalence is less clearly established.[9,10] Data are even more scarce for the mutational spectrum of less frequent histiocytoses such as juvenile xanthogranuloma (JXG), Rosai-Dorfman disease (RDD), and malignant histiocytosis (MH).[7,11,12]

The mutation status related to the MAP-kinase pathway is of therapeutic interest because of implications for which targeted therapy is indicated for chemotherapy-resistant disease: specific inhibitors of BRAF in the context of the *BRAF*^{V600E} mutation and MEK inhibitors for other *BRAF* or *MAP2K1* mutations.[1] Moreover, we have established a genotype–phenotype correlation in childhood LCH between the *BRAF*^{V600E} mutation and multisystem (MS) risk organs positive (RO+) LCH, resistance to first-line vinblastine-steroid chemotherapy, reactivations, and late sequelae, in particular neurodegenerative ramifications.[13,14] For pediatric LCH, these data have paved the way to developing tailored strategies of therapeutic stratification and long-term adapted follow-up based on molecular findings.

Molecular analysis in histiocytoses represents a difficulty compared with other neoplasia pathologies because of the low infiltration into lesions of pathological cells bearing the oncogenic mutation and the limited material from diagnostic biopsies in young children.[4,15] Centralized analysis in a reference pathology laboratory is of interest in order to benefit from an optimized process that includes histological review, macrodissection if necessary to enrich the sample in pathological histiocytosis, and finally the choice of analytical method, with digital PCR if necessary.[13]

This study used data from a representative cohort of children treated for pediatric histiocytosis, and whose molecular analysis was centralized in a national expert reference laboratory. The aim was to define the spectrum of somatic mutations for each histiocytosis subtype and characterize genotype–phenotype correlations, with a specific focus on non-*BRAF*^{V600E} mutations in childhood LCH and on mutations associated with JXG, RDD, and MH (i.e., non-LCH subtypes).

2 | MATERIALS AND METHODS

2.1 | Patients

Among patients with pediatric histiocytosis included in the French National histiocytoses registry,[16] 484 had benefited from the histologic review over the last 12 years (2010–2021) in the histiocytosis French reference laboratory. The inclusion criteria were age <18 years at diagnosis, presence of histiocytes in analyzed material, and amplifiable DNA detected after extraction. The exclusion criteria were the absence of histiocytosis infiltration in samples or non-amplifiable DNA. This study was approved by the Ethics Committee, Sud-Ouest et Outre-Mer II (#2019-A01814-53), and was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent (clinical trial registration NCT04437381 [Molecular Targets for the Treatment of Histiocytosis TARGET-HISTIO]). Among the 484 pediatric biopsy samples received during the covered period, 415 were from cases that met the inclusion criteria (Figure 1).

Demographic data, clinical characteristics, extent of disease, type of treatment and response to therapy, reactivation, and permanent sequelae were recorded as previously described[13] and according to classifications established by the Histiocyte Society.[17] Treatment efficacy was evaluated according to the classification used by the Histiocyte Society. When tumorous lesions were present, we used the Response Evaluation Criteria in Solid Tumors version 1.1, as previously described.[18] Among childhood, LCH patients included here, 214/366 (58.5%, Supplementary Material 1A) were already included

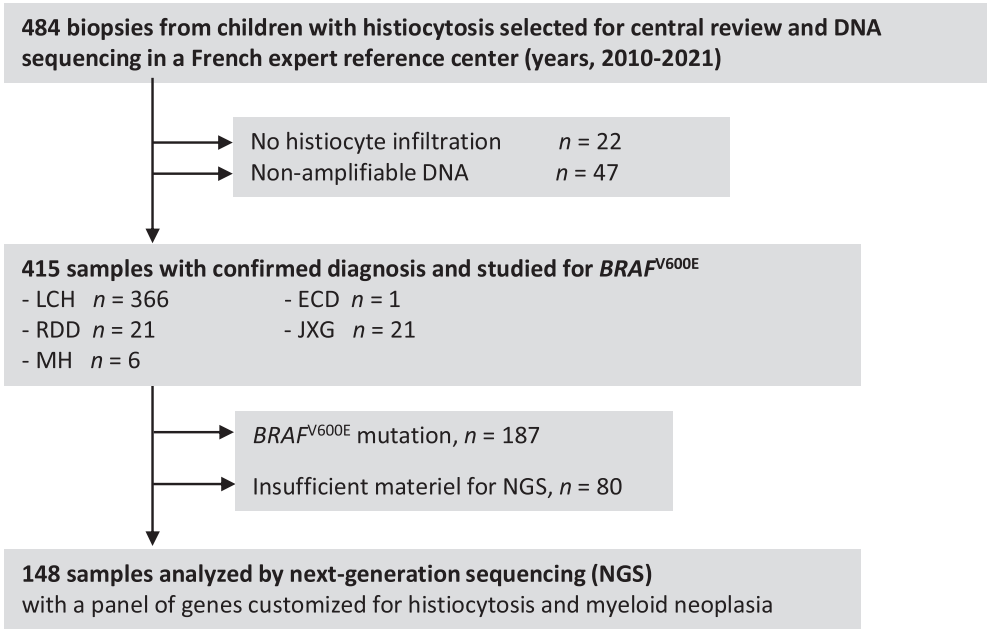

FIGURE 1 Workflow of recruitment of histiocytosis patients into the study. ECD, Erdheim-Chester disease; JXG, juvenile xanthogranuloma; LCH, Langerhans cell histiocytosis; MH, malignant histiocytosis; NGS, next-generation sequencing; RDD, Rosai-Dorfman disease.

in the *BRAF*^{V600E} correlation study published in 2016 by our group, with extended follow-up.[13]

2.2 | Histological review and DNA extraction

Histologic control with hematoxylin and eosin staining and CD1a (Dako, clone 010, #IR069), CD207 (Leica, #NCL-L-Langerin), and CD163 (Leica, clone 10D6, #NCL-CD163) antibody analysis was performed to review the histiocytic disorder diagnosis and identify a region suitable for DNA extraction. Tissue samples were macrodissected and the percentage of histiocytes was evaluated for each patient. Since 2016, pERK (Cell Signaling, CD13.14.4E, XP®, #8544) antibody has progressively been applied to complete immunohistochemistry (IHC) analysis.

