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CICLO XXX

COORDINATORE Prof. Renato Corradetti

# Fighting against neurotoxic oxysterols: new insights from cerebrotendinous xanthomatosis and spastic paraplegia type 5 (SPG5)

Settore Scientifico Disciplinare MED-26


**Dottorando**
Dott. Mignarri Andrea

_____
*(firma)*

**Tutore**
Prof. Dotti Maria Teresa

_____
*(firma)*

**Coordinatore**
Prof. Corradetti Renato

_____
*(firma)*


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# Index

# 1. A brief introduction to the two disorders of bile acid synthesis

## 1.1 Cerebrotendinous xanthomatosis

Cerebrotendinous xanthomatosis (CTX) is an autosomal recessive lipid storage disease due to mutations of *CYP27A1* resulting in deficiency of sterol-27hydroxylase, which plays a key role in the conversion of cholesterol to bile acids (Cali and Russell 1991). The enzyme defect is responsible for a sharp decrease in chenodeoxycholic acid (CDCA) and a compensatory increase in the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis (Fig. 1): this leads to accumulation of cholestanol and other bile acid precursors in plasma and tissues, as well as increased urinary excretion of bile alcohols (Björkhem and Hansson 2010). CTX patients have been reported from all over the world, and the prevalence of the disease is considered to be underestimated (Lorincz et al 2005). The clinical picture is characterized by a variable association of systemic signs, such as infantile-onset chronic diarrhea, juvenile cataracts, tendon xanthomas and premature osteoporosis, and neurological symptoms including low intelligence, spasticity, ataxia, epilepsy, parkinsonism, and polyneuropathy (Federico and Dotti 1996). CTX patients also show variable psychiatric manifestations, including personality, affective and psychotic disorders: they can appear either early in the course of the disease or late complicating the other neurologic disturbances (Fraidakis 2013). Magnetic resonance imaging (MRI) evidence of bilateral signal abnormalities of the cerebellar dentate nuclei is the major neuroradiological hallmark (Barkhof et al 2000; De Stefano et al 2001). Since elevated concentrations of serum cholestanol are found in all untreated patients (Pilo de la Fuente et al 2011), plasma cholestanol assessment is an extremely useful test for CTX diagnosis.

Definitive diagnosis is obtained by molecular analysis of the CYP27A1 gene. The pathogenetic mechanisms underlying neurological damage in CTX have not been completely clarified. Large amounts of cholestanol are known to induce apoptosis, with particular involvement of cerebellar neuronal cells (Inoue et al 1999). However, the origin of brain cholestanol is not obvious: it has been suggested that cerebral storage of cholestanol may be a consequence of the flux of its precursor 7α-hydroxy-4-cholesten-3-one (7αC4) across the blood-brain barrier (Panzenboeck et al 2007; Bavner et al 2010). The lack of 27-OHC could also play a role: indeed, cerebral 27-OHC originates from the circulation and represents an important regulator of cholesterol metabolism in the brain (Heverin et al 2005; Ali et al 2013). Administration of CDCA strongly decreases synthesis of toxic bile acid intermediates, exerting negative feedback on 7α-hydroxylase (Fig. 2) and limiting the formation of newly synthesized cholesterol by the liver (Batta et al 1985). Long-term oral administration of CDCA in CTX patients has been reported to stabilize or improve clinical and laboratory parameters without toxic side-effects (Berginer et al 1984; Salen et al; Samenuk and Koffman 2001; Mignarri et al 2011; Ginanneschi et al 2013; Martini et al 2013). CDCA may arrest disease progression and prevent neurological deterioration, particularly when started in childhood (Van Heijst et al 1998; Federico and Dotti 2003; Berginer et al 2009), whereas poor response to CDCA treatment may be recorded when treatment is started once neurological impairment is manifest (Pilo de la Fuente et al 2008; Mignarri et al 2012a; Rubio-Agusti et al 2012; Yahalom et al 2013). Replacement therapy with other bile acids such as ursodeoxycholic acid and taurocholic acid has failed to correct biochemical abnormalities (Koopman et al 1985). Cholic acid is biochemically effective and has sometimes been preferred to CDCA because it is considered safer,

especially in children (Pierre et al 2008), but long term clinical data on its efficacy in CTX are not yet available. Inhibitors of HMG-CoA reductase associated with CDCA have also been tried with contradictory results (Nakamura et al 1991; Salen et al 1994; Verrips et al 1999). In our experience they do not provide any real long term benefit. Oral CDCA therefore remains the elective treatment for CTX.

### 1.2 Spastic paraplegia type 5 (SPG5)

Hereditary spastic paraplegia (HSP) is a degenerative disorder primarily affecting the spinal cord and especially its corticospinal tracts. The clinical hallmark of HSP is a progressive spastic gait disturbance starting between the first year of age and the 8th decade1. HSP is a rare disease affecting about 4-7 in 100.000 individuals. Although rare, HSP splits up in at least 75 genetic subtypes annotated as spastic paraplegia genes (SPG), most of them representing ultra-rare diseases. Therapy focusses on symptomatic treatment as up to now no interventions addressing causal pathogenic factors have been established for any subtype of HSP. The Mendelian heredity of HSP pinpoints the source of the specific pathophysiological process to the respectively affected gene and thus directs the view on the underlying pathogenesis. Of major importance, understanding molecular disease pathology has the potential to be translated into an effective therapy for rare diseases like HSP or even individual patients. Spastic paraplegia type 5 (SPG5) is a rare subtype of HSP representing about 1.3% of HSP patients in a large consecutive cohort study. SPG5 follows an autosomal recessive trait and is caused by loss of function mutations in *CYP7B1* coding for the 7α-hydroxylase CYP7B1. Both pure and complicated clinical forms are possible. Electrophysiology reveals abnormal conduction along the central pathways and peripheral nervous system

sparing, and brain MRI might show white matter disease (Goizet et al. 2009). The clinical course of SPG5 is progressive. CYP7B1 is a key enzyme of the so-called "acidic" pathway in the synthesis of primary bile acids from cholesterol in the liver (Fig. 1), but is widely expressed in the body including the brain. CYP7B1 catalyses the 7α-hydroxylation of 25-hydroxycholesterol (25-OHC) and 27-hydroxycholesterol (27-OHC). Oxysterols accumulate in SPG5 with levels of 27-OHC and 25-OHC found to be markedly increased in serum and cerebrospinal fluid (CSF) of SPG5 patients compared to controls (Schule et al 2010): high CSF levels of 25-OHC and 27-OHC induce a significant reduction in neurite outgrowth, metabolic activity and viability of motor neuron-like cells and human cortical neurons derived from induced pluripotent stem cells. Taken together this data strongly suggests that oxysterols, and namely 27-OHC are not only a biomarker but also a factor driving disease pathology in SPG5. Concentrations of oxysterols are considerably higher in serum than in the CNS. As oxysterols are able to pass the blood brain barrier there is a net flux of 25-OHC and 27-OHC from the circulation to the brain (Heverin et al 2005; Ali et al 2013). The CNS is the main site where pathology occurs in SPG5; therefore, this influx of oxysterols from the circulation into the CNS is likely to be a key contributor to SPG5 pathogenesis. As cholesterol and 27-OHC levels in the circulation are closely correlated, SPG5 patients may benefit from a cholesterol lowering-therapy which may in turn also lower elevated oxysterol levels.

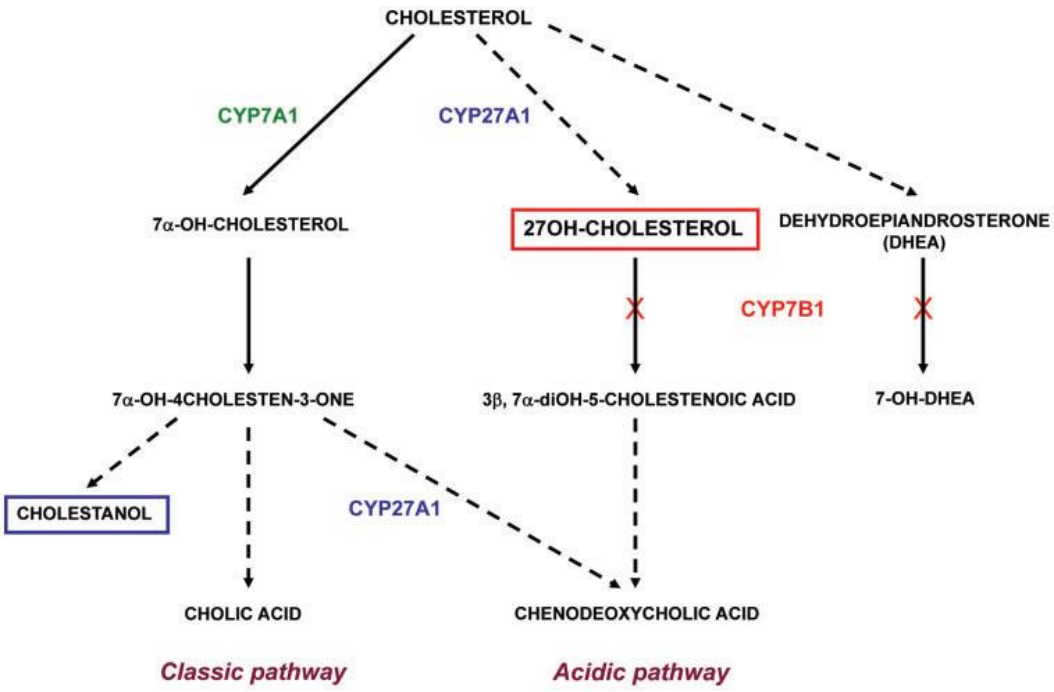



**Fig. 1.** Bile acid synthesis pathways and biochemical pathogenesis of CTX and SPG5.

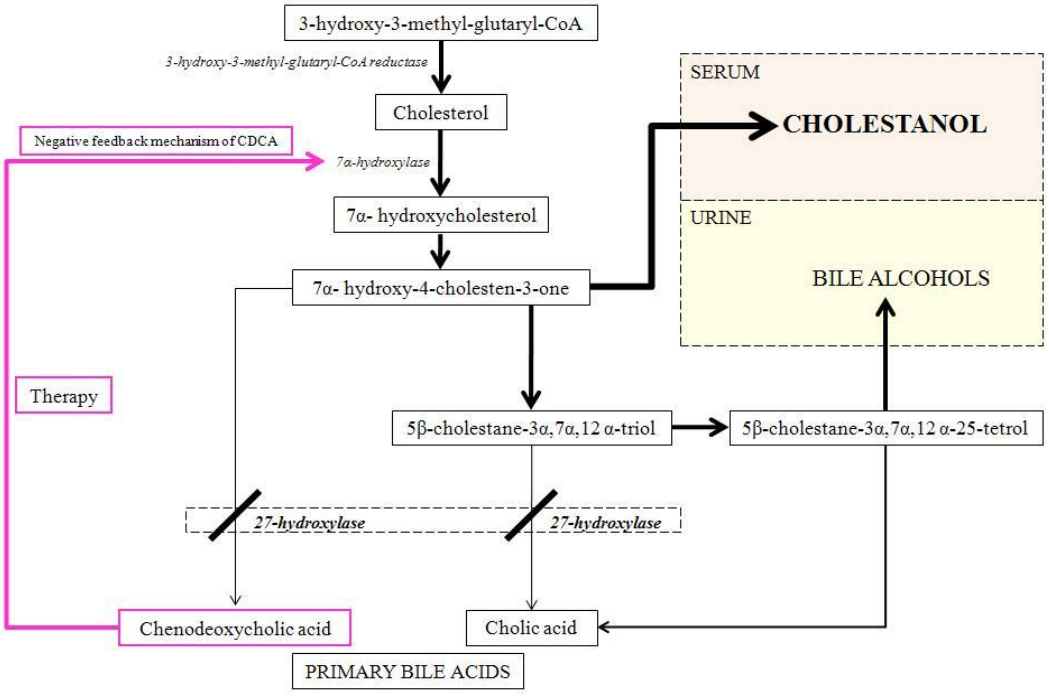



**Fig. 2.** Rationale for CDCA treatment in CTX.

## 2. The importance of early diagnosis and treatment: a suspicion index for cerebrotendinous xanthomatosis

### 2.1 Introduction

Authors agree on the need for early diagnosis and timely introduction of replacement therapy, since CTX is a chronic disorder leading to progressive deterioration and premature death if not treated. However, as there is considerable variation in disease onset, systemic involvement and neurological impairment, its recognition at an initial stage may be challenging in the clinical setting. This prompted us to develop a simple practical diagnostic algorithm for early identification of CTX patients.

### 2.2 Materials and methods

The study was conducted in three phases. In the first, we performed a pooled analysis of selected CTX series to obtain data to use for building a suspicion index (SI). In the second phase, we developed a diagnostic tool consisting of the SI and an associated flow chart. In the third phase, we applied the SI retrospectively to our population to assess the degree to which diagnosis could be brought forward. The study was approved by the local ethics committee. All patients gave informed consent for data collection. We carried out a MEDLINE search from 1982 to 2013 using the key word "cerebrotendinous xanthomatosis", and reviewed all the published articles. To include data in the pooled analysis, studies were selected on the basis of the following criteria: 1) CTX diagnosis based on the finding of CYP27A1 gene mutations and/or clinical and biochemical criteria; 2) availability of clinical and laboratory data; 3) sample size ≥10 patients. In the case of articles from the same group, we selected the one reporting the

greatest number of patients and/or the most complete information. Articles from which it was not possible to extract crude data were excluded. We also performed a retrospective evaluation of the clinical data of 55 CTX patients (28 females, 27 males) belonging to 39 unrelated families. The median age of our subjects was 36 years (range 1-67). All patients were diagnosed in our Unit for Neurometabolic Disorders in the period 1986 to 2012 and were evaluated clinically by three neurologists experienced in CTX (AF, AM and MTD). The medical records were available for consultation in our archives, enabling us to establish frequency and age of onset of symptoms, age at diagnosis and genotype. In 37 out of 55 patients, serum cholestanol levels at diagnosis were assessed and compared with those of normal controls. When present, instrumental tests such as brain CT and/or MRI scans, electrophysiological studies and bone densitometry were analyzed. Articles by our group reporting part of the patients analyzed herein and fulfilling the criteria for inclusion in the pooled analysis were not considered because of the reappraisal made in the present study. To build the SI we itemized family history characteristics as well as systemic and neurological features. We attributed weighted scores to each parameter on the basis of three criteria: frequency, peculiarity and age at manifestation, as pointed out in the review. We assigned the following scores: 100 for very strong indicators, 50 for strong indicators and 25 for moderate indicators. To bring diagnosis forward, we ascribed more strength to childhood-onset indicators and less strength to adult-onset indicators. We also aimed at indicators which are easy to evaluate in clinical practice and do not require expensive tests. After creating the SI, we built a diagnostic flow chart based on its application. The diagnostic algorithm obtained in the second phase was applied retrospectively to our 55 patients with a genetic diagnosis of CTX. For each subject we calculated age

and SI score at diagnosis, as well as the age at which diagnosis could have been established using the SI. All indicators were detailed. We did not apply the diagnostic tool to the other international CTX series selected for the review, because of the lack of complete information regarding the presence and/or the age at manifestation of each indicator. We tested all data for normal distribution with the Kolmogorov–Smirnov distance method. Cholestanol concentrations were compared with those of normal controls, using Student's t-test. Ages at diagnosis were compared with those obtained applying the diagnostic tool, using one-way analysis of variance (ANOVA). If ANOVA showed a significant difference, Bonferroni's multiple comparison test was performed. Statistical significance was assumed for $p < 0.01$.

### 2.3 Results

Table 1 summarizes the results of the review of the selected CTX populations including our cohort and other four international series (Berginer et al 1984; Verrips et al 2000; Lee et al 2001; Pilo de la Fuente et al 2011). We analyzed a total of 170 patients from Europe (Italy, Netherlands, Spain, Belgium, Germany, UK), North America (USA), Africa (Tunisia) and Asia (Israel, China). We documented a substantial diagnostic delay (20-25 years): patients were referred to clinicians during childhood or adolescence (9-19 years), but diagnosis of CTX was made during the fourth decade (34-38 years). At the time of diagnosis, all patients who underwent serum cholestanol assessment (130/130) showed elevated concentrations. Among systemic manifestations, tendon xanthomas and cataract were the most frequent (69% and 88%, respectively): age at presentation was earlier for cataract, which was usually diagnosed in the second decade, while xanthomas were observed later during the third decade.

Chronic unexplained diarrhea was present in about half of patients, with onset in childhood and frequent referral to pediatricians. We also observed an increased incidence of prolonged unexplained neonatal cholestatic jaundice. Osteoporosis was frequent in our population, but was usually revealed by bone densitometry in the fourth decade at the time of the diagnosis. Neurological manifestations covered a broad range of signs and symptoms. Motor disturbances including spastic paraparesis and ataxia were very frequent (77% and 62%, respectively), and typically presented at the end of the third decade. We also observed parkinsonism in some cases, with later onset. Notably, more than half the subjects had mild/moderate intellectual disability: early childhood developmental milestones were achieved punctually, but patients showed learning difficulties with poor school performance, sustained infantile behavior and lack of age-appropriate self-care skills. Psychiatric disturbances, including depression, bipolar disorder, anxiety, panic disorder and psychosis, were reported in about half the patients, typically manifesting in the third decade. Epilepsy was reported in one third of cases, with variable age of onset. Axonal or demyelinating polyneuropathy was documented by neurophysiological study in 66% (71/108) of cases; however, neuropathy was often subclinical and was usually diagnosed in the third/fourth decade. Brain MRI revealed dentate nuclei signal abnormalities in 75% (62/83) of patients, but this typical neuroradiological finding was usually documented in the fourth decade at the time of diagnosis. Family history showed consanguinity in one third of families.

| | Our cohort | Verrips et al 2000a (Table 1) | Pilo de la Fuente et al 2011b | Lee et al 2001 | Berginer et al 1984 |
|---|---|---|---|---|---|
| **Number of patients** | 55 (28 F, 27 M) | 54 | 25 (15 F, 10 M) | 19 (12 F, 7 M) | 17 (12 F, 5 M) |
| **Age at onset** | 9.5±9.0 years | 14±11.6 years | 19.2±11.2 years | not reported | not reported |
| **Age at diagnosis** | 35.5±11.8 years | 33.5±10.9 years | 38.0±10.7 years | not reported | not reported |
| **Consanguineous parents** | 33% (13/39 families) | 6% (2/32 families) | not reported | 0% (0/12 families) | not reported |
| **Tendon xanthomas** | 78% (43/55); 25.6±9.6 years | 50% (27/54); 24.7±11.3 years | 56% (14/25) | 100% (19/19) | 88% (15/17) |
| **Cataract** | 89% (49/55); 17.0±14.5 years | 96% (52/55); 19.7±11.2 years | 92% (23/25) | 74% (14/19) | 71% (12/17) |
| **Chronic diarrhea** | 40% (22/55); 3.7±6.8 years | 35% (19/54); 9.8±9.9 years | 92% (23/25) | not reported | not reported |
| **Neonatal jaundice** | 15% (8/55) | not reported | not reported | not reported | not reported |
| **Osteoporosis (BD)** | 67% (20/30); 38.6±10.1 years | not reported | not reported | 21% (4/19) | not reported |
| **Cerebellar signs** | 36% (20/55); 28.7±9.3 years | 65% (35/54); 27.8±10.5 years | 83% (20/24) | 89% (17/19) | 76% (13/17) |
| **Pyramidal signs** | 64% (35/55); 27.4±10.1 years | 72% (39/54); 27.6±8.9 years | 92% (23/25) | 89% (17/19) | 100% (17/17) |
| **Intellectual disability** | 60% (33/55); 6.5±3.1 years | 61% (33/54) | 48% (12/25) | not reported | not reported |
| **Psychiatric disturbances** | 44% (24/55); 23.4±13.3 years | not reported | 61% (14/23) | not reported | not reported |
| **Epilepsy** | 33% (18/55); 10.3±9.9 years | 26% (14/54); 24.0±15.5 years | 32% (8/25) | not reported | not reported |
| **Parkinsonism** | 9% (5/55); 38.4±16.2 years | not reported | not reported | not reported | not reported |
| **Polyneuropathy (EMG)** | 70% (21/30); 35.4±9.2 years | 79% (19/24); 28.3±6.6 years | 67% (12/18) | 63% (12/19) | 41% (7/17) |
| **Dentate lesions (MRI)** | 77% (20/26); 34.1±8.6 years | 82% (28/34); 38.7±11.1 years | 61% (14/23) | not reported | not reported |
| **High serum cholestanol** | 100% (37/37); 2.9±1.2 mg/dl | 100% (43/43); 3.9±3.0 mg/dl | 100% (14/14); 4.1±2.2 mg/dl | 100% (19/19); 3.8±1.6 mg/dl | 100% (17/17); 5.0±1.2 mg/dl |

**Table 1.** Results of the systematic review carried out in the selected CTX series. Signs and symptoms are expressed as percentage; when reported, age at manifestation/diagnosis (mean ± standard deviation) of the sign or symptom is also indicated.

| Indicators | Family history | Systemic | Neurological |
|---|---|---|---|
| **(A) Very strong**<br><br>**(score = 100)** | A1) Sibling with CTX | A2) Tendon xanthomas | |
| **(B) Strong**<br><br>**(score = 50)** | B1) Consanguineous parents | B2) Juvenile cataract<br><br>B3) Childhood-onset chronic diarrhea<br><br>B4) Prolonged unexplained neonatal jaundice or cholestasis | B5) Ataxia (a) and/or Spastic paraparesis (b)<br><br>B6) Dentate nuclei signal alterations at MRI<br><br>B7) Intellectual disability (a) and/or Psychiatric disturbances (b) |
| **(C) Moderate**<br><br>**(score = 25)** | | C1) Early osteoporosis | C2) Epilepsy<br><br>C3) Parkinsonism<br><br>C4) Polyneuropathy |

**Table 2.** Suspicion index.

Table 2 shows the proposed suspicion index (SI) for use by clinicians to calculate the total CTX prediction score. We divided the diagnostic indicators into three categories:

I) Family history: sibling with CTX; consanguineous parents.

