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## Cardiac pathology in neuronal ceroid lipofuscinoses (NCL): More than a mere co-morbidity

### Journal Item

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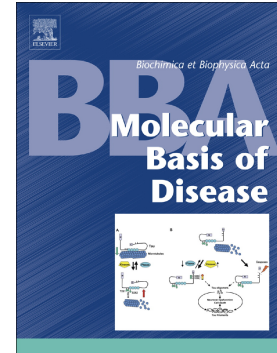
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**Cardiac pathology in neuronal ceroid lipofuscinoses (NCL): more than a mere co-morbidity**

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**Abstract:**

The neuronal ceroid lipofuscinoses (NCLs) are mostly seen as diseases affecting the central nervous system, but there is accumulating evidence that they have co-morbidities outside the brain. One of these co-morbidities is a decline in cardiac function. This is becoming increasingly recognised in teenagers and adolescents with juvenile CLN3, but it may also occur in individuals with other NCLs. The purpose of this review is to summarise the current knowledge of the structural and functional changes found in the hearts of animal models and people diagnosed with NCL. In addition, we present evidence of structural changes that were observed in a systematic comparison of the cardiomyocytes from CLN3<sup>Δex7/8</sup> mice.

**Keywords:**

Batten Disease

Juvenile CLN3

Cardiac Pathology

ECG

Echocardiogram

Cardiomyocyte Structure

**Declaration of Interest**

The authors declare no conflict of interest for this study.

### Article Highlights

- NCLs have a cardiac co-morbidity, best described for CLN3
- This review summarises cardiac phenotypes in animal models and NCL patients
- The cardiac phenotype is variable and to date not well studied
- Bradycardia, left ventricular hypertrophy and conduction problems are common

### Abbreviations:

NCL - Neuronal ceroid lipofuscinoses

PPT1- Palmitoyl protein thioesterase 1

TPP1 - tripeptidyl peptidase 1

ER - endoplasmic reticulum

SA node - sinoatrial node

AV node - atrioventricular node

Ca<sup>2+</sup> - Calcium ion

ECG - electrocardiogram

KO - knockout

ASM – autofluorescent storage material

GROD – granular osmophilic deposits

iPSC - induced pluripotent stem cell

WT - wild-type

**Main Text:**

Neuronal ceroid lipofuscinoses (NCLs; sometimes referred to as Batten disease) is the generic name for a group of debilitating and life-shortening, neurodegenerative diseases that involve lysosomal dysfunction and accumulation of cellular storage material ('ceroid'; sometimes called lipofuscin) [1]. Impaired lysosomal function leads to the clinical hallmarks, such as progressive loss of vision, behavioural changes, epilepsy, and psychomotor decline, seen in NCL patients. The accumulation of storage materials within cells may also occur, but its contribution to cellular pathology is uncertain. NCL diseases are linked to mutations in 13 different genes, which encode for a diverse range of proteins that impact on lysosomal function. These proteins include lysosomal enzymes (e.g. Palmitoyl protein thioesterase 1, PPT1 and tripeptidyl peptidase 1, TPP1), transmembrane proteins localised to the lysosomal (e.g. CLN3) or endoplasmic reticulum (ER; e.g. CLN6 and CLN8) membranes. Although symptoms can arise in adult life, the majority of NCL-causing gene mutations lead to an infantile, late infantile or juvenile disease onset, with devastating consequences for patients and their families. Mutations in one of the 13 genes give rise to forms of NCL that may have both distinctive and common aspects. For example, mutations in *PPT1* or *TPP1* cause forms of NCL denoted CLN1 and CLN2 respectively, which both commonly affect infants in early life. Whereas, mutations in *CLN3* lead to similar a similar loss of vision and motor control, but the disease typically has a later onset. For most of the NCL diseases, it is not fully clear how the altered functions of proteins encoded by the mutated genes lead to the development of the specific diseases. Although, there is increasing understand of the functions and locations of some of the wild type proteins at the cellular level [2, 3]. NCL is the most common form of inherited infantile/juvenile dementia, and considerable effort is focussed on understanding its etiology and treatment options [2, 4].

