Kufs disease due to mutation of *CLN6*: clinical, pathological and molecular genetic features

Samuel F Berkovic[1], Karen L Oliver[1], Laura Canafoglia[2], Penina Krieger[1], John A Damiano[1], Michael S Hildebrand[1], Michela Morbin[3], Danya F Vears[1], Vito Sofia[4], Loretta Giuliano[4], Barbara Garavaglia[5], Alessandro Simonati[6], Filippo M Santorelli[7], Antonio Gambardella[8], Angelo Labate[8], Vincenzo Belcastro[9], Barbara Castellotti[10], Cigdem Ozkara[11], Adam Zeman[12], Julia Rankin[13], Sara E Mole[14], Umberto Aguglia[15], Michael Farrell[16], Sulekha Rajagopalan[17], Alan McDougall[18], Susan Brammah[19], Frederick Andermann[20], Eva Andermann[20], Hans-Henrik M Dahl[1], Silvana Franceschetti[2] and Stirling Carpenter[21]

1 Epilepsy Research Centre, Department of Medicine, University of Melbourne, Austin Health, Heidelberg, Victoria, Australia.
2 Department of Neurophysiology, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy
3 Deceased, previously Neuropathology, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy
4 Department of Medical and Surgical Sciences and Advanced Technologies "G.F. Ingrassia", Section of Neurosciences, University of Catania, Catania, Italy
5 Medical Genetics and Neurogenetics Unit, Bicocca Laboratories, Fondazione IRCCS Istituto Neurologico "Carlo Besta", Milan, Italy
6 Department of Neuroscience, Biomedicine, Movement-Neurology and Neuropathology, Policlinico GB Rossi, P.le LA Scuro, 37134 Verona, Italy
7 Molecular Medicine, IRCCS Fondazione Stella Maris, Pisa, Italy
8 Institute of Neurology, University Magna Græcia Catanzaro, Italy; Institute of Molecular Bioimaging and Physiology of the National Research Council (IBFM-CNR) Germaneto, CZ, Italy
9 Neurology Unit, S. Anna Hospital, Como, Italy
10 Unit Genetics of Neurodegenerative and Metabolic Diseases, IRCCS Foundation C. Besta Neurological Institute, Milan, Italy
11 Istanbul University-Cerrahpaşa, Medical Faculty, Department of Neurology, Istanbul
12 University of Exeter Medical School, St Luke's Campus, Magdalen Road, Exeter EX1 2LU, United Kingdom
13 Clinical Genetics, Royal Devon and Exeter Hospital, Gladstone Road, Exeter EX1 2ED, United Kingdom
14 MRC Laboratory for Molecular Cell Biology and UCL GOS Institute of Child Health, Department of Genetics, Evolution and Environment, University College London, London WC1E 6BT, United Kingdom
15 Department of Medical and Surgical Sciences, University Magna Græcia Catanzaro, Italy; Institute of Molecular Bioimaging and Physiology of the National Research Council (IBFM-CNR) Germaneto, CZ, Italy
16 Department of Neuropathology, Beaumont Hospital, Dublin 9, Ireland
17 Department of Clinical Genetics, Liverpool Hospital, Liverpool, New South Wales 1871 Australia
18 Department of Neurology, Liverpool Hospital, Liverpool, New South Wales 1871 Australia
19 Central Sydney Electron Microscope Unit, Concord Repatriation General Hospital, Concord, New South Wales, Australia
20 Epilepsy Research Group, Montreal Neurological Hospital and Institute, Montreal, Quebec, Canada; Departments of Neurology & Neurosurgery and Peadiatrics, McGill University, Montreal, Quebec, Canada
21 Consultant in Neuropathology, Centro Hospitalar São João, Porto. Portugal

Corresponding author:
Prof Samuel Berkovic
245 Burgundy St
Heidelberg, VIC
Australia
Phone: +61 3 9035 7093
Email: s.berkovic@unimelb.edu.au