For most samples, DNA was extracted as described by Colomba et al.[19] Since 2020, DNA extraction has been automated on a Maxwell® RSC Instrument (Promega, France), with extraction performed according to the supplier's recommendations. For formalin-fixed paraffin-embedded (FFPE) and frozen biological materials, the Maxwell® RSC DNA FFPE Kit and Maxwell® RSC Tissue DNA Kit were used, respectively. For targeted *BRAF*^{V600} analysis, DNA was quantified by a spectrophotometric method (ND-100, Nanodrop®, or Multiscan Go, Thermo Fisher Scientific, France). For next-generation sequencing (NGS) analysis, DNA quantity was evaluated by the Qubit Fluorometer (Thermo Fisher Scientific, France).

2.3 | *BRAF*^{V600} analysis

BRAF^{V600} status was defined using first-line detection methods (qPCR or pyrosequencing), as previously described,[13,19] and reviewed using a digital PCR approach if the first result was doubtful.[20] Since 2021, we have applied a new digital PCR method using a QIAcuity One, 5plex Instrument (Qiagen) with a commercially available BRAF^{V600E} assay (#DMH0000004, Qiagen). The reaction conditions for this digital PCR are reported in Supplementary Material 1B.

2.4 | DNA-Seq NGS

The DNA-Seq panel included almost 60 genes covering hot spots or all exons previously reported to be mutated in histiocytoses and genes involved in the MAP-kinase pathway and myeloid neoplasia. The sequencing technique used and the regions covered by panels used in this study are presented in Supplementary Material 1C–E. The quality sequencing data and target coverage quality data are also included. The sequencing data were analyzed depending on the applied technique. Mutations detected by DNA sequencing were interpreted according to standards and guidelines as described by Richards et al.,[21] and Sanger sequencing and length analysis of PCR products (LAPP) methods were used to confirm unusual and/or low-frequency *BRAF* and *MA2K1* alterations, as described in Supplementary Material 1F,G.

2.5 | Statistical analysis

Differences between groups were tested with the Mann–Whitney *U* test for quantitative variables and Fisher's exact test for qualitative variables. For statistical analysis, the threshold significance was 0.01, and for univariate analyses (multitests) of LCH presentation according to mutational status, $p < .002$ was considered statistically significant (Bonferroni correction). Survival analyses included the interval between diagnosis and an event (reactivation or death) or the last examination. Survival rates were estimated with the Kaplan–Meier

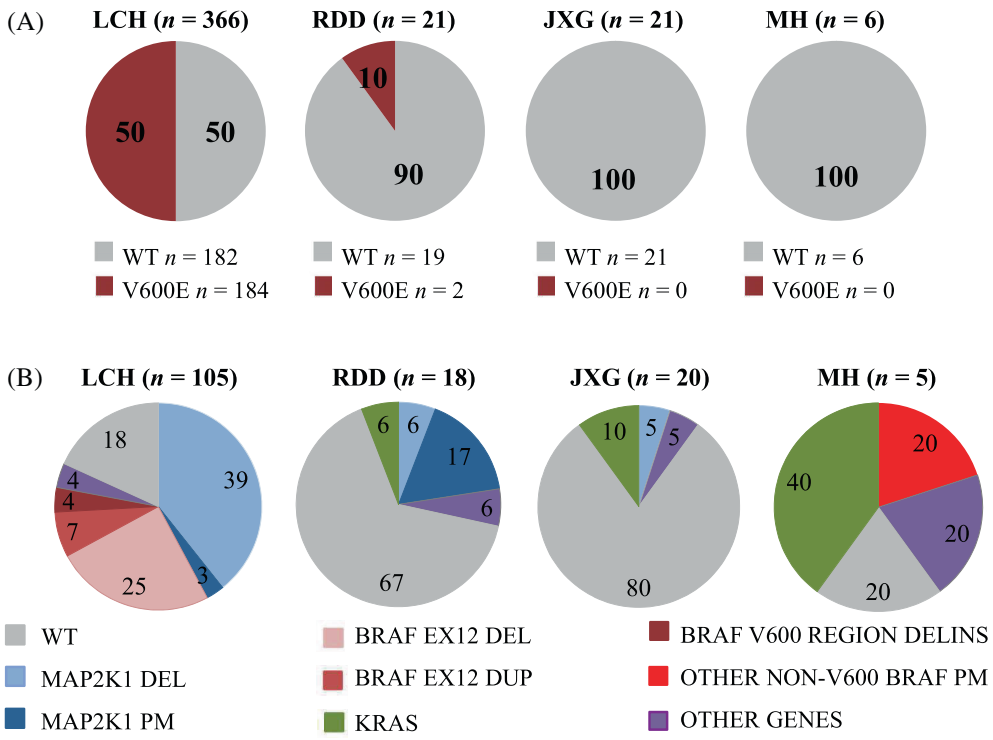

FIGURE 2 Molecular overview of pediatric histiocytoses. (A) Frequency of *BRAF*^{V600E} mutation in different histiocytosis disorders. The sole ECD *BRAF*^{V600E} patient is not represented. (B) Landscape and frequency of different mutations observed by DNA-Seq in histiocytosis cases that were wild-type (WT) for *BRAF*^{V600}. The section results are given in %. DUP, duplication; INS, insertion; PM, point mutation.

method, and subgroups were compared with the log-rank test. All statistical analyses were performed with Stata 13 software. The cut-off date for these analyses was June 31, 2022.