Potentially life-extending therapies for NCLs are becoming available ([5]; also reviewed in [2]), in particular for those NCLs in which the cellular dysfunctions caused by gene mutations are known. For example, CLN1 and CLN2 are due to mutations in enzymes involved in the processing of proteins by lysosomes, and hence enzyme replacement therapy could be a viable option. Intraventricular enzyme replacement therapy has been approved for CLN2 disease. The effects of the treatment are encouraging so far, but it is not known what the long-term consequences of this treatment will be and natural history studies are underway to assess this. Due to the progressive neurodegeneration associated with all forms of NCL, the central nervous system has been a primary focus of research and therapeutic strategies. However, alterations in the peripheral nervous system and other organs, such as the heart, are known and lead to incapacitating co-morbidities [6]. It is therefore important to understand the co-morbidities arising outside the CNS in patients with the various NCLs. In particular, accumulating evidence suggests that changes in heart function affects NCL patients' quality of life and their levels of activity and mobility. It is plausible that therapies developed to treat CNS-related dysfunctions in NCLs may also have benefit for peripheral tissues [7, 8].

Whilst it is clear that the heart is affected in NCLs, there are substantial gaps in understanding what goes wrong, and in particular why and when. Anecdotal case studies from juvenile CLN3 patients reported changes in heart function and accumulation of storage material from as early as 1979 [9], and a first systematic review was published in 2011 [10]. To date, the majority of co-morbidities associated with the heart were described for juvenile CLN3 patients. This could point to a difference in the penetrance of *CLN3* mutation on the

heart relative to other NCL mutations, perhaps because juvenile CLN3 has a later onset than the infantile forms. However, the various NCLs progress through similar depreciative phases, and it is likely that cardiac pathologies are a feature of all NCLs, and as yet they may have been overlooked or not investigated in some instances. To our knowledge, neither the causes nor the timescale in which the cardiac pathology develops has been systematically studied for the various NCLs. There is an abundance of NCL animal models that recapitulate many aspects of the diseases. These models could provide a basis for understanding cardiac issues associated with the NCLs, but so far, such models have rarely been used to study the changes in structure or function of the heart.

### **Function of the normal heart**

The mammalian heart is a muscular pump that consists of two atrial chambers and two ventricular chambers. The atrial chambers are located above the ventricles and receive blood coming into the heart from the systemic and pulmonary circulations. Blood passes through the atrial chambers into the ventricles, where the major force for blood pumping is developed [11]. Each beat of the heart arises from a highly coordinated series of events that involves electrical excitation, contraction and electrical/mechanical relaxation; collectively these events are referred to as the cardiac cycle [12, 13]. The heart contains cells that display automaticity, which is the capacity to generate spontaneous action potentials. The sinoatrial node (SA node), a specialised group of cardiomyocytes located in the right atrial chamber, is the healthy heart's principle pacemaker because it has a relatively high intrinsic frequency of action potential generation [14]. Other cells within the heart, notably the atrioventricular node (AV node), which is another group of specialised cardiomyocytes



located towards the lower back aspect of the septum between the right and left atria, can act as a secondary pacemaker and trigger action potentials albeit with a lower frequency.

When an action potential is generated by the SA node it rapidly propagates across the right and left atrial chambers, thereby causing electrical excitation of the contractile cardiomyocytes in the atrial walls that leads to blood being squeezed into the ventricles. Under sedentary conditions, the pressure of venous blood return accounts for the majority of ventricular blood filling. During periods of increased oxygen demand, such as when exercising, the atrial chambers can be stimulated to contract more forcefully and thereby contribute significantly to ventricular filling. This increased atrial blood pumping capacity is sometimes referred to as atrial kick, and its contribution can decline in certain cardiac pathologies such as atrial fibrillation. It takes approximately 30 milliseconds for the action potential to propagate across the atrial chambers and reach the AV node, which acts as a conduit for the transmission of the electrical signal to the ventricular chambers. On reaching the AV node, there is a short pause (~100 milliseconds) in the propagation of the action potential to ensure completion of atrial systole and to prevent the transfer of aberrant electrical activity such as the spontaneous depolarisations that occur during atrial fibrillation. The lower portion of the AV node is designated the bundle of His, which then splits into the left and right branches, allowing activation of the left and right ventricles [15]. The bundle branches culminate in Purkinje fibres that pass action potentials to groups of contractile cardiomyocytes, thereby causing the heart to contract upwards from the ventricular base and expel blood in a coordinated manner to the lungs and body. Backflow of blood between the atrial and ventricular chambers, or from the pulmonary and aortic vessels is prevented by atrioventricular and semilunar valves.