II) Systemic: tendon xanthomas; juvenile cataract; childhood-onset chronic diarrhea; prolonged unexplained neonatal jaundice or cholestasis; early osteoporosis.

III) Neurological: cerebellar ataxia; spastic paraparesis; MRI evidence of dentate nuclei signal alterations; intellectual disability; psychiatric disturbances; epilepsy; parkinsonism; polyneuropathy.

A) Very strong indicators: A1) sibling with CTX; A2) tendon xanthomas.

B) Strong indicators: B1) consanguineous parents; B2) juvenile cataract; B3) childhood-onset chronic diarrhea; B4) prolonged unexplained neonatal jaundice or cholestasis; B5) ataxia (a) and/or spastic paraparesis (b); B6) MRI evidence of dentate nuclei signal alterations; B7) intellectual disability (a) and/or psychiatric disturbances (b).

C) Moderate indicators: C1) early osteoporosis; C2) epilepsy; C3) parkinsonism; C4) polyneuropathy.

The following scores were assigned to weigh the variables: 100 for each very strong indicator; 50 for each strong indicator; 25 for each moderate indicator. Although indicators B5 and B7 included two different signs (a and b), one or both signs in the same patient (a and/or b) scored 50 alike. After creating the SI, we built a diagnostic flow chart based on its application (Fig. 1). A total score ≥100 warranted assessment of serum cholestanol concentrations, which had to be measured in the absence of treatment with bile acids and statins as well as corticosteroids, since steroids can lower plasma cholestanol by inducing residual CYP27A1 activity or cholestanol elimination

(Siman-Tov et al 2006). Evidence of increased plasma cholestanol suggested *CYP27A1* gene sequencing, while normal serum cholestanol levels indicated a different diagnosis. However, two other conditions were established to be sufficient to sequence *CYP27A1*, irrespective of cholestanol assessment: i) a total score ≥200 with at least one very strong or four strong indicators; ii) a sibling with genetically confirmed CTX. According to our flow chart, CTX can only be diagnosed definitively on the basis of *CYP27A1* molecular analysis.

We applied the SI to our patient population consisting of 55 patients. For each subject we determined: I) age, serum cholestanol (available in 37/55), SI score and indicators at the time of diagnosis; II) age and indicators at the time when SI score reached 100; III) age and indicators at the time when SI score reached 200 (with presence of at least one very strong or four strong indicators). Fig. 2 shows age at diagnosis (minimum, $25^{th}$ percentile, median, $75^{th}$ percentile, maximum) of each clinical indicator. At diagnosis, median age was 36 years (mean 35.5 ± 11.8 standard deviation [SD]) and the median SI score was 300 (mean 298.2 ± 66.6 SD). Serum cholestanol concentrations were elevated in 35/35 patients: mean 2.94 ± 1.21 mg/dl against 0.22 ± 0.08 mg/dl in 17 age-matched controls ($p<0.0001$). For each patient, we calculated when SI score reached 100 and 200: mean age (±SD) at SI ≥100 was 10.6 ± 9.8 years, and mean age at SI ≥200 was 24.1 ± 11.4 years. When we compared the age at actual diagnosis with the ages at SI ≥100 and SI ≥200, we found statistically significant differences between the three groups ($p<0.01$). The mean difference between age at actual diagnosis and age at SI ≥100 and SI ≥200 was 25.1 ± 11.8 years and 12.0 ± 9.5 years, respectively. Fig. 3 shows age at diagnosis, age at SI ≥100 and age at SI ≥200 (all expressed as minimum, $25^{th}$ percentile, median, $75^{th}$ percentile, and maximum). Indicators at diagnosis, SI ≥100

and SI ≥200 are detailed in Fig. 4. Notably, disabling neurological indicators such as ataxia, spastic paraparesis and psychiatric disturbances were very frequent at diagnosis and almost absent at SI ≥100. On the other hand, intellectual impairment and epilepsy were found at an earlier stage of the disease. Among systemic indicators, tendon xanthomas and cataract were present in most patients at diagnosis, but xanthomas were found in few subjects at SI ≥100 while cataract manifested earlier. Chronic diarrhea and neonatal jaundice were not as frequent as the above systemic indicators, but their early appearance made them very important for timely diagnosis. Other indicators such as MRI evidence of dentate nuclei signal alterations, osteoporosis, parkinsonism and polyneuropathy were never found at SI ≥100.

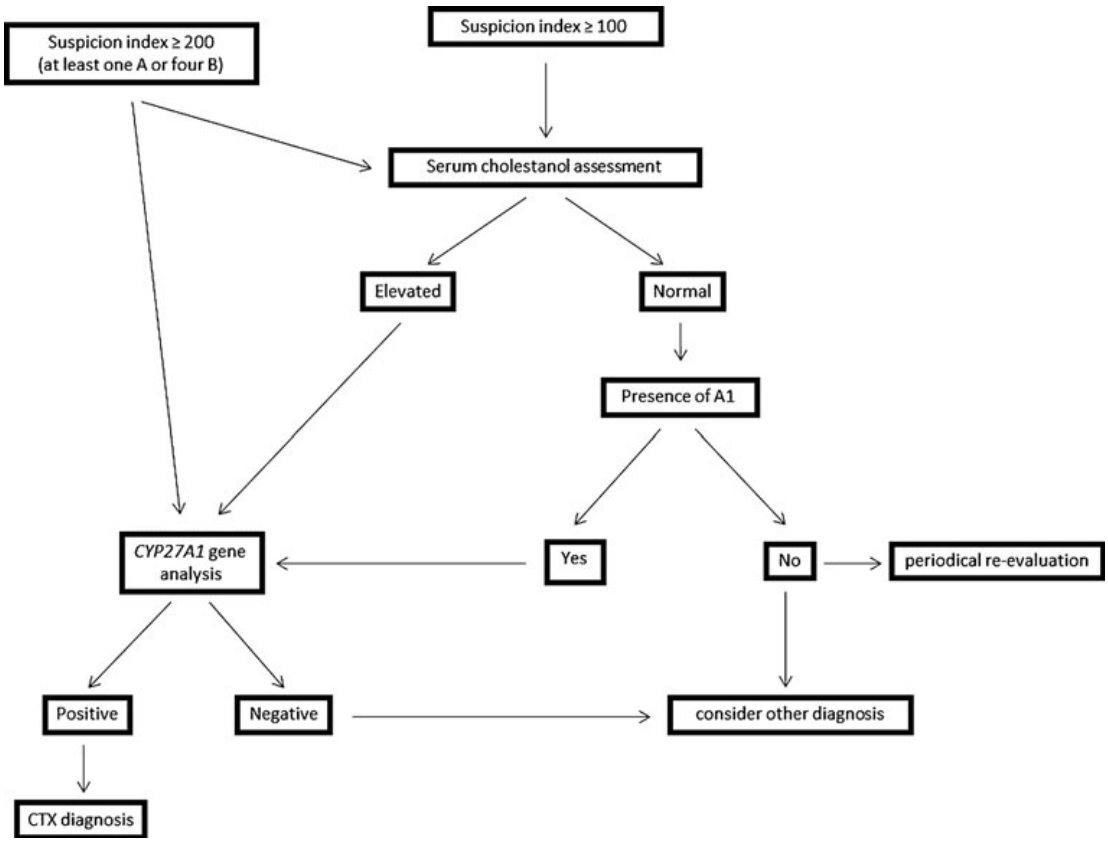



**Fig. 1.** Diagnostic flow chart based on application of the suspicion index.

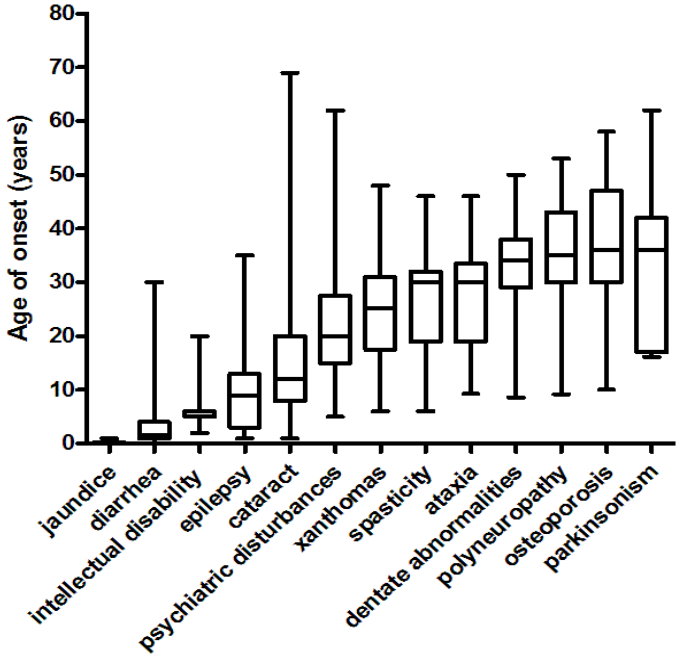



**Fig. 2.** Box plot representing age at diagnosis (minimum, 25$^{th}$ percentile, median, 75$^{th}$ percentile, maximum) of each clinical indicator in our 55 CTX patients.

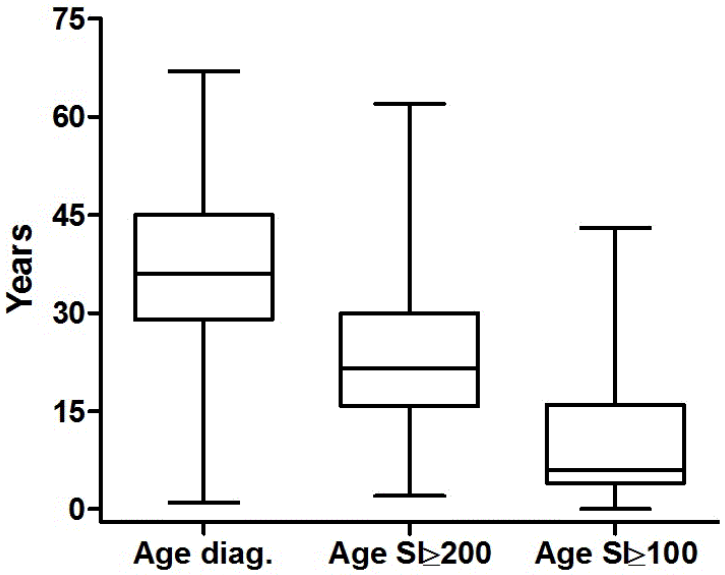



**Fig. 3.** Box plot representing age at diagnosis, age at SI ≥100 and age at SI ≥200 (all expressed as minimum, 25$^{th}$ percentile, median, 75$^{th}$ percentile, and maximum) in our 55 CTX patients. A statistically significant difference was observed between the three groups ($p<0.01$).

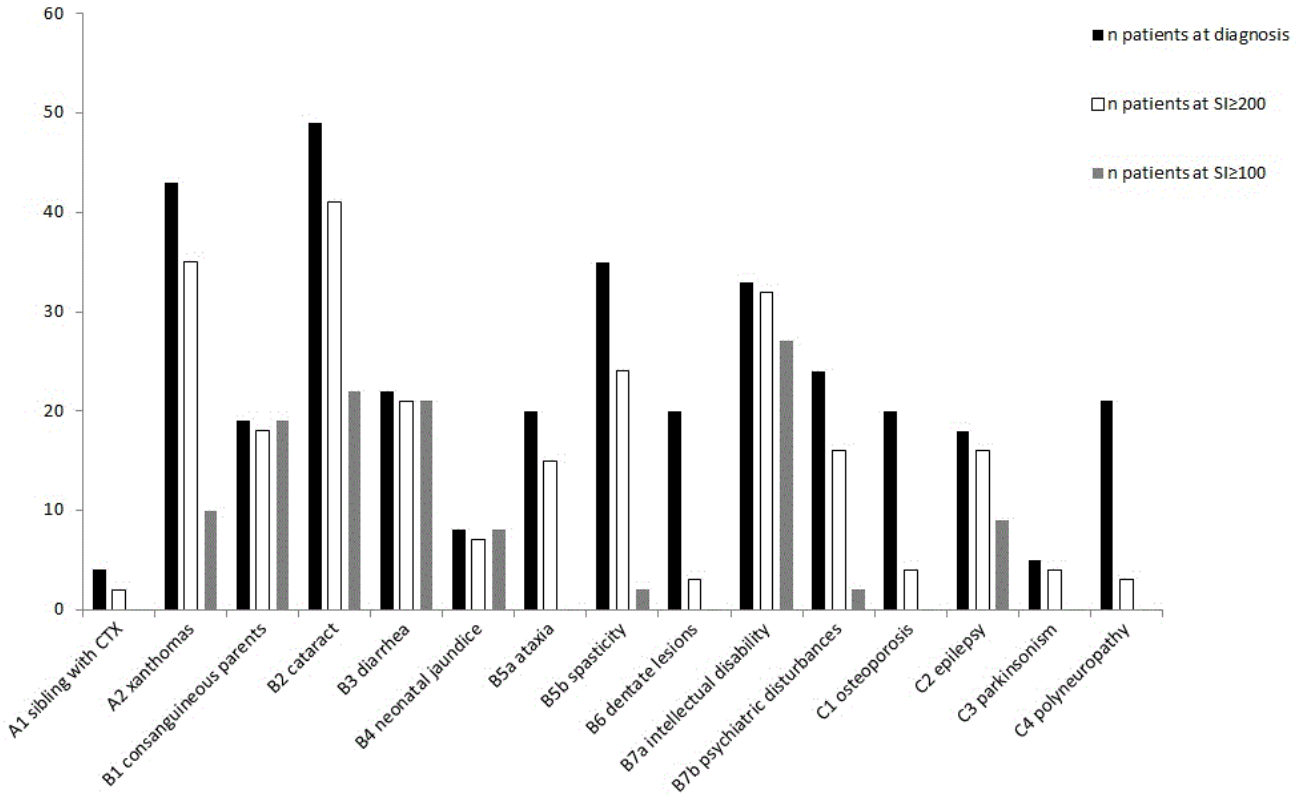



**Fig. 4.** Frequency of the indicators at diagnosis, at SI ≥200 and at SI ≥100 in our patient population.

## 2.4 Discussion

In the present study we reviewed the largest available series of CTX cases analyzed together, and we developed a suspicion index (SI) to achieve early diagnosis of this treatable metabolic disorder. We assigned a higher score to tendon xanthomas than to juvenile cataract, despite the higher frequency of the latter. Indeed, tendon xanthomas point strongly to CTX, although they may also be observed in sitosterolemia, which is very rare and does not share other phenotypical manifestations with CTX, and occasionally in familial hypercholesterolemia. On the other hand, juvenile cataract may also occur in a number of disorders causing neurological manifestations and requiring differential diagnosis with CTX, such as galactosemia (which shares also diarrhea and jaundice with CTX), Marinesco-Sjögren syndrome and myotonic dystrophy type 1. Our review highlighted the frequency and childhood manifestation of chronic diarrhea,

confirming the findings of previous studies (Cruysberg et al 1991; Verrips et al 2000; Cruysberg et al 2009; Berginer et al 2009) and further emphasising the importance of this symptom for early diagnosis. We also considered prolonged unexplained neonatal jaundice or cholestasis as a strong diagnostic indicator. Nevertheless, several single case reports have described neonatal cholestatic jaundice as a possible early manifestation of CTX (Clayton et al 2001; von Bahr et al 2005; Pierre et al 2008). Of note, unexplained neonatal cholestatic jaundice can also be observed in Niemann-Pick disease type C, another autosomal recessive lipid storage disorder. Early osteoporosis is a common systemic manifestation of CTX (Berginer et al 1993; Martini et al 2013), but since it is often subclinical and only detectable by bone densitometry, its importance as diagnostic indicator is limited. We considered spastic paraparesis, cerebellar ataxia, intellectual disability and psychiatric disturbances to be strong diagnostic indicators, due to their high frequency in CTX. Intellectual disability, usually presenting in the school age, is particularly important to consider for early diagnosis. Epilepsy was classified as a moderate CTX diagnostic indicator: this early-onset symptom is not very frequent and also occurs in a multitude of neurometabolic disorders. Parkinsonism was found in some patients of our population but was not reported in the other four series: we included it in the SI as a moderate indicator because of increasing evidence that it could be an underestimated neurological manifestation of CTX (Su et al 2010; Mignarri et al 2012; Rubio-Agusti et al 2012). MRI evidence of dentate nuclei signal alterations was considered a strong diagnostic indicator because in spite of its elevated frequency and peculiarity, it appears relatively late. Finally, we included polyneuropathy as a moderate indicator: neurophysiologically confirmed neuropathy is very frequent in CTX, but signs or symptoms are often absent or difficult to appreciate

because central nervous system involvement usually dominates the clinical picture (Pilo et al 2011; Ginanneschi et al 2013). We proposed a diagnostic flow chart based on our SI, and established a total SI score ≥100 as sufficient to assess serum cholestanol. We assigned an important diagnostic role to serum cholestanol assessment for three main reasons: first, our review confirmed that all untreated CTX patients show very elevated plasma cholestanol levels; second, we are not aware of high cholestanol concentrations in normal controls and patients with other diseases; third, plasma cholestanol assessment is easy to perform. Although serum cholestanol analysis in patients with SI ≥100 could involve a considerable overall cost, early diagnosis and treatment may prevent neurological disability and high related social and health costs in the long term. The diagnostic gold standard is molecular analysis of the CYP27A1 gene. Our flow chart recommends genetic analysis for patients with a total SI score ≥200 and at least one very strong indicator or four strong indicators, and for all siblings of CTX patients, even those with normal cholestanol values. This ensures that diagnosis is not missed in cases with a clinical picture highly suggestive of CTX or an elevated statistical probability of being affected. Notably, our SI is very easy to apply in the clinical setting: indeed, most of the indicators can be determined from medical history and clinical examination. Although our diagnostic algorithm was proved to be useful for early diagnosis, a pediatric screening program may be also needed. Assessment of serum cholestanol in children with neonatal cholestatic jaundice, cataract or unexplained chronic diarrhea may allow very early diagnosis and treatment of CTX, but could involve a great number of patients as well as considerable costs. This aspect deserves further epidemiological studies with a pediatric perspective. A limitation of this study was due to the fact that we could not test our diagnostic tool in

false positive cases, as the number of subjects who underwent testing of cholestanol levels and/or CYP27A1 analysis in our laboratory in the last 20 years was limited and therefore not statistically significant. However, this point may be less important because the aim of the present study was to enable earlier diagnosis rather than to improve differential diagnosis. Early diagnosis and timely introduction of replacement therapy are unanimously considered to be crucial in CTX patients in order to prevent neurological disability. However, our review pointed out a marked delay between symptoms onset and diagnosis. A recent cross-sectional observational study evaluating the long-term neurological outcome of CTX patients treated with CDCA revealed that subjects who started therapy after the age of 25 years had worse outcome and were significantly more limited in ambulation and cognition (Yahalom et al 2013). Our results show that the present suspicion index could allow early diagnosis of CTX, so that treatment can be started before disability occurs. We therefore propose its use in clinical practice and hope for validation and discussion in future studies.

