



Two Brothers with Atypical *UNC13D*-Related Hemophagocytic Lymphohistiocytosis Characterized by Massive Lung and Brain Involvement

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Hemophagocytic lymphohistiocytosis (HLH) is a potentially fatal hyperinflammatory condition. Variants in different genes have been associated with the familial forms of the syndrome (FHL), usually presenting within the first 2 years of life. Due to increasing awareness of the signs and symptoms of HLH and a better understanding of the genetic basis of the disease, FHL has been increasingly diagnosed in patients presenting beyond infancy. Here, we report on two brothers with atypical, late-onset HLH in which whole exome sequencing revealed a homozygous pathogenic *UNC13D* variant. In the first brother, the clinical phenotype was dominated by a massive lung involvement. In the second brother a progressive neurological deterioration was observed. In both cases, the clinical manifestations at symptom onset were misleading, making the diagnosis difficult to achieve. This report expands the spectrum of clinical presentations of FLH3. Moreover, it highlights the importance to warn clinicians to keep a high level of suspicion in patients presenting with fever, cytopenia, splenomegaly of unknown origin, and unresponsiveness to conventional treatment even beyond early childhood. Moreover, this report emphasizes that insidious neurologic symptoms may represent the initial or sole presenting sign of FHL, even in the absence of peripheral signs of activation.

Keywords: *UNC13D*, FHL3, atypical FHL, CNS-HLH, extracorporeal membrane oxygenation

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is a potentially fatal hyperinflammatory condition characterized by prolonged and unexplained fever, unresponsiveness to conventional treatment, hepatosplenomegaly, cytopenia, hypertriglyceridemia, and hypofibrinogenemia (1, 2). Variants in four different genes have been associated with the familial forms of the syndrome (FHL). FHL-2 is due to variations in the *PRF1* gene, encoding perforin 1, implicated in target cell lysis (3). *UNC13D*

encoding MUNC13-4, *STX11* encoding SYNTAXIN11, and *STXBP2* encoding MUNC18-2 are implicated in the trafficking and exocytosis of lytic granules, and mediate the release at the cell surface of perforin1 and other effector molecules implicated in cytotoxicity (4–7). The impairment of the cytotoxic activity may lead to uncontrolled activation of cytotoxic T cells and macrophages, which finally result in a hyperinflammation, T-cell, and macrophage infiltration of various organs including bone marrow, liver, and the central nervous system (8).

FHL usually presents within the first 2 years of life (9). However, the increasing awareness of the signs and symptoms of HLH and the better comprehension of the genetic basis of the disease allow the identification of FHL in patients presenting beyond infancy and sometimes before the development of the HLH (10). Atypical presentations have been reported in adolescents and even in adults, characterized by milder and often recurrent HLH episodes and prolonged survival in the absence of hematopoietic stem cell transplantation (HSCT), unusual in patients with the typical disease. Here, we report on two brothers with atypical, late-onset HLH in which whole exome sequencing (WES) revealed a homozygous pathogenic *UNC13D* variant. In both cases, the clinical manifestations at symptom onset were misleading, making the diagnosis difficult to achieve.

CASE REPORT

Written informed consent was obtained from the parents for the publication of both the cases. The patients were dizygotic twins born preterm (33 weeks) following conception by *in vitro* fertilization, yielding a triplet pregnancy complicated by *in utero* death of the third fetus. Parents were not consanguineous and familial history was unremarkable, apart from a history of recurrent abortions in the mother. At the age of 6.2 years, after an uneventful childhood, the first brother developed fever resistant to antibiotics and severe, rapidly progressive dyspnea, requiring the admission to a pediatric intensive care unit for mechanical ventilation. The rapid progression of such a severe lung involvement raised the suspicion of a primary immunodeficiency (11). Clinical examination revealed splenomegaly and macular truncal rash. Laboratory examinations revealed trilineage cytopenia, hypertriglyceridemia, hypofibrinogenemia, and hyperferritinemia (Table 1). The analysis of the bone marrow aspirate revealed hypocellularity, but lymphocytes and histiocytes infiltration and hemophagocytosis were not observed. Lung Rx and CT scan revealed massive bilateral pulmonary consolidations with alveolar involvement, only sparing the apical lobes (Figures 1A–C). *Klebsiella pneumoniae* and *Candida albicans* were isolated from the bronchial aspirate while the pleural fluid culture was negative. Nasal, pharyngeal and rectal swab, urine culture, mannan, galactomannan and PCR for *HSV1/2*, *Parvovirus B19*, *HHV8*, *HHV6*, *CMV*, *EBV*, and other respiratory viruses in the blood and bronchial aspirate were negative. Cytological examination of the pleural fluid showed an increase of the protein levels and of the leukocytes, in particular lymphocytes. Basal immunological examination revealed hypogammaglobulinemia (IgG 315 mg/dL, IgA 46 mg/dL, IgM 58 mg/dL) with normal production of specific antibodies and a slight