An increase in cytosolic calcium ion ( $\text{Ca}^{2+}$ ) concentration is the link between electrical excitation of cardiomyocytes and contraction [13, 16, 17]. When a cardiomyocyte is depolarised by the arrival of an action potential, voltage-activated  $\text{Ca}^{2+}$  channels in the sarcolemma (the myocyte cell membrane) are opened, and there is an influx of  $\text{Ca}^{2+}$  from the extracellular space. This initial surge of  $\text{Ca}^{2+}$  is not sufficient to trigger contraction to any significant extent, but is amplified by a process of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release via ryanodine receptors (a family of  $\text{Ca}^{2+}$ -activated,  $\text{Ca}^{2+}$  permeable channels) [18] located on the sarcoplasmic reticulum (a  $\text{Ca}^{2+}$ -storing organelle in muscle cells) [19]. The  $\text{Ca}^{2+}$  signal arising from activation of ryanodine receptors diffuses throughout the cardiomyocyte. Upon reaching the contractile actomyosin fibres,  $\text{Ca}^{2+}$  binds to troponin C, which consequently allows the engagement of actin and myosin so that they can slide past each other and cause shortening of the cell [20, 21]. The simultaneous contraction of the millions of cardiomyocytes that make up the walls of the heart generates the force for pumping of blood. To allow the heart to relax and be ready for the next beat, the mechanical change, and the electrical and  $\text{Ca}^{2+}$  signals have to recover. Alterations in the conduction of action potentials within the heart, or in the excitation or recovery of cardiomyocytes, can lead to abnormal and reduced cardiac performance. For example, aberrant  $\text{Ca}^{2+}$  signals, which arise with cardiomyocytes under various conditions, can prevent cells from responding to incoming action potentials [22]. Moreover, aberrant  $\text{Ca}^{2+}$  signals, along with numerous other factors, can cause the heart to undergo hypertrophic growth [16]. Initially, hypertrophy may support cardiac function and support blood pumping, but it can progress to heart failure. The cyclical electrical activity of the heart is typically monitored via an

electrocardiogram, using electrodes placed on the chest and body. The phases of heart depolarisation and repolarisation can be recorded, and any abnormalities in their occurrence or timing can be used to diagnose cardiac problems such as blocks in the conduction of action potentials within the heart. Healthy patients are typically said to be in 'sinus rhythm' where their heart beats at an expected rate (~70 beats per minute) and with the anticipated order of electrical events. Arrhythmias occur when heart beats in an unexpected manner, which may be too slow (bradycardia), too fast (tachycardia) or irregularly.

The volume of blood pumped by the heart depends on the beating frequency (heart rate) and stroke volume (the volume of blood pumped by the heart during a cardiac cycle). Both factors can be altered by neuronal and hormonal signals. Heart rate is principally controlled by the autonomic nervous system, via efferent neurons emanating from the cardiovascular regulation centres in the medulla within the brainstem [23]. The medulla integrates electrical, chemical and mechanical signals and consequently adjusts heart rate. The parasympathetic nervous system, via the vagal nerves, slows heart rate through the release of acetylcholine at the SA and AV nodes. In contrast, the sympathetic nervous system increases heart rate by releasing epinephrine and norepinephrine at the SA and AV nodes. In addition to their chronotropic effects (i.e. rate of heart beating), both the parasympathetic and sympathetic nervous systems can impact on inotropy (i.e. the force of contraction) by releasing neurotransmitters in the vicinity of contractile cardiomyocytes. The overall heart rate and force of contraction is determined by a balance between the input from the parasympathetic and sympathetic nervous systems, in addition to effects of circulating hormones released by other organs and paracrine factors produced by the heart

itself [24]. Moreover, the heart has its own intrinsic nervous system that send afferent signals to the brain that can also impact on heart rate [23]. Interestingly, there are links between the cardiac nervous system and cognitive functions [12].

### **Cardiac phenotypes in NCL animal models**

Animal models available for the various NCLs have been reviewed elsewhere [25-27]. Small animal models include murine models, zebrafish and *Drosophila* [26]. Relatively few studies have characterised heart function in these model systems (outlined below and summarised in Table 1). Accumulation of storage material (either autofluorescent ceroid or the subunit C of the mitochondrial ATPase) was found in all NCL animal models apart from the cathepsin D knockout mouse that is used as a model for CLN10 [28] (summarised in Table 1).

### ***Small animal models***

A detailed phenotypic characterisation of the cardiac function (blood pressure, heart rate, markers for hypertrophy, electrocardiogram) was published for the CLN3 <sup>$\Delta$ ex7/8</sup> mouse. The only difference observed was a slight increase of the heart weight at 12-19 months of age [29]. An enlarged heart was also found in a zebrafish model of CLN3, but no changes in the heart's function were reported [30]. Similarly, a CLN1 mouse model showed no differences in electrocardiogram (ECG) parameters at 7 months of age. Analysis of specific markers indicated a trend towards hypertrophy, but no significant differences were found. Storage material was mostly present in endothelial cells inside the heart and in valve stromal cells, and only to a small extent in cardiomyocytes [31]. No reports of changes in the heart structure or function are available for the *Drosophila* models of NCLs.

