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Clinical Immunology 265 (2024) 110299

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

Histological evidence of MAPK pathway activation across subtypes of adult orbital xanthogranulomatous disease irrespective of the detection of oncogenic mutations

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ARTICLE INFO

Keywords:
Adult orbital xanthogranuloma
Adult-onset asthma and periocular xanthogranuloma
Necrobiotic xanthogranuloma
Erdheim-Chester disease
MAPK pathway
Histiocytic neoplasms

ABSTRACT

Adult orbital xanthogranulomatous disease (AOXGD) is a spectrum of histiocytoses with four subtypes. Mitogen-activated protein kinase (MAPK) pathway mutations have been detected in various histiocytic neoplasms, little is known about this in AOXGD. Targeted regions of cancer- and histiocytosis-related genes were analyzed and immunohistochemical staining of phosphorylated ERK (pERK), cyclin D1 and PU.1 was performed in 28 AOXGD and 10 control xanthelasma biopsies to assess MAPK pathway activation.

Mutations were detected in 7/28 (25%) patients. Positive staining for pERK and/or cyclin D1 was found across all subtypes in 17/27 (63%) patients of whom 12/17 (71%) did not harbour a mutation. Xanthelasma tissue stained negative for pERK and cyclin D1. Relapse occurred in 5/7 (71%) patients with a MAPK pathway mutation compared to 8/21 (38%) patients in whom no mutation could be detected.

Molecular analysis and evaluation for systemic disease is warranted to identify patients at risk of recurrent xanthomatous disease.

1. Introduction

Adult orbital xanthogranulomatous disease (AOXGD) is a spectrum of histiocytoses, with variable clinical features. Based on these features, four subtypes are identified: adult-onset xanthogranuloma (AOX), adult-onset asthma and periocular xanthogranuloma (AAPOX), necrobiotic xanthogranuloma (NBX) and Erdheim-Chester disease (ECD). [1,2] All AOXGD subtypes share hallmark histopathological features, including tissue infiltration by foamy histiocytes that are positive for CD68 and CD163, but negative for CD1a and CD207. Spread among these foamy histiocytes are Touton giant cells, characterized by centrally localized eosinophilic cytoplasm and an annulus of nuclei surrounded by pale, foamy cytoplasm. Aggregates of lymphocytes and fibrosis are also frequently seen. In contrast, necrobiosis - defined as packed collagen deposits - is only seen in patients with NBX. [3,4]

Additional clinical evaluation, including laboratory investigations and total body imaging are necessary in AOXGD, since various subtypes may have either systemic manifestations outside the periorbital region or associated diseases, including IgG4-related disease and various hematologic malignancies. [1,2,5–7] Systemic evaluations and long-term follow-up in patients with AOXGD are, therefore, essential. [8]

Historically, histiocytoses were roughly classified as disorders of Langerhans and non-Langerhans cell origin. [9,10] A revised classification by experts from the Histiocyte Society arranged the various histiocytoses into five separate groups: the L, C, R, M and H group. [11] Langerhans cell histiocytosis (LCH) and ECD, both frequently associated

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https://doi.org/10.1016/j.clim.2024.110299
Received 22 May 2024; Received in revised form 19 June 2024; Accepted 21 June 2024
Available online 25 June 2024

with a somatic mitogen-activated protein kinase (MAPK) pathway mutation, were classified as L-group histiocytosis. [11–13] Since then, multiple other mutations in the MAPK pathway have been found in histiocytosis subtypes originally not classified as L-group; these include juvenile xanthogranuloma and Rosai-Dorfman disease (RDD). Mutations that have been found involve *MAP2K1* (*MEK1*), *MAP2K2* (*MEK2*), *ARAF*, *BRAF*, *KRAS* and *NRAS* genes. [12–24]

The MAPK pathway is a complex signaling pathway. It regulates, among others, cell proliferation and survival. This pathway is activated through tyrosine kinase receptors transferring extracellular signals that activate the proto-onco-gene *RAS* in a complex multistep process. Activated *RAS* leads to a cascade of phosphorylation events of three consecutive kinases, RAF, MEK and ERK. [25] Gain-of-function mutations in this pathway can lead to RAS-independent activation of the downstream kinases, causing autonomous growth and transformation. [26] Activated phosphorylated ERK (pERK) translocates to the nucleus where it activates transcription factors such as cyclin D1. Cyclin D1 is a protein that promotes cell proliferation and survival and has been shown to be a neoplastic marker in both LCH and other *BRAF*-mutated neoplasms. [27–29] A recent study also showed that cyclin D1 immunostaining can be used as an additional marker to diagnose periocular histiocytoses. [16] Hence, expression of pERK and cyclin D1 are two separate immunohistochemical indicators of MAPK pathway hyperactivation. [27] PU.1 was earlier shown to be a useful nuclear marker for histiocytic differentiation. [30]

Besides ECD, little is known about MAPK pathway-activating mutations in other AOXGD subtypes. Recently, the first two patients with AAPOX have been described with a somatic *KRAS* mutation. [16] We investigated MAPK pathway activation in a consecutive series of biopsies from patients with histologically proven AOXGD treated in the Rotterdam Eye Hospital or in the Erasmus Medical Center by assessing the presence of pathogenic MAPK mutations and positive immunohistochemical staining for pERK and cyclin D1.