TABLE 1 | Comparison of clinical and laboratory markers in the two twins carrying the same *UNC13D* mutation.

	Patient 1	Patient 2
<i>UNC13D</i> mutation	Homozygous 1847A>G	Homozygous 1847A>G
Age at onset	6.2 years	3 years
Symptoms at the onset	Fever and severe dyspnea	Pervasive developmental disorder
Fever	Yes	No
Splenomegaly	Yes	Yes
CNS involvement	No	Yes
Lung involvement	Massive bilateral consolidations	Signs of pulmonary hypertension
WBC (cells/mm ³)	800	7,750
Neutrophil count (cells/mm ³)	430	5,450
Lymphocyte count (cells/mm ³)	320	1,880
Platelet count (cells/mm ³)	27,000	241,000
Hemoglobin levels (g/dL)	7.8	14.9
Fibrinogen (mg/dL)	89	n.a.
Triglycerides (mg/dL)	1,556	110
Ferritin (ng/mL)	2,550	84
Albumin (gr/dL)	2.4	5
IgG/IgA/IgM (mg/dL)	315/46/58	487/45/120
IgE (IU/mL)	249	n.a.
CD3+ % (cells/mm ³)	57% (182)	77.5%
CD4+ % (cells/mm ³)	34% (109)	33%
CD8+ % (cells/mm ³)	13% (42)	33.2%
CD19+ % (cells/mm ³)	8% (26)	21%
CD16+ CD56+ % (cells/mm ³)	22% (70)	1.5%
CD4+ CD45RA+ % (cells/mm ³)	n.a.	6.6%
CD4+ CD45RO+ % (cells/mm ³)	n.a.	26.4%
CD8+ CD45RA+	n.a.	14.9%
CD8+ CD45RO+	n.a.	18.3%
CD3+ HLADR+	27%	n.a.
Granule release assay	n.a.	Absent degranulation
Perforin expression	n.a.	Normal
Bone marrow aspirate	Hypocellularity	Lymphohistiocytic infiltration

CNS, central nervous system; WBC, white blood cells; n.a., not available.

increase of IgE levels (249 IU/mL). A marked lymphopenia was observed, despite normal T, B, and NK cells percentages, apart from an increase of the CD3+ HLADR+ cells (Table 1). WES was performed due to suspicion of a CID with secondary HLH. Treatment with intravenous immunoglobulins, antibiotics, and antifungals was started. Venous extracorporeal membrane oxygenation (ECMO) was initiated due to the development of bilateral massive pneumothorax, but unfortunately the patient died of cardiopulmonary collapse.

The second brother developed a pervasive developmental disorder at the age of 3 years. By age 5, his neurological condition deteriorated with the development of asthenia, ataxia, and nystagmus, facilitating a neurological evaluation. Brain MRI revealed areas of demyelination enhanced by contrast agent, involving pons, cerebellar peduncles, cerebellum, and white matter of the left temporal lobe (Figures 1D,E). CSF evaluation revealed pleocytosis and oligoclonal bands. Based on the radiological findings and favorable response to steroid treatment, a diagnosis of Chronic Lymphocytic Inflammation with Pontine Perivascular Enhancement Responsive to Steroids was suspected and the patient was started on Azathioprine. However, clinical and radiological findings deteriorated despite the immunosuppressive treatment