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Abstract

Kufs disease is the major adult form of neuronal ceroid lipofuscinosis, but is rare and difficult to diagnose. Diagnosis was traditionally dependent on the demonstration of characteristic storage material, but distinction from normal age-related accumulation of lipofuscin can be challenging. Mutation of *CLN6* has emerged as the most important cause of recessive Kufs disease but, remarkably, is also responsible for variant late infantile ceroid lipofuscinosis. Here we provide a detailed description of Kufs disease due to *CLN6* pathogenic variants. We studied 20 cases of Kufs disease with *CLN6* pathogenic variants from 13 unrelated families. Mean age of onset was 28 years (range 12-51) with bimodal peaks in teenage and early adult life. The typical presentation was of progressive myoclonus epilepsy with debilitating myoclonic seizures and relatively infrequent tonic-clonic seizures. Patients became wheelchair bound with a mean 12 years post onset. Ataxia was the most prominent motor feature. Dementia appeared to be an invariable accompaniment, although it could take a number of years to manifest and occasionally cognitive impairment preceded myoclonic seizures. Patients were usually highly photosensitive on EEG. MRI showed progressive cerebral and cerebellar atrophy. The median survival time was 26 years from disease onset. Ultrastructural examination of the pathology revealed fingerprint profiles as the characteristic inclusions, but they were not reliably seen in tissues other than brain. Curvilinear profiles, which are seen in the late infantile form, were not a feature. Of the 13 unrelated families we observed homozygous *CLN6* pathogenic variants in four and compound heterozygous variants in nine. Compared to the variant late infantile form, there was a lower proportion of variants that predicted protein truncation. Certain heterozygous missense variants in the same amino acid position were found in both variant late infantile and Kufs disease. There was a predominance of cases from Italy and surrounding regions; this was partially explained by the discovery of three founder pathogenic variants. Clinical distinction of Type A (progressive myoclonus epilepsy) and Type B (dementia with motor disturbance) Kufs disease was supported by molecular diagnoses. Type A is usually caused by recessive pathogenic variants in *CLN6* or dominant variants in *DNAJC5*. Type B Kufs is usually associated with recessive *CTSF* pathogenic variants. The diagnosis of Kufs remains challenging but, with the availability of genetic diagnosis, this will largely supersede the use of diagnostic biopsies, particularly as biopsies of peripheral tissues has unsatisfactory sensitivity and specificity.

Keywords
neuronal ceroid lipofuscinosis, neuropathology, neurodegeneration, Kufs disease, *CLN6*, ataxia

Abbreviations

CGH – comparative genomic hybridization
ddPCR – droplet digital polymerase chain reaction
DRPLA – dentatorubral-pallidoluysian atrophy
FPP – fingerprint profiles
GRODs - granular osmiophilic deposits
MEAK – myoclonus epilepsy and ataxia due to *KCNC1* mutation
MERRF – myoclonic epilepsy with ragged red fibers
MLPA – multiplex ligation-dependent probe amplification
NCL - neuronal ceroid lipofuscinosis
PME – progressive myoclonus epilepsy
PMA – progressive myoclonus ataxia
SNP – single nucleotide polymorphism
TCS – tonic-clonic seizure

Introduction

The diagnosis of slowly progressive degenerative disorders in teenagers and adults remains challenging. Kufs disease, the paradigmatic form of late-onset neuronal ceroid lipofuscinoses, is one important cause, however, the clinico-pathologic literature is confusing and accurate diagnosis is difficult. In an earlier detailed review of cases reported as Kufs disease (Berkovic *et al.*, 1988), we found the diagnosis could be supported in only 50/118 cases and defined two types on the basis of the prominent clinical features; Type A presenting as a progressive myoclonus epilepsy, and Type B presenting as dementia with motor disturbance. Kufs disease differs from the more common childhood neuronal ceroid lipofuscinosis (NCL) forms, not only by its late onset, but also by the absence of retinal involvement. Contemporary challenges in diagnosis remain, as a recent analysis of 47 cases of putative Kufs disease showed that approximately a third had alternate diagnoses and, for many others, no diagnosis could be definitively substantiated (Berkovic *et al.*, 2016). The diagnosis traditionally has rested on characteristic pathology, which is most clearly shown in brain tissue, but is now rarely available.

With recent discoveries of the molecular genetic basis of many cases and families with Kufs disease, the diagnostic landscape is changing. Well established genes for recessive Kufs disease are *CLN6* (Arsov *et al.*, 2011; Andrade *et al.*, 2012; Ozkara *et al.*, 2017) and *CTSF* (Smith *et al.*, 2013; Di Fabio *et al.*, 2014; Bras *et al.*, 2016; van der Zee *et al.*, 2016) and for some dominant families, *DNAJC5* (Benitez *et al.*, 2011; Noskova *et al.*, 2011; Velinov *et al.*, 2012; Cadieux-Dion *et al.*, 2013; Moro *et al*., 2014; Jarrett *et al*., 2018). Recessive pathogenic variants of the progranulin gene (*GRN*) causes an adult form of NCL with visual impairment (Smith *et al.*, 2012; Canafoglia *et al.*, 2014; Almeida *et al.*, 2016), while heterozygous *GRN* variants are an important cause of fronto-temporal dementia. Additionally, there are other NCL genes where the presentation is normally in childhood, however, protracted or late onset forms have also been described that might present to the adult neurologist (Kousi *et al.*, 2012; http://www.ucl.ac.uk/ncl/mutation.shtml).

CLN6 pathogenic variants are the most common identified cause of recessive Kufs disease and, remarkably, also causes a late-infantile form of NCL. We initially reported seven *CLN6* Kufs families (Arsov *et al.*, 2011) and a handful of others have subsequently been reported (https://www.ucl.ac.uk/ncl/mutation.shtml, last updated 26/02/2018). Here we present an analysis of 13 personally evaluated families with emphasis on the natural history, ultrastructural features in available pathology material and molecular genetics allowing a more complete description of *CLN6*-related Kufs disease. We compare the *CLN6* molecular findings in those with teenage or adult onset Kufs disease versus late-infantile NCL to determine whether a genotype-phenotype correlation exists. Finally, we review available data on all Kufs disease cases with a molecular

diagnosis and evaluate the utility of the Type A/Type B classification of Kufs disease in light of molecular genetic advances.