2. Materials and methods

This is a descriptive, retrospective study of 28 patients with histologically proven AOXGD, diagnosed between 1989 and 2022. Data including baseline demographics and clinical parameters were retrospectively collected from medical records. Detailed clinical data of a subset of patients have been reported previously. [8] The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam, number MEC-2021-0510, and the study was conducted according to criteria set by the declaration of Helsinki. Informed consent was not obtained, since this was a retrospective study and histopathological findings were determined in archived diagnostic biopsies.

Haematoxylin and eosin-stained slides prepared from archived formalin fixed paraffin embedded (FFPE) (*peri*)orbital tissue biopsies were reviewed by an ophthalmic pathologist (RVE) for correct histopathological diagnosis. Specifically, biopsies were scored for the presence of classic Touton giant cells, foamy histiocytes, lymphocytic infiltrate and fibrosis in the presence or absence of necrobiosis. Additional immunohistochemical staining was performed for pERK (Nuclear Fast Red 1:250; polyclonal rabbit, Sigma-Aldrich), cyclin D1 (3,3′-diaminobenzidine ready to use; SP4-R monoclonal rabbit, Ventana Medical System Inc) and PU.1 (1:800; EPR3158Y monoclonal rabbit, Abcam) on 27 available biopsies of patients with AOXGD. Ten xanthelasma lesions obtained from patients without a histiocytosis history served as control periorbital skin tissue containing only reactive histiocytes. Automated immunostaining was performed using an IHC staining system (Ventana Benchmark ULTRA) operated in an ISO 15189:2012 certified laboratory. The pERK staining was scored positive when clusters of cytoplasmic and/or nuclear positive histiocytes were observed in >10%. For cyclin D1, positivity was scored when nuclear expression was present in >10% of histiocyte nuclei and PU.1 stained all histiocyte nuclei.

Standard next-generation sequencing (NGS) was performed on 12 tissue samples. To this end, we selected samples containing >60% of PU.1 positive histiocytes which comprised an estimated 5–25% of neoplastic histiocytes based on our experience from routine histopathological evaluation of histiocytosis biopsies. DNA was enriched using the Agilent SureSelectXT Human All Exon capture kit and paired-end sequenced on the Illumina platform. Sequencing data were demultiplexed with bcl2fastq2 Conversion Software from Illumina. Illumina DRAGEN Bio-IT Platform was used for read mapping to the hg19 genome and sequence mutation detection. The detected sequence mutations were annotated and filtered with Alissa Interpret software and classified with Alamut Visual. Copy number mutation detection was performed using the BAM multiscale reference method using depth of coverage analysis and dynamical bins in NexusClinical. The detected copy number mutations were annotated and filtered with the NexusClinical software and classified using UCSC Genome Browser (NCBI37/hg19). Targeted regions of 36 to 57 cancer-related genes were tested for mutations. The number of targeted genes was dependent on the year the NGS panel was performed, since the number of genes increased with the advancement of the panel. Supplementary table 1 displays the details of the NGS panels used. All panels included recurrently reported MAPK pathway activating genes, such as *MAP2K1* (also known as *MEK1*), *ARAF*, *BRAF*, *RAF1* (also known as *CRAF*), *KRAS* and *NRAS*. *MAP2K2* (also known as *MEK2*) was only covered by the targeted locus capture (TLC) NGS panel.

TLC NGS was performed on 24 samples that were either not subjected to or yielded negative results in standard NGS. These samples were analyzed for the presence of gene fusions in 54 genes as well as a limited analysis of other small mutations in a subset of genes, including *BRAF, HRAS, KRAS, NRAS, PIK3CA, MAP2K1* and *CSF1R*, see supplementary table 1. To this end, DNA samples were enriched using a capture panel after a KAPA library prep on the gDNA. [31] NGS reads were aligned to the human GRCh37/hg19 genome. Gene fusions in the FFPE samples are detected based on the identification of breakpoint sequence between the target gene and fusion gene partner, automated identification of regions enriched for proximity ligated products linked to a given target gene and/or genomic coverage profiles in a genome browser. [32] Standard NGS using the same panel as described above was performed on two samples with only DNA left available.

Descriptive analyses are reported for patient characteristics, clinical features, molecular genetics, and histopathological findings.