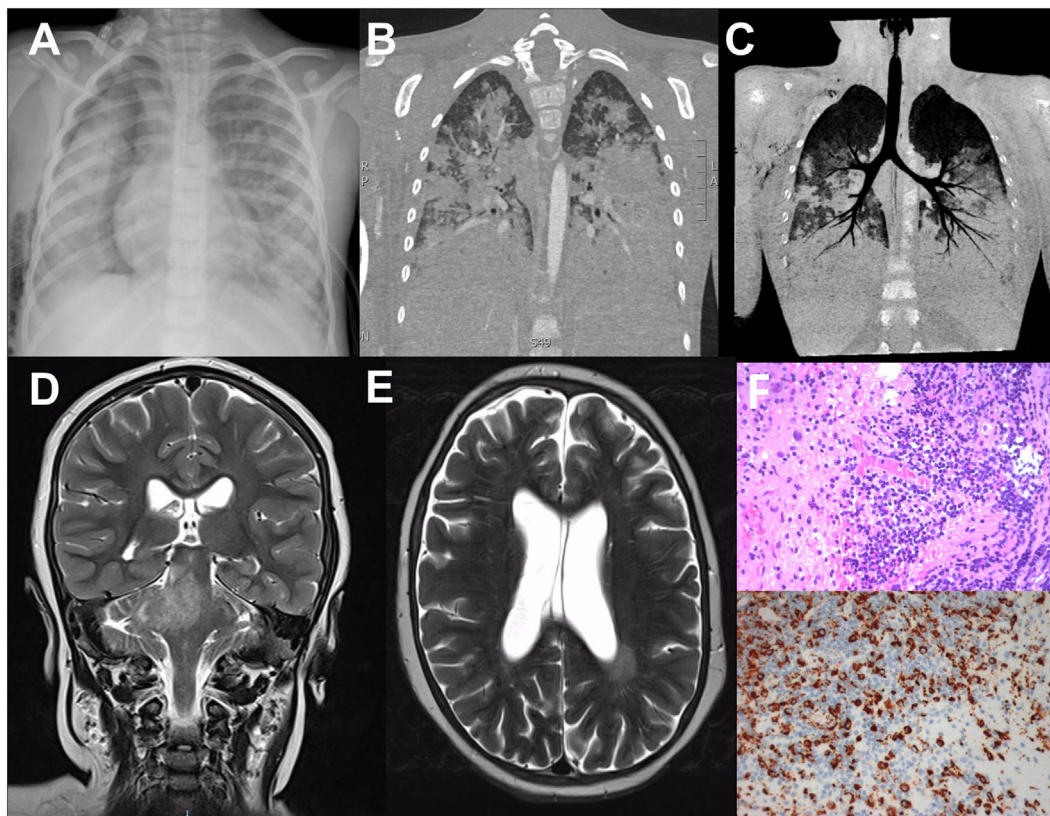


FIGURE 1 | Radiological findings in the dizygotic twins homozygous for 1847A>G (p.Glu616Gly) in *UNC13D*. **(A)** Thoracic Rx of the patient 1; broad area of opacity involving the right mediobasal lobe and the left basal lobe, with bilateral hilar and perihilar infiltration. **(B,C)** Pulmonary CT scan of the patient 1; massive bilateral pulmonary consolidations with alveolar involvement, only sparing the apical lobes. **(D,E)** Brain MRI scan of the patient 2; T2w image with hyperintense signal in the left periventricular region, in pons, right cerebellar peduncles, and right cerebellar hemisphere. **(F)** Hematoxylin-eosin staining of the brain biopsy showing lymphohistiocytic perivascular and intraparenchymal inflammatory infiltrate (upper panel); immunohistochemical staining for the histiocytic marker CD68 (lower panel).

and a brain biopsy was performed showing a lymphohistiocytic perivascular and intraparenchymal inflammatory infiltrate (**Figure 1F**). A CNS-HLH was suspected. Peripheral markers of activation were evaluated and found to be normal (**Table 1**). Splenomegaly and bone marrow lymphohistiocytic infiltration were detected. Immunological studies revealed normal IgG, IgA, and IgM levels and slightly increased CD3+ cells, mainly showing a memory phenotype, absent degranulation of the NK cells after the stimulus with K562 cells, normal perforin expression, suggesting a diagnosis of HLH (**Table 1**).

In the first brother, WES revealed a homozygous pathogenic *UNC13D* variant, 1847A>G (p.Glu616Gly). This variant has been previously reported in affected individuals, and functionally demonstrated to result in deranged splicing of the gene (12, 13). It is absent from population databases. The second twin was genotyped for the variant by Sanger sequencing, revealing homozygosity for the same *UNC13D* variant. The parents were both heterozygous. A diagnosis of isolated CNS-HLH was made, and the patient was started on the HLH-2004 protocol. The patient unfortunately died of transplant-related complications.