# 3. Old and new biochemical markers in cerebrotendinuos xanthomatosis: evaluation of their utility for diagnosis and treatment follow up

## 3.1 Introduction

In the present study, we evaluated the serum profile of bile acids intermediates (cholestanol and its precursor 7αC4, 27-OHC, 24S-hydroxycholesterol), cholesterol, lathosterol, and plant sterols (campesterol, sitosterol) in a significantly large CTX population: we performed a baseline assessment and a long-term follow up during therapy with oral CDCA, comparing biochemical data with neurological outcome. We aimed at (a) clarifying the biochemical abnormalities and their response to CDCA treatment, (b) identifying reliable diagnostic and prognostic markers, and (c) understanding if a distinctive biochemical pattern exists in patients with neurological progression despite treatment.

## 3.2 Materials and methods

We studied 19 CTX patients (11 males, 8 females) aged 13-54 years (median age 32 years, mean±standard deviation 32.5±10.4 years), belonging to 15 unrelated Italian families. In all cases the diagnosis was confirmed by point mutations or deletions in the CYP27A1 gene. Demographic, clinical and molecular details of patients are summarized in Table 1. On enrolment in the study, all patients were untreated. After baseline (t0) clinical examination and biochemical analyses, they started oral CDCA therapy at a daily dose of 750 mg (250 mg three times a day). Then a long-term clinical and biochemical follow up was performed (t1 = 0-1.5 years; t2 = 1.5-2.5 years; t3 = 2.5-3.5 years; t4 = >4 years).

| Demographic info | | | | Clinical picture and disability scores | | | | | | | *CYP27A1* gene analysis | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Fm** | **Pt** | **S** | **Age** | **Catar** | **Xant** | **Cogn impair** | **Psych disturb** | **Spast** | **Ataxia** | **RS/EDSS** | **First mutation** | **Second mutation** |
| a | 1CP | M | 43 | yes | yes | yes | no | yes | yes | 3/6 (=) | c.646 G>C | c.646 G>C |
| b | 2CG | M | 21 | yes | yes | yes | yes | no | no | 1/ 3 (=) | c.752 C>A | c.1263+5 G>T |
| | 3CR | F | 18 | yes | yes | no | yes | no | no | 0/2 (=) | c.752 C>A | c.1263+5 G>T |
| c | 4DGS | M | 30 | yes | yes | yes | no | yes | yes | 2/4 (=) | c.776 A>G | c.776 A>G |
| d | 5DL | F | 34 | yes | no | yes | no | yes | yes | 3/5 (↑) | c.1263+1 G>A | c.1263+1 G>A |
| | 6DL | M | 36 | no | yes | yes | no | yes | yes | 3/7 (↑) | c.1263+1 G>A | c.1263+1 G>A |
| e | 7FG | M | 45 | yes | yes | yes | yes | yes | yes | 3/5 (↑) | c.752 C>A | c.752 C>A |
| f | 8FP | M | 37 | yes | yes | yes | no | yes | yes | 3/4 (↑) | c.1184+1 G>A | c.1184+1 G>A |
| g | 9GL | M | 25 | yes | yes | no | no | no | no | 1/1.5 (=) | c.863 delA | c.1183 C>T |
| | 10GM | M | 13 | no | no | no | no | no | no | 0/0 (=) | c.863 delA | c.1183 C>T |
| h | 11IS | M | 31 | yes | yes | yes | yes | yes | yes | 2/3 (=) | c.647-1 G>T | c.1183 C>T |
| i | 12PC | F | 33 | yes | no | yes | no | yes | no | 2/3 (↑) | c.752 C>A | c.752 C>A |
| j | 13RS | F | 54 | no | yes | no | yes | yes | no | 2/3.5 (=) | c.646 G>C | c.1538 G>A |
| k | 14RA | F | 31 | yes | yes | yes | yes | yes | yes | 2/3.5 (=) | c.1263+81_1596+?del | c.1263+81_1596+?del |
| l | 15RM | F | 22 | yes | yes | no | yes | no | no | 0/2 (=) | c.1183 C>T | c.646 G>C |
| m | 16SD | M | 32 | yes | yes | yes | yes | yes | yes | 2/3.5 (↑) | c.1184+1 G>A | c.1184+1 G>A |
| n | 17SR | F | 36 | yes | yes | yes | no | yes | no | 3/3.5 (=) | c.646 G>C | c.1184+1 G>A |
| | 18SV | M | 29 | yes | yes | yes | yes | yes | no | 2/3.5 (↑) | c.646 G>C | c.1184+1 G>A |
| o | 19VS | F | 48 | yes | no | yes | no | no | no | 1/1.5 (=) | c.1016 C>T | c.1016 C>T |

**Table 1**. Demographic information, clinical picture (including neurological disability scores), and molecular analysis of the 19 CTX patients enrolled for the study.

All patients underwent neurological examination including Rankin Scale (van Swieten et al 1988) and Expanded Disability Status Scale (Kurtzke 1983) administration at baseline and during the follow up period: subjects with unchanged Rankin Scale (RS) and Expanded Disability Status Scale (EDSS) scores over time were considered "neurologically stable", while those with increased RS and/or EDSS scores at follow up were classified as "neurologically worsening". We analyzed cholesterol metabolism both at baseline and during CDCA treatment. Moreover, we compared biochemical data of "neurologically stable" patients with those of "neurologically worsening" patients. The study was approved by the Local Ethics Committee, Faculty of Medicine, University of Siena, and written informed consent was obtained from all patients. All

solvents were obtained from Merck (Darmstadt, Germany) and were of analytical grade. BHT, piperidine, 2,3,4,5,6-pentafluorobenzoyl chloride and trimethylsilylimidazole were purchased from Sigma-Aldrich (St Louis, MO, USA). Silica cartridge columns (Supelclean LC-Si, size 1 ml) were obtained from Supelco Inc. (Bellefonte, PA, USA). Total Cholesterol Assay Kit (Colorimetric) was purchased from Cell Biolabs Inc. (San Diego, CA, USA). All deuterated sterols used as internal standards (cholestanol-d4, 27-hydroxycholesterol-d9, lathosterol-d4) were synthesized in our laboratory as described previously (Galli Kienle et al 1980; Alessandrini et al 2004). Cholestane was purchased from Sigma-Aldrich (St Louis, MO, USA) and 19-hydroxycholesterol was obtained from Steraloids (Newport, RI, USA). Cholestanol, lathosterol, campesterol, β-sitosterol were purchased from Sigma-Aldrich (St Louis, MO, USA), 7αC4, 27-OHC and 24S-hydroxycholesterol (24-OHC) were obtained from Steraloids (Newport, RI, USA). Deuterated cholestanol was added to 0.2-ml plasma samples as solutions of 0.2 µg/µl (5µl) in ethyl acetate. Alkaline hydrolysis was carried out with 1 ml 1 N NaOH in 90% ethanol at 60 °C for 90 minutes under nitrogen; physiologic saline solution (1 ml) was then added, and sterols were extracted with 2 ml of petroleum ether and taken to dryness under a stream of nitrogen. HPLC separation was performed in order to remove excess cholesterol from lipid extract, as previously described (Kuriyama et al 1991). The extracted sterols were converted into their benzoyl derivatives by addition of 0.2 ml of a dichloromethane reaction mixture containing 0,2% triethylamine (TEA) and 0.5% 2,3,4,5,6-pentafluoro-benzoyl chloride (PFB). After incubation (20 minutes at room temperature) reaction mixture was evaporated under nitrogen. HPLC separation was performed with a Jasco HPLC system (880-PU, 801-SC ,880-02) equipped with a Jasco 875-UV detector (Jasco, Tokyo,

Japan). Analyses were carried out with an Inertsil® ODS-2 (4,6 mm x 150 mm) column with guard column (GL Sciences Inc., Tokyo, Japan) using a mobile phase of acetonitrile/water/acetic acid (100:3:0.2, v/v/v) at a flow rate of 1 ml/min. Absorbance was monitored at λ 228 nm. Samples were injected dissolved in 0,2 ml of eluent and the retention time of PFB derivatives of cholesterol and cholestanol was verified by injection of pure derivatized standards. The PFB-cholestanol fraction of each sample was collected and, after solvent evaporation, dissolved in 25 µl of toluene for GC-MS analysis. Extraction and purification of plasma 7αC4, 27-OHC, 24-OHC, lathosterol and plant sterols (campesterol and β-sitosterol) was performed as previously described (Kuriyama et al 1991; Alessandrini et al 2004). As internal standards, we used 19-hydroxycholesterol for 7αC4 and 24-OHC, deuterated 27-OHC for 27-OHC, deuterated lathosterol for lathosterol and cholestane for plant sterols. Before GC-MS analysis all sterols were converted into their trimethylsilyl ethers (TMS) with a mixture of trimethylsilylimidazole:piperidine (1:1) (by volume), as already reported (Del Puppo et al 1998; Bertolotti et al 2008). Analysis of sterols was carried out using a Thermo Finnigan GC-Q instrument (Waltham, MA, USA). The spectrometer was set at 70 eV ion energy, 0.1 mA emission current, and 300 °C transfer line temperature. Separation of sterols was achieved by a J&W HP5 capillary column (Agilent technologies, Santa Clara, CA, USA) 0.32 mm i.d., 0.25 mm film thickness, 30 m long, operating at 1 ml/min helium flow rate. Column temperature was programmed from 180 °C to 300 °C. For sterols quantification, we focused specific ions: m/z 215 and 427 for PFB-cholestanol, m/z 219 and 431 for deuterated PFB-cholestanol (IS), m/z 382 and 472 for 7αC4-TMS, m/z 353 for 19-hydroxycholesterol-TMS (IS), m/z 456 for 27-OHC-TMS, m/z 413 for 24-OHC -TMS, m/z 465 for deuterated 27-OHC-TMS (IS), m/z 255 for

lathosterol-TMS, m/z 382 for campesterol-TMS, m/z 396 for β-sitosterol-TMS, 259 for deuterated lathosterol-TMS (IS) and 372 for cholestane (IS). Calibration curves were prepared by spiking plasma with a fixed amount of each internal standard and increasing amounts of the above-mentioned sterols. These samples were treated and analyzed as the experimental samples. Concentrations were calculated on the basis of the slope of the standard curve as well as of the peak area ratio (sterol/IS) found in the sample. The assay results were linear ($r>0.98$) in the tested ranges. To measure total plasma cholesterol levels, a commercial assay kit (colorimetric) was used according to the manufacturer's instructions (Cell Biolabs Inc., San Diego, CA, USA). Absorbance at 570 nm was read with a BGM Labtech spectrophotometric microplate reader (Cary, NC, USA). Sample cholesterol concentrations were determined by interpolation from a standard curve. We tested data for normal distribution with the Anderson–Darling test. In the case of normally distributed data samples, we used parametric tests for comparisons (Student's t-test to compare two groups, Duncan's new multiple range parametric to compare three or more groups). In the case of not normally distributed data samples, we used non parametric tests for comparisons (Mann-Whitney U test to compare two groups, Kruskal-Wallis test with Dunn's post hoc test to compare three or more groups). We also performed analysis of correlations between variables by calculation of Spearman coefficient and relative p-value. Statistical significance was assumed for $p<0.05$.

### 3.3 Results

Plasma concentrations of cholestanol, 7αC4, 27-OHC, 24-OHC, lathosterol, and plant sterols of our 19 CTX patients before and during CDCA therapy are reported in Table 2

and Figures 1 and 2. Serum cholestanol levels at baseline were on average 10-fold higher than normal, and a statistical significance was found between CTX patients and control subjects ($p<0.01$). Once treatment was started, plasma cholestanol sharply decreased until normalization after 18 months of CDCA intake, and no loss of efficacy was detectable in the long-term. Untreated CTX patients had significantly elevated (from 100-fold to 200-fold increased) plasma concentration of 7αC4 compared to controls ($p<0.01$), indicating marked hyperstimulation of the first part of the "classic" pathway of bile acid synthesis. Serum levels of 7αC4 consistently decreased after CDCA treatment; however, 7αC4 never normalized in half subjects, and some degree of accumulation appeared to persist in most patients. Notably, a significant positive correlation was found between plasma values of 7αC4 and cholestanol ($\rho = 0.78$; $p<<0.01$). As expected, serum 27-OHC was below the limit of detection in almost all CTX plasma samples, and no changes were observed under CDCA treatment. Serum 24-OHC was slightly but significantly ($p<0.05$) increased in untreated CTX patients, and mildly but not significantly decreased during long-term treatment. In order to complete our investigation of cholesterol metabolism, we also assayed plasma levels of cholesterol, lathosterol (as biomarker of hepatic and whole body cholesterol "de-novo" synthesis) and phytosterols (as biomarkers of cholesterol intestinal absorption). Serum cholesterol was normal in most patients (only few patients showed mild hypercholesterolemia), and did not substantially change during CDCA treatment. Plasma lathosterol levels were significantly increased in untreated CTX patients ($p<0.01$), indicating that cholesterol synthesis was incremented, and normalized in the course of therapy. Moreover, plasma levels to lathosterol and 7αC4 resulted to be strictly correlated ($\rho = 0.8$; $p<<0.01$). Similarly to lathosterol, baseline serum levels of

the plant sterols campesterol and sitosterol were high in CTX patients, indicating increased cholesterol intestinal absorption; however, only sitosterol increase was statistically significant ($p<0.05$). CDCA treatment strongly reduced plasma concentration of plant sterols. We performed neurological examination and disability assessment with RS and EDSS at baseline and during the entire follow up period: on the basis of unchanged disability scores over time, 12 patients were classified as "neurologically stable"; on the other hand, 7 patients showed increased RS and/or EDSS scores at follow up and were considered as "neurologically worsening". Age at diagnosis was not significantly different between the two groups. Disability at diagnosis was higher in the "neurologically worsening" group; in particular a statistically significant difference was found between baseline RS of the two samples ($p<0.05$). When we compared biochemical data of "neurologically stable" patients with those of "neurologically worsening" subjects, we did not find significant differences between the two groups, both before therapy and during long-term follow up. Notably, serum concentration of cholestanol, 7αC4, and lathosterol at baseline tended to be higher in those patients who later presented neurological worsening despite therapy, but no statistical significance was observed. Given the small number of biochemical data available at long-term follow up on "neurologically worsening" patients, it was difficult to perform a statistical comparison between the two groups. Finally, no correlation was observed between genotype and biochemical pattern, as well as between genotype and phenotypical characteristics or response to therapy.

| | | t0 (n=19) | t1 (n=13) | t2 (n=11) | t3 (n=11) | t4 (n=12) | Normal values |
|---|---|---|---|---|---|---|---|
| **Cholestanol** (mg/dl) | mean±sd | 3.42±1.28 | 0.81±0.59 | 0.69±0.60 | 0.53±0.30 | 0.38±0.15 | 0.34±0.16 (Kuriyama et al 1991) |
| | IQR (median) | 2.46-4.01 (3.72) | 0.55-0.84 (0.72) | 0.28-0.84 (0.48) | 0.33-0.68 (0.40) | 0.30-0.42 (0.38) | |
| **7αC4** (μg/dl) | mean±sd | 368.0±221.0 | 34.9±51.1 | 25.9±53.6 | 20.0±18.0 | 22.9±29.0 | 2.2±2.0 (Camilleri et al 2009) |
| | IQR (median) | 200.0-527.0 (297.0) | 12.7-33.4 (21.0) | 2.4-12.9 (8.2) | 7.6-28.0 (13.7) | 3.1-44.4 (7.3) | |
| **27-OHC** (μg/dl) | mean±sd | 1.0±1.2 | 1.1±0.8 | 0.7±0.6 | 0.7±0.7 | 0.6±0.7 | 15.4±4.3 (Dzeletovic et al 1995) |
| | IQR (median) | 0.0-1.2 (0.8) | 0.8-1.4 (1.2) | 0.4-1.0 (0.6) | 0.0-1.3 (0.5) | 0.0-1.2 (0.3) | |
| **24-OHC** (μg/dl) | mean±sd | 8.6±4.5 | 7.8±3.8 | 7.2±1.7 | 7.3±3.2 | 7.2±2.4 | 6.4±2.4 (Dzeletovic et al 1995) |
| | IQR (median) | 5.5-10.4 (7.4) | 4.9-12.3 (6.5) | 6.5-8.4 (7.4) | 5.2-8.6 (6.6) | 4.9-9.1 (7.2) | |
| **Cholesterol** (mg/dl) | mean±sd | 187±67 | 196±97 | 176±37 | 187±41 | 165±42 | 166±32 (Kempen et al 1988) |
| | IQR (median) | 149-215 (160) | 149-191 (166) | 150-186 (176) | 172-209 (191) | 132-187 (172) | |
| **Lathosterol** (μg/dl) | mean±sd | 934±347 | 178±102 | 158±82 | 168±92 | 136±76 | 206±79 (Kempen et al 1988) |
| | IQR (median) | 677-1200 (842) | 123-480 (141) | 118-176 (124) | 124-213 (133) | 85-159 (113) | |
| (μg/100mg cholesterol) | mean±sd | 526±160 | 105±159 | 78±21 | 86±36 | 83±48 | 96±34 (Kempen et al 1988) |
| | IQR (median) | 458-615 (499) | 65-114 (86) | 70-83 (79) | 68-98 (80) | 52-94 (70) | |
| **Campesterol** (μg/dl) | mean±sd | 561±286 | 226±76 | 182±86 | 186±62 | 169±77 | 399±218 (Kuriyama et al 1991) |
| | IQR (median) | 346-734 (525) | 161-249 (233) | 115-256 (166) | 149-209 (178) | 111-210 (154) | |
| (μg/100mg cholesterol) | mean±sd | 334±203 | 141±95 | 104±61 | 107±57 | 108±64 | 240±120 (Kuriyama et al 1991) |
| | IQR (median) | 209-328 (283) | 89-141 (105) | 52-127 (89) | 77-134 (83) | 74-111 (81) | |
| **Sitosterol** (μg/dl) | mean±sd | 1119±595 | 491±148 | 418±202 | 430±184 | 402±223 | 629±171 (Kuriyama et al 1991) |
| | IQR (median) | 651-1628 (938) | 413-599 (468) | 245-533 (435) | 330-492 (370) | 245-445 (355) | |
| (μg/100mg cholesterol) | mean±sd | 637±293 | 301±159 | 244±140 | 253±163 | 256±170 | 410±90 (Kuriyama et al 1991) |
| | IQR (median) | 434-682 (552) | 226-361 (278) | 124-348 (218) | 152-333 (201) | 160-254 (183) | |

**Table 2 (previous page).** Determination of plasma levels of cholestanol, oxysterols (7αC4, 27-OHC, 24-OHC), cholesterol, lathosterol, and plant sterols (campesterol, sitosterol) at baseline and during CDCA treatment. Data were reported as mean±sd and interquartile range (IQR) with median. Evaluation times: t0 = baseline; t1 = 0-1.5 years; t2 = 1.5-2.5 years; t3 = 2.5-3.5 years; t4 = >4 years.