3. Results

3.1. Patient characteristics

Patient characteristics, molecular genetics and immunohistochemistry of patients with AOXGD are summarized in Table 1. This study included 28 patients, 15 males and 13 females. Of these 28 patients, 11 (39%) were diagnosed with AOX, 6 (21%) with AAPOX, 7 (25%) with NBX and 4 (14%) with ECD. Patients diagnosed with AOX and AAPOX had a mean age of 56 years (range 31–82 and 45–72, respectively). Patients with NBX had a mean age of 59 years (range 49–75) and ECD a mean age of 58 years (range 47–67).

3.2. Molecular studies on AOXGD biopsies

DNA testing demonstrated MAPK pathway mutations in 7/28 (25%) patients (Fig. 1). Two biopsies from patients with AAPOX showed mutations, one in NRASp.G12D and the second patient presented with a KRASp.K117N mutation. The latter patient was also found to have a PIK3CAp.R992Q mutation. Four patients with ECD all showed a pathological mutation in the MAPK pathway, with two patients presenting with a BRAFp.V600E mutation and two with either KRASp.G12A or KRASp.K117N. In contrast, none of the patients with NBX showed molecular alterations in the aforementioned genes of the MAPK pathway.

Table 1
Characteristics, molecular genetics and immunohistochemistry of patients with adult orbital xanthogranulomatous disease.

Patient	Age at diagnosis (years)	Sex	Diagnosis	Year of biopsy	Mutation analysis (VAF)	pERK	Cyclin D1	PU.1	Disease localization	Medical history	Therapy	Disease course	Localization recurrence or progression	Follow-up (months)	Alive at last FU
1	31	M	AOX	2018	-‡ and ¥	–	–	+	LUE, LG	None	Debulking	Remission		4	Yes
2	51	M	AOX	2011	-¥	–	–	+	RLE, EOM	None	Debulking	Remission		6	Yes
3	48	M	AOX	2006	-¥	+	+	+	DO, EOM	None	Debulking	Progression after 4 months	DO, conjunctiva after incomplete debulking	99	Yes
4	50	F	AOX	1994	-¥	–	+	+	RUE, RLE, LUE, LLE, sublingual	None	Debulking	Recurrence after 60 months	RUE, RLE, LUE, LLE, EOM	74	Yes
5	61	F	AOX	2011	-¥	–	+	+	RUE	Hypertension, TIA	Debulking	Remission		19	Yes
6	82	F	AOX	2004	-¥	–	+	+	LUE	Hypertension	Debulking	Remission		5	Yes
7	49	M	AOX	2005	-¥	–	+	+	DO	None	Debulking, prednisolone 35 mg/day, azathioprine 150 mg/day	Remission		15	Yes
8	80	F	AOX	1989	-¥	–	–	+	RUE, RLE, EOM, LG, sinus	Hypertension, psoriasiform dermatitis	Prednisolone 60 mg/day. After recurrence prednisolone 60 mg/day and azathioprine 50 mg/day	Recurrence after 48 months	RUE, RLE, EOM, LG	120	Yes
9	70	M	AOX	2003	-¥	+	+	+	RUE, LN	TIA, CVA, CEA, DM	Debulking, prednisolone 20 mg/day and azathioprine 100 mg/day	Remission		7	Yes
10	42	M	AOX	2021	-‡¥	+	+	+	RLE	HNP	Debulking	Remission		13	Yes
11††	48	M	AOX	2021	KRASp. G12R (23%)‡ BRAFp. G466E (21%)‡	–	+	+	RUE, skin, bone	Melanoma, thyroid carcinoma (KRAS-; BRAF-), prostate carcinoma	Debulking and laser ablation	Progression after 1 month	Skin	302	Yes
12	50	M	AAPOX	2015	-¥	–	–	+	RUE, RLE, LUE, LLE, EOM, LG, LN	IgG4-RD, constrictive lung disease, lung adenocarcinoma, pancreatic carcinoma with liver metastases	Prednisolone 60 mg/day, debulking	Remission		132	Deceased§
13	53	F	AAPOX	2010	KRASp. K117N (23%)¥ PIK3CAp. R992Q (5%)¥	+	+	+	RUE, LUE, LG	Asthma	Debulking, prednisolone 60 mg/day and methotrexate 10 mg/week	Remission		168	Yes
14	45	F	AAPOX	2010	NRASp. G12D (31%)¥	Focal	Focal	+	RUE, RLE, LUE, LLE, EOM, LG, LN	Asthma, fibromyalgia, hypercholesterolemia, iron-deficiency anemia, nephrotic syndrome, ALL	Debulking, prednisolone 60 mg/day and azathioprine 75 mg/day. Switch to mycophenolic acid 1440 mg/day	Progression after 30 months	LG	160	Deceased±
15	68	F	AAPOX	2003	-¥	+	+	+	RUE, RLE, LUE, LLE, EOM, LG	Asthma, M. Graves, TED, corneal melt	Debulking, prednisolone 30 mg/day and mycophenolic acid 720 mg/day. After recurrence prednisolone 30 mg/day	Recurrence after 12 months	RUE, RLE, LUE	249	Yes