3 | RESULTS

A total of 415 biopsies were analyzed from 415 children with confirmed diagnoses as follows: LCH, 366 patients; ECD, 1 patient; RDD, 21 patients; JXG, 21 patients; and MH, 6 patients. The *BRAF*^{V600E} mutation was detected in 45.1% of 415 samples ($n = 187/415$) and almost exclusively in cases of LCH. Three non-LCH histiocytosis samples showed *BRAF*^{V600E} mutations, two from RDD and the ECD case (Figure 2A).

Because of DNA quantity and quality, DNA-Seq analysis was not performed in 80 *BRAF*^{WT} (wild-type) samples. Among 148 samples that were wild-type at the *BRAF*^{V600E} locus and analyzed by DNA-Seq, 26.4% still had other aberrations in *BRAF* ($n = 39/148$), mostly deletions and small duplications localized in exon 12, and 33.1% had mutations in the *MAP2K1* gene ($n = 49/148$). Among *MAP2K1* alterations, exon 2 deletions were more frequent than exon 3 deletions (57.2% vs. 30.6% of all *MAP2K1* alterations), whereas point mutations were rare although seen in both exons (6.1% in exon 2 and 6.1% in exon 3, of all *MAP2K1* alterations). In 9.5% of samples ($n = 14/148$), we detected a mutation in genes other than *BRAF* and *MAP2K1*, and in three cases, these mutations coexisted with *BRAF* or *MAP2K1* variants. For both RDD and JXG samples, *MAP2K1* and *KRAS* mutations were occasionally found, but for most of these histiocytosis subtypes, no mutation was identified despite our large NGS panel. Finally, almost all patients analyzed by NGS for MH had at least one non-recurrent molecular alteration (Figure 2B). A detailed description of all detected mutations is presented in Supplementary Table 1.

3.1 | Molecular status among children with LCH and clinical correlation

Among 366 children with LCH investigated for *BRAF*^{V600E}, 184 (50.3%) harbored this mutation. Among those with non-*BRAF*^{V600E}-mutated LCH ($n = 182$), 105 samples were subjected to further molecular analysis. In this subgroup, the prevalence of other somatic mutations was 41.9% for *MAP2K1* exon 2 or 3 mutations ($n = 44$), 24.8% for a small deletion in *BRAF* exon 12 ($n = 26$), and 7.6% for a small duplication in *BRAF* exon 12 ($n = 8$) (Figure 2B, Supplementary Table 1). Four patients had a *BRAF* mutation localized at the V600 codon that was different from V600E (V600_W604delinsKTDG, V600_K601delinsEISMEWVP, V600delinsDL, and V600D). Four other patients had non-recurrent mutations without other molecular alterations: *CSF1R*^{Q965*} (variant allele frequency [VAF], 3.4%) together with *EZH2*^{P751S} (VAF, 15.0%), *ETV6*^{R369W} (VAF, 8.2%), *NF1*^{R1250W} (VAF, 4.2%), and *TET2*^{Q810*} (VAF, 12.2%). In two children, *MAP2K1* deletion was found together with either *JAK2*^{R677R} (VAF, 10.2%) or *GATA2*$^{G135\_P137del}$ (VAF, 10.0%) mutation. Thus, we sometimes but rarely observed alterations in genes commonly mutated in myeloid malignancies or clonal hematopoiesis, in contrast to what is reported in adults with ECD.[22]

Finally, no significant mutation was identified after NGS analysis in 19/105 samples (18.1% of non-*BRAF*^{V600E}-mutated samples). *BRAF*^{V600E} mutation was associated with multisystem (MS) disease

TABLE 1 Characteristics of LCH patients according to *BRAF*^{V600E} status; values in % of *n* unless otherwise indicated.

Characteristics	With *BRAF*^{V600E} ($n = 184$)	Without *BRAF*^{V600E} ($n = 182$)	*p*
Male	51.6	64.8	.01
Female	48.4	35.2	
Age at diagnosis (median)	1.9 years	3.3 years	.002
SS-LCH	51.1	72.5	<.001
RO− MS LCH	21.7	19.8	
RO+ MS LCH	27.2	7.7	
Bone involvement	72.3	73.6	.81
Skin involvement	56.0	30.2	<.001
Pituitary involvement	19.6	11.5	.04
Tumor of central nervous system involvement	5.4	3.3	.44
Liver involvement	21.7	4.9	<.001
Hematologic involvement	22.3	3.8	<.001
Spleen involvement	19.6	4.4	<.001
Lung involvement	12.5	12.1	1
Lymph node involvement	10.3	11.0	.87
Follow-up (median)	5.1 years	3.3 years	.008
VLB steroid regimen	60.9	48.9	.03
Response to VLB steroid	76.8	94.4	.001
Receipt of second-line therapy	30.4	7.1	<.001
Receipt of targeted therapy	18.5	3.3	<.001
5-year cumulative incidence of reactivations	42.0	30.3	.06
Permanent consequence	25.5	15.9	.03
Neurodegeneration	7.6	0	<.001
Sclerosing cholangitis	3.3	3.3	1
Severe lung involvement	1.6	2.7	.50
Death	2.7	1.6	.72

Abbreviations: LCH, Langerhans cell histiocytosis; MS, multiple system; RO, risk organs; SS, single system, VLB, vinblastine.

and RO involvement: 27.2% of patients with *BRAF*^{V600E} had MS RO+ LCH compared with 7.7% of patients without *BRAF*^{V600E} ($p < .001$), and hematologic, spleen, and liver involvement was present in 22.3%, 19.6%, and 21.7% of cases with *BRAF*^{V600E} compared with 3.8%, 4.4%, and 4.9% of cases without this mutation ($p < .001$) (Table 1).