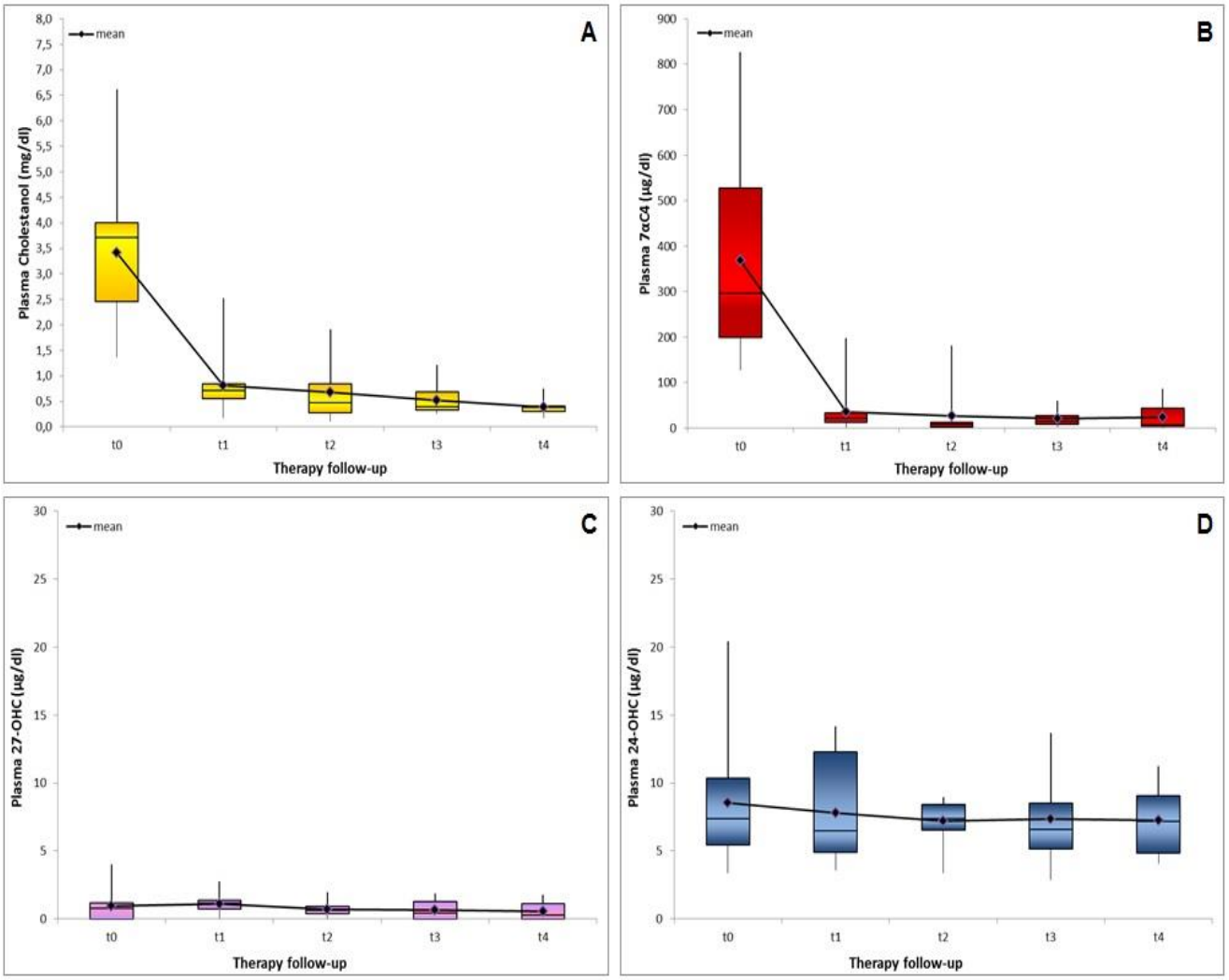



**Figure 1.** Box plots representing plasma levels of cholestanol, 7αC4, 27-OHC, and 24-OHC before therapy and during CDCA treatment. Data are expressed as minimum, 25th percentile, median, 75th percentile, and maximum; mean values are also indicated. Evaluation times: t0 = baseline; t1 = 0-1.5 years; t2 = 1.5-2.5 years; t3 = 2.5-3.5 years; t4 = >4 years.

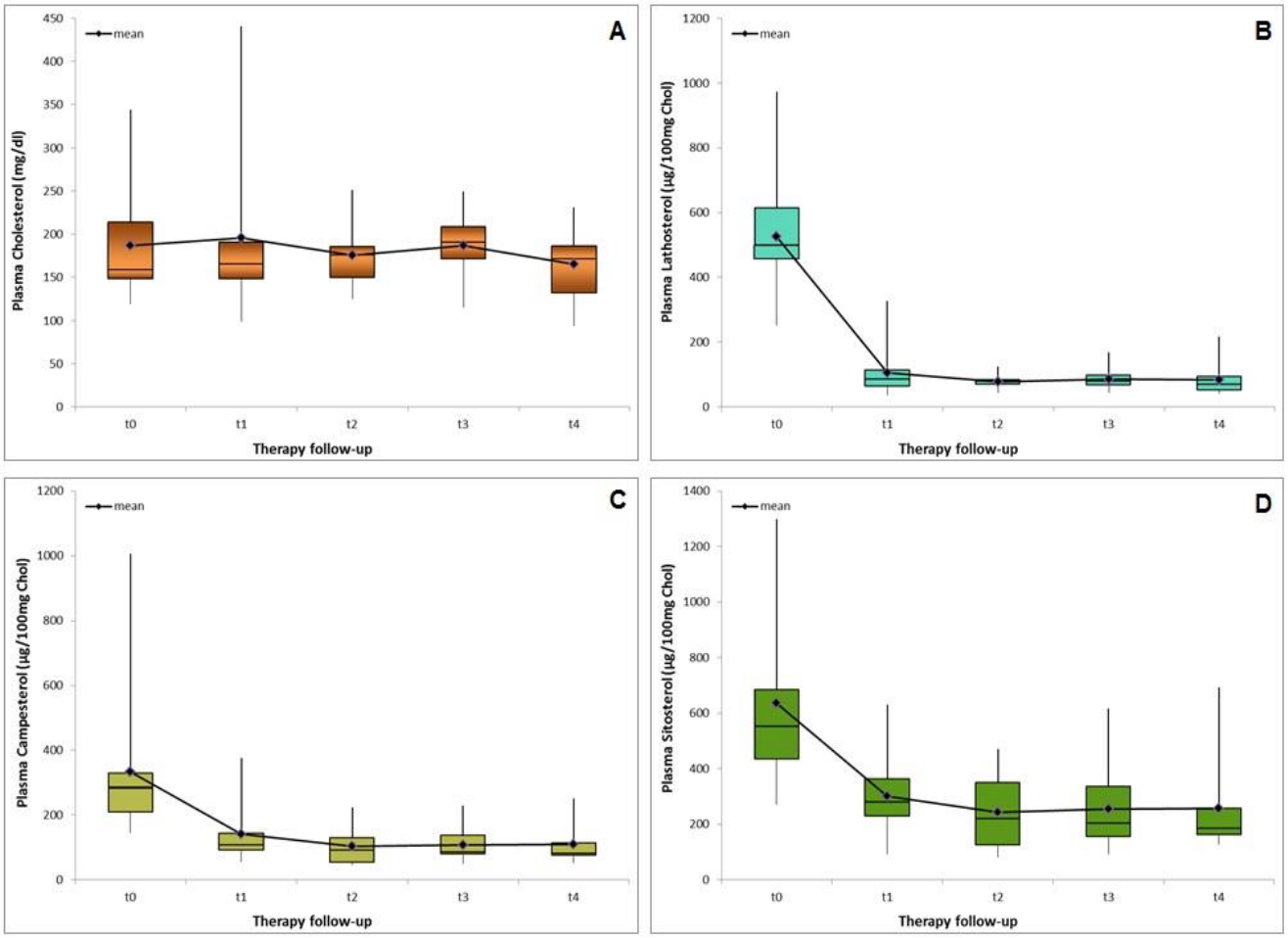



**Figure 2.** Box plots representing plasma levels of cholesterol, lathosterol, campesterol, and sitosterol before therapy and during CDCA treatment. Data are expressed as minimum, 25th percentile, median, 75th percentile, and maximum; mean values are also indicated. Evaluation times: t0 = baseline; t1 = 0-1.5 years; t2 = 1.5-2.5 years; t3 = 2.5-3.5 years; t4 = >4 years.

## 3.4 Discussion

In spite of the undoubted improvement in the diagnosis and therapy and the better understanding of pathogenetic mechanisms, several points of criticism on CTX pathogenesis, diagnosis, and treatment still remain to be solved (Björkhem et al 2010). In this respect, our long-term study of cholesterol metabolism may help to clarify some relevant aspects. As previously observed in small series of untreated CTX patients, this study confirmed pre-treatment increase of cholestanol, lathosterol, and plant sterols.

Moreover, 7αC4 was markedly increased, whereas 27-OHC was generally absent or extremely low. Total cholesterol levels were substantially normal. After CDCA treatment normalization of all biochemical parameters was observed with the exception of serum 7αC4, whose level was sharply reduced but still higher than normal in most patients, and plasma 27-OHC which was not modified. Each of the above reported biochemical findings suggests some considerations both in itself and in relation to clinical follow up. As expected, plasma cholestanol was elevated in all untreated patients, thus confirming its utility as diagnostic, easy to dose marker. However, cholestanol may be reduced by several drugs (bile acids, statins, and steroids) whereas increased levels can be found in sitosterolemia (Salen et al 1985). Treatment with CDCA normalized serum cholestanol, and we did not observe any loss of efficacy at follow up. In this respect, our data differ from previous observation of cholestanol increase (after initial normalization) during long-term treatment in two children reported by de Sain-van der Velden et al (2008), which could be due, for instance, to too low dose of CDCA. Serum 7αC4 has been proposed as a very important marker for both diagnosis and monitoring of replacement therapy in CTX (DeBarber et al 2010; Björkhem et al 2014). Circulating 7αC4 closely mirrors cholesterol 7α-hydroxylation rate, thus reflecting the activity of the "classic" pathway of bile acid synthesis (Bertolotti et al 2008). Furthermore, 7αC4 is strictly correlated with accumulation of cholestanol in the brain in both CTX subjects (Panzenboeck et al 2007) and mice with disruption of sterol 27-hydroxylase (Bavner et al 2010). However, so far data on 7αC4 in CTX patients are very few. We found extremely high plasma 7αC4 at baseline, while under CDCA 7αC4 consistently decreased; however, a mild 7αC4 increase appeared to persist in most patients. Probably, CDCA dose may be incremented until normal 7αC4

values are reached. In this respect, a pilot study in CTX patients should be performed, increasing CDCA and monitoring the 7αC4 levels in serum as well as in the cerebrospinal fluid. Finally, the positive correlation between plasma values of 7αC4 and cholestanol strengthens the value of 7αC4 as an adjunctive diagnostic biomarker. Serum 27-OHC was very low or absent in all CTX patients, regardless of CDCA treatment confirming the substantial inactivity of sterol 27-hydroxylase, and the inability of CDCA to correct deficiencies of intermediates in the "alternative" pathway of bile acid synthesis. Assessment of serum 27-OHC may be considered the best analysis for CTX diagnosis being reliable at any stage of disease, irrespective of treatment. Furthermore, dosage of plasma 27-OHC could be considered of choice in the view of a possible neonatal screening. According to a recent paper the preferred nomenclature for 27-hydroxylation and 27-hydroxycholesterol should be (25R)26-hydroxylation and (25R)26-hydroxycholesterol, respectively (Fakheri and Javitt 2012).

Under in vitro conditions 27-OHC is an activator of liver X receptor (LXR). The role of this activation under in vivo conditions is controversial, however. The activation of some LXR-regulated genes as a consequence of feeding mice with high dietary cholesterol has been demonstrated to be mediated by side-chain oxidized cholesterol. If the oxysterol responsible for the activation is 24-, 25- or 27-hydroxycholesterol has not been shown, however (Chen et al 2007). A knockout of CYP27 in mice does not change the expression of a number of LXR-target genes in the brain. The situation is similar in mice with an overexpression of the enzyme. We must conclude that 27-OHC is of little importance for LXR signalling in the brain, at least under normal conditions, while it may be of some importance for regulation of cholesterol synthesis by mechanisms not involving LXR. Indeed, the flux of 27-OHC from the circulation into

the brain is of some importance for cholesterol homeostasis in the brain (Ali et al 2013). Decreased serum 24-OHC levels have been found to be related to the rate of neuronal degeneration in Huntington's disease and Alzheimer's disease (Leoni and Caccia 2011). Unexpectedly, 24-OHC was normal or slightly increased in our patients and poorly influenced by therapy. Moreover, the levels of 24-OHC were not significantly changed when corrected for cholesterol levels. Therefore, in CTX it does not seem to have an important role on pathogenesis or monitoring of neurodegeneration over time. Lathosterol is a well-known marker of cholesterol "de-novo" synthesis. In our patients, serum lathosterol was markedly increased at baseline, and normalized during CDCA treatment. Moreover, lathosterol and 7αC4 levels were strictly correlated. Most likely, the hyperactivation of the "classic" pathway of bile acid synthesis due to the lack of CDCA causes a very high consumption of cholesterol, which is compensated by incrementing "de-novo" synthesis. CDCA therapy inactivates 7α-hydroxylation strongly reducing cholesterol consumption. As a result, cholesterol "de-novo" synthesis decreases with corresponding lathosterol normalization. Increase of lathosterol may be considered a valuable diagnostic marker which parallels elevation of 7αC4 and cholestanol levels in untreated CTX subjects. Moreover, the assessment of serum lathosterol may help the clinician in deciding whether to add a statin to CDCA or not. Plant sterols are considered markers of cholesterol intestinal absorption. Sitosterol, and to a lesser extent campesterol, were increased at baseline and decreased during treatment with CDCA. Since cholesterol absorption and synthesis are usually inversely correlated, one could expect that the values of plant sterols were reduced. The elevated concentration of plant sterols in CTX subjects may be linked to the percentage increase of cholic acid (CA), produced

via the “alternative” pathway, in bile: indeed, CA is much more effective in stimulating absorption of cholesterol and plant sterols than CDCA. If that was the case, the decrease of plant sterols occurring during treatment would be explained by reduced absorption due to replacement of CA in bile with CDCA as the dominating bile acid. With the exception of few patients showing mild hypercholesterolemia, serum cholesterol was substantially normal, and no changes were observed under CDCA treatment. These data may mean that, in absence of altered dietary intake, cholesterol synthesis is equivalent to cholesterol consumption in CTX. Biochemical evaluation did not evidence statistically relevant differences between “neurologically stable” and “neurologically worsening” patients. Nevertheless, some potentially interesting aspects emerged. Serum levels of cholestanol, 7αC4, and lathosterol at baseline tended to be higher in those patients who later presented neurological worsening suggesting that higher levels of toxic bile acid intermediates can predict a poor response to therapy. Regarding follow up, one could expect that 7αC4 decrease to a lesser extent during CDCA treatment in worsening patients: we were not able to confirm this hypothesis, but the small number of biochemical data available at long-term follow up on “neurologically worsening” patients may have led us to underestimate possible differences. In this respect assessment of 7αC4 in cerebrospinal fluid may be very helpful. Moreover, since 27-OHC was absent or very low in all of our patients both at baseline and under CDCA treatment, we do not have data pointing to a pathogenetic role of 27-OHC deficiency in neurological evolution. Finally, it is worth noting the higher baseline disability in the “neurologically worsening” group suggesting that the presence of significant neurological impairment before treatment may predict poorer response to therapy. In conclusion, our metabolic evaluation of CTX patients allowed

us to clarify some pathogenetic aspects of the disease, to specify the role of the various biochemical parameters in the diagnostic setting, and to better assess the effects of CDCA treatment on cholesterol metabolism, also in the light of clinical evolution.

# 4. Brain MRI in cerebrotendinous xanthomatosis: redefinition of the diagnostic and prognostic role and evidence of new markers of disease progression

## 4.1 Introduction

The first MRI studies in CTX patients revealed only mild brain atrophy and demyelination in the supratentorial white matter (Pedley et al 1985; Swanson and Cromwell 1986). In 1990, two short reports first described cerebellar abnormalities (Bencze et al 1990; Fiorelli et al 1990). Further studies confirmed the presence of diffuse cerebral and cerebellar lesions, the latter involving dentate nuclei and surrounding white matter (Hokezu et al 1992; Berginer et al 1994; Dotti et al 1994). The largest MRI study published so far analysed 24 CTX patients using T1-weighted (T1W) and T2-weighted (T2W) sequences (Barkhof et al 2000): besides nonspecific supratentorial abnormalities, lesions in the dentate nuclei, which were in most cases T2W-hyperintense and T1W-isointense, were described in most patients and considered a peculiar finding; abnormalities of cerebral peduncles and cerebellar white matter were also observed. Further studies pinpointed to the fluid attenuated inversion recovery (FLAIR) sequence sensitivity in detecting dentate nuclei alterations and white matter signal abnormalities (De Stefano et al 2001; Lionnet et al 2014). In the last 15 years MRI studies on CTX patients focused on in vivo quantification of brain damage. Magnetic resonance spectroscopy (MRS) demonstrated a decrease of N-Acetylaspartate (NAA), which correlated with clinical disability. Magnetization transfer MRI also provided quantitative measures related to disability scores (Inglese et al 2003). Finally, regional brain volumes were found to be decreased in CTX and to correlate closely with clinical status (Guerrera et al 2010). Unfortunately, all these studies lacked to

provide information on follow up. In this paper we report the clinical and MRI findings of 38 CTX patients, and the follow up data of 16 of them who were untreated at baseline. The purposes of our study were (a) to better characterize MRI features in CTX by analysing the largest population ever reported, (b) to identify peculiar diagnostic patterns in the light of disease phenotype, and (c) to search for correlations between neuroimaging changes and clinical disability and/or disease outcome.