(*continued on next page*)

Table 1 (*continued*)

Patient	Age at diagnosis (years)	Sex	Diagnosis	Year of biopsy	Mutation analysis (VAF)	pERK	Cyclin D1	PU.1	Disease localization	Medical history	Therapy	Disease course	Localization recurrence or progression	Follow-up (months)	Alive at last FU
16	72	M	AAPOX	2022	-[‡ and ¥]	–	–	+	LUE, LG, LN, bone, periaortic, mesenteric, pancreas, SMG	Constrictive lung disease	and mycophenolic acid 360 mg/day Debulking, triamcinolone injection, prednisolone 80 mg/day. After tapering progression, start methotrexate 15 mg/week and prednisolone 20 mg/day	Progression after 5 months	LUE	28	Yes
17	45	M	AAPOX	2021	-[‡ and ¥]	–	–	+	RUE, RLE, LUE, LLE	Hemangioma liver	Prednisolone 30 mg, azathioprine, mycophenolic acid 1440/day. After tapering, intravenous methylprednisolone 1000 mg monthly, reduced to 500 mg monthly	Stable with treatment		30	Yes
18	43	M	NBX	1999	-[‡ and ¥]	+	+	+	RUE, RLE, LUE, LLE, LG, LN	Primary sclerotic cholangitis, IgG4-RD	Debulking, prednisolone 20 mg/day after progression	Progression after 48 months	DO, EOM, left m. temporalis	292	Yes
19	73	F	NBX	2002	-[¥]	+	–	+	LLE, LN	Hypertension, COPD, tuberculosis	Debulking, prednisolone 25 mg/day azathioprine 100 mg/day	Remission		262	Yes
20	49	F	NBX	2019	-[¥]	–	–	+	LUE, LLE	None	Debulking and subcutaneous triamcinolone 4–12 mg/injection	Progression after 6 months	LUE, LLE	52	Yes
21	67	M	NBX	2017	-[¥]	+	+	+	RLE Skin, parotid gland	CLL, thymoma, hypertension, hypercholesterolemia	Debulking	Stable without treatment		78	Yes
22	57	F	NBX	2014	-[¥]	+	+	+	RUE, LG, LN, skin	Hypertension, hypercholesterolemia, sarcoidosis	Debulking. Prednisolone 30 mg/day and methotrexate 20 mg/week. Switch to dexamethasone 6 mg/day, switch to azathioprine 150 mg/day. Progression: switch to mycophenolate mofetil 3 g/day. Switch to cyclophosphamide 2 g/month, without effect. Treatment was ceased.	Progression after 34 months	LG	127	Yes
23	52	M	NBX	2018	-[‡ and ¥]	Failed	–	Failed	RUE, LUE	M. Graves, T cell lymphoma, pneumococcal sepsis, EBV infection	R-CHOP	Rapid decline due to T cell lymphoma		2	Deceased[£]
24	75	M	NBX	2022	-[‡ and ¥]	Failed	Failed	Failed	RUE, RLE, LUE, LLE, periaortic	TIA, COPD, uveitis, depression	Prednisonlone 30 mg/day and tocilizumab 162 mg/week subcutaneous	Stable with treatment		15	Yes
25	47	M	ECD	2000	Failed[‡] BRAFp.V600E (9%)[¥]	+	+	+	DO, bone, brain, periaortic, RP, skin	None	Prednisolone 70 mg/day. Debulking and radiotherapy 34Gy. Methotrexate 10 mg/	Progression after 6 months.		120	Deceased[£]

(*continued on next page*)

Table 1 (*continued*)