### ***Large animal models***

Large animals are considered to be physiologically more relevant to the human pathology because of the size of their hearts, similar heart rate and life-span [32]. NCLs occur spontaneously in large animals such as dogs and sheep. For some more frequently occurring NCLs, breeding stocks have been established and the relevant NCL animal models are available (reviewed in [25, 27]). The largest number of NCLs has been described in dogs: CLN 1, 2, 6, 7, 8, 10 [33-40]. Several ovine models exist for CLN5 and 6 [41], and a bovine model for CLN5 [42]. In addition to these, a non-human primate model (macaque) is available for CLN7 [43]. The changes in cardiac structure and function reported for these models are described below and summarised in Table 1.

Similar to the small animal models, changes in cardiac structure and function were rarely studied systematically in the large animal models. After more frequent reports showing a cardiac pathology in CLN3 patients, Palmer et al. investigated whether they were also present in CLN6 Merino sheep [41], but found neither changes in heart function nor an accumulation of storage material. In contrast, pronounced cardiac pathologies were found in canine CLN7 and CLN8 models. The English Setter was originally described as model for NCLs in 1982 [44], and later confirmed to carry a mutation causing CLN8 [45]. Armstrong et al. performed a detailed study of the cardiac pathology on the English Setter in 1986 [46] and found that the animals showed sinus arrhythmias, conduction problems and signs of left ventricular hypertrophy. The characteristic accumulation of autofluorescent storage material (ASM), presence of curvilinear bodies and calcification was found in the Setters' hearts. An accumulation of ASM was also found in the hearts of canine and macaque CLN7

models [35, 36, 43]. The macaques showed bradycardia and abnormal ECGs with prolonged P wave width and PR interval, potentially indicating conduction problems [43], whilst cardiac function was not studied in the canine model.

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NCL	System (Gene, mutation or model)	Observations	
CLN1	Mouse (PPT1 <sup>-/-</sup> )	GROD and storage material in hearts from 7 month-old animals, mostly in endothelial cells and only to a lesser extent in cardiomyocytes. ECGs were used to measure cardiac function (filling, ejection fraction, size), but no significant differences found. Trend towards hypertrophy.	[31]
CLN2	Mouse	CLN2 <sup>-/-</sup> mouse shows accumulation of ASM in the heart without any gross alteration of the cells' structure	[47]
CLN2	Canine	Gene therapy extends survival in a TPP1 <sup>-/-</sup> canine model, at which point a cardiac phenotype becomes apparent. Functional changes include an increased heart rate, enlarged ventricles and a reduced ejection fraction. The heart shows increased fibrosis and accumulation of ASM.	[48]
CLN3	Mouse ( $\Delta^{ex7/8}$ )	Slight increase in heart weight, but no changes in heart rate, ECG parameters or markers of hypertrophy observed. Accumulation of mitochondrial subunit C storage material inside the heart.	[29]
CLN3	Mouse	Most prominent lacZ staining, indicating the CLN3 localisation, in the cardiac endothelium, not the myocardium.	[49]
CLN3	Zebrafish	Elongated heart that lacked pigmented erythrocytes. No functional differences were reported.	[30]
CLN6	Ovine	ECGs revealed no functional differences in mid- or late-stage Merino sheep. No accumulation of ASM was observed in cardiomyocytes, the heart valves, the SA or the AV node.	[41]
CLN7	Mouse (CLN7 KO)	Accumulation of mitochondrial ATPase subunit C in hearts from CLN7 KO mice. Heart function not studied.	[50]
CLN7	Mouse (CLN7 KO)	Accumulation of ASM in cardiomyocytes from CLN7 KO mice. Heart function not studied.	[51]
CLN7	Canine; Chihuahua	Storage material found, without mention of accumulation in the conduction system or of cardiac deficits in the animals.	[35]
CLN7	Canine; Chihuahua	ASM accumulation in cardiomyocytes. No report of its accumulation in the conduction system or of cardiac deficits in the animals.	[36]
CLN7	Macaque	Accumulation of ASM in the heart. ECGs showed a prolonged P wave width and PR interval (potentially indicating delayed conduction to the AV node) and bradycardia.	[43]
CLN8	Canine; English Setter	Six dogs were studied. Sinus arrhythmias, conduction problems, QRS-notches and signs of left ventricular hypertrophy were found. Accumulation of ASM, presence of curvilinear bodies and calcification was found in the hearts.	[46]
CLN10	Cathepsin D (KO mouse)	No histological changes were reported for cardiomyocytes. Cardiac tissue was enriched with subunit C of the mitochondrial F <sub>1</sub> F <sub>0</sub> ATPase, but it was not investigated in which cell type or part of the heart this enrichment occurred.	[28] [52]

**Table 1: Summary of studies using animal NCL models that investigated changes in the structure and/or function of the heart.** ASM – autofluorescent storage material, GROD – granular osmophilic deposits.