## 4.2 Materials and methods

We retrospectively studied 38 CTX patients (20 females, 18 males) aged 13-67 years (median age 41 years, mean±standard deviation 38.9±11.7 years), belonging to 30 families, who were consecutively evaluated in our reference centre for neurometabolic disorders between 1999 and 2016. Demographic and clinical details are summarized in Table 1. All patients underwent physical and neurological examination, including Rankin Scale (van Swieten et al 1988) and Expanded Disability Status Scale (Kurtzke et al 1983) administration, and brain MRI at 1.5T. Brain CT scan was also performed to rule out or confirm the presence of calcifications. Spinal cord MRI at 1.5T was performed in 6 patients. At baseline evaluation 11 patients were already on oral CDCA therapy at a daily dose of 750 mg whereas 27 were still untreated. In 16 drug-free subjects (median age 32.5 years, mean±standard deviation 33.5±11.9 years), we were able to perform follow up in course of treatment after a period of 24-48 months (median 42 months, mean±standard deviation 38.3±10.9 months). Patients with unchanged Rankin Scale (RS) and Expanded Disability Status Scale (EDSS) scores over time were considered “neurologically stable”, while those with increased RS and EDSS scores at follow up were regarded as “neurologically worsening”.

| Demographic info | | | Clinical picture, neurological disability scores, treatment duration | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pt/Fm | S | Age | Xant | Catar | Cogn impair | Psych disturb | Ataxia | Spast | Park | Seiz | RS/ EDSS | CDCA (years) |
| 1AL/a | F | 31 | yes | yes | yes | yes | no | yes | no | yes | 1/3 | 0 |
| 2AN/a | F | 24 | no | yes | yes | yes | yes | yes | no | no | 2/3.5 | 0 |
| 3BMC/b | F | 43 | yes | yes | yes | yes | no | yes | no | yes | 1/3 | 13 |
| 4BMG/b | F | 49 | yes | yes | yes | yes | no | yes | yes | yes | 4/6 | 13 |
| 5BPR/b | F | 38 | yes | yes | yes | no | no | yes | no | yes | 2/3.5 | 16 |
| 6BG/c | M | 40 | no | yes | yes | no | yes | yes | no | yes | 4/6.5 | 4 |
| 7CP/d | M | 43 | yes | yes | yes | no | yes | yes | no | no | 3/6 | 0 |
| 8CF/e | F | 29 | yes | yes | yes | yes | no | no | no | yes | 1/2 | 0 |
| 9CU/f | M | 42 | yes | yes | no | yes | no | yes | no | no | 3/4 | 11 |
| 10CG/g | M | 21 | yes | yes | yes | yes | no | no | no | no | 1/3 | 0 |
| 11CR/g | F | 18 | yes | yes | no | yes | no | no | no | no | 0/2 | 0 |
| 12DSC/h | M | 49 | yes | yes | yes | yes | no | no | yes | no | 4/5.5 | 0 |
| 13DFB/i | F | 67 | yes | no | no | yes | no | no | yes | no | 2/3 | 0 |
| 14DGM/j | F | 29 | yes | yes | yes | yes | no | no | no | no | 1/2.5 | 0 |
| 15DGS/j | M | 32 | yes | yes | yes | no | yes | yes | no | no | 2/4 | 0 |
| 16DMLo/k | F | 34 | no | yes | yes | no | yes | yes | no | no | 3/5 | 0 |
| 17DMLu/k | M | 36 | yes | no | yes | no | yes | yes | no | no | 3/7 | 0 |
| 18FG/l | M | 45 | yes | yes | yes | yes | yes | yes | no | yes | 3/5 | 0 |
| 19FP/m | M | 40 | yes | yes | yes | no | yes | yes | no | no | 3/4 | 0 |
| 20GL/n | M | 25 | yes | yes | no | no | no | no | no | no | 1/1.5 | 0 |
| 21GM/n | M | 13 | no | no | no | no | no | no | no | no | 0/0 | 0 |
| 22IS/o | M | 35 | yes | yes | yes | yes | yes | yes | no | no | 2/3 | 0 |
| 23LM/p | F | 45 | yes | yes | yes | yes | no | yes | no | yes | 1/3 | 18 |
| 24LA/q | M | 53 | yes | yes | yes | no | yes | yes | no | no | 3/5 | 0 |
| 25LC/r | M | 43 | no | yes | yes | no | yes | yes | no | no | 2/3.5 | 0 |
| 26LR/s | M | 55 | yes | yes | yes | no | no | yes | yes | no | 3/3.5 | 6 |
| 27PG/t | M | 57 | yes | yes | yes | yes | yes | yes | no | no | 4/7 | 13 |
| 28PC/u | F | 33 | no | yes | yes | no | yes | no | no | no | 2/3 | 0 |
| 29RS/v | F | 54 | yes | no | no | yes | no | yes | no | no | 2/3.5 | 0 |
| 30RA/w | F | 31 | yes | yes | yes | yes | yes | yes | no | no | 2/3.5 | 0 |
| 31RM/x | F | 22 | yes | yes | no | yes | no | no | no | no | 0/2 | 0 |
| 32SD/y | M | 32 | yes | yes | yes | yes | yes | yes | no | no | 2/3.5 | 0 |
| 33SR/z | F | 49 | yes | yes | yes | no | no | yes | no | no | 3/3.5 | 13 |
| 34SV/z | M | 42 | yes | yes | yes | yes | no | yes | no | yes | 2/3.5 | 13 |
| 35TC/aa | F | 44 | yes | yes | yes | yes | no | yes | no | no | 2/3 | 0 |
| 36TMR/ab | F | 42 | yes | yes | no | yes | no | yes | no | no | 2/3.5 | 9 |
| 37VS/ac | F | 48 | no | yes | yes | no | no | no | no | no | 1/1.5 | 0 |
| 38/ZD/ad | F | 46 | yes | yes | yes | no | yes | yes | no | no | 3/4.5 | 0 |

**Table 1.** Demographic information, clinical picture (including neurological disability scores and treatment duration), and molecular analysis of the 38 CTX patients enrolled for the study.

MRI was performed by using 2 types of MRI 1.5T machines (Philips Gyroscan NT, Philips Medical Systems, Best, The Netherlands; Magnetom Avanto, Siemens AG Healthcare Sector, Erlangen, Germany) and included 5 mm T1W spin-echo imaging, T2W imaging, FLAIR imaging, T2*-weighted gradient echo (GRE) imaging, susceptibility weighted imaging (SWI), diffusion weighted imaging (DWI). Four neuroradiologists were involved in imaging analysis: three of them (NDS, PG, and LM) had 25-year experience and one (IG) 10-year experience with neuroimaging; they were aware of the diagnosis, but blinded to clinical status and treatment. To assess the severity of parenchymal signal alterations, we used a 5-point scale: score 0 (absent) if signal intensity abnormalities were absent; score 1 (mild) if signal intensity abnormalities were present and had a maximum diameter larger than 1 mm and smaller than 2 mm in only 1 acquired axial slice; score 2 (moderate) if signal abnormalities were present and had a diameter larger than 2 mm and smaller than 4 mm in more than 1 acquired axial slice; score 3 (marked) if signal abnormalities were present and had diameter larger than 4 mm and smaller than 6 mm in more than 1 acquired axial slice; score 4 (severe) if signal abnormalities were present and had a diameter larger than 6 mm in more than 1 acquired axial slice. The presence/absence of cortical atrophy was established qualitatively by observing the following linear distances: a) the bifrontal span of the lateral ventricle, b) the width of the lateral ventricles at the head of the caudate nucleus, c) the sum of the separate widths of the left and right sylvian fissures, d) the minimum width of the bodies of the lateral ventricles at the waist, and e) the width of the third ventricle (the minimum distance between the thalamic inner boundaries). To evaluate if a progression of brain atrophy occurred over time in a single patient, the ventricular enlargement in relation to the atrophy of the surrounding

structures was assessed and the width of the third ventricle was measured. Cerebellar atrophy was assessed qualitatively based on enlargement of the peri-mesencephalic-subarachnoid spaces, cerebellar cisterns, and fourth ventricle. To evaluate if a progression of cerebellar atrophy occurred over time in a single patient, a qualitative examination was performed. At baseline, a Spearman correlation was evaluated between the MRI outcomes and both the disability scores and the clinical expression of the disease. Similarly, at follow-up, a Spearman correlation was evaluated between baseline MRI outcomes and changes in disability scores and, when it was possible, between changes in MRI outcomes and in disability scores. All the analyses were corrected for age and gender and performed by using R (www.r-project.org). Given the exploratory nature of this study and the limited size of the population, the correlation was considered significant for $p<0.05$.

### 4.3 Results

We found some kind of alteration in all brain MR exams: the main pathological findings were supra- and infratentorial atrophy, subcortical and periventricular white matter abnormalities, brainstem lesions, and cerebellar parenchymal alterations involving the dentate nuclei and the surrounding white matter. Brain MRI findings in all the 38 patients are summarized in Table 2. Cerebral cortical and central atrophy was observed in 26/38 (68%) patients: it correlated with age but not with global disability. We did not observe a higher frequency of cortical atrophy in patients with cognitive impairment compared to those without cognitive disturbances. Parenchymal signal alterations were present in all patients, and white matter abnormalities were disclosed in 37/38 (97%) subjects. Signal intensity increase in the periventricular white matter on

T2W and/or FLAIR images was observed in 34/38 (89%) patients: it was mild in 19 cases, moderate in 8, marked in 6, and severe in only 1 subject. Although very frequent, the presence and the extent of periventricular signal intensity changes did not correlate with age, disability, or other MRI abnormalities, and was not associated with a particular phenotype. Subcortical white matter T2W and/or FLAIR hyperintensity was observed in 22/38 (58%) patients: it was mild in 16 cases, moderate in 5, and marked in only 1 subject. Subcortical white matter abnormalities did not correlate to demographic, clinical or MRI parameters. Brainstem T2W and/or FLAIR hyperintense lesions were present in 20/38 (53%) patients: they were mild in 11 cases, moderate in 5, marked in 2, and severe in 2 subjects. The presence of brainstem lesions positively correlated with disability expressed by RS ($r=0.52$; $p<0.002$) and EDSS ($r=0.52$; $p<0.002$); however, it was not associated to a peculiar clinical expression. Cerebellar atrophy was present in 27/38 (71%) patients and was significantly more frequent in subjects with ataxia compared to those without cerebellar disturbances ($p<0.05$). Cerebellar dentate nuclei T2W and/or FLAIR hyperintensity was found in 32/38 (84%) patients: it was mild in 14 cases, moderate in 9, marked in 5, and severe in 4 subjects. We found a significant correlation between the extent of dentate hyperintense lesions and disability expressed by RS ($r=0.45$; $p<0.007$) and EDSS ($r=0.63$; $p<0.00001$). Dentate nuclei hyperintensity correlated with ataxia ($p<0.00001$), but also with spasticity ($p<0.001$) and cognitive impairment ($p<0.003$). Calcifications of the dentate nuclei were observed on MRI (SWI and/or GRE) and confirmed by CT scan in 8/38 (21%) patients: they were mild in 5 cases and moderate in 3 subjects. We observed a correlation with disability expressed by RS ($r=0.4$; $p<0.02$) and EDSS ($r=0.52$; $p<0.002$), as well as with ataxia ($p<0.05$). Cerebellar white matter T2W and/or FLAIR

hyperintensity was observed in 21/38 (55%) patients: it was mild in 9 cases, moderate in 4, marked in 5, and severe in 3 subjects. We observed a correlation with disability expressed by EDSS ($r=0.34$; $p<0.05$), as well as with ataxia ($p<0.005$). Cerebellar vacuolation with hypointensity in T1W and FLAIR images was observed in 10/38 (26%) patients: it was mild in 5 cases, moderate in 3, and marked in 2 subjects. The development of vacuoles was not age-related, and was significantly related to the extent of dentate nuclei hyperintensity ($r=0.52$; $p<0.001$). Cerebellar vacuolation correlated with disability expressed by RS ($r=0.42$; $p<0.02$) and EDSS ($r=0.49$; $p<0.003$), and strongly correlated with ataxia ($p<0.0003$). Figure 1 shows the wide MRI spectrum of cerebellar signal alterations, and figure 2 shows the grading of dentate nuclei FLAIR hyperintensity. Spinal cord MRI was performed in 6 patients (3, 11, 27, 30, 31, 38): no spinal atrophy was found, while bands of T2W hyperintensity restricted to the lateral and dorsal columns were observed in 2 patients (30, 38). Figure 3 summarizes the main brain and spinal MRI findings. In 16 patients who were untreated at baseline and then started CDCA, we performed clinical and MRI follow up after 24-48 months (Table 3). Clinically, 10 subjects were considered “neurologically stable”, while 6 patients were regarded as “neurologically worsening” since their disability scores increased. Brain MRI was completely unchanged in 6/10 “neurologically stable” patients and in 1/6 “neurologically worsening” patients, while it revealed some slight changes in 4/10 “neurologically stable” patients and important increase in atrophy and/or signal alteration in 5/6 “neurologically worsening” subjects. Figure 4 shows the main brain MRI changes at follow up in a “neurologically worsening” patient. Interestingly, we observed that different clinical and neuroradiological evolution was associated with the presence of different patterns of cerebellar involvement at baseline: a) in all the 6

subjects who at baseline showed cerebellar vacuolation in addition to dentate nuclei T2W/FLAIR hyperintensity, MRI worsened at follow up, and in 5 of them also neurological disability worsened; b) in the 6 patients presenting dentate nuclei T2W/FLAIR hyperintensity without vacuolation at baseline, MRI changes were absent or very mild at follow up and only 1 subject clinically worsened; c) all the 4 patients showing absence of dentate nuclei lesions at baseline were clinically and neuroradiologically stable at follow up evaluation. None of these 16 patients had or developed dentate nuclei calcification. The pattern of progression of white matter lesions at follow up was also very interesting: a) subcortical white matter involvement was unchanged in all patients regardless of clinical evolution; b) periventricular white matter T2W/FLAIR hyperintensity slightly increased in only 2 worsening subjects; c) cerebellar white matter alterations increased in 5 patients, 4 of which presented neurological progression. The presence of brainstem lesions at baseline did not seem to be associated in itself to a worse clinical outcome. However, at follow up brainstem lesions increased in 2 patients with neurological worsening. Both cortical and cerebellar atrophy increased at follow up in 5 patients, but a clear correlation with clinical outcome was not observed. Statistically, we were able to establish a strong correlation between the presence of cerebellar vacuolation at baseline and a clinical progression at follow up as documented by RS ($r=0.74$; $p<0.003$) and EDSS ($r=0.77$; $p<0.002$).

| Pt/Fm | Cortical atrophy | Subcortical WM T2/FLAIR hyperintensity | Periventricular WM T2/FLAIR hyperintensity | Brainstem T2/FLAIR hyperintensity | Cerebellar atrophy | Cerebellar WM T2/FLAIR hyperintensity | Dentate nuclei T2/FLAIR hyperintensity | Dentate nuclei calcification | Cerebellar vacuolation |
|---|---|---|---|---|---|---|---|---|---|
| 1AL/a | No | 0 | 1 | 0 | No | 0 | 1 | 0 | 0 |
| 2AN/a | No | 1 | 1 | 0 | No | 0 | 1 | 0 | 0 |
| 3BMC/b | No | 0 | 0 | 0 | Yes | 1 | 2 | 1 | 0 |
| 4BMG/b | Yes | 2 | 3 | 2 | Yes | 0 | 2 | 1 | 0 |
| 5BPR/b | Yes | 0 | 1 | 0 | No | 0 | 1 | 0 | 0 |
| 6BG/c | Yes | 0 | 4 | 4 | Yes | 4 | 4 | 2 | 2 |
| 7CP/d | Yes | 0 | 1 | 1 | Yes | 3 | 4 | 1 | 0 |
| 8CF/e | Yes | 2 | 2 | 0 | No | 0 | 1 | 0 | 0 |
| 9CU/f | Yes | 1 | 2 | 1 | Yes | 3 | 2 | 0 | 0 |
| 10CG/g | No | 0 | 1 | 0 | No | 1 | 2 | 0 | 0 |
| 11CR/g | No | 1 | 0 | 0 | Yes | 0 | 1 | 0 | 0 |
| 12DSC/h | Yes | 1 | 1 | 0 | Yes | 0 | 0 | 0 | 0 |
| 13DFB/i | Yes | 0 | 3 | 2 | Yes | 0 | 0 | 0 | 0 |
| 14DGM/j | No | 1 | 1 | 0 | Yes | 1 | 1 | 0 | 0 |
| 15DGS/j | Yes | 1 | 1 | 1 | Yes | 2 | 2 | 0 | 1 |
| 16DMLo/k | Yes | 3 | 3 | 3 | Yes | 3 | 3 | 2 | 3 |
| 17DMLu/k | Yes | 1 | 1 | 2 | Yes | 3 | 3 | 1 | 2 |
| 18FG/l | Yes | 0 | 1 | 0 | Yes | 0 | 3 | 0 | 1 |
| 19FP/m | Yes | 1 | 1 | 3 | Yes | 3 | 1 | 0 | 3 |
| 20GL/n | No | 0 | 1 | 0 | No | 0 | 0 | 0 | 0 |
| 21GM/n | No | 0 | 1 | 0 | No | 0 | 0 | 0 | 0 |
| 22IS/o | Yes | 0 | 2 | 0 | Yes | 0 | 1 | 0 | 0 |
| 23LM/p | Yes | 1 | 2 | 0 | Yes | 0 | 1 | 0 | 0 |
| 24LA/q | Yes | 1 | 2 | 1 | Yes | 0 | 1 | 0 | 0 |
| 25LC/r | Yes | 0 | 2 | 0 | Yes | 0 | 3 | 0 | 0 |
| 26LR/s | Yes | 1 | 1 | 1 | Yes | 1 | 1 | 0 | 0 |
| 27PG/t | Yes | 2 | 2 | 2 | Yes | 4 | 4 | 1 | 0 |
| 28PC/u | Yes | 1 | 1 | 1 | Yes | 2 | 2 | 0 | 1 |
| 29RS/v | Yes | 2 | 1 | 1 | Yes | 2 | 2 | 0 | 1 |
| 30RA/w | No | 0 | 2 | 1 | No | 1 | 2 | 0 | 0 |
| 31RM/x | No | 1 | 1 | 1 | No | 1 | 0 | 0 | 0 |
| 32SD/y | Yes | 2 | 3 | 4 | Yes | 4 | 4 | 2 | 2 |
| 33SR/z | Yes | 1 | 1 | 1 | Yes | 1 | 1 | 0 | 0 |
| 34SV/z | No | 0 | 0 | 0 | No | 0 | 1 | 0 | 0 |
| 35TC/aa | Yes | 1 | 3 | 1 | Yes | 1 | 2 | 0 | 0 |
| 36TMR/ab | No | 1 | 0 | 0 | No | 1 | 1 | 0 | 0 |
| 37VS/ac | Yes | 0 | 1 | 0 | Yes | 0 | 0 | 0 | 0 |
| 38ZD/ad | Yes | 0 | 3 | 2 | Yes | 2 | 3 | 0 | 1 |