Methods

Patients

Patients were ascertained via centres with a known interest in Kufs disease and by direct referral. We collated clinical data on ancestry, sex, family history, birth and development, age of disease onset, behavioural and cognitive changes, seizure types, motor symptoms, vision, disease progression and outcome. Additionally, electrophysiological and neuroimaging data were analysed. As the cases had been ascertained over many years, the quality and completeness of the data varied.

This study was approved by the Ethics Committee at the Austin Hospital (Melbourne, Australia) and written informed consent was obtained from all patients following local IRB requirements.

Pathological review

Persistent attempts were made to source pathological material for all patients who had had previously reported biopsies (including skin, muscle, rectal, brain). All available images were subsequently re-analysed by the same expert neuropathologist (S.C.) who reported specifically on the presence or absence of curvilinear profiles, fingerprint profiles and GRODs (granular osmiophilic deposits).

Molecular genetic analysis

Genomic DNA was extracted from brain specimens using an All Prep DNA/RNA Kit or QIAamp DNA FFPE Tissue Kit, or peripheral blood using a QIAamp DNA Maxi Kit (Qiagen, Hilden, Germany).

Single nucleotide and small indel variants were identified by standard PCR on a Veriti Thermal Cycler (Applied Biosystems, Carlsbad, CA) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing protocol on an ABI 3730XL DNA Analyzer (Life Technologies Corporation, Carlsbad, CA). All exons, exon-intron boundaries, and untranslated regions of *CLN6* (NM_017882.2) were sequenced as previously described (Arsov *et al.*, 2011).

Suspected deletions were investigated by SNP genotyping, array CGH, MLPA and ddPCR analyses. SNPs in and around *CLN6* were sequenced; heterozygous SNPs were deemed to be outside the deleted region. Array CGH analyses were performed with Human CGH 3x720K Whole-Genome Tiling Array (Roche-NimbleGen) and Whole- Genome Cytogenetics 2.7M DNA chip (Affymetrix). MLPA probes in and around *CLN6* were also designed and used for copy number estimations as described in the MRC-Holland protocol (MRC-Holland, Amsterdam, The

Netherlands). Droplet Digital PCR probes and primers were designed to assess gene copy number if deletions were suspected, or to interrogate specific *CLN6* pathogenic variants, as we previously described (Damiano *et al.*, 2017; Hildebrand *et al.*, 2018). Probe and primer sequences used in molecular genetic assays are available on request.

CLN6 phenotype/genotype correlation

We extracted a list of pathogenic *CLN6* variants from the NCL mutation database (http://www.ucl.ac.uk/ncl/mutation.shtml) including those reported in Kousi *et al.*, 2012. The list was further curated following a review of the scientific literature in PubMed on 2nd July 2018 using search term "CLN6". Pathogenic variants were then divided into two groups: those causing late-infantile NCL (onset ≤8 years) (Canafoglia *et al.*, 2015, Kousi *et al.*, 2012) versus teenage or adult-onset Kufs disease (onset ≥11 years and no visual failure) (Berkovic *et al*., 1988). Kufs disease cases were further divided into those with teenage versus adult onset, where we defined teenage as ≤19 years. Variants were excluded when limited clinical data precluded such clinical classification or when pathogenicity was uncertain. One typical teenage-onset Kufs case could not be analysed as molecular data were not published (Lv *et al*., 2018). Additionally, homozygous *CLN6* missense variants were extracted from control database Genome Aggregation Database (gnomAD, version r2.0.2) (http://gnomad.broadinstitute.org/).

All pathogenic variants were classified and grouped into those predicted to result in protein truncation (e.g., nonsense, frameshift indels, canonical +/- splice site, single or multi-exon deletion) versus those not (e.g., missense, non-frameshift indels). This enabled us to compare the number of unrelated patients reported to carry two truncation variants (as either homozygous or compound heterozygous pathogenic variants) versus one or zero truncation variants between the two disease groups. Multiple late-infantile NCL families with the same homozygous pathogenic variant due to a known founder effect (Kousi *et al.*, 2012) were considered distantly related and therefore only counted once (e.g., p.Glu72* in Costa Rica, p.Ile154del in Portugal, p.Val91Glufs*42 in Newfoundland and p.Arg106Profs*26 in Pakistan).

Further, the position for each variant was plotted along the length of the *CLN6* gene. Each variant was colour-coded per its clinical classification (i.e., late-infantile NCL, teenage/adult Kufs or control).

Data availability statement

Data supporting the genotype-phenotype correlation findings of this study are openly available in the NCL mutation database at http://www.ucl.ac.uk/ncl/mutation.shtml and from the gnomAD

control database (version r2.0.2) at http://gnomad.broadinstitute.org/. Source clinical and pathological data are not publicly available due to their containing information that could compromise the privacy of research participants.