As discussed above, most reports of cardiac dysfunction in NCL human patients have been associated with CLN3, which typically has a juvenile onset. A plausible explanation for this apparent connection is that cardiac phenotypes may be overlooked in the more rapidly degenerating infantile NCL diseases (e.g. CLN1, CLN2 etc) compared to the more slowly developing juvenile CLN3. Evidence that supports this hypothesis was provided by a study in which CLN2 dogs were treated with gene therapy (injection of adeno-associated virus encoding TPP1 into the cerebrospinal fluid) [48] that delayed the disease onset and prolonged the animals' life span [8]. Cardiac function in the treated animals was measured at a time when untreated animals would be in the end stage of the disease or have deceased (i.e. >10 months of age). At this time, the treated animals had impaired cardiac function compared to healthy age-matched animals. They showed an increased heart rate, enlarged ventricles with an increased end-systolic volume and a reduced ejection fraction. Structural changes included the appearance of ASM (although less than in untreated animals), and the occurrence of lesions and fibrosis [48]. Whilst more evidence is required, the study by Katz et al. could indicate that cardiac problems may develop in all forms of NCLs if there is enough life-span. This may have implications for therapeutic approaches that seek to alleviate neuronal aspects of NCLs without addressing deterioration in peripheral tissues and organs.



### Cardiac phenotypes in human NCLs

Changes in the heart function of teenagers and adolescents with juvenile CLN3 were described in early case studies [9, 53] and in autopsy studies [54]. However, it was not until 2011 that a systematic clinical investigation of cardiac involvement in juvenile CLN3 was reported by clinicians in Denmark [10]. The Danish cross-sectional and follow-up study comprised twenty-nine children and adolescents with juvenile CLN3, and showed progressive cardiac impairment with repolarisation disturbances, expressed by abnormally deeply inverted T waves, ventricular hypertrophy, and sinus node dysfunction ultimately leading to a severe bradycardia and/or other conduction abnormalities [10]. The inverted T waves were present as early as 14 years of age and were associated with an increased risk of death during the 7½ years follow-up period. At increasing age, heart rate and heart rate variability, expressed as the vagal index, were significantly reduced, suggesting an age-dependent bidirectional effect of the heart rate: one through decreasing parasympathetic activity on the heart and the other through a direct negative influence on sinus node automaticity [10]. Four of seven patients beyond 20 years of age had hypertrophy of the left ventricular walls, but the ejection fractions were within normal limits. Recently, consecutive follow-up studies of heart rate variability in juvenile CLN3 patients up to their third decade of life have been published [55]. The parasympathetic activity decreased significantly with increasing age, and beyond 15-20 years of age the parasympathetic activity levels were very low, nearly totally abolished, whereas there were no similar age-dependent changes in the sympathetic activity on the heart [55].

A similar longitudinal study is ongoing at the University Medical Center Hamburg-Eppendorf, Germany. For this latter study, 42 juvenile CLN3 patients are examined every 6 months as part of standard of care follow-up in the NCLs specialty clinic at the University Medical Center Hamburg-Eppendorf. Preliminary results from this ongoing data collection showed that more than 60% of patients developed a cardiac pathology. These results are in line with the Danish cohort reported by Ostergaard et al. [10]. In both cohorts, ECGs and echocardiograms showed various cardiac abnormalities, which became more obvious with increasing age of the patients and were present in every individual over the age of 20 years in the Danish study. Left ventricular hypertrophy and bradycardia were the most common pathologies, found in approximately 20-30% of the patients in each study (Table 2 and unpublished personal communication) [10, 56], but no consistent pattern of changes was seen. Patients in the German study were typically examined longitudinally starting at disease diagnosis, before the onset of changes in ECGs and echocardiograms, which will provide insight into the age when the cardiac pathology starts. This was not possible in the study conducted in Denmark.

	<b>Denmark</b>
Total number juvenile CLN3 patients in the study	29
Patients with any cardiac pathology observed:	15 (52% of all patients)
Of which, <ul style="list-style-type: none"> <li>• Patients with diagnosed bradycardia:</li> <li>• Patients with diagnosed left ventricular hypertrophy:</li> </ul>	5 (33% of patients showing a cardiac pathology)  4 (27% of patients showing a cardiac pathology)

**Table 2: Numbers of juvenile CLN3 patients displaying cardiac abnormalities in ECG recordings and echocardiograms.** Based on [10, 56].