Patients with *BRAF*^{V600E} LCH were more likely to have skin involvement (56.0%, *BRAF*^{V600E}; 30.2%, non-*BRAF*^{V600E}; $p < .001$) and to require more vinblastine-steroid chemotherapy (60.9%, *BRAF*^{V600E}; 48.9%, non-*BRAF*^{V600E}; $p = .03$). Response to vinblastine-steroid chemotherapy was lower with *BRAF*^{V600E} (76.8%, *BRAF*^{V600E}; 94.4%, non-*BRAF*^{V600E}; $p = .001$), and 30.4% of those harboring *BRAF*^{V600E} were treated with a second-line therapy compared with 7.1% without *BRAF*^{V600E}. Only 3.3% ($n = 6$) of children without *BRAF*^{V600E} received targeted therapy, compared with 18.5% ($n = 34$) bearing *BRAF*^{V600E}. Response to targeted therapy was constant when administered for chemo-refractory active LCH disease. Patients with *BRAF*^{V600E} tended to experience a higher 5-year LCH reactivation risk (42.0%) compared with those lacking *BRAF*^{V600E} (30.3%; log-rank test, $p = .06$). Regarding long-term consequences of the disease, 7.6% of patients with *BRAF*^{V600E} developed a clinical neurodegenerative LCH, but no patients without this mutation did so ($p < .001$). For the less frequent recurrent mutations (*MAP2K1*, *BRAF* exon 12 small deletions, *BRAF* exon 12 small duplication), we found no significant difference related to characteristics of LCH disease and outcome between these subgroups (Table 2).

Although cases involving non-*BRAF*^{V600E}-mutated LCH were globally less severe, some involved a life-threatening presentation. One patient with a *BRAF* exon 12 small duplication had an MS RO+ LCH (hematological involvement, disease activity score [DAS] = 6, diagnosis at age 0.3 years) but with a favorable outcome as the disease responded to vinblastine-corticosteroid therapy. Two patients with a *BRAF* exon 12 small deletion had a severe disease course, one with severe destructive lung involvement and the other with sclerosing cholangitis. Both underwent solid organ transplantation (lung and liver, respectively) with continuous MEK inhibitor therapy before, during, and after the procedure, with good graft function and no reactivation under targeted therapy. Three patients with a *MAP2K1* mutation had a severe disease presentation. One of these children

TABLE 2 Characteristics of LCH samples that were wild-type for *BRAF*^{V600E} and studied by NGS, according to molecular status; values in % of *n* unless otherwise specified.

Characteristics	*MAP2K1* mutation ($n = 44$)	*BRAF* exon 12 small deletion ($n = 26$)	*BRAF* exon 12 small duplication ($n = 8$)	WT ($n = 19$)	*p*
Male	72.7	61.5	75.0	52.6	.40
Female	27.3	38.5	25.0	47.4	
Age at diagnosis (median)	3.4 years	3.2 years	4.1 years	2.2 years	NS
SS-LCH	72.7	80.8	75.0	73.7	
RO- MS LCH	25	11.5	12.5	21.0	.60
RO+ MS LCH	2.3	7.7	12.5	5.3	
Bone involvement	88.6	61.5	100	63.2	.008
Skin involvement	18.2	30.8	12.5	47.4	.08
Pituitary involvement	9.1	11.5	0	15.8	.59
Tumor of the central nervous system involvement	0	3.8	0	5.3	.21
Liver involvement	2.3	7.7	0	0	.56
Hematologic involvement	2.3	0	12.5	5.3	.37
Spleen involvement	2.3	0	0	0	1.0
Lung involvement	9.1	15.4	12.5	10.5	.87
Lymph node involvement	22.7	3.8	12.5	5.3	.10
Follow-up (median)	1.6 years	2.6 years	2.2 years	1.6 years	NS
VLB steroid regimen	47.7	50.0	75.0	57.9	.54
Response to VLB steroid	95.2	91.7	80.0	90.9	.92
Receipt of second-line therapy	6.8	7.7	12.5	10.5	.81
Receipt of targeted therapy	4.5	7.7	0	5.2	.89
5-year cumulative incidence of reactivations	40.8	20.1	31.4	35.4	.60
Permanent consequence	11.4	19.2	0	15.8	.58
Neurodegeneration	0	0	0	0	-
Sclerosing cholangitis	0	7.7	0	0	.19
Severe lung involvement	4.5	7.7	0	0	.77
Death	0	0	0	5.3	.28

Note: When specified, *p* value was obtained by comparison among all four groups of patients.
Abbreviations: LCH, Langerhans cell histiocytosis; MS, multiple system; NS, not significant; RO, risk organs; SS, single system, WT, wild-type, VLB, vinblastine.

(*MAP2K1*$^{p.F53C}$) had MS RO+ LCH (DAS = 13, diagnosis at age 0.4 years) with diffuse lung lesions refractory to vinblastine-corticosteroid therapy, but experienced a clinical complete remission with MEK inhibitor therapy. The second child with *MAP2K1*$^{F53\_Q58delinsL}$ underwent a left complete pneumonectomy at age 1 month because of complete destruction of the unilateral lung parenchyma leading to serious mechanical complications, and the third of these patients with *MAP2K1*$^{Q56\_G61delinsR}$ was successfully treated with MEK inhibitor after experiencing no response to vinblastine-corticosteroid first-line chemotherapy and severe diffuse lung lesions. The four patients treated with MEK inhibitors were still on therapy at the last visit, including one who experienced reactivation after discontinuation.