Patient	Age at diagnosis (years)	Sex	Diagnosis	Year of biopsy	Mutation analysis (VAF)	pERK	Cyclin D1	PU.1	Disease localization	Medical history	Therapy	Disease course	Localization recurrence or progression	Follow-up (months)	Alive at last FU
											week, switch to cyclosporine 200 mg/day, switch to octreotide 300μg/day, switch to cyclophosphamide 150 mg/day, switch to etanercept 50 mg/week, switch to gammaglobulines 140 g/month. ^{177}Lutetium-octreotate 5.6GBq and azathioprine 100 mg/day	Therapy resistant			
26[*]	59	F	ECD	2018	KRASp.G12A (52%)[‡]				RUE, RLE, LUE, LLE, bone, skin	Hypertension, diabetes mellitus	Intravenous methylprednisolone 1 g for 3 days, prednisolone 60 mg/day	Progression after 1 month	RUE, RLE, LUE, LLE	3	Yes
27	59	F	ECD	2022	BRAFp.V600E (9.5%)[‡]	+	Failed	Failed	RLE, bone, skin	TIA, glaucoma, HNP, duodenal ulcer	Tocilizumab 162 mg/week	Stable with treatment		19	Yes
28	67	F	ECD	2020	KRASp.K117N (28%)[‡]	−	+	+	RUE, RLE, LUE, LLE, bone, skin	Hypertension, asthma, fibromyalgia, M. Sjögren, borderline, depression, Barrett's esophagus	Debulking, laser ablation, prednisolone 30 mg. Radiotherapy 10 × 2 Gy on the ilium	Progression after 1 month	Skin	50	Yes

AAPOX: adult-onset asthma and periocular xanthogranuloma, AOX: adult-onset xanthogranuloma, CEA: carotid endarterectomy, CLL: chronic lymphocytic leukemia, COPD: chronic obstructive pulmonary disease, DO: diffuse orbit, DM: diabetes mellitus, EBV: Epstein-Barr virus, ECD: Erdheim-Chester disease, EOM: extra-ocular muscle, F: female, FU: follow up, HNP: herniated nucleus pulposus, IgG4-RD: IgG4-related disease, LG: lacrimal gland, LLE: left lower eyelid, LN: lymph nodes, LUE: left upper eyelid, M: male, N/A: not applicable, NBX: necrobiotic xanthogranuloma, pERK: phosphorylated ERK, R-CHOP: rituximab/cyclophosphamide/hydroxydaunorubicine/oncovin/prednisolone, RLE: right lower eyelid, RP: retroperitoneal, RUE: right upper eyelid, SMG: submandibular gland, TED: thyroid eye disease, TIA: transient ischemic attack; VAF: variant allele frequency.

Recurrence is measured from the start of the treatment or start of diagnosis in patients where no treatment was commenced.

[†] : In retrospect, the AOX lesion turned out to be one of many xanthogranulomatous skin lesions developed during a prolonged period of time. PET-CT imaging showed metabolic active sclerotic bone lesions. Follow-up scans did not show progression. A biopsy could not definitively confirm histiocytic disorder.

[*] No left-over tissue available for immunohistochemical staining.

[‡] : next-generation sequencing.

[¥] : formalin-fixed paraffin-embedded-targeted locus capture next-generation sequencing.

[§] : Death duo to metastatic pancreatic carcinoma.

[±] : Death duo to B cell acute lymphocytic leukemia.

[°] : Death due to T cell lymphoma, almost simultaneously diagnosed with ECD.

[£] : Death due to cerebral vascular accident.