DISCUSSION

Hemophagocytic lymphohistiocytosis represents the clinical and immunological manifestation of several diseases including FHL, characterized by a well-known pathogenic mechanism and a large number of disorders in which pathogenesis is still poorly understood. Indeed, HLH has been found in association with inborn errors of the metabolism (14–16), autoimmune disease (17), congenital immunodeficiencies, such as chronic granulomatous disease (18), DiGeorge syndrome (19), severe combined immunodeficiency (20), and X-linked agammaglobulinemia (21, 22), and other disorders (23–25). These last cases, described as “secondary” HLH, usually present in older children. More recently, the increasing awareness of the presenting signs of the disease and the development of new tools for the diagnosis of these conditions have made possible the identification also in patients presenting later in life with atypical symptoms (26). Considering the similarities between atypical FHL and secondary HLH, it can be difficult in some cases to achieve a rapid diagnosis. Moreover, in many cases of atypical FHL, the peripheral signs of HLH may be completely absent, making the diagnosis even

more difficult to achieve. In a recent paper, Gray et al reported on a case of late-onset FHL3 in which the clinical presentation was characterized by growth arrest, inflammatory arachnoiditis, and dysgammaglobulinemia, without any signs of HLH (10). Similarly, the hypomorphic A91V variation has been recently described in association with atypical late-onset HLH in a patient with neuromyelitis optica (27).

In this report, we describe two male dizygotic twins with atypical FHL3 (12, 13, 28). In both patients, the onset was delayed compared to the median age of 4.1 months reported in a wide cohort of FHL3 patients (13). Missense variants in *UNC13D* are associated with a later onset as compared with loss of function variants and median age at the diagnosis was 117.5 months in four patients with homozygous c.1847A>G (13). The two brothers presented with very different clinical manifestations. In the first brother the clinical picture was dominated by a massive lung involvement and laboratory features suggested a secondary HLH in the context of a CID (24). WES failed to detect mutations other than *UNC13D*. However, even though primary immunodeficiencies have been traditionally considered as monogenic disorders, in the recent years high throughput sequencing techniques have highlighted that atypical phenotypes may be explained by the coexistence of multiple mutations (29–33). Since WES may not be able to detect large deletion/insertion and deep intronic mutations, we cannot exclude that a second mutation may have had a role in shaping such a complex clinical phenotype. On the other hand, even though immunological alterations are not usually found in patients with typical FHL, recent evidence suggests that different immunological alterations including antibody deficiency, low B-cell numbers, reduced percentage of marginal-zone like and class-switched memory B cells, and susceptibility to bacterial sinopulmonary infections may be detected in patients with different forms of FHL (34). In some cases, hypogammaglobulinemia and defects of specific antibodies developed after recurrent mild episodes of HLH (34). Interestingly, the immunological alterations are not usually found in patients with typical FHL, suggesting that in atypical forms a silent chronic T cell activation may impair the B cell development leading to the development of hypogammaglobulinemia (34). For this reason, the association of a clinical picture of common variable immunodeficiency/CID, with splenomegaly, cytopenia, and episodes of unexplained fever should address the diagnosis also toward atypical FHL (34).

Lung involvement is not well characterized in HLH, especially in pediatric patients and guidelines on the treatment of these conditions are also lacking (35). In a cohort of adult patients, lung involvement, due to lymphocytic infiltration or infection, was reported in about 54% of cases and associated with a worse prognosis (36). However, adult HLH may have a different pathogenesis compared to children affected with FHL. In the patient described, *Klebsiella pneumoniae* and *Candida albicans* may have played a role in triggering the lung involvement and, moreover, the hypogammaglobulinemia along with the marked lymphopenia may have had a role in the rapid deterioration of the clinical conditions. However, it should be noted that five out of eight HLH diagnostic criteria were present at the diagnosis (fever, splenomegaly, trilineage cytopenia, hyperferritinemia, hypofibrinogenemia/

hypertriglyceridemia), suggesting that HLH also had a role in shaping the clinical picture. Moreover, the patient did not show any sign of improvement despite 3 months of intravenous treatment with immunoglobulins, different antifungals, and antibiotics, suggesting an inflammatory more than infectious condition. Even though lung biopsy was not performed in our patients, all cultures, including pleural fluid were negative, with pleural fluid showing increased levels of proteins and lymphocytes, suggesting lung infiltration by activated lymphocytes and histiocytes. In our patient, ECMO was initiated due to the development of bilateral massive pneumothorax. However, in a recent study on a small cohort of patients, survival of pediatric HLH patients on ECMO was worse than patients without HLH on ECMO (37). It should be noted that the severity of the clinical manifestations and the high mortality related to the lung involvement may themselves lead to a reduced estimation of the incidence of the lung involvement in FHL, since the patient may die without receiving a genetic diagnosis.