Among four patients with mutations localized at the V600 codon of *BRAF* other than V600E, one patient, with *BRAF*$^{V600\_W604delinsLTDG}$, had infant MS RO+ that responded to vinblastine-corticosteroid therapy with a favorable outcome, while the three other patients had benign single-system (SS)-LCH disease.

Among patients with non-recurrent mutations, one with *TET2*$^{Q810^*}$ and one with *ETV6*^{R369W} had SS-LCH of skin and bone, respectively, with unremarkable presentation and outcome. Conversely, a patient with *CSF1R*$^{Q965^*}$ and *EZH2*^{P751S} combined mutations had SS bone LCH diagnosed at age 8.3 years but had an uncommon reactivation with ECD features (osteosclerosis of long bones, coated aorta) at age 14.5 years, managed with interferon-α therapy. A patient with *NF1*^{R1250W} had MS RO+ LCH diagnosed at age 6.2 years, with a favorable outcome after vinblastine-corticosteroid treatment together with 6-mercaptopurine therapy. Finally, 19 patients had wild-type samples after DNA-targeted NGS investigation, and pERK IHC was positive in all cases. Of note, in this subgroup of patients, one had infant MS RO+ LCH refractory to vinblastine-steroid therapy but responsive to MEK inhibitor, and one

had indolent skin SS-LCH associated with severe inherited osteopetrosis, which ended in death.

3.2 | Molecular study of childhood RDD

Samples of 21 children with RDD were included in this study (Supplementary Table 2A), 10 boys and 11 girls with a median age of 7.9 years (range, 0.8–15.4 years) at diagnosis, 7 (33%) of whom had sub-Saharan African ancestry. Six patients (18.6%) had significantly associated disease: R6 and R9 had primary immunodeficiency, R3 and R15 had developmental delay, which was associated with a congenital heart defect for R15; R20 had Steinert myotonic dystrophy, and R21 had fragile X syndrome. Nine (43%) patients had classic nodal RDD, and 12 (57%) had extranodal involvement: seven in the head and neck (nasal cavity and paranasal sinuses mass, $n = 5$; thickened mucosa of larynx, $n = 1$; orbit, $n = 1$), two in the skin, two with central nervous system tumors, and one with multifocal bone involvement.

Molecular studies showed significant mutation in samples from eight patients (Figure 2, Supplementary Table 1). Patients R1 and R6 had classical nodal RDD mutated *BRAF*^{V600E} and *KRAS*^{G13D}, respectively. *BRAF*^{V600E} was also detected in patient R21, who had spinal cord involvement. *MAP2K1* was the most mutated gene reported in this cohort, with four mutations identified that represented 22% of cases subjected to NGS (D58_E62del, V60E, I103S, and C121S). The four RDD patients with *MAP2K1* mutations (R3, R9, R15, and R18) had lymph node involvement, and patient R3 also had a paranasal sinus mass. Regarding clinical syndromic features of patient R15 and the allelic *MAP2K1*^{I103S} frequency at 51%, a germline mutation was suspected and confirmed, and a diagnosis of cardiofaciocutaneous syndrome was made (de novo mutation). After targeted NGS, 61.1% of studied samples were found to be wild-type. Of note, the *DNMT3A*^{R749C} mutation was identified in patient R9 with a unifocal skin lesion. IHC analyses of biopsy samples showed pERK positivity for all tested samples, including nine that were wild-type.

In this cohort, 12 patients had benefited from an initial course of corticosteroid therapy, five of whom also had cytotoxic chemotherapy. Of the 12, 83% ($n = 10/12$) experienced a response to this first-line therapy, and five of them had reactivation after discontinuation. Nine patients had benefited from second-line chemotherapy: 2CDA ($n = 3$), MEK inhibitor ($n = 2$), BRAF inhibitor ($n = 1$), mercaptopurine ($n = 1$), methotrexate-azathioprine ($n = 1$), interferon alpha ($n = 1$), and rapamycin associated with rituximab in a patient with associated primary immunodeficiency ($n = 1$). Responses were variable, but of note, the three patients treated with targeted therapy also experienced a response. Seven patients received no systemic therapy, with favorable outcomes.

3.3 | Molecular study of JXG

The group of 21 children with JXG (9 boys and 12 girls) had mostly a severe presentation with multifocal skin or extracutaneous involvement (Supplementary Table 2B). Median age at diagnosis was 0.6 years (range, 0–11.8 years). Fifteen patients had skin involvement (solitary skin lesions, $n = 2$; multifocal skin lesions, $n = 13$). Moreover, 15 patients had extra-cutaneous involvement. Four had orbital or ophthalmologic localization (iris involvement, $n = 1$; infiltrative orbital lesion, $n = 3$), and 11 had a tumor mass with various localizations: liver ($n = 5$), soft tissue ($n = 5$), bone ($n = 4$, including two patients with a compressive base skull tumor and two patients with multifocal bone localizations), spleen ($n = 3$), lung ($n = 2$), mediastinal ($n = 1$), and kidney ($n = 1$). Finally, five patients had hematologic involvement with severe cytopenia (JX3, JX7, JX8, JX12, and JX20).