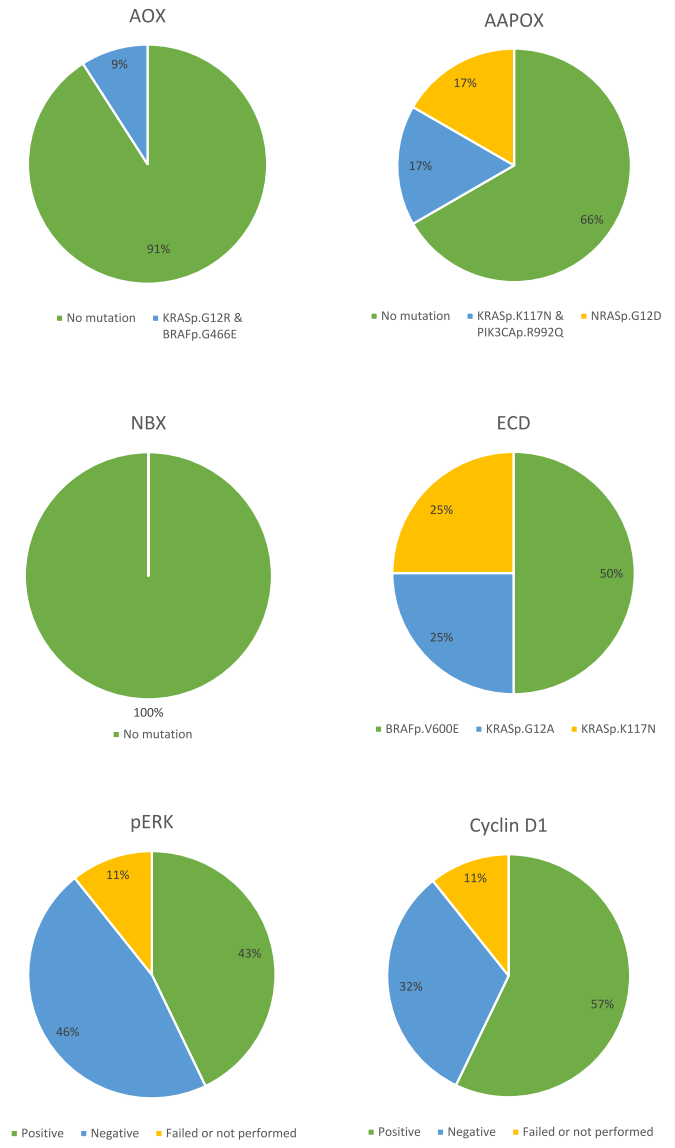

Fig. 1. Pie charts of the mutations and immunohistochemical staining in subtypes of AOXGD.
AAPOX: adult-onset asthma and periocular xanthogranuloma, AOX: adult-onset xanthogranuloma, ECD: Erdheim-Chester disease, NBX: necrobiotic xanthogranuloma, pERK: phosphorylated ERK.

One patient showed a concurrent KRASp.G12R and BRAFp.G466E mutation with comparable variant allele frequency in an AOX lesion. In retrospect, this AOX lesion turned out to be one of many xanthogranulomatous skin lesions developed during a prolonged period of time.

3.3. Immunohistochemistry for pERK, cyclin D1 and PU.1

Immunohistochemical staining for pERK, cyclin D1 and PU.1, was performed in 27/28 (96%) patients (Fig. 2). Results of immunohistochemical staining for respectively pERK ($n = 2$), cyclin D1 (n = 2) and PU.1 ($n = 3$) could not be interpreted despite adequate positive controls. Positive immunohistochemical staining for PU.1 was found in 24/24 (100%) patients. In 11/25 (44%) patients, immunohistochemical staining for pERK was positive. Immunohistochemical staining for cyclin D1 was positive in 15/25 (60%) patients. Focal staining for pERK and cyclin D1 was seen in 1/25 (4%) patients. The staining pattern was never uniformly positive, rather disperse clusters of positive staining cells were observed, which often showed overlapping staining for pERK and cyclin D1 when pERK was expressed. Positive staining for either pERK or cyclin D1 was found in 17 patients of which 12 did not show kinase-activating mutations (Table 1). PU.1 was positive in 10/10 (100%) xanthelasma lesions. In contrast, cyclin D1 and pERK were negative in these control samples (Fig. 2).

4. Discussion

In this study, indications for the activation of the MAPK pathway were found in all subtypes of AOXGD, but not in control xanthelasma lesions. Positive staining for either pERK or cyclin D1 was found in >60% of AOXGD patients. Yet, mutations in the MAPK pathway were found only in 7/28 (25%) patients.

Mutations in the MAPK pathway were first discovered in LCH and consecutively in other histiocytoses. [12–15,19,24] Currently, a mutation in the MAPK pathway is found in over 80% of patients with ECD. [33–35] Indeed, all 4 patients with ECD showed a known MAPK pathway-activating mutation. [13,20,36] So far, few MAPK pathway mutations have been described in the other subtypes of AOXGD. [16] In our study, we found histological evidence supporting the assumption that a large proportion of AOXGD patients in our cohort has MAPK-related mutations. MAPK pathway-activating mutations were found in 2/6 (33%) of our patients with AAPOX. One had a KRASp.K117N mutation and a concurrent PIK3CAp.R992Q mutation with a low variant allele frequency. KRASp.K117N mutations have been described in RDD. [16,20,22,37] In AAPOX, 2 KRAS mutations have been described. [16] *PIK3CA* mutations have been described in ECD. [20,35] *PIK3CA* is the gene that encodes for the catalytic subunit p110α of phosphatidylinositol 3-kinase (PI3K). PI3Ks are lipid kinases that control cellular activities, such as proliferation and survival. Mutations in *PIK3CA* can lead to gain-of-function of PI3Ks that subsequently activate the PI3K/AKT/mTOR pathway that promotes cell survival, proliferation and growth. [38,39] However, this specific PIK3CAp.R992Q mutation has only been previously described in squamous cell carcinoma and thyroid cancer. [40,41] The second patient with AAPOX showed a NRASp.G12D mutation. This mutation has been described in various histiocytoses, including ECD, LCH and RDD. [20,42] None of the 7 patients with NBX showed mutations in the MAPK pathway. This is concurrent with the literature as, to the best of our knowledge, mutations in the MAPK pathway have not been described in NBX patients. Nonetheless, 4/7 (57%) showed positive immunohistochemical staining for either pERK or cyclin D1.