**Table 2.** Brain MRI findings (baseline evaluation) in all the 38 CTX patients enrolled for the study.

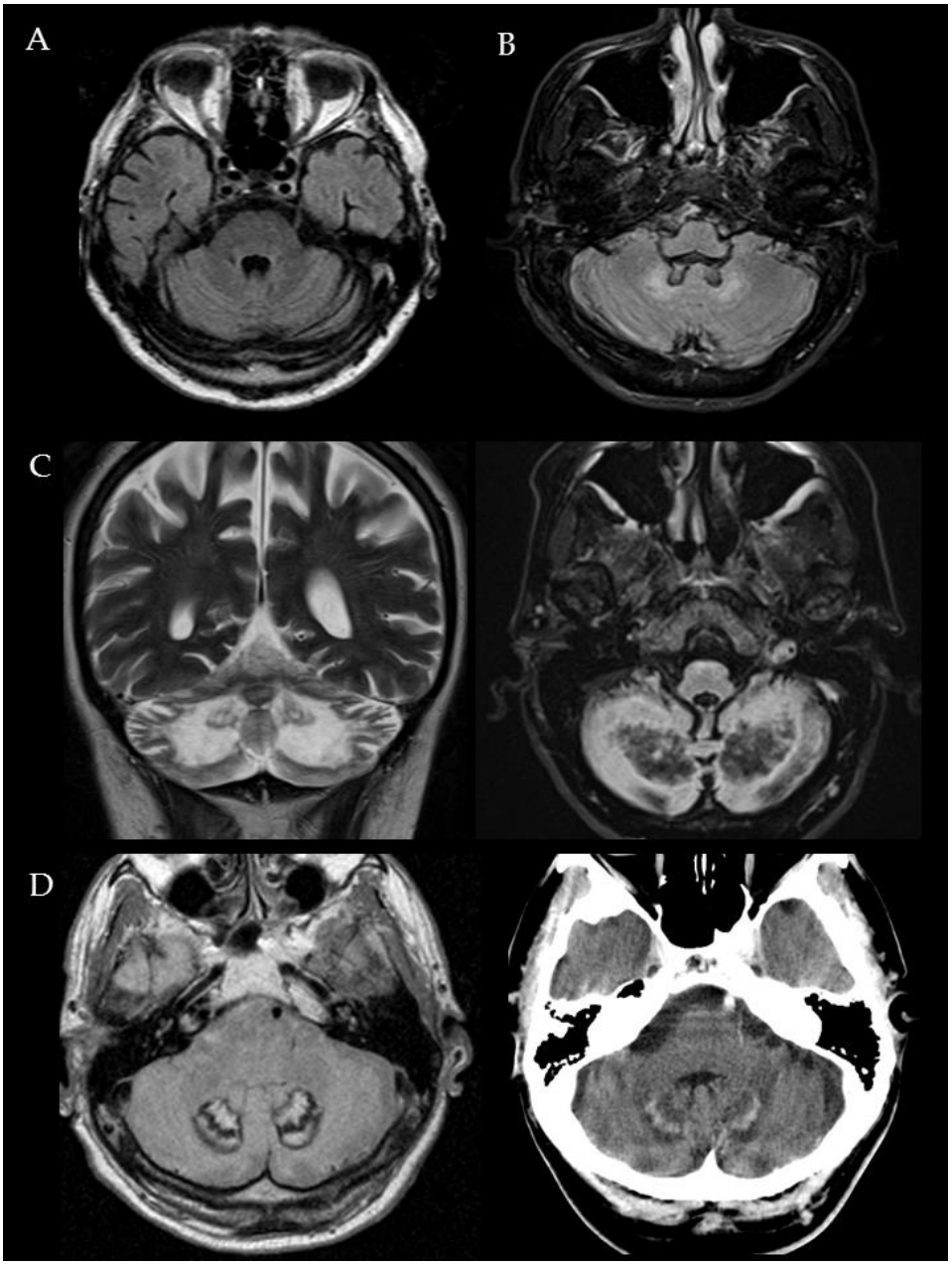



**Fig. 1.** The MRI spectrum of cerebellar parenchymal abnormalities in CTX. A) Axial FLAIR image shows absence of cerebellar signal abnormalities (patient 13). B) Axial FLAIR image reveals bilateral dentate nuclei hyperintensity (patient 10). C) Coronal T2W image (left) shows bilateral hyperintensity of dentate nuclei and surrounding white matter, and axial FLAIR image (right) shows in the same areas low signal intensity consistent with cerebellar vacuolation (patient 28). D) Axial FLAIR image (left) shows bilateral hyperintensity of the dentate nuclei with a central stripe of low signal suggesting calcification, and brain CT (right) shows in the same areas hypodense signal surrounding hyperdense lesions which are consistent with calcification (patient 7).

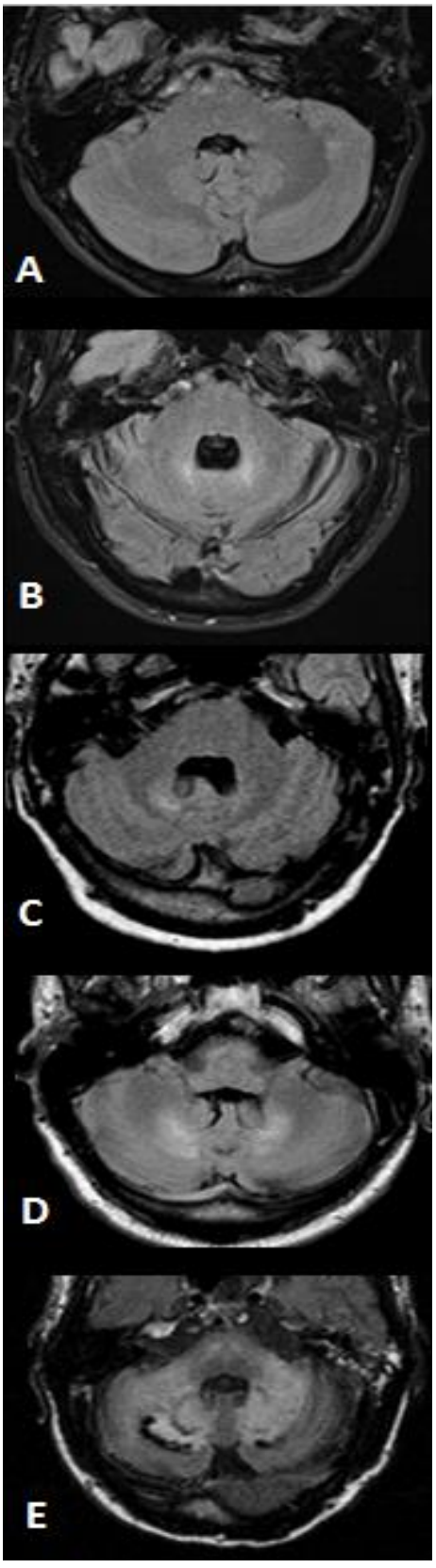



**Fig. 2.** Grading of dentate nuclei hyperintensity using axial FLAIR images: A) score 0 (patient 21); B) score 1 (patient 24); C) score 2 (patient 10); D) score 3 (patient 25); E) score 4 (patient 32).

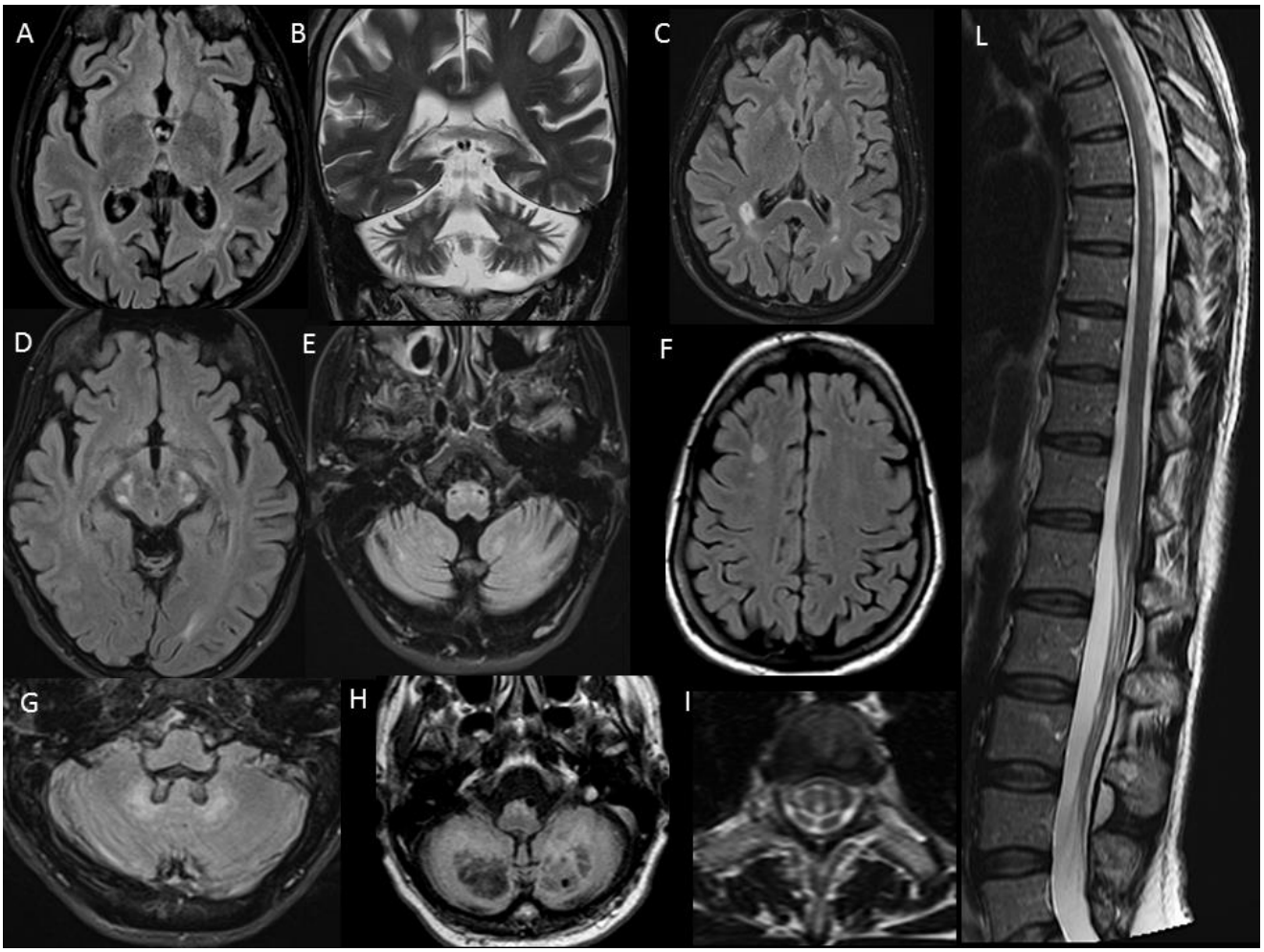



**Fig. 3.** A summary of the main MRI findings in CTX. A) Axial FLAIR image shows cortical atrophy (patient 37). B) Coronal T2W image demonstrates cortical atrophy, marked cerebellar atrophy, and hyperintensity of dentate nuclei and surrounding white matter (patient 19). C) Axial FLAIR image shows posterior periventricular white matter lesions (patient 23). D) Axial FLAIR image shows bilateral hyperintense lesions of the midbrain (patient 16). E) Axial FLAIR image shows bilateral small hypointense lesions (vacuoles) in the medulla oblongata (patient 16). F) Axial FLAIR image demonstrates white matter lesions in the subcortical white matter (patient 8). G) Axial FLAIR image demonstrates bilateral dentate nuclei hyperintensity (patient 10) H) Axial FLAIR image shows areas of low signal intensity consistent with cerebellar vacuolation (patient 38). I) Axial and L) sagittal T2W images show cervical spinal cord involvement with white matter abnormalities in the lateral and dorsal columns (patient 38).

| BASELINE EVALUATION (UNTREATED PATIENTS) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Pt/Fm** | **Age** | **RS/ EDSS** | **Cortical atrophy** | **Subcortical WM T2/FLAIR hyperintensity** | **Periventricular WM T2/FLAIR hyperintensity** | **Brainstem T2/FLAIR hyperintensity** | **Cerebellar atrophy** | **Cerebellar WM T2/FLAIR hyperintensity** | **Dentate nuclei T2/FLAIR hyperintensity** | **Dentate nuclei calcification** | **Cerebellar vacuolation** |
| 10CG/g | 21 | 1/3 | No | 0 | 1 | 0 | No | 1 | 2 | 0 | 0 |
| 11CR/g | 18 | 0/2 | No | 1 | 0 | 0 | Yes | 0 | 1 | 0 | 0 |
| 14DGM/j | 29 | 1/2.5 | No | 1 | 1 | 0 | Yes | 1 | 1 | 0 | 0 |
| 15DGS/j | 32 | 2/4 | Yes | 1 | 1 | 1 | Yes | 2 | 2 | 0 | 1 |
| 18FG/l | 45 | 3/5 | Yes | 0 | 1 | 0 | Yes | 0 | 3 | 0 | 1 |
| 19FP/m | 40 | 3/4 | Yes | 1 | 1 | 3 | Yes | 3 | 1 | 0 | 3 |
| 20GL/n | 25 | 1/1.5 | No | 0 | 1 | 0 | No | 0 | 0 | 0 | 0 |
| 21GM/n | 13 | 0/0 | No | 0 | 1 | 0 | No | 0 | 0 | 0 | 0 |
| 22IS/o | 35 | 2/3 | Yes | 0 | 2 | 0 | Yes | 0 | 1 | 0 | 0 |
| 28PC/u | 33 | 2/3 | Yes | 1 | 1 | 1 | Yes | 2 | 2 | 0 | 1 |
| 29RS/v | 54 | 2/3.5 | Yes | 2 | 1 | 1 | Yes | 2 | 2 | 0 | 1 |
| 30RA/w | 31 | 2/3.5 | No | 0 | 2 | 1 | No | 1 | 2 | 0 | 0 |
| 31RM/x | 22 | 0/2 | No | 1 | 1 | 1 | No | 1 | 0 | 0 | 0 |
| 35TC/aa | 44 | 2/3 | Yes | 1 | 3 | 1 | Yes | 1 | 2 | 0 | 0 |
| 37VS/ac | 48 | 1/1.5 | Yes | 0 | 1 | 0 | Yes | 0 | 0 | 0 | 0 |
| 38/ZD/ad | 46 | 3/4.5 | Yes | 0 | 3 | 2 | Yes | 2 | 3 | 0 | 1 |
| **FOLLOW UP EVALUATION (UNDER CDCA TREATMENT)** | | | | | | | | | | | |
| **Pt/Fm** | **CDCA (months)** | **RS/ EDSS** | **Cortical atrophy** | **Subcortical WM T2/FLAIR hyperintensity** | **Periventricular WM T2/FLAIR hyperintensity** | **Brainstem T2/FLAIR hyperintensity** | **Cerebellar atrophy** | **Cerebellar WM T2/FLAIR hyperintensity** | **Dentate nuclei T2/FLAIR hyperintensity** | **Dentate nuclei calcification** | **Cerebellar vacuolation** |
| 10CG/g | 24 | 1/3 (-) | No (-) | 0 (0) | 1 (0) | 0 (0) | No (-) | 1 (0) | 2 (0) | 0 (0) | 0 (0) |
| 11CR/g | 24 | 0/2 (-) | No (-) | 1 (0) | 0 (0) | 0 (0) | Yes (-) | 0 (0) | 1 (0) | 0 (0) | 0 (0) |
| 14DGM/j | 48 | 2/4 (+) | No (-) | 1 (0) | 1 (0) | 0 (0) | Yes (+) | 2 (1) | 1 (0) | 0 (0) | 0 (0) |
| 15DGS/j | 48 | 3/6 (+) | Yes (-) | 1 (0) | 1 (0) | 1 (0) | Yes (-) | 2 (0) | 2 (0) | 0 (0) | 1 (0) |
| 18FG/l | 24 | 4/6 (+) | Yes (+) | 0 (0) | 2 (1) | 0 (0) | Yes (-) | 1 (1) | 3 (0) | 0 (0) | 2 (1) |
| 19FP/m | 24 | 4/6 (+) | Yes (+) | 1 (0) | 1 (0) | 3 (0) | Yes (+) | 4 (1) | 1 (0) | 0 (0) | 3 (0) |
| 20GL/n | 48 | 1/1.5 (-) | No (-) | 0 (0) | 1 (0) | 0 (0) | No (-) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 21GM/n | 48 | 0/0 (-) | No (-) | 0 (0) | 1 (0) | 0 (0) | No (-) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 22IS/o | 36 | 2/3 (-) | Yes (-) | 0 (0) | 2 (0) | 0 (0) | Yes (-) | 0 (0) | 1 (0) | 0 (0) | 0 (0) |
| 28PC/u | 48 | 4/7 (+) | Yes (+) | 1 (0) | 1 (0) | 4 (3) | Yes (+) | 2 (0) | 4 (2) | 0 (0) | 4 (3) |
| 29RS/v | 36 | 2/3.5 (-) | Yes (-) | 2 (0) | 1 (0) | 1 (0) | Yes (-) | 3 (1) | 3 (1) | 0 (0) | 2 (1) |
| 30RA/w | 48 | 2/3.5 (-) | No (-) | 0 (0) | 2 (0) | 1 (0) | Yes (+) | 1 (0) | 3 (1) | 0 (0) | 0 (0) |
| 31RM/x | 48 | 0/2 (-) | No (-) | 1 (0) | 1 (0) | 1 (0) | No (-) | 1 (0) | 0 (0) | 0 (0) | 0 (0) |
| 35TC/aa | 48 | 2/3 (-) | Yes (+) | 1 (0) | 3 (0) | 1 (0) | Yes (-) | 1 (0) | 2 (0) | 0 (0) | 0 (0) |
| 37VS/ac | 24 | 1/1.5 (-) | Yes (+) | 0 (0) | 1 (0) | 0 (0) | Yes (+) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 38/ZD/ad | 36 | 4/7 (+) | Yes (-) | 0 (0) | 4 (1) | 3 (1) | Yes (+) | 4 (2) | 4 (1) | 0 (0) | 3 (2) |

**Table 3.** Disability scores and brain MRI findings (baseline and follow up evaluations) in 16 patients who were initially untreated and then were put on oral therapy with CDCA 750 mg/day.