Results

Patients

We studied 20 patients (7 male) with Kufs disease due to recessive pathogenic variants in *CLN6*. Twelve of these have been previously published in varying detail (Berkovic *et al.*, 1988; Callagy *et al.*, 2000; Arsov *et al.*, 2011; Muona *et al.*, 2015; Ozkara *et al.*, 2017) and eight cases are unpublished. New follow-up data was available on 9/12 previously published cases who were alive at last publication. One case published earlier (Ku8 (Arsov *et al.*, 2011)) was excluded from the clinical analysis because details were not available.

Six were sporadic cases and 14 were familial, comprising sibling pairs from 7 unrelated families. Of the 13 unrelated cases and families, nine were of Italian ancestry from Italy (n=8) and Canada (n=1); one was a family of Maltese ancestry from Australia; and the remaining three families were from the United Kingdom, Ireland and Turkey. In two families, the parents were reported as first cousins and in another two, the parents were known to be from the same or adjacent villages in Malta and Italy respectively.

Clinical features

The mean age of onset was 28 years (range 12-51) on a background of normal development with no significant antecedent factors reported. Onset appeared bimodal, as previously reported for Kufs overall (Berkovic *et al.*, 1988), with 7 adolescent (onset 12-19 years) and 13 adult cases (onset 26-51 years) (Fig. 1).

The typical presentation was of progressive myoclonus epilepsy, with myoclonic and tonic-clonic seizures, although in four patients (Cases 6b, 8a, 8b, 10) there were definitive cognitive/behavioural changes or ataxia 3-8 years before the development of seizures; in Case 5 scholastic difficulties from age 12 years likely represented disease onset (Table 1).

Myoclonus was debilitating and was sometimes associated with a report of transient impaired awareness. ‘Massive’ myoclonus resulting in falls and myoclonic status were also reported. Generalised tonic-clonic seizures were rare in most patients and were not observed at all in three patients (Cases 4b, 6b, 11b). Repetitive tonic seizures in clusters lasting 20 minutes or more were described in three patients (Cases 1a, 3a, 3b).

Ataxia was the most prominent motor impairment and typically required a walking aide. Dysarthria was also prominent. Cerebellar signs in the limbs were difficult to measure due to action

myoclonus. Later features included pyramidal signs and extra-pyramidal features including bradykinesia and dystonia.

EEG data was available for 18/20 cases. Epileptiform discharges that were generalised, multifocal or posteriorly predominant were observed. Data on photic stimulation were reported for all 18 cases; 17/18 were photosensitive on testing, often including at low frequency (1-5 Hz); this is consistent with what has previously been described (Berkovic *et al.*, 1988; Guellerin *et al.*, 2012; Canafoglia *et al.*, 2015). EEG background rhythms varied from normal to slow and irregular.

MRI was available for 17/20 cases. Cerebral and to a lesser extent cerebellar atrophy was evident for all. Case 9b had two normal brain MRI reports during the first 4 years following diagnosis; but mild atrophy was evident 7 years post-disease onset. Brain atrophy was reported as progressively worsening over time in multiple patients that had serial MRIs performed (Cases 1a, 6a, 9a, 9b, 10, 11a). No other specific brain abnormalities were consistently noted across the cohort.

Disease course

The course was slowly progressive, involving ataxia with loss of mobility and cognitive decline resulting in dementia. Two siblings had no evident cognitive decline at 20 (Case 9a) and 7 (Case 9b) years post disease onset (Ozkara *et al.*, 2017). Vision loss was not observed in any patient.

Progressive ataxia and unrelenting myoclonus led to patients becoming wheelchair bound; this occurred on average 12 years into the disease course (range 3-25; median 13). At last follow-up, six patients remained ambulant 3-20 years after disease onset (Table 1).

Seven patients were deceased (Cases 1a, 3a, 3b, 6a, 6b, 7, 10). Death occurred 8 – 31 years after onset. Thirteen cases were still alive 5-27 years after symptom onset with the oldest (Case 4b) 65 years of age (disease onset at 51). Whilst survival was often prolonged (median survival time 26 years), these patients were bed-ridden for many years.

Inter and intra-family variation

Age of onset and survival outcome varied even within the same family (Table 1). No significant differences were evident between sexes, with mean onset age 26 years for females (range 12-46) and 31 years for males (range 12-51) ($p = 0.46$, two-tailed t-test). Median survival time was 26 years for females and 22 years for males ($p = 0.7$, log rank test).

Pathological findings

Thirteen of twenty patients had at least one biopsy as part of their clinical work-up (Table 1). Fingerprint profiles were the prominent diagnostic inclusions observed, although they were usually accompanied by rectilinear complex – irregular straight profiles, occasionally resolvable into pentalaminar structures (Anderson *et al*., 2011). Curvilinear profiles were not seen, although they are described in the late infantile form of *CLN6* disease. Banal age-related lipofuscin was often present, and had to be distinguished from disease-related granular osmiophilic deposits (GRODS). Definite fingerprint profiles were found in 4/8 skin biopsies, in one neuron in 1/3 rectal biopsies, in vascular smooth muscle of 1/5 muscle biopsies and in 3/4 brain biopsies (Fig. 2).