All cases were negative for *BRAF*^{V600E} mutation. NGS showed significant mutations in samples from four patients (Figure 2B, Supplementary Table 1). Two different mutations of *KRAS* were identified: *KRAS*$^{Q70\_Y71insWEYSAMRDQ}$ in patient JX1 with multifocal skin involvement; and *KRAS*^{G12R} in patient JX12 with multifocal skin involvement and an infiltrative orbital lesion. This child presented with Burkitt leukemia at age 2.7 years (2 years after the multifocal skin JXG diagnosis), and the treatment of the leukemia resulted in effacement of cutaneous lesions but did not prevent the development of infiltrative orbital lesions. A *MAP2K1*$^{Q58\_E62del}$ was identified in a newborn girl (JX3) with a voluminous neck tumor and hematologic involvement. Finally, a *CSF1R*$^{Q970^*}$ mutation was identified in a boy aged 11.8 years (patient C4) with a solitary skin lesion, and the histologic diagnosis was a C group histiocytosis without typical features of JXG. After targeted NGS, 80% of the studied samples remained wild-type. IHC analyses of biopsy samples showed pERK positivity for 12 samples, and 4 were negative (C4, JX6, JX10, JX17).

Twelve patients had benefited from a first-line systemic chemotherapy, and among them, 11 were treated with an LCH-like regimen with vinblastine associated with corticosteroid or mercaptopurine therapy. An objective response was observed for six patients, and one patient had stabilization of the disease. Four patients were treated with a second-line therapy: three (JX1, JX9, and JX12) received MEK inhibitors, with a frank clinical response for two and stabilization of the disease for one; and JX7 experienced clinical remission and favorable outcome after 2CDA-aracytine second-line therapy in the context of very severe systemic disseminated disease. Finally, one other patient (JX20), also with very severe systemic disseminated disease, was treated with 2CDA-aracytine as first-line therapy but died of infectious complications in a situation with persistently active disease. This patient was the only one among those with JXG who died.

3.4 | Malignant histiocytosis

Six children (three boys and three girls) with MH were included in this study (Supplementary Table 2C). Median age at diagnosis was 12.4 years (range, 1.4–16.2 years). For three children, MH was diagnosed secondary to the management of B-cell acute lymphoblastic leukemia (patients M1 and M5) and Burkitt leukemia (patient M3). At the initial diagnosis, bones were involved in three patients (multifocal lesions) treated for a previous lymphoid malignancy, while one patient

had disseminated central nervous system tumor lesions (M1), one had a soft tissue tumor with inguinal lymph node localization (M4), and one patient had a cervical lymph node localization (M6). For two patients (M4 and M5), no follow-up was available. The systemic chemotherapy scheme was variable for the four patients for whom follow-up was available. Three of them experienced a poor response to chemotherapy, with death at a median of 11 months from diagnosis (range, 9.6–24.9 months). M6 was the only survivor and experienced a response to topotecan–temozolomide (TOTEM) combined chemotherapy.

IHC for pERK was performed for three cases (M2, M5, and M6), and all were positive. All cases were negative for *BRAF*^{V600E} mutations (Figure 2A). NGS was performed on samples from five cases, and mutations were highlighted in four (Figure 2B, Supplementary Table 1): a novel *BRAF*^{G469R} mutation associated with *NF1*^{E1266*} in a girl age 12.4 years with disseminated central nervous system tumor lesions (patient M2); *KRAS*^{Q61H} in a boy age 12.8 years with MH of bone secondary to Burkitt leukemia (patient M3); *ETV6*^{M389I} in a boy age 1.4 years with a soft tissue tumor lesion (patient M4); and *KRAS*^{A146P} in a boy age 16.2 years with MH of bone secondary to B-cell acute lymphoblastic leukemia (patient M5). No *PTPN11* alteration was detected in our patients, in contrast to what has been reported in adults with malignant histiocytoses,[23] whereas two of our three patients with previous B-cell lymphoid malignancies had *KRAS* mutations, as reported in adults with histiocytic sarcoma.[24]

4 | DISCUSSION

Here, we present the results of the molecular and clinicopathological characterization of 415 cases of pediatric histiocytosis, most of them (88%) of the LCH subtype. Analyses of clinical features, response to treatment, and prognosis were conducted in relation to the molecular profiles of the cases. To our knowledge, this group is the largest homogeneous cohort of children representing all histiocytosis subtypes published to date.

As in other cancers, the most frequent *BRAF* alteration that we found in our pediatric cohort was the *BRAF*^{V600E} mutation. In the present study, *BRAF*^{V600E} was found in 45.1% of all histiocytoses and in 50.3% of childhood LCH. This result is in accordance with previously published findings showing *BRAF*^{V600E} in 19%–67.6% of pediatric LCH.[3–6] In four children with LCH in the current study, we observed more complex alterations (small deletion/duplication) around codon V600 (Supplementary Table 1). These mutations altered the physicochemical properties of this hydrophobic domain, leading to the activation of downstream effectors of the MAP-kinase pathway. Such complex rearrangements of the V600 region have rarely been described in histiocytosis[25] and are more frequent in other cancers.[26,27] The *BRAF*^{V600E} mutation has rarely been detected in non-LCH pediatric histiocytosis.[24,28–30] In our series, we found this mutation in only two RDD patients and in one with ECD, which is a rare condition in childhood.[31–33] Deletions and duplications in exon 12 of *BRAF* were exclusively found in the LCH group and were present in 24.8% (deletions) and 7.6% (duplications) of non-*BRAF*^{V600E}-mutated cases. A pathogenic role of these deletions and duplications in MAP-kinase pathway activation has been confirmed,[9,10] and they can be targeted by BRAF or MEK inhibitors; however, the efficacy of MEK inhibitors needs further investigation in the context of *MAP2K1* exon 3 deletions (also called class 3 mutants[34]) and BRAF exon 12 small duplications.[10] Finally, in a patient with MH, we report a novel *BRAF*^{G469R} mutation of unknown pathogenic function; pERK staining was positive in this MH case.