**Where is the cardiac pathology in NCL- the conduction system, the myocytes, or everywhere?**

The findings discussed above point to a range of cardiac issues in CLN3, and in particular bradycardia and left ventricular hypertrophy. The bradycardia is due to a decrease in automaticity of the sinus node most likely because of a lysosomal dysfunction of the involved nervous cells. Accumulation of storage material is also seen within the atrioventricular node and bundle of His. The hypertrophic growth of the heart is due to a lysosomal dysfunction in the contractile cardiomyocytes. What role the accumulation of storage material plays is less well known. Indeed, deposition of ceroid lipopigment has been shown to occur extensively in the sinus node, as well as in the atrioventricular node and in the bundle of His [54], and to a lesser extent inside the cardiomyocytes [53, 54, 57]. However, as pointed out by the animal studies mentioned above, other cell types, such as endothelial cells, may also be subjected to phenotypic and functional changes. Fibrosis and calcification were frequently observed in histological heart sections from juvenile CLN3 patients [53, 54].

In ECG recordings, severe bradycardia including sinus arrests for periods as long as 22 seconds has been demonstrated for a juvenile CLN3 patient [53]. Several case studies exist that report pacemaker implantation in individual patients, and some of them showed an improvement in their life quality. Following pacemaker-implantation in four Danish patients, all individuals expressed that they felt markedly less physically tired, and accordingly, the parents noted that their adolescents became more active. In two of the four cases, the parents spontaneously said that their youngsters had significantly warmer hands and feet (personal communication). In one case study from Germany, a 30 year-old male juvenile

CLN3 patient was diagnosed with recurrent episodes of asystoly. This diagnosis happened in parallel with a sudden loss of motor and language function. It should be noted that this patient had an unusually slowly progressing phenotype prior to this diagnosis despite being homozygous for the most common mutation in the *CLN3* gene: a 1 kb deletion. After implantation of a cardiac pacemaker, the patient's motor and language function rapidly improved, resulting in a change of rating from 0 to 1 scoring unit in each of the motor and language functions using the Hamburg juvenile NCL score [58].

Cardiac complications have also been described in CLN1, CLN2 and CLN6 patients. CLN1 patients had no apparent cardiac problems during their lifetime, but showed a mild dilation of the ventricles and accumulation of storage material in a post-mortem analysis [53]. One CLN2 patient who had an atypically slow disease progression developed ventricular tachycardia and an AV block after the age of 23 years [59]. Two sibling CLN6 patients developed cardiac abnormalities of different degrees. The more prominent pathology included arrhythmia and conduction blocks. Post-mortem hearts from both patients showed fibrosis, accumulation of ASM and curvilinear bodies [60].

NCL	Age of onset and number of patients	Observations	Reference
CLN1	Juvenile	CLN1 patient with c.287G>A mutation (not mentioned whether homo- or heterozygous). No cardiac symptoms during the lifetime. During a post-mortem mild dilation of the ventricles was found, and accumulation of ASM within cardiomyocytes. Cells of the conduction system also showed strong accumulation of ASM and deterioration (vacuolisation of the cytoplasm, GROD accumulation).	[53]
CLN2	Late infantile	CLN2 patient with a c.857A>G mutation (heterozygous). Normal ECGs at 14 and 20 years of age. Right and left bundle blocks and bradycardia observed from the age of 23. Two episodes of tachycardia at the age of 23 and 27 and a complete AV block, leading to the death at 28 years of age.	[59]
CLN3	Juvenile	Cardiac hypertrophy and thickened mitral valves observed. EM images show curvilinear bodies, fingerprint patterns and lipofuscin deposits.	[61] Review
CLN3	Juvenile	Patient homozygous for 1.02 kb deletion with severe bradycardia, reduced ejection fraction, repolarisation abnormalities and T wave inversion were observed, together with left ventricular hypertrophy.	[62]
CLN3	Juvenile (n=2)	First patient (homozygous for the 1.02 kb deletion) with left ventricular hypertrophy and repolarisation problems. Second patient (homozygous for the 1.02 kb deletion) with severe arrhythmia (bradycardia). Cardiomyocytes from both patients showed vacuolisation of the cytoplasm and accumulation of ASM. Fibrosis and infiltration of fat was observed in both hearts. ASM accumulation was stronger in the conduction system than in cardiomyocytes.	[53]
CLN3	Juvenile (n=2)	Siblings diagnosed by evidence of vacuolated lymphocytes and a typical skin biopsy. Patient 1 with bradycardia and ventricular hypertrophy. Periods of sinus arrest, atrial and ventricular tachycardia. Patient 2 with shortened PR intervals and only mild ventricular hypertrophy. Both patients showed accumulation of ASM mostly in the conduction system (SA and AV node), and to a lesser extent in the cardiomyocytes.	[57]
CLN3	Juvenile, slow progression (n=2)	Brothers with CLN3 (homozygous for the p.Gly165Glu mutation). Both patients were diagnosed with sick sinus syndrome and cardiac conduction defects.	[63]
CLN3	Juvenile (n=13)	Frequently observed changes were abnormal P waves, bradycardia, right bundle or other forms of conduction blocks. Cardiomyocytes were slightly hypertrophic and showed the most prominent lipopigment accumulation near the nucleus. Fibrosis and calcification were found in both, the endocardium and the interstitium. Strong accumulation of ASM was found in the conduction system.	[54]
CLN6	Adult (n=2)	Diagnosed by a skin biopsy with a typical fingerprint of Kufs disease, no genetic testing that confirms the diagnosis. Severity of cardiac problems different in the 2 patients. AF and conduction blocks in one patient, followed by ventricular	[60]