Molecular genetic analysis

Four unrelated patients had homozygous and nine had compound heterozygous pathogenic variants in *CLN6* (Table 1). In two cases the genotype was inferred as DNA was unavailable (Case 3b genotype inferred from affected sister; Case 7 inferred from variants in parents). The four families with homozygous pathogenic variants were those with known or suspected consanguinity.

Most pathogenic variants were predicted to result in amino-acid substitutions; however, there were four heterozygous pathogenic variants predicting protein truncation, including two small deletions and/or insertions (indels), one large deletion, and one canonical splice site change.

We previously reported that Case 5 (Ku6) was homozygous for the *CLN6* pathogenic variant p.Phe238Thr (c.712_713TT>AC) (Arsov *et al.*, 2011). However, when the mother's DNA subsequently became available we unexpectedly discovered that she did not carry the p.Phe238Thr variant. Additional SNP, CGH, MLPA and ddPCR analyses suggested a deletion located within the 10.7 kb region chr. 15: 68,497,500-68,508,200 (GRCh37/hg19) of *CLN6*. DNA sequencing across this region identified a deletion with breakpoints at nucleotides 68,498,676 and 68,504,456. The 5,781bp deletion, c.297+256_936+1802del, removes *CLN6* exons 4 – 7 (Fig. 3). Further analysis confirmed that this deletion was present in the affected proband and in her unaffected sister and mother.

CLN6 phenotype/genotype correlation

We compared the number of unrelated families with two, one or zero predicted truncating variants in Kufs disease (including the 13 families evaluated here plus 3 from the literature (Arsov *et al.*, 2011; Andrade *et al.*, 2012; Kousi *et al.*, 2012) versus 42 previously published late-infantile NCL families (Table 2; Supplementary Table 1). The distribution of variants differed ($p = 0.01$, Fisher's exact test); 15/42 late-infantile cases had two alleles with truncation variants (predicting absence of intact CLN6 protein) whereas this was not seen in any of the 16 Kufs families.

5/16 Kufs families had one heterozygous variant predicting protein truncation. Cases 8a and 8b with teenage–onset Kufs disease have a missense change on one allele with the other allele harbouring a heterozygous variant predicting protein truncation (splice donor variant, *CLN6* c.486+1G>T); in the homozygous state, this same splice donor variant has been previously reported in a late-infantile NCL family (Teixeira *et al.*, 2003). Further, our literature review identified a homozygous *CLN6* missense variant p.Asp256Glu that results in siblings with teenage-onset Kufs (Andrade *et al.*, 2012), but when coupled with truncation variant p.Tyr84* results in late-infantile NCL with onset <12 years (Faruq *et al.*, 2014) (Supplementary table 1).

Heterozygous truncation variants did not appear to be preferentially seen in teenage versus adult-onset Kufs; of 4 cases with clear age of onset information, 2 began in teenage years and 2 had adult onset (Table 1).

Display of the positions for pathogenic variants along the *CLN6* gene did not reveal an obvious difference in variant location for the two disease groups (late-infantile NCL versus Kufs) (Fig. 3), although no variants resulting in Kufs disease are present in exon 6. The potential for mutation hotspots along *CLN6* has previously been raised (e.g., Tyr221) (Kousi *et al.*, 2012); here, we can also appreciate how variants affecting the same amino acid residue can occur in either disease phenotype (i.e., Tyr84, Arg103, Arg136, Tyr142, Met241, Asp256 and Pro297) (Fig. 3 and Supplementary Table 1). However, as highlighted earlier, it is important to consider the type of variant on the second allele. Consistent with our observation for variant p.Asp256Glu, missense changes at Arg103, Arg136 and Met241 when reported in late-infantile NCL cases were coupled with a truncation variant whereas in Kufs disease the second variant was another missense change (http://www.ucl.ac.uk/ncl/mutation.shtml).

Although some pathogenic variants are limited to regions of the protein devoid of natural variation in the homozygous state (e.g., exons 5 and 6), this is not the case for all (Fig. 4).

Founder mutations

Across our 13 unrelated cases, three pathogenic variants were present in multiple families. The p.Arg103Gln variant was present in Cases 1a,b, Case10 and Case 13; all of Italian ancestry. The p.Met241Val variant was present in Cases 11a,b of Italian ancestry and Case 12 of Italian/Sicilian ancestry. Case 12 had a second variant, p.Phe238Thr, and this variant was also present in Cases 4a,b of Maltese and Case 5 of Sicilian origin. These observations are consistent with a founder effect for p.Arg103Gln and p.Met241Val variants in the mainland Italian population and p.Phe238Thr in both Sicily and Malta, which is geographically plausible.