Notably, there was not a clear difference in the clinical course of AOXGD patients with or without mutations in the MAPK pathway in this study. However, 5/7 (71%) patients with a proven MAPK pathway-activating mutation relapsed as compared to 8/21 (38%) patients in whom we could not detect such mutations. This is illustrated by a patient with AOX who presented with a *peri*orbital skin lesion containing two pathogenic mutations (KRASp.G12R and BRAFp.G466E). In retrospect, this lesion turned out to be one of many RAS mutated xanthogranulomatous skin lesions developed over time (P.G. Kemps et al., *manuscript in preparation*). Hence, molecular analysis of (peri)orbital tissue biopsies seems helpful for the identification of patients at risk of recurrent xanthomatous disease.

Mutations in the MAPK pathway lead to activation and subsequent expression of pERK and cyclin D1. Therefore, pERK and cyclin D1 could serve as surrogate indicators of MAPK pathway activation. [16,27–29] Expression of pERK or cyclin D1 was found in all subtypes of AOXGD. In most patients, a mutation functionally affecting the MAPK pathway was not found. This might be due to the fact that not all genes of the MAPK pathway were covered by the NGS assays available. Another plausible explanation for the discrepancy between the pERK/cyclin D1 staining results and the mutational status lies in the actual number of mutation-bearing histiocytes present in the AOXGD biopsies investigated. If only few cells harboring a MAPK mutation are present, the variant allele frequency may be too low to be reliably detected by NGS. [43] This

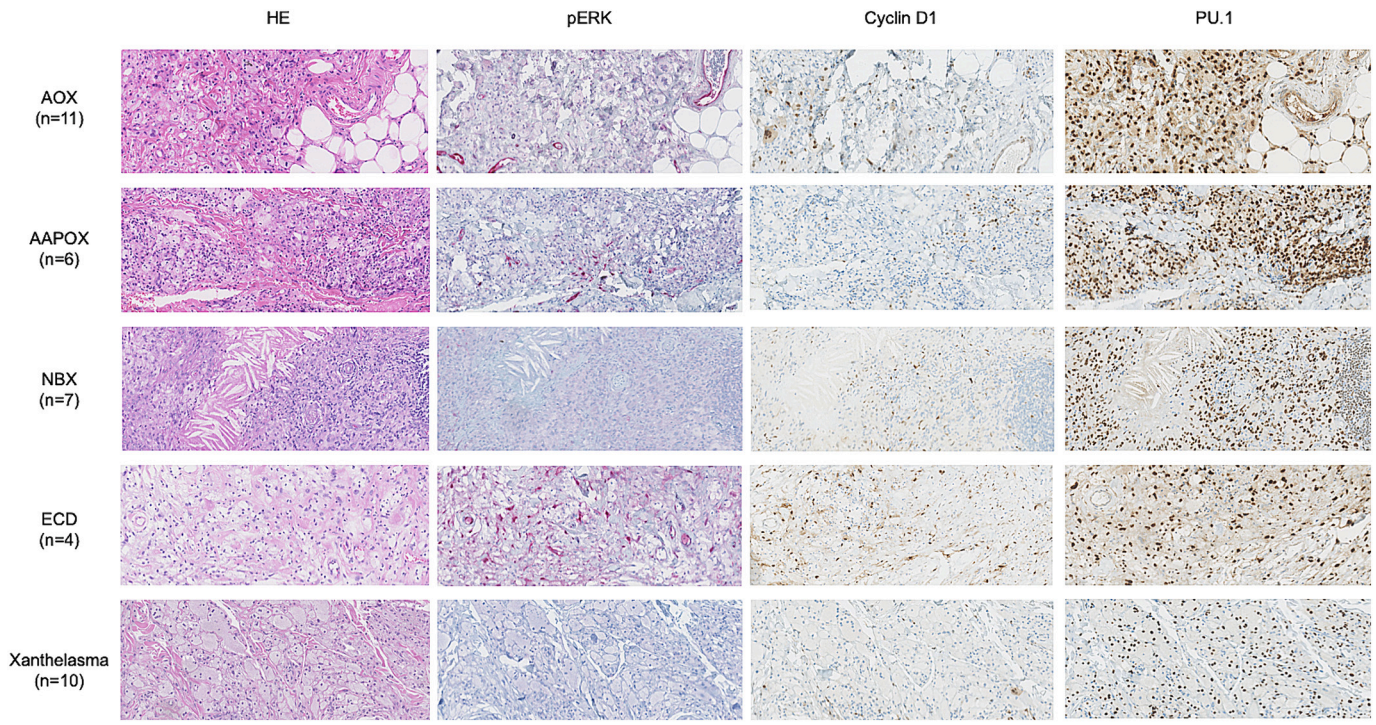

Fig. 2. The variety of immunohistochemical staining for haematoxylin and eosin, pERK, cyclin D1 and PU.1 in patients with AOX (patient 5), AAPOX (patient 14), NBX (patient 21), ECD (patient 25) and xanthelasma.
AAPOX: adult-onset asthma and periocular xanthogranuloma, AOX: adult-onset xanthogranuloma, ECD: Erdheim-Chester disease, HE: haematoxylin and eosin, NBX: necrobiotic xanthogranuloma, pERK: phosphorylated ERK.