		tachycardia. AF and inverted T waves in the other patient, with mild ventricular hypertrophy but no arrhythmia. Cardiomyocytes from both patients showed fibrosis, accumulation of ASM and curvilinear bodies.	
?	Adult	No genotyping to confirm the type of NCL. Severe loss of mitochondrial integrity and cristae structure, appearance of inclusions inside mitochondria, GROD and other types of storage material inside cardiomyocytes	[64]

**Table 3: Summary of case studies indicating changes in the cardiac structure and/or function in NCL patients.** ASM – autofluorescent storage material, GROD – granular osmophilic deposits.

### How can we study changes in the cardiac structure and function in NCLs?

The fact that a cardiac pathology is a co-morbidity in juvenile CLN3 is clear from the patient records discussed above. Moreover, animal studies indicate that cardiac defects might affect other NCL patients if their lifespan is prolonged by a successful treatment [48]. The hope that currently approved and experimental therapies might indeed be able to extend patients' life span is high. However, it is too early for the impact of these recently developed treatments to be fully known [2, 5]. Thus, it is becoming critical to understand the causes of the cardiac problems in NCLs and to see if they could be ameliorated by early treatment, thus improving patients' quality of life. To better understand the changes occurring in patients, a regular non-invasive monitoring of cardiac function would be useful and allow clinicians to intervene before changes in heart function cause problems, e.g. by implanting cardiac pacemakers to prevent arrhythmia [10]. On the other hand, if we want to understand the cellular basis for the changes observed and establish the order in which they occur, we will need to utilise animal models or iPSC-derived cardiomyocytes.

iPSC-derived cells offer the possibility to study the effects of NCL-causing mutations on cardiomyocytes (and other cell types) in the absence of neuronal input. iPSCs with mutations in the *CLN1*, *CLN2*, *CLN3*, *CLN5*, *CLN7*, *CLN8* and *CLN10* genes are available, either derived from patients [2, 65-67] or generated via CRISPR technology [68-70] and can be differentiated into several different cell types. Two main problems arise when using iPSC-derived cardiomyocytes: the resulting cells usually have an immature phenotype, and differentiation is difficult, expensive and not possible with every iPSC clone. Adult cardiomyocytes are terminally differentiated cells with a precise morphology and ultrastructure, and their proper function relies on this differentiation. The shape and size of iPSC-derived cardiomyocytes changes over time in culture, but does not fully recapitulate adult cell morphology [71]. Cytoskeletal structure in iPSC-derived cardiomyocytes, particularly expression of cardiac troponin isoforms, has been used as a marker of cardiomyocyte maturity; demonstrating a shift towards adult structure over time post-differentiation, but it does not fully recapitulate adult cardiomyocytes [72, 73]. Other differences are in the number of mitochondria [74] or number of nuclei [71]. As discussed above, calcium handling and contraction are highly relevant to cardiomyocyte function and disease, but are not fully matured in iPSC-derived cells [75]. Differentiation protocols have improved to yield a more matured phenotype through methods such as electrical stimulation, 3D culture or co-culture [76, 77]. We are currently optimising the protocols to differentiate cardiomyocytes from *CLN3* patient-derived iPSCs, to compare their structure and function to that seen in cardiomyocytes from the corresponding mouse models.