Discussion

Kufs disease due to pathogenic variants in *CLN6* usually presents as a progressive myoclonus epilepsy (Type A Kufs) with a teenage or adult onset (onset age range 12-51 years) as previously reported (Arsov *et al.*, 2011). Our more extensive analysis herein highlighted that although myoclonic epilepsy is the usual presentation, a quarter of the cases had cognitive, behavioural or motor changes a few years before the first seizure. Myoclonus was documented in all, except Case 6b where data was incomplete. Frequent action myoclonus was the most debilitating clinical symptom. Photosensitivity was common, and sometimes extreme. Prominent photosensitivity is rare in adult-onset epilepsies, so it is a clinical clue to consider Kufs disease. Similarly, prolonged tonic seizures were reported in some cases, again a rare phenomenon in adult epilepsies. Tonic-clonic seizures were typically infrequent. Cognitive impairment usually emerged early and, as noted above, could be a presenting symptom but was sometimes delayed. Disease progression occurred at variable rates, even within families, with a mean time from onset to requiring a wheelchair being 12 years (median = 13) and death occurring 8-31 years after onset (median survival time = 26).

In teenage years the differential diagnosis of PME is wide, including Unverricht-Lundborg disease, Lafora disease, MERRF, sialidosis, MEAK, *SCARB2* etc. In adult life the differential is narrower and includes MERRF, neuroserpinopathy and early onset Alzheimer disease (Minassian *et al.*, 2016).

While most cases had a PME presentation, three cases did not have convulsive seizures and the clinical syndrome was that of a progressive myoclonic ataxia (PMA) (Marsden *et al.*, 1990). While PME and PMA are useful broad clinical syndromes to narrow down considerations for specific diagnoses, they do have some overlap (Marseille Consensus Group, 1990). Indeed, although only two of the PMA cases had EEG studies, one had abundant generalized epileptiform discharges. A number of other disorders can have both PME and PMA presentations including MERRF (DiMauro *et al.*, 2013), DRPLA (Koide *et al.*, 1994), North Sea myoclonus (*GOSR2* mutation) (Boisse Lomax *et al.*, 2013), action myoclonus renal failure syndrome (*SCARB2* mutation) (Dibbens *et al.*, 2009) and myoclonus epilepsy and ataxia due to *KCNC1* mutation (Oliver *et al.*, 2017).

The prominent ultrastructural finding was fingerprint profiles (Fig. 2). These are highly stereotyped and with sufficient experience easily recognized at low magnifications, even though the exact criteria for their identification demand high resolution (Carpenter *et al*., 1977). The other component of the lysosomes consists of rectilinear complex. It is harder to recognize and harder to describe than the fingerprint profiles. Short pentalaminar structures are the most characteristic

feature (Anderson *et al.*, 2013). Rectilinear complex is likely more basic to the storage process, since it probably does not occur nonspecifically.

Biopsy of peripheral tissues in Kufs' disease is not reliable for diagnosis. After the second decade, fingerprint profiles occur in eccrine secretory cells and vascular smooth muscle of people who clearly do not have any form of ceroid lipofuscinosis (Ferlazzo *et al.*, 2012; Berkovic *et al.*, 2016). Reliance on findings in peripheral tissues has been an important source of misdiagnosis in Kufs' disease (Berkovic *et al.*, 1988; Pasquinelli *et al.*, 2004). Skin biopsy remains useful, however, for diagnosis in children. The one negative brain biopsy in this series can be ascribed to the limited number of neurons involved in Kufs' disease and the necessarily limited size of brain biopsies. Importantly, genetic analysis of *CLN6* in adults may obviate the need for an invasive brain biopsy in cases with a pertinent presentation.

A literature review was conducted to identify all published Kufs cases with a molecular diagnosis of either *CTSF* or *DNAJC5* mutation and these were compared to our analysis of 20 *CLN6* cases. Compatibility for each molecular diagnosis with either Kufs sub-type A or B was then made, provided enough clinical data was available. Kufs Type A and Type B sub-types were first proposed in 1988, not to imply the presence of two separate diseases, but rather to aid clinical recognition and diagnosis (Berkovic *et al.*, 1988). Thirty years later, with recent molecular genetic discoveries we can see that in fact the sub-types do generally hold for different genetic etiologies. Kufs disease due to mutation in *CLN6* and *DNAJC5* typically result in Type A phenotype with Type B predominantly accounted for by mutations in *CTSF* (Table 3).

Comparison of median survival times across the three genetic aetiology groups (Table 3) suggests that *DNAJC5* may have a more rapid clinical progression. However, further research with an increased sample size would be required to confirm this observation. Also, as only two recurrent *DNAJC5* missense pathogenic variants have been published to date, it may be that the clinical spectrum associated with this gene is yet to be fully appreciated.

Our observation that three pathogenic *CLN6* variants were present in more than one Kufs family, from similar geographical regions, is consistent with multiple founder effects. The large proportion of cases in our cohort from the Italian region is therefore not simply explained by ascertainment bias, but is also likely a product of these rare pathogenic variants being founder mutations in mainland Italy, Sicily and its neighbouring country Malta.

The question of whether the position or type of genetic variant may be a predictor of phenotype (late-infantile NCL versus Kufs) is an obvious one. Not only is the age of onset strikingly different

(mean 6 years for late-infantile NCL (Mole *et al.*, 2005) and mean 28 years for Kufs), but retinal involvement with visual failure is usual in late-infantile NCL and never observed in *CLN6*-related Kufs. Within Kufs cases, the bimodal age distribution also suggests there may be molecular differences between teenage and adult onset cases. Clinical support for the late-infantile NCL and Kufs distinction also comes from the observation that there are no reported families with both phenotypes; however, within Kufs families, age of onset can be variable (Table 1), including two families (families 1 and 9) with both teenage and adult onset. Sex did not appear to influence age of onset or survival time within the Kufs cohort.