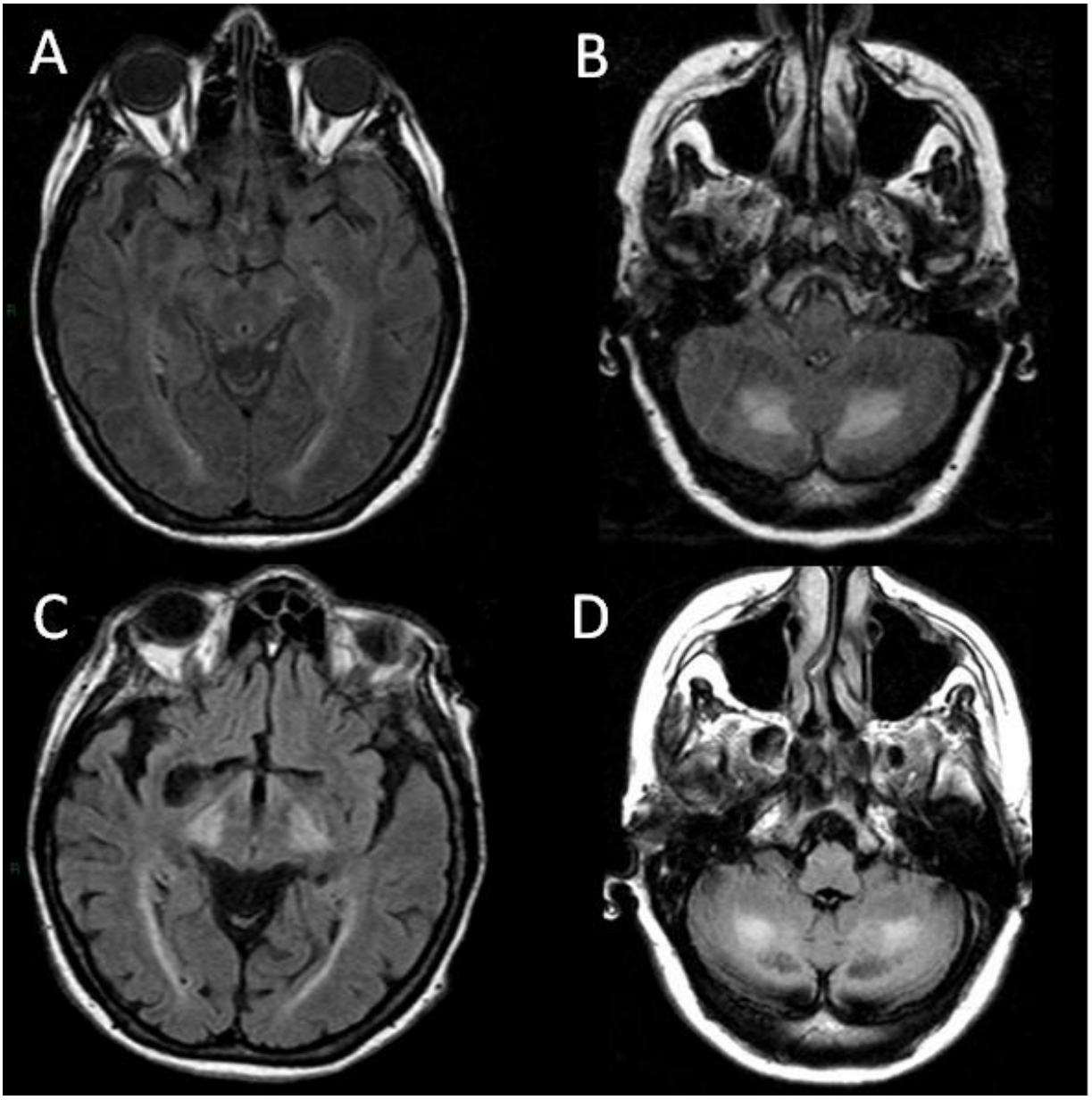



**Fig. 4.** Brain MRI findings in a "neurologically worsening" CTX subject (patient 38). Axial FLAIR images at baseline show hyperintense signal in the periventricular white matter and brainstem (A), and bilateral hyperintensity of the dentate nuclei and surrounding white matter which also shows areas of slightly hypointense signal (B); axial FLAIR images at 36-months follow up show marked increase of periventricular and brainstem hyperintense lesions (C), and increase of hyperintensity in the dentate nuclei and surrounding white matter with cerebellar vacuolation replacing part of the hyperintense areas (D).

## 4.4 Discussion

Since the first case reports, the idea took shape that brain MRI could become a very useful clinical tool in CTX diagnosis and, possibly, prognosis. In the early nineties, neuroimaging studies on different CTX populations (Hokezu et al 1992; Berginer et al 1994; Dotti et al 1994) draw the following conclusions: (a) MRI was the gold standard to show diffuse or focal lesions of cerebral white matter and cerebellum; (b) lesions of the dentate nuclei and surrounding white matter could reflect demyelination, necrosis or cystic spaces; (c) there was no correlation between biochemical abnormalities and the severity of MRI alterations. On the other hand, controversies emerged on the possible correlation between clinical phenotype and neuroradiological findings. In particular, since an MRI follow up during CDCA treatment did not reveal substantial changes in a group of 11 patients, MRI was considered little helpful for treatment monitoring. More recent MRI studies focused on patients with peculiar cerebellar features consisting on T1W and FLAIR hypointensities suggestive of neuroaxonal loss (Mignarri et al 2012; Androdias et al 2012). We hypothesized to consider cerebellar vacuolation, which we observed in a long-term follow up of a patient with worsening in spite of treatment, as a possible marker of poor outcome. Since MRI is usually performed in CTX patients before the diagnosis is made, our detailed description of the complex spectrum of MRI abnormalities in CTX brains should contribute to the earlier recognition. A remarkable preference for some grey matter nuclei with extension to the adjacent white matter is typical and therefore strongly suggestive of CTX: infratentorial lesions involving the dentate nuclei and surrounding white matter, and less frequently the brainstem, should lead to suspect CTX. However, this finding is not invariably present, since cerebellar signal alterations can be subtle or absent in some CTX patients

who may show only slight supratentorial abnormalities. While the latter seem to be nonspecific and do not correlate with disability or particular phenotypes, the lesions of the infratentorial regions, and especially those of the cerebellum, could offer insights into the pathological process underlying neurological impairment and the natural history of the disease. We found a significant correlation between the presence and the extent of cerebellar parenchymal alterations and the clinical disability, in spite of the presence or not of cerebellar symptoms. In particular, dentate nuclei T2W/FLAIR hyperintensity correlated not only with ataxia, but also with spasticity and dementia. These findings are in line with the growing evidence that the cerebellum has a complex role in movement and cognition, and alteration of cerebellar structures may lead to a large spectrum of dysfunctions (Koziol et al 2014). We were also surprised to observe in a considerable number of patients, in addition to the "classical" T2W/FLAIR hyperintensity of dentate nuclei and surrounding white matter, the presence of vacuolation and calcification. In particular, cerebellar vacuoles seemed to be relevant in clinical respect since they were not age-related and positively correlated to the extent of dentate nuclei hyperintensity. The variable appearance of cerebellar abnormalities seems to provide a snapshot into the neuropathology underlying CTX. Lipid accumulation results in a neurotoxic effect on the metabolic apparatus of neurons with subsequent demyelination and axonal degeneration, the latter being predominant especially in the later stages of the disease (Soffer et al 1995; Pilo de la Fuente et al 2008). Our MR images of the cerebellum may reflect the histopathological findings: T2W/FLAIR hyperintensities are probably the initial result of abnormal lipid storage, while vacuolation and calcification could be the result of secondary degeneration caused by cholestanol-induced apoptosis (Inoue et al 1999) or by other yet undefined

mechanisms leading to axonal damage. Our long-term follow up evaluation revealed important information on lesions evolution and suggested to consider some MRI markers that can be related to clinical progression. Broadly, we noticed that clinical and neuroradiological stability goes hand in hand over time, while increased atrophy and/or signal alterations at follow up was almost invariably present in "neurologically worsening" patients. What is more important, we obtained very useful information comparing MRI appearance of the cerebellar parenchyma at baseline with clinical and neuroradiological evolution: the presence of cerebellar vacuolation was predictive of clinical and MRI worsening despite CDCA therapy, while the sole presence of dentate nuclei T2W/FLAIR hyperintensity was associated to a low risk of progression, and the absence of signal alterations was related to clinical and neuroradiological stability over time. Notably, cerebellar vacuolation can be regarded as the first available biomarker of disease progression. In fact, biochemical studies failed to detect reliable markers of disease outcome (Mignarri et al 2016). Our data may suggest that patients showing MRI evidence of cerebellar vacuolation should be monitored more strictly over time and could be the ones to select for additional or experimental therapies.The limitations of this work were (a) its retrospective nature, (b)  (e) the unavailability of a specific disability scale for CTX, (f) the absence of an untreated control group at follow up, and (g) the absence of autopsy data. Having in mind these limits, we can affirm that our study widely improves the knowledge on neuroimaging profile in CTX and identifies peculiar MRI patterns related to clinical status and especially to the disease outcome, which is a crucial endpoint in a treatable disease.

# 5. Very early treatment with chenodeoxycholic acid can prevent neurological damage in cerebrotendinous xanthomatosis: a paediatric case report

## 5.1 Introduction

A wide range of clinical manifestations of CTX is described according to the different age of presentation. In infancy the first manifestation may be cholestatic jaundice: it usually improves spontaneously and if no further investigation is perfomed the disease remains undiagnosed. Few cases of cholestatic presentations are described and most cases are retrospectively reported from adult patients (Mignarri et al 2014). In the first decade of life CTX may presents with intellectual disability and diarrhoea. The diagnosis is often delayed till the second/third decade, when cataract and tendon xanthomas present, or till the fourth decades, when neurological involvement (spastic paresis, ataxia, polyneuropathy) becomes manifest. As it is possible to treat CTX patients with CDCA, an early diagnosis is crucial to prevent further progression of the disease. Here, we report a case of a child presenting with neonatal cholestasis and diagnosed as CTX in the first year of life. She was promptly treated with CDCA and presented a normal neurological development.

## 5.2 Case report

The patient was born after an uncomplicated pregnancy and delivery. The parents are not consanguineous and did not present any ophthalmologic, neurologic, cardiac and hepatic diseases. Breast-feeding was begun and the female child showed normal growth and development till the second month of life, when jaundice with pale stools were noticed. Physical examination showed no dysmorphic features, severe jaundice, a

slightly firm liver. Normocholic stools and normal urines were present. Ophtalmoscopic examination was normal. Liver function tests showed direct hyperbilirubinemia (maximum value 8,79 mg/dl, total bilirubin 13,56 mg/dl), ALT and AST elevation (473 UI/l, normal values 5-56 U/L; 530 UI/l, normal values 5-77 U/L), with normal gamma-GT, alkaline phosphatase and clotting factors. A percutaneous liver biopsy showed aspecific aspects of idiopathic neonatal cholestasis, such as pronounced lobular disarray, oedema and giant-cells, bile pigment within hepatocytes, with slightly enlarged portal tracts. A parenteral supplementation with fat soluble vitamins was initiated and a therapy with ursodeoxycholic acid (UDCA) at the dosage of 15mg/Kg was started at about 3 months of age. The most frequent causes of neonatal cholestasis were excluded with a complete workup. Urinary biliary acids analysis was performed by liquid chromatography tandem-mass-spectrometry and showed the presence of cholestanepentols glucuronide 38,4 ratio/mM creat (normal value: 0,86 ratio/mM creat), allowing to suspect CTX. The diagnosis was confirmed by the dosage of cholestanol in serum, showing a value of 3140 +/- 55 μg/dl (normal control value 470 μg/dl). The molecular analysis of *CYP27A1* showed homozygousity for a deletion of 1,9 Kb with loss of exon 7,8,9 on chromosome 2q33-qter. In the following months jaundice slowly disappeared, with a normalization of bilirubin and transferases respectively at 6 and 8 months. After the diagnosis of CTX, therapy with UDCA was stopped and CDCA was started at 8 months of age. The initial dose was 10 mg/Kg/day, increasing until 14 mg/Kg/day. A parallel, constant decrease of cholestanol in plasma was noticed under therapy: from 3140 μg/dl till 600 μg/dl after 14 months of treatment (Fig. 1).

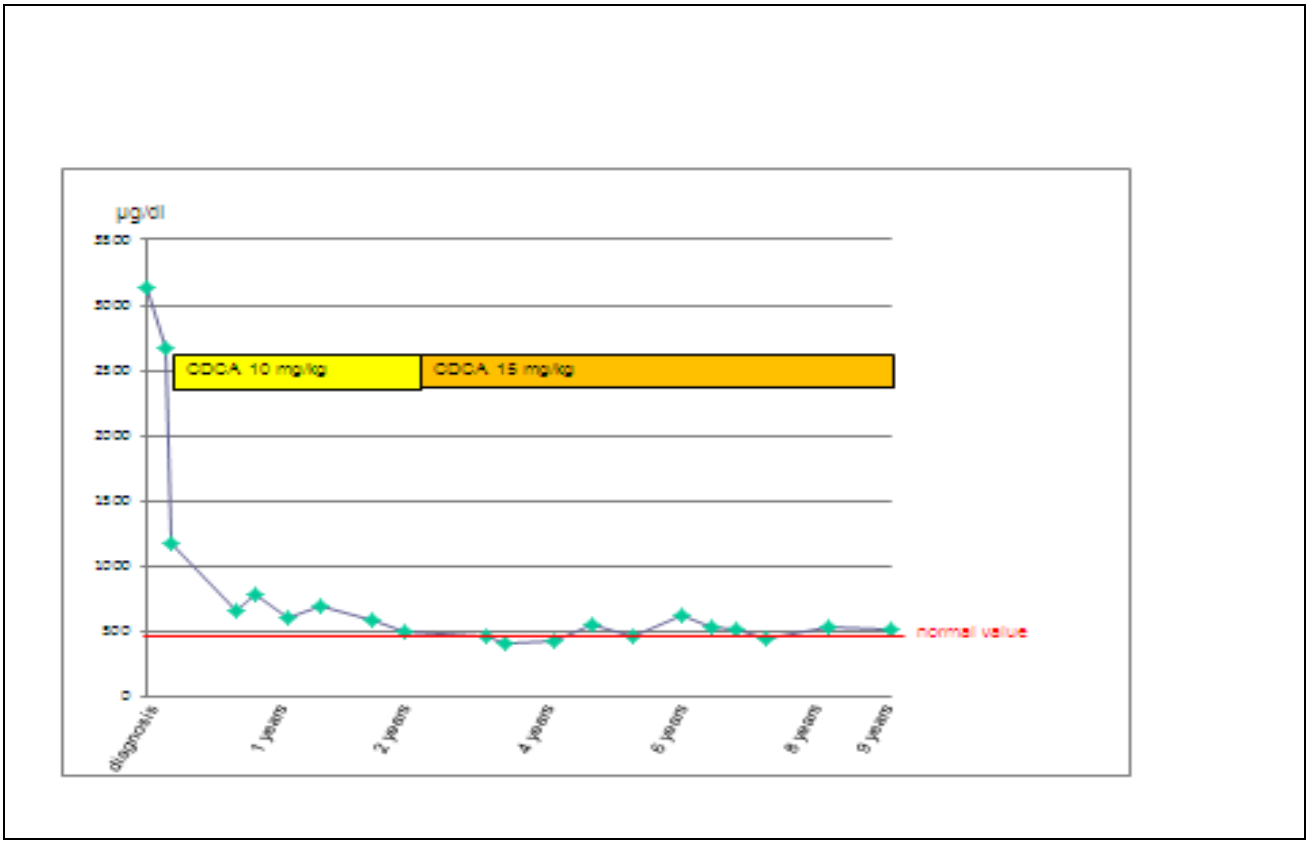



**Fig. 1.** Cholestanol values during follow-up.

In the absence of clinical manifestations and in the presence of normal laboratory values, the patient was strictly monitored with periodic assessment of urinary bile acids and plasma cholestanol to define the minimum dose capable to prevent toxic accumulation of abnormal metabolites. During the ten-year follow-up liver function tests were always in the normal limits and therapy was well tolerated with no evidence of clinical or biochemical side effects. The patient never presented cataract or diarrhoea. Concerning neurological involvement, she was clinically silent, with normal neurological examination. At age 8 brain MRI showed slight T2/FLAIR hiperintensity of the periventricular white matter, while other regions (including dentate nuclei) were completely spared (Fig. 2).

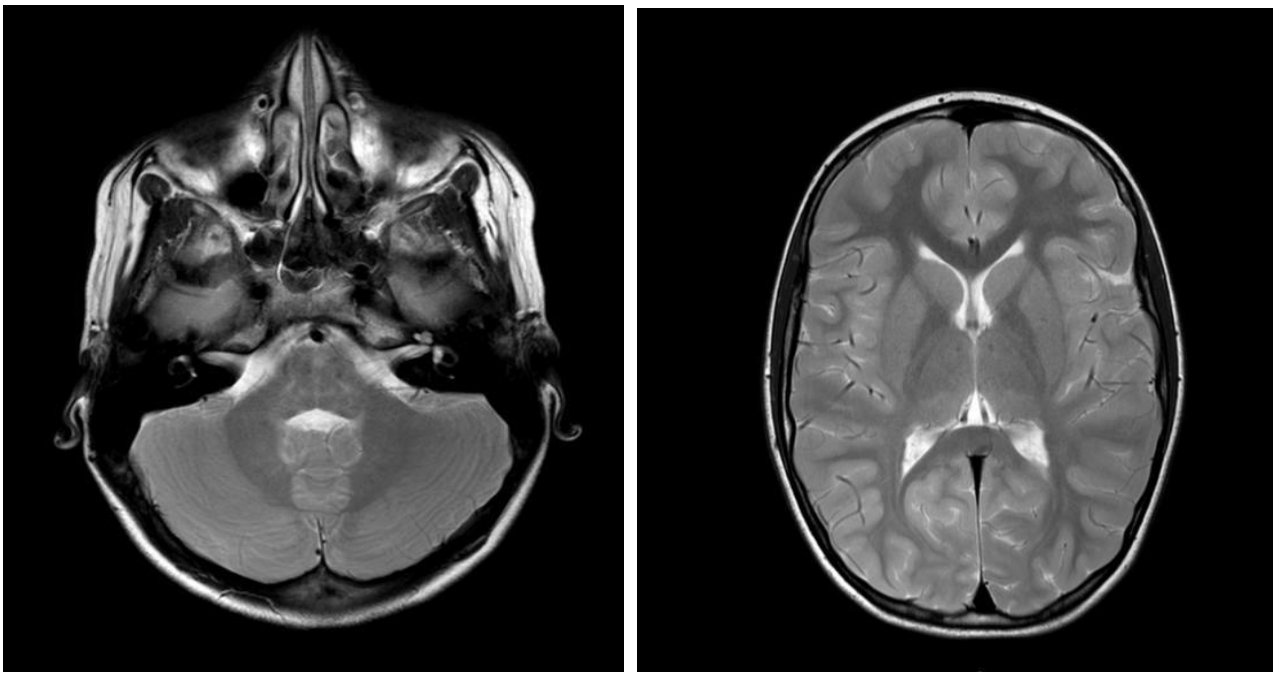


**Fig. 2.** Brain MRI showing sparing of the dentate nuclei (left) and slight hyperintensity of the periventricular white matter (right).

## 5.3 Discussion

CTX has been associated with neonatal cholestasis, which usually improves spontaneously and does not require further investigations. On the other hand, the past medical history of many patients with CTX reveals a prolonged neonatal cholestatic jaundice (Mignarri et al 2014). Cataracts, chronic diarrhea and psychomotor retardation can be the first manifestations of CTX in childhood, usually followed by tendon xanthomas and progressive neurologic impairment. Therapy with CDCA is considered the best treatment, as it inhibits defective bile acid synthesis in CTX and reduces elevated plasma and hepatic concentration of abnormal metabolites. The therapy is quite effective both in childhood and adulthood and it can lead to remarkable improvement in clinical and biochemical manifestations (Van Heijst et al 1998). Prompt preclinical administration in early childhood may prevent the cerebrotendinous

xanthomatosis phenotype. Our patient presented neonatal cholestasis, which regressed by the age of 6 months; however, at this time, her cholestanol level was already very high. The early beginning of the therapy with CDCA, with the declining of the toxic bile acid intermediates, prevented clinical deterioration. Considering the high frequency of severe clinical manifestations in CTX and the availability of an effective therapy, early diagnosis and treatment are crucial to prevent irreversible systemic and neurological damage. Since accumulation of toxic metabolites is progressive but may be clinically silent, a strict monitoring of cholestanol and cholestanepentols during the whole duration of therapy is recommendable. Our long term experience, with an early beginning of the treatment with CDCA, not only confirms the efficacy of the drug in preventing neurologic damage, but also demonstrates its total safety.