In our cohort, *MAP2K1* alterations were the most frequently detected mutations in non-*BRAF*^{V600E} childhood LCH, occurring in 42% of these patients. In contrast to *BRAF*, where the V600E point mutation is predominant, deletions were more common in the *MAP2K1* gene, especially in exon 2 (57.2% of all *MAP2K1* alterations). Furthermore, we identified *MAP2K1* alterations in 20% of RDD cases and in one patient with JXG. Previous publications have reported *MAP2K1* mutations in 11%–30% of pediatric LCH patients[5–7] and more rarely in other histiocytosis types.[8,12,35,36] Because non-*BRAF*^{V600E}-mutated LCH cases constitute half of our LCH cohort, we can estimate that *MAP2K1* mutations are found in 21% of childhood LCH, in accordance with previously published data.

In 12 samples without *BRAF* or *MAP2K1* alteration, NGS revealed a mutation in genes other than *BRAF* or *MAP2K1* (Supplementary Table 1). Additionally, samples from two *MAP2K1*-mutated LCH cases and one *BRAF*^{G469R}-mutated MH case harbored a second mutation in another gene. The "less common" mutated genes in our series were *KRAS* ($n = 5$), *CSF1R*, *ETV6*, and *NF1* (each $n = 2$), and *DNMT3A*, *EZH2*, *GATA2*, *JAK2*, and *TET2* (each $n = 1$), all of which could have pathological consequences.[21] According to previous studies, the following genes (already found in cancer cases) also are rarely found to be mutated in pediatric histiocytosis: *ARAF*,[7,37,38] *CSF1R*,[7] *KRAS*,[11,39] *KIT*,[7,40] *MET*,[3,7] *NF1*,[7] *NRAS*,[7] and *TP53*,[3,11] and quite rarely with *MAP2K1* or *BRAF* alteration.[3,35]

KRAS mutations were found in JXG ($n = 2$), RDD ($n = 1$), and MH ($n = 2$) types. *KRAS* is a well-known oncogene, and regarding histiocytoses, *KRAS* mutations have been reported in secondary MH[23,24,41] and occasionally in RDD or JXG.[11] In contrast to the activating mutations in *CSF1R* reported by Durham et al.,[7] both *CSF1R* mutations detected in our series (LCH, $n = 1$; XGJ, $n = 1$) generate the stop codon in the c-CBL binding domain, which might lead to abnormal CSF1R ubiquitination and degradation by the interruption of the c-CBL–CSF1R association.[42,43] The pathological mechanism of these mutants needs further investigation.

In contrast to reports concerning adults with ECD,[22] we observed very rare alteration in genes commonly mutated in myeloid malignancies or clonal hematopoiesis: the *DNMT3A*^{R749C} mutation highlighted here in a child with RDD and the *TET2*^{Q810*} mutation identified in a child with LCH were the only ones found in this cohort that are involved in clonal hematopoiesis.

Together with genetic analysis, we evaluated the correlation between the molecular profile and the clinical characteristics and outcomes for each histiocytosis subtype. Given the small number of cases representing the non-LCH histiocytosis subtypes and no recurrent

molecular profile found among them, a correlation analysis could not be performed in these groups. Nevertheless, we describe a substantial cohort of children with RDD, JXG, or MH with detailed clinical phenotype and outcomes, whereas most relevant previous publications have been case reports.

We note that the 21 children with JXG reported here typically had a severe presentation with multifocal skin or extracutaneous involvement. Only two patients had a solitary skin lesion, which is the most common presentation of JXG, with a favorable prognosis and spontaneous regression. To date, the largest study included 55 JXG patients,[7] most with skin involvement only ($n = 51$), and with various molecular alterations reported but no clear predominance of any of them. The most common were changes in *MAP2K1* ($n = 6$), *CSF1R* ($n = 5$), and *KRAS* ($n = 4$), and *NTRK1* ($n = 6$) and *BRAF* ($n = 4$) gene fusions. In the current work, we also found associations with molecular changes in *MAP2K1*, *CSF1R*, and *KRAS*. Of interest, we identified no *BRAF*^{V600E} mutations, in keeping with the findings of Durham et al., although this mutant has previously been reported in the central nervous system JXG.[29,30] Further detailed clinico-molecular studies are needed to gain more insights into JXG. Furthermore, we report here one of the largest cohorts of childhood LCH cases with molecular analysis data. In this current study, we again show the clinical correlation of *BRAF*^{V600E} mutation with MS RO+ LCH and resistance to first-line vinblastine-steroid chemotherapy that we reported in 2016,[13] now with a patient cohort enriched by more than one-third with new patients, and a centralized molecular analysis, in contrast to our previous study. The risk of permanent sequelae, in particular clinical neurodegenerative LCH, is also significantly higher in *BRAF*^{V600E}-mutated cases.[14] Others have reported similar findings for pediatric LCH, in part, but in smaller cohorts.[4,37,44] One exception is a recently published international cohort study by Kemps et al., which included an equivalent number of patients to ours.[5] Regarding these data for children diagnosed with *BRAF*^{V600E}-mutated LCH, specific attention should be paid to young children regarding MS-RO+ LCH risk. We suggest a close follow-up at least during the first year after the diagnosis, and parents should be made aware of symptoms that are suggestive of disease progression towards MS OR+ LCH (prolonged fever and/or diarrhea, signs of cytopenia, enlarged abdomen), with instructions to consult quickly if symptoms arise. Also, for children with *BRAF*^{V600E}-mutated LCH, especially those who have experienced pituitary, skull base, or orbit bone involvement, follow-up after remission of LCH should be extended, if possible, throughout the primary and secondary school years to identify suggestive early signs of neurodegeneration (academic delay, attention, and concentration difficulties).