assumption is illustrated by a recent study wherein *KRAS* mutations could only by detected by digital droplet PCR, showing presence of mutation-specific amplicons in only 0.4–2.3% of the droplets. [16] We indeed observed that often only a small percentage of xanthogranuloma-associated histiocytes stain positive for pERK and/or cyclin D1. Noteworthy, our conventional NGS assays perform with a lower limit of sensitivity of 5% mutant allele reads. Alternative mechanisms driving the upregulation of pERK and cyclin D1 in situ must, however, be considered as the MAPK pathway can be activated by 1) various inflammation-promoting growth factors, 2) activating mutations in alternative tyrosine kinase receptors or 3) structural alterations in the CCND1 gene. [20,28,44]

The orbit has a particular susceptibility to immune activation. Orbital fibroblasts are regulated by several inflammatory mediators, including transforming growth factor-β, insulin-like growth factor-1 receptor and interleukin-6. These mediators are all known to be able to activate the MAPK pathway subsequently resulting in pERK expression in various orbital inflammatory disorders. [45–47]

Conversely, one patient with a known mutation in the MAPK pathway did not show pERK hyperactivation, but did show upregulation of cyclin D1. Notably, this patient displayed a KRAS mutation. *RAS* interacts with multiple downstream effectors and some KRAS mutation do not lead to pERK activation. [48–51] In contrast, upregulation of cyclin D1 is seen since this is also a downstream effector of *KRAS*. [52]

As expected, no positive staining for pERK or cyclin D1 was seen in control peri-orbital xanthelasma lesions. PU.1 staining was positive in all successfully stained AOXGD and all control peri-orbital xanthelasma lesions. PU.1 is a transcription factor expressed in myeloid lineage-derived cells and is an immunohistochemical marker for histiocytosis. [30] It has an intense nuclear staining, that is easily interpreted compared to the sometimes-difficult interpretation seen in other immunohistochemical markers, including CD68 or CD163. [53,54]

Hence, combination of pERK and cyclin D1 staining seems helpful to distinguish AOXGD from benign xanthelasma.

Since multiple mutations in the MAPK pathway have been found in histiocytosis patients other therapeutic options to intervene in the MAPK pathway are BRAF and MEK inhibitors. Both cobimetinib and trametinib have been used successfully in patients with ECD lesions expressing *ARAF*, *BRAF*, *RAF1*, *NRAS*, *KRAS*, *MEK1* or *MEK2* alterations. [55,56] This likely also applies to lesions expressing the BRAFp.G466E mutation, given that this gain-of-function mutation is responsive to MEK inhibition (www.genomenexus.org).

However, BRAF and MEK inhibitors have various side effects, including skin cancer, rash, arthralgia, diarrhea, hypertension. [57]. A second unknown issue is the minimum duration of targeted therapy. Hence, unlike in patients with systemic granulomatous disease including the (*peri*)orbital region, application of targeted therapy in patients with AOXGD is questionable.

This study has several limitations. It was not possible to perform a standard NGS on all stored tissue samples. The FFPE-TLC NGS was performed on tissue samples that were not subjected to standard NGS or had negative results. These panels overlap at all relevant MAPK mutations except for *MAP2K2* (also known as *MEK2*) which was only present in the FFPE-TLC NGS panel (Supplementary Table 1). Furthermore, gene fusions - not identified in this AOXGD cohort - can only be detected at the DNA level with FFPE-TLC NGS. Moreover, not all immunohistochemical staining could be performed on all tissue samples, as some cases were seen in consultation and tissue samples were, therefore, not available.

5. Conclusion

Molecular analysis revealed somatic MAPK pathway mutations in over one-fifth of AOXGD patients. While histological indicators of MAPK pathway activation were seen in all subtypes, these mutations were confined to ECD and AAPOX cases. This suggests that MAPK pathway activation plays a broader role in the pathophysiology of AOXGD than previously thought. Our results further imply that clinical and radiologic

evaluation for systemic disease is required in all AOXGD patients presenting with positive histological indicators of MAPK pathway activation.

Funding

This work was supported by Stichting Wetenschappelijk Onderzoek Het Oogziekenhuis – Flieringa [grant number 2021S01]. The funding source had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

CRediT authorship contribution statement

S.E. Detiger: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis. **D. Paridaens:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **P.G. Kemps:** Writing – review & editing. **A.G.S. van Halteren:** Writing – review & editing, Supervision. **P.M. van Hagen:** Writing – original draft, Supervision, Conceptualization. **J.A.M. van Laar:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **R.M. Verdijk:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2024.110299.

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