Mouse models are invaluable to study the time-line in which phenotypic changes occur and to test whether interventions could affect the typical disease progression. During the

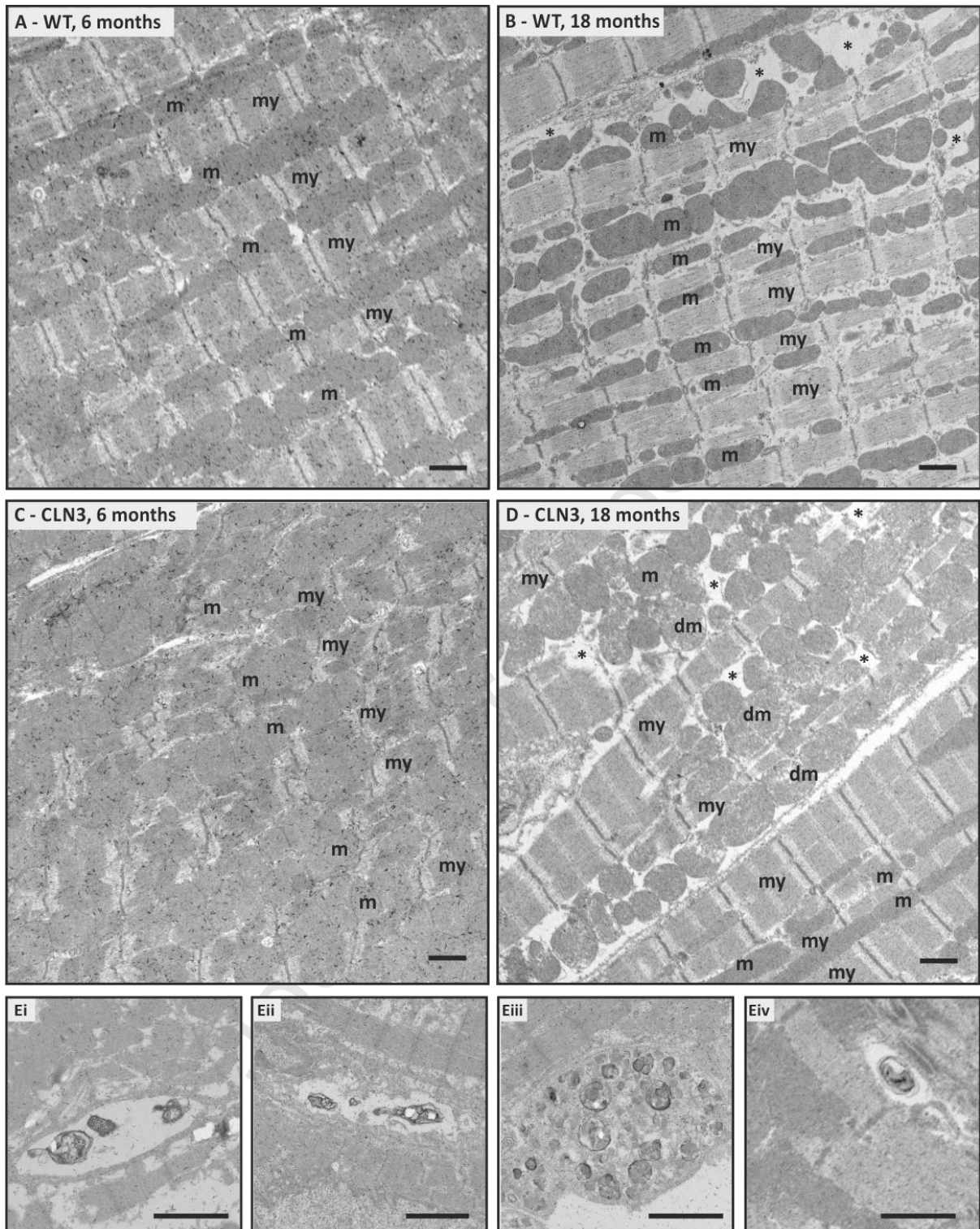
progression of juvenile CLN3, the control of heart rhythm by the parasympathetic and sympathetic nervous systems is altered [10, 55]. The consequences arising from these changes can be observed in animal models (see Table 1), but it can be difficult to decipher which cell type is the cause of a particular cardiac pathology. Deposition of storage material in the conduction system is likely to be the cause of the observed arrhythmia and disrupted propagation of electrical signals (conduction and bundle blocks), whilst the hypertrophy could be caused by the deposition of storage material in the cardiomyocytes themselves. However, cardiomyocytes will undergo hypertrophic growth in response to a number of stimuli and stressors [13]. Changes in contractility, calcium homeostasis or signalling could therefore cause hypertrophy independent of ASM accumulation.

### **Structural Changes in Cardiomyocytes from CLN3<sup