Despite the large cohort in the current study, groups with *MAP2K1* mutations or *BRAF* exon 12 small deletions or duplications encompassed fewer than 50 patients each, which is a limitation for correlation analysis between these molecular subgroups. These cases are generally less severe than those involving *BRAF*^{V600E} but without important differences between them. A few patients may have rare but life-threatening presentations, such as MS RO+ or severe lung involvement, or sclerosing cholangitis. In patients with *MAP2K1* mutations (non-class 3 *MAP2K1* mutants) or a *BRAF* exon 12 small deletions, MEK inhibitors were occasionally used with good clinical response, as previously reported with BRAF inhibitors for chemo-refractory *BRAF*^{V600E}-mutated LCH.[45] Despite this broad molecular study, no mutation was detected in many cases. We found estimated wild-type rates of 9% for LCH, 60% for RDD, 80% for JXG, and 20% for MH, based on targeted NGS results. It should be pointed out that for LCH and RDD, activation of the MAPK pathway appears to be universal, as attested by ERK phosphorylation in all samples studied here. Unidentified genetic alterations or extracellular signals may be involved in wild-type samples. Further molecular analyses are needed (e.g., whole genome sequencing or fusion gene analyses performed on biopsy samples, or peripheral blood mononuclear cell NGS analyses for cases with very low infiltration of pathological histiocytes) to elucidate the genetic basis of this disease and improve genotype–phenotype correlations. As an example, a few publications have reported the presence of tyrosine kinase receptor fusion genes[7,46] or BRAF fusion[9] in some pediatric histiocytosis, which is an avenue worth pursuing. Also, because of the rarity of pediatric histiocytosis or disseminated JXG, international teamwork probably will be necessary to accelerate progress in understanding this histiocytosis subtype.

5 | CONCLUSIONS

Our molecular analysis revealed a very heterogeneous mutational profile across histiocytosis subtypes. The genotype–phenotype correlation analysis confirmed our previous observations and showed a significant correlation of *BRAF*^{V600E} mutation with MS RO+ LCH and neurodegeneration. Correlation analyses of non-*BRAF*^{V600E}-mutated LCH cases harboring other genetic alterations in *BRAF* and *MAP2K1* genes were harder to perform because of the small number of cases in each mutation subgroup; however, the prognosis appears to be better in these cases. Molecular and clinical information about these diseases offers important knowledge for clinicians to better evaluate disease prognosis and use in clinical management and therapeutic decision-making. Collaboration between groups of the Histiocytosis Society and among members of the European Consortium for Histiocytosis or North American Consortium for Histiocytosis may accelerate the application of the molecular knowledge to clinical practice and validate its usefulness.

AUTHOR CONTRIBUTIONS

Conception and design: Jean Donadieu, Jean-François Emile, Sébastien Heritier, Zofia Hélias-Rodzewicz. *Development of methodology*: Jean-François Emile, Zofia Hélias-Rodzewicz. *Acquisition of data (Collection and examination of patient data)*: Mohamed-Aziz Barkaoui, Anne Lambilliotte, Despina Moshous, Caroline Thomas, Saba Azarnoush, Marlène Pasquet, Ludovic Mansuy, Nathalie Aladjidi, Eric Jeziorski, Perrine Marec-Berard, Marion Gilibert-Yvert, Alexandra Spiegel, Paul Saultier, Isabelle Pellier, Anne Pagnier, Sophie Pertuisel, Maryline Poirée, Damien Bodet, Frédéric Millot, Florentina Isfan, Jean-Louis Stephan, Amaury Leruste, Charlotte Rigaud, Bruno Filhon, Liana

Carausu, Yves Reguerre, Isabelle Kieffer, Bénédicte Brichard, Ahmed Idbaih, Christine Bodemer, Fleur Cohen-Aubart, Julien Haroche, Abdellatif Tazi, Sabah Boudjemaa, Sylvie Fraitag, Jean Donadieu, Jean-François Emile, Sébastien Heritier. *Experiments*: Nathalie Terrones, Rim Ben Jannet, Mariama Bakari, Zofia Hélias-Rodzewicz. *Analysis and interpretation of data (e.g., statistical analysis, sequencing data analysis)*: Jean-François Emile, Zofia Hélias-Rodzewicz. *Writing, review, and/or revision of the manuscript*: Sébastien Heritier, Zofia Hélias-Rodzewicz. All authors approved the final version of the manuscript.

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ACKNOWLEDGMENTS

The authors thank the patients and their families for their participation in this study, and all pediatricians and pathologists from the Société Française de lutte contre les Cancers de l'Enfant et de l'Adolescent (SFCE).

FUNDING INFORMATION

This work was supported by grants from the Programme de Recherche Translationnelle en Cancérologie (grant reference 19-143), and from the Conquer Cancer Foundation ASCO Young Investigator Award funded by the Strike 3 Foundation (grant reference #11413). This project received constant, unlimited support from the Association Histiocytose France, and support from the Association pour le Recherche et l'Enseignement en Pathologie, and from the Association Recherche et Maladie Hématologiques de l'Enfant.

CONFLICT OF INTEREST STATEMENT

Ahmed Idbaih received Research grants from Carthera, Transgene, Sanofi, Air Liquide, Servier, and Nutritheragene. He is on the advisory board for Novartis, Leo Pharma, Novocure, and Boehringer Ingelheim Int. Other authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available within the article and its supplementary materials. Derived data supporting the findings of this study are available from the corresponding author Sébastien Heritier on request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Hélias-Rodzewicz Z, Donadieu J, Terrones N, et al. Molecular and clinicopathologic characterization of pediatric histiocytoses. *Am J Hematol*. 2023;1-12. doi:10.1002/ajh.26938