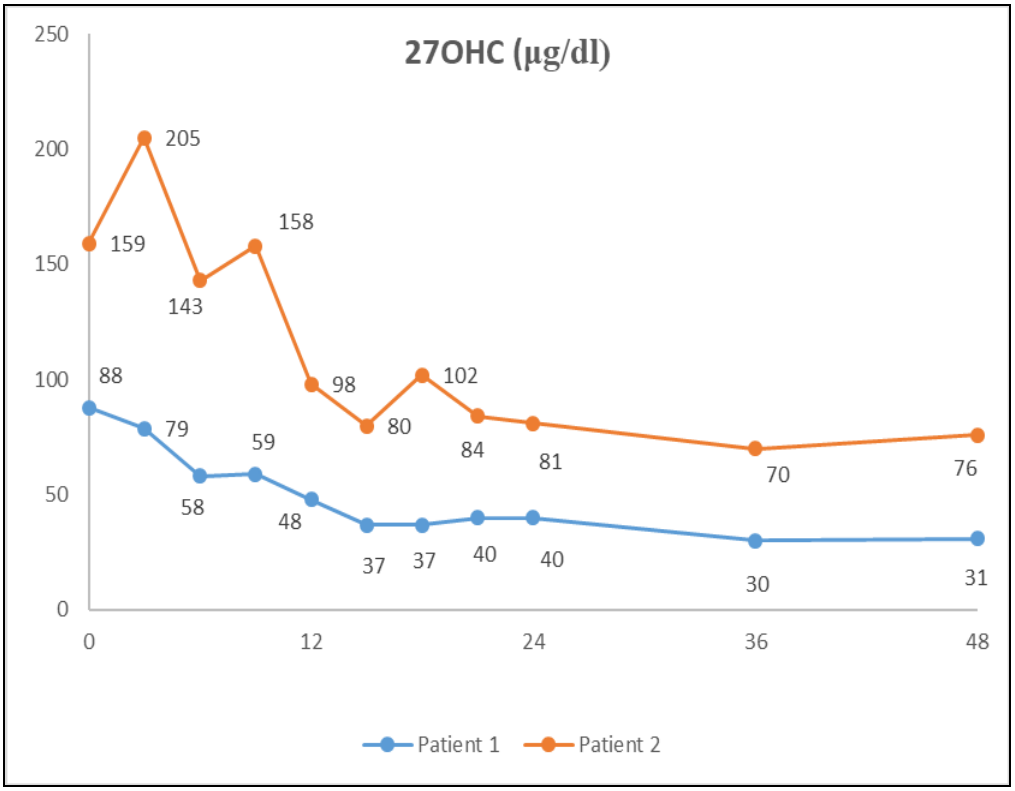
## 6. SPG5 is the first treatable form of hereditary spastic paraplegia

### 6.1 Long-term follow up data on the first SPG5 patients treated with cholesterol-lowering drugs

Spastic paraplegia type 5 (SPG5) is an autosomal recessive hereditary spastic paraparesis (HSP) caused by mutations in *CYP7B1*, which is responsible for a key step in the alternative pathway of bile acid synthesis (Tsaousidou et al 2008). Both pure and complicated clinical forms are possible, and brain magnetic resonance imaging (MRI) might show periventricular and subcortical white matter involvement (Goizet et al 2009). Electrophysiology reveals abnormal conduction along the central pathways and peripheral nervous system sparing (Criscuolo et al 2009). SPG5 patients have increased levels of 27-hydroxycholesterol (27OHC) in plasma and cerebrospinal fluid (CSF) (Schüle et al 2010). Cerebral accumulation of 27OHC may be an important pathogenetic event in SPG5, with relevant therapeutic implications: indeed, since the substrate availability is a limiting factor for CYP27A1 activity (Pandak et al 2002) and brain 27OHC has an extracerebral origin (Heverin et al 2005), HMG CoA reductase inhibitors might reduce 27OHC thus preventing neurological impairment. Here we describe two SPG5 patients and report the follow up data including evaluation of response to therapy with cholesterol-lowering drugs. Patient 1 was a 29-year-old woman with a two-year history of gait disturbances whereas her 24-year-old brother referred walking difficulties and lower limbs stiffness since he was age 20. Past medical history revealed prolonged neonatal jaundice in both sibs. Neurological examination disclosed a pure clinical phenotype with spastic paraplegia rating scale (SPRS) (Schüle et al 2010) values of 15/52 and 10/52 in patients 1 and 2, respectively.

Neuropsychological assessment did not detect cognitive impairment. Brain MRI showed slight FLAIR hyperintense signal in the periventricular and centrum ovale white matter, and spectroscopy (1H MRS) on those areas showed a mild reduction of the relative ratio N-acetyl aspartate/creatine and the presence of a small lipid peak in both patients. Spinal cord MRI was normal. Motor evoked potentials (MEPs) study uncovered abnormal central motor conduction times (CMCTs) recording from both upper and lower limbs, and electromyography was normal. Gene analysis detected two compound heterozygous mutations (c.333_334delTC and c.806delA) in CYP7B1. Serum 27OHC levels were markedly elevated (Fig. 1). Both subjects underwent neurological evaluation including SPRS scoring, routine blood tests, cholesterol and 27OHC determinations, and instrumental follow up including MEPs, conventional brain MRI, and MR spectroscopy. Patient 1 received different oral doses of simvastatin (from 20 mg/day to 60 mg/day) during the first 12 months, and then oral ezetimibe 10 mg/day was added to simvastatin 40 mg/day for 36 months. Treatments were well tolerated. Serum 27OHC concentration progressively decreased under treatment with simvastatin either alone or when in combination with ezetimibe (Fig. 1), and cholesterol tended to decrease in parallel with 27OHC. Association of simvastatin and ezetimibe was more effective in reducing 27OHC than the increase of simvastatin dosage up to 60 mg/day. No changes of neurological disability as well as brain MRI and spectroscopic pattern were observed. MEPs revealed a trend toward improvement or stabilization of central motor conduction times (CMCTs). Patient 2, who did not tolerated simvastatin because of cramps and marked hyperCKemia, received ezetimibe 10 mg/day for 39 months, and tolerated well this therapy. We observed a persistent decrease of serum 27OHC values (Fig. 1) whereas cholesterol levels initially decreased

and then returned to baseline. Alike his sister, SPRS and MRS scores as well as brain MRI and spectroscopy were unchanged. MEPs showed substantially stable CMCTs values over time.




| Month | Patient 1 | Patient 2 |
|---|---|---|
| 0 | no treatment | no treatment |
| 3 | simvastatin 20 mg | no treatment |
| 6 | simvastatin 40 mg | no treatment |
| 9 | simvastatin 60 mg | no treatment |
| 12 | simvastatin 40 mg | ezetimibe 10 mg |
| 15 | simvastatin 40 mg + ezetimibe 10 mg | ezetimibe 10 mg |
| 18 | simvastatin 40 mg + ezetimibe 10 mg | no treatment (1 month) |
| 21 | simvastatin 40 mg + ezetimibe 10 mg | ezetimibe 10 mg |
| 24 | simvastatin 40 mg + ezetimibe 10 mg | ezetimibe 10 mg |
| 36 | simvastatin 40 mg + ezetimibe 10 mg | ezetimibe 10 mg |
| 48 | simvastatin 40 mg + ezetimibe 10 mg | ezetimibe 10 mg |

**Fig. 1.** Serum 27OHC over follow up in Patient 1 and Patient 2. N.V. of 27OHC = 16 ± 3 µg/dl.

Since chenodeoxycholic acid (CDCA) has been reported to be beneficial in a child with liver disease associated with a missense variant in CYP7B1 (Dai et al 2014), we added oral CDCA 500 mg/day to the therapy of our patients. However, CDCA was discontinued after a month due to lack of 27OHC level variation and significant side effects (marked elevation of liver enzymes in patient 1 and diarrhea in patient 2), which disappeared upon drug withdrawal. SPG5 represents a nice model of neurodegeneration due to defective cholesterol metabolism. Our case reports deserve discussion both on diagnosis and possible treatment. The presence of neonatal jaundice in our patients could be not casual since CYP7B1 mutations have been associated with severe neonatal cholestatic liver disease (Setchell et al 1998). Prolonged neonatal jaundice may represent a systemic manifestation of SPG5, as observed in other cholesterol metabolism disorders such as cerebrotendinous xanthomatosis (Clayton et al 2002) and Niemann-Pick disease type C (Wijburg et al 2012). Serum 27OHC represents an extremely useful diagnostic biomarker allowing differential diagnosis between SPG5 and other autosomal recessive HSPs. Yield of 27OHC assessment in newborns with prolonged unexplained neonatal jaundice should be also considered in the light of a possible disease modifying treatment. In our patients long-term administration of cholesterol-lowering drugs reduced serum levels of 27OHC by over 50%. Although 27OHC never normalized, we achieved a marked decrease, without loss of biochemical effectiveness over time. Occurrence of cramps and hyperCKemia in patient 2 points out the importance of taking into account statin-associated myopathy (SAM). Indeed, early introduction of coenzyme Q10 supplementation may lead to symptomatic improvement in patients with SAM. Clinical stability was observed at follow up in both patients, and repeated neurophysiological and imaging examinations did not reveal disease

progression. Even if further studies are needed to prove that 27OHC is the "bad guy" in SPG5 pathogenesis, we think that administration of cholesterol-lowering drugs should be considered in HSP patients harboring *CYP7B1* mutations. Not only HMG-CoA reductase inhibitors but also ezetimibe might be attempted, especially when statins are not tolerated, and combination therapy could also be given. However, more clinical meaningful endpoints should be defined before embarking in double-blind, placebo-controlled clinical trials assessing the efficacy of cholesterol-lowering drugs in SPG5.

## 6.2 SPG5 siblings with different phenotypes showing reduction of 27-hydroxycholesterol after simvastatin-ezetimibe treatment

Spastic paraplegia type 5 (SPG5) is an autosomal recessive hereditary spastic paraplegia (HSP) due to mutations in CYP7B1 and characterized by pure or complicated phenotypes (Goizet et al 2009). CYP7B1 catalyses the 7α-hydroxylation of 27-hydroxycholesterol (27OHC) and 25-hydroxycholesterol (25OHC), which are markedly increased in serum and cerebrospinal fluid of SPG5 patients (Schüle et al 2010). In particular, 27OHC may be a key contributor to SPG5 pathogenesis since it is able to enter the brain from the blood flow. As circulating levels of cholesterol and 27OHC are closely correlated, SPG5 patients should benefit from statins and/or ezetimibe which lower 27OHC and may prevent disease progression. Here we describe two SPG5 siblings with different phenotypes, and report the follow up data after cholesterol-lowering therapy. Patient 1 was a 48-year-old woman who presented episodes of diplopia since age 18 and progressive gait disturbances from age 25, forcing her to the wheelchair at age 37. Brain MRI showed marked leukoencephalopathy involving periventricular and subcortical white matter, brainstem,

and cerebellum, as well as brain atrophy and thickness of corpus callosum (Fig. 1A-D). Due to the initial relapsing-remitting course of the disease, she had been diagnosed with multiple sclerosis (MS) and treated accordingly without beneficial effects. From age 30 she also developed dysarthria and cognitive deterioration. Past history revealed prolonged neonatal jaundice. Neurological examination disclosed a severe pyramidal syndrome complicated by dementia and cerebellar signs, with a spastic paraplegia rating scale (SPRS) score of 37/52. Patient 2 was a 41-year woman who presented since age 20 a slowly-progressive gait impairment with spasticity and sensory ataxia. Brain MRI at age 25 was normal, while from age 36 periventricular white matter signal alteration was evident (Fig. 1E-H). Neurological examination revealed moderate spastic paraparesis and altered vibration and position sense, with SPRS 21/52.

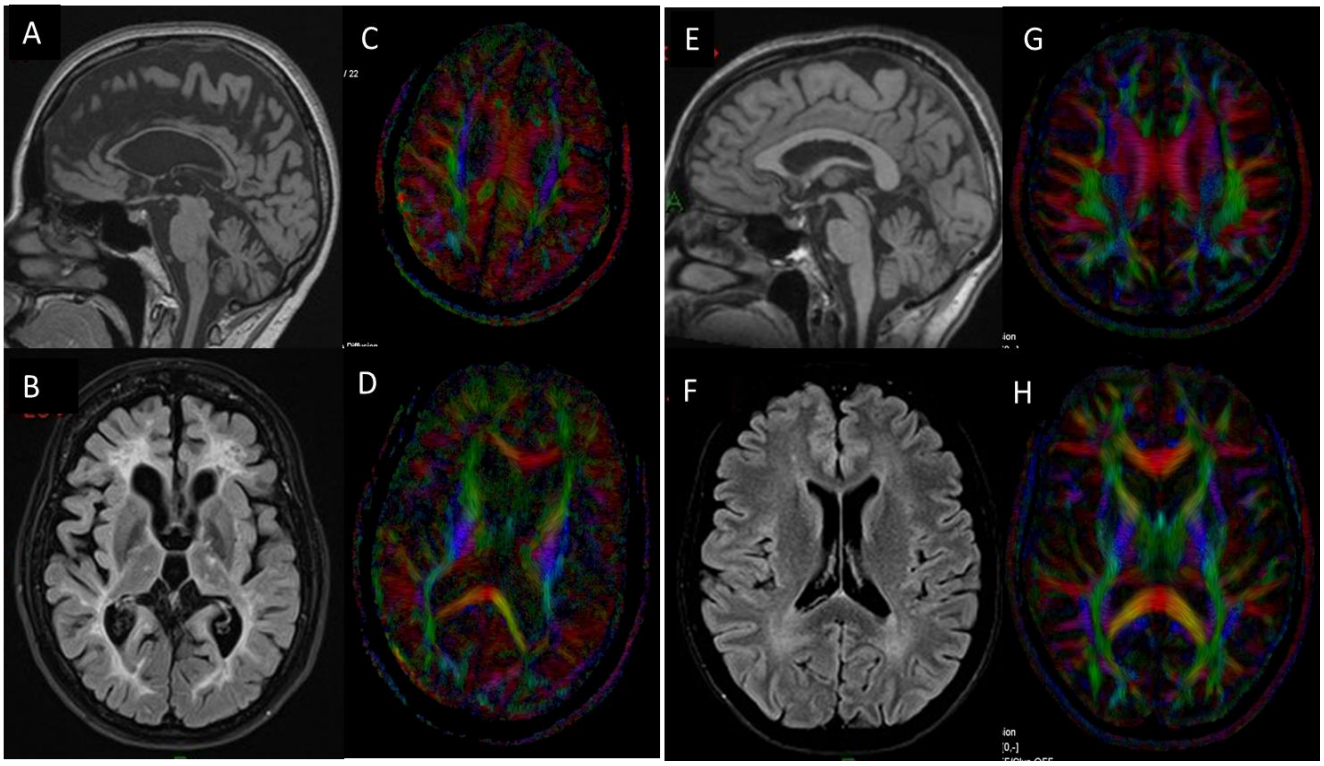



**Fig. 1.** Brain MRI in Patient 1 (A-D) and Patient 2 (E-H). See the text for explanation.

In both patients CYP7B1 analysis detected the homozygous missense mutation c.889A>G (p.T297A) and serum levels of 27OHC and 25OHC were markedly elevated, while cholesterol was increased only in patient 1 (Fig. 2). Simvastatin 40 mg/day and ezetimibe 10 mg/day were started being well tolerated, and after 12 months we performed clinical, biochemical, and radiological follow up: in both subjects 27OHC concentration decreased in parallel with cholesterol, while 25OHC decreased only in patient 1 (Fig. 2); no changes of SPRS and MRI findings were noticed.

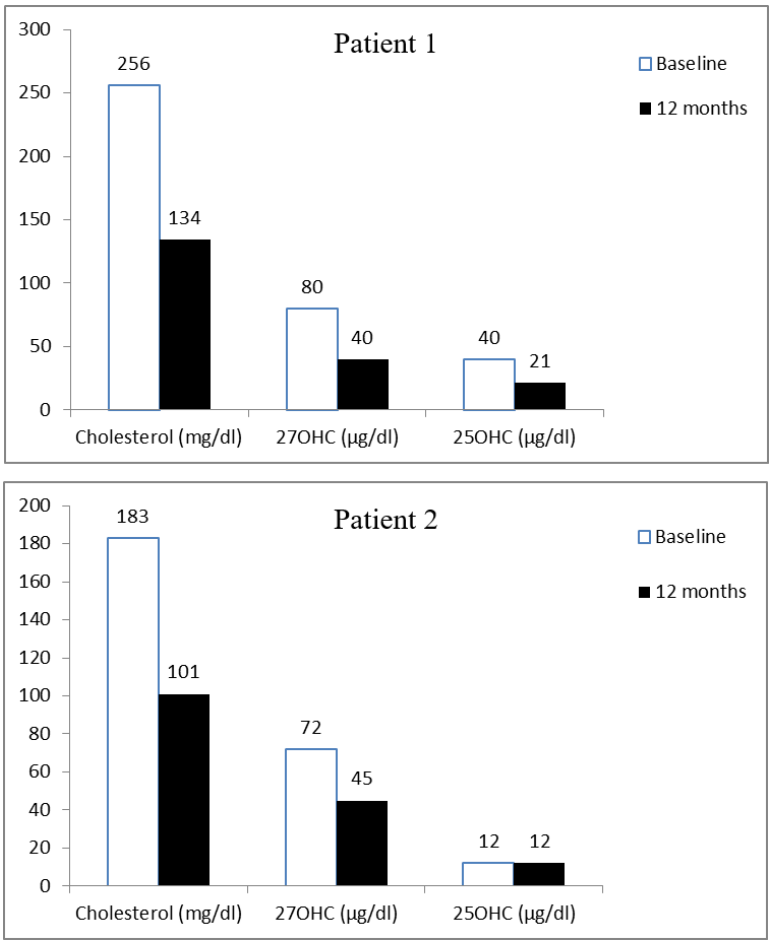



**Fig. 2.** Serum levels of cholesterol, 27-hydroxycholesterol (27OHC), and 25-hydroxycholesterol (25OHC) at baseline and after 12-month treatment with simvastatin 40 mg/day and ezetimibe 10 mg/day. Normal values: cholesterol = 130-200 mg/dl; 27OHC = 16 ± 3 µg/dl; 25OHC < 1 µg/dl.

The profound difference between clinical and MRI features of our patients emphasises the possible heterogeneity of SPG5 phenotype. Patient 1 was affected by a very complex form with cerebellar and cognitive impairment, and MRI evidence of severe leukoencephalopathy, marked brain atrophy, and thin corpus callosum which are atypical for SPG5: she was misdiagnosed with MS as previously occurred in other cases (Criscuolo et al 2009), highlighting the importance of differential diagnosis between SPG5 and MS; also, she presented neonatal jaundice which may be a systemic manifestation of the disease having been described in other SPG5 patients and being CYP7B1 mutations associated also with neonatal cholestatic liver disease. Patient 2 showed spastic paraparesis complicated by impaired deep sensation, which should be regarded as a possible feature of SPG5 and can be sometimes the main clinical presentation. Therapy of HSPs essentially focuses on symptomatic treatment. In this regard, SPG5 may represent the first form with an available disease-modifying therapy. In our patients simvastatin and ezetimibe reduced serum levels of cholesterol and 27OHC by 40-50%, in line with our expectations; notably, 25OHC decreased only in the patient who had hypercholesterolemia at baseline. Despite oxysterols did not normalize, clinical and MRI stability was observed. In conclusion, our findings support the use of simvastatin-ezetimibe in SPG5, which however should be further evaluated with a double-blind, placebo-controlled clinical trial.

## 7. Publications related to the thesis


- Mignarri A, Carecchio M, Del Puppo M et al (2017) SPG5 siblings with different phenotypes showing reduction of 27-hydroxycholesterol after simvastatin-ezetimibe treatment. J Neurol Sci 383: 39-41.
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