The function of CLN6 remains enigmatic. It is a 311 amino acid transmembrane endoplasmic reticulum protein, which unlike most of the other NCL proteins is not localized to the lysosome (Cárcel-Trullols *et al*., 2015). Consistent with an earlier analysis reporting that mutation severity is associated with onset age (Kousi *et al.*, 2012), we found that presumed complete loss of function of CLN6 (inferred by two variants predicting protein truncation) was only observed in late-infantile NCL (Table 2). Whilst pathogenic variants were observed within all 7 exons of *CLN6*, no homozygous variants in the gnomAD control database were seen in exons 5 or 6 (Fig. 4) suggesting this region may be of specific functional importance. Further, the only variants present in exon 6 result in the more severe phenotype of late-infantile NCL. Interestingly, heterozygous pathogenic variants at four amino acid positions were observed with both phenotypes of late-infantile onset and Kufs (Fig. 4). Again, in these instances the mutation severity of the second allele was predictive of phenotypic outcome – missense variants were seen in Kufs and truncation variants in late-infantile cases. Further understanding of genotype-phenotype correlation involving missense variants will require discovery of the functional role of CLN6 and a robust functional assay.

The diagnosis of Kufs disease remains a challenge. It should be suspected in teenagers or young to middle aged adults with progressive myoclonus epilepsy or dementia with motor features. The age of onset is wide and the rarity of the disorder adds to the diagnostic difficulty. It is clear that there are many pitfalls to traditional pathological diagnosis. Indeed, the "gold standard" of brain biopsy, including careful ultrastructural examination, is rarely available but now can be largely circumvented by molecular diagnosis. Pathogenic variants in *CLN6* are the commonest molecular cause of Kufs disease presenting with progressive myoclonus epilepsy in sporadic cases or siblings, but *DNAJC5* variants are the likely cause in dominant pedigrees. Pathogenic variants in *CTSF* are the major known cause of Type B Kufs; there appear to be a number of presently unsolved cases although the question of the veracity of pathological diagnosis is always an issue. For *CLN6*-related Kufs, the phenotype and molecular architecture are now clear. Discovery of the functional role of the protein would likely explain the mysteries of why this gene can have late-infantile and

teenage-adult presentations and why the retina is spared in the latter form and perhaps provide clues to novel therapeutic strategies.

Figure legends

Figure 1

Histogram of onset ages of *CLN6* related Kufs disease (n = 20). There is a bimodal distribution with teenage and early adult peaks.

Figure 2

Ultrastructural features.

A. Brain biopsy (Case 2; aged 35 years). This electron micrograph shows a cytoplasmic area of a neuron that is filled with lysosomes where the fingerprint profiles (white arrow) stand out because of their osmiophilia. The other material inside the lysosomes is rectilinear complex. Bar = 1.0 micron.

B. Skin biopsy (Case 7; aged 20 years). The characteristic paired parallel lines that make up the fingerprint profiles become visible only at high magnification, as seen here. Bar = 0.1 micron.

Figure 3.

The *CLN6* deletion in Case 5.

A. The panel shows the gene structure of *CLN6* with numbered exons and the location of the 5,781 bp deletion. Filled boxes flanking exons 1 and 7 are the 5' and 3' untranslated regions, respectively. The chromosome 15 nucleotide numbering is from the GRCh37/hg19 assembly.

B. The DNA sequences at the deletion breakpoints. Deleted DNA is shown in a smaller font size and coloured blue.

Figure 4.

CLN6 pathogenic variants. Comparison of the distribution for pathogenic NCL variants along *CLN6* according to disease group and in reference to homozygous control variation from gnomAD. The size of the symbol is relative to the number of times the variant has been reported.

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Supplementary material

Supplementary Table 1.

Complete list of late-infantile NCL and Kufs *CLN6* pathogenic variants contributing to the phenotype-genotype analyses.

Supplementary Figure 1.
Kaplan-Meier estimates of survival for Kufs due to *CLN6*, *CTSF* or *DNAJC5* pathogenic variants.

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Table 1. Clinico-pathological and genetic summary of 20 patients (13 unrelated) with Kufs disease due to *CLN6* pathogenic variants.