The second brother presented at the age of 3 years with signs of a pervasive developmental disorder. His neurological conditions deteriorated progressively over a very long period. While lung infiltration is an unusual presentation of FHL (12, 13), neurologic signs are present in about 2/3 of the patients with FLH at the onset and may also develop later in the course of the disease (38, 39). Common neurological manifestations include irritability, seizures, bulging fontanel in infants, neck stiffness, hypotonia, and hypertonia (9, 40, 41). Other less common signs include nonspecific signs of increased intracranial pressure, cranial nerve palsy, ataxia, hemiplegia, quadriplegia, blindness, and coma (9, 40, 41). CSF examination usually shows elevated protein, increased number of mononuclear cells, and occasionally hemophagocytosis (9, 40, 41). Typical radiologic findings include demyelination or impaired myelination, gray and white matter multifocal inflammation mimicking neuroinflammatory disorders including multiple sclerosis and acute disseminated encephalomyelitis (41, 42). FHL3 patients seem to be even more prone to develop CNS involvement. In a recent study, 60% of the patients with FHL3 showed a CNS involvement compared with the 32% in patients with FHL2 (13). Moreover, the variant identified in these twins has been frequently associated with CNS involvement (13). Of note, in our patient, more typical clinical neurological manifestations associated with HLH, including demyelination, ataxia, and cerebral palsy (43), were preceded by a pervasive developmental disorder, suggesting a very slow and insidious process which delayed the diagnosis. Moreover, the patient never showed any sign of systemic HLH. Previous evidence suggest that primary HLH may also represent the underlying cause of unknown CNS inflammation even in the absence of overt clinical signs of HLH, suggesting that FHL should be included in the differential diagnosis of unclassified progressive neuroinflammatory disease (44–50). T and NK cell degranulation assays and the evaluation of the expression of intracellular perforin can be used for the prompt identification of such patients, allowing a targeted treatment (51). Corticosteroids, etoposide, cyclosporine A, commonly used for the treatment of systemic HLH, along with intrathecal methotrexate and corticosteroids represent the standard, first-line therapeutic approach also for

the treatment of CNS-HLH (1, 9, 38). Antithymocyte globulin has also been used, in association with steroids, cyclosporin A, and intrathecal methotrexate injection to achieve remission in HLH (52). However, the efficacy of this association on CNS is comparable to the HLH-94 protocol (38, 52). HSCT also represents an important tool for the treatment of CNS-HLH especially in patients with familial forms, or with severe, persistent or reactivated HLH (38). Recently, novel therapeutic approaches are emerging as effective in the treatment of the refractory and complicated HLH even though many of these are still considered as second line or salvage experimental treatments. Among these, alemtuzumab has shown promising results in the treatment of the refractory HLH even though its effectiveness in the treatment of refractory CNS-HLH has not been clarified yet (53). Based on encouraging preclinical data from animal model, a clinical trial (NCT01818492) is currently ongoing to evaluate the efficacy of anti-IFN γ monoclonal antibody NI-0501 in the treatment of refractory HLH. Finally, the JAK1/2 inhibitor ruxolitinib has been recently shown to be effective in reducing the CNS involvement in a Rab27a^{-/-} mouse model (54).

CONCLUDING REMARKS

In conclusion, this report expands the spectrum of clinical presentations of FLH3. It is important to warn clinicians to keep a high level of suspicion in patients presenting with fever, cytopenia, splenomegaly of unknown origin, and unresponsiveness

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ETHICS STATEMENT

The study was carried out in accordance with the Declaration of Helsinki and was approved by the institutional ethics committee of the University Federico II of Naples. Informed consent was obtained from all patients before the study.

AUTHOR CONTRIBUTIONS

CP, CC, GG, ML, and EC organized, collected, and analyzed the data and wrote the manuscript. PP, RR, MV, and LP followed up the patients, collected the data, and performed the experiments. CS performed whole exome sequencing; all authors reviewed and approved the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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