Case	Onset | sex	Initial symptom	Later symptoms	Photo-sensitive	Outcome [current age]	Biopsy review	*CLN6* pathogenic variants (hg19)
1a	16y | F[a,b]	Myoclonus	TCS (24y) Prolonged tonic seizures Pyramidal-extrapyramidal rigidity Dystonia Dementia	Yes	Deceased [47y]	Negative (skin, muscle)	c.200T>C, p.L67P; c.308G>A, p.R103Q
1b	36y | M[a]	TCS	Myoclonus Unstable gait, Pyramidal signs Cognitive impairment	Yes	Bedridden [58y]	FPP (rectal) Negative (muscle)	c.200T>C, p.L67P; c.308G>A, p.R103Q
2	28y | F[a,b]	TCS, Myoclonus	Ataxia, Dysarthria Dementia, Aggression, Psychosis	Yes	Wheelchair [55y]	FPP (skin, brain)	c.139C>T, p.L47F (homozygous)
3a	31y | F[a,c]	TCS	Myoclonus (33y) Prolonged tonic seizures Late ataxia and cerebellar signs Dementia (38y), Psychosis	Yes	Deceased [57y]	FPP (skin)	c.17G>C, p.R6T (homozygous)
3b	30y | F[c]	TCS	Myoclonus (32y) Myoclonic status Prolonged tonic seizures Ataxia, Dysarthria Dementia	Yes	Deceased [44y]	FPP (brain, skin, liver) Negative (muscle)	Not tested; older sister of 3a
4a	46y | F[a]	Myoclonus	TCS, Non-convulsive status Ataxia, Tremor, Dysarthria Dementia, Depression	Yes	Wheelchair [62y]	FPP (muscle) Negative (skin)	c.712_713delinsAC, p.F238T (homozygous)
4b	51y | M[a]	Myoclonus Ataxia	Tremor, Dysarthria, Dystonia Depression Memory problems	Yes	Unstable gait; independent [65y]	ND	c.712_713delinsAC, p.F238T (homozygous)
5	17y | F[a,b]	Myoclonus	TCS Ataxia, Tremor, Dysarthria Bradykinesia, Exaggerated tendon reflexes Cognitive impairment (12y)	Yes	Bedridden [35y]	Negative (skin, rectal)	c.712_713delinsAC, p.F238T; c.297+256_936+1802del (5781bp deletion)
6a	35y | F[a,d]	TCS	Myoclonus Ataxia, Tremor Dementia, Aggression, Psychosis	Yes	Deceased [43y]	FPP (brain)	c.446G>A, p.R149H; c.890delC, p.P297Lfs*53
6b	43y | M[a,d]	Ataxia	Dysarthria Dementia	No data	Deceased [52y]	Negative (rectal, brain)	c.446G>A, p.R149H; c.890delC, p.P297Lfs*53
7	12y | M	TCS	Myoclonus Ataxia, Dysarthria Dementia	Yes	Deceased [34y]	FPP (skin)	c.226 C>T; p.L76F c.403 C>G; p.H135D
8a	16y | M	Dementia Tremor	TCS (24y), Myoclonus (24y) Pyramidal signs Psychosis	No	Wheelchair [30y]	Lipofuscin; more than expected for age (skin)	c.278C>T, p.T93M; c.486+1G>A
8b	19y | F	Dementia	Myoclonus (25y), TCS (28y) Pyramidal signs Psychosis (28y)	No data	Wheelchair [28y]	ND	c.278C>T, p.T93M; c.486+1G>A
9a	18y | F[e,f]	TCS	Myoclonus, Absences Eyelid myoclonia Ataxia Depression, OCD	Yes	Ambulant; no cognitive decline [38y]	ND	c.509A>G, p.Y170C (homozygous)
9b	26y | M[e,f]	TCS	Myoclonus Ataxia, Dysarthria Depression, OCD	Yes	Ambulant; no cognitive decline [33y]	ND	c.509A>G, p.Y170C (homozygous)
10	34y | F	Pyramidal rigidity, Depression	TCS (37y), Myoclonus Severe spasticity Wheelchair (39y) Dementia	Yes	Deceased [54y]	Negative (skin)	c.308G>A, p.R103Q; c.425insT, p.Y142Lfs*8
11a	32y | M	Myoclonus	TCS (41y) Ataxia, Tremor Bradykinesia, Hypomimia Cognitive decline	Yes	Ambulant with support [43y]	ND	c.814C>G, p.L272V; c.721A>G, p.M241V
11b	30y | F	Myoclonus	Ataxia, Tremor, Dysarthria Cognitive decline	Yes	Ambulant with support [45y]	ND	c.814C>G, p.L272V; c.721A>G, p.M241V
12	13y | F	Myoclonus	TCS (15y) Ataxia, Dysarthria Cognitive decline	Yes	Ambulant [17y]	ND	c.712_713delinsAC, p.F238T; c.721A>G, p.M241V
13	29y | F	TCS	Myoclonus Ataxia, Dysarthria Cognitive decline	Yes	Wheelchair [49y]	Negative (muscle)	c.407G>A, p.R136H; c.308G>A, p.R103Q

Abbreviations: TCS = tonic-clonic seizure; OCD = obsessive compulsive disorder; ND = not done; FPP = fingerprint profiles

[a]Previously published in Arsov *et al*., 2011; [b]Previously published in Canafoglia *et al*., 2015; [c]Previously published in Berkovic *et al*., 1988; [d]Previously published in Callagy *et al*., 2000; [e]Previously published in Muona *et al*., 2015; [f]Previously published in Ozkara *et al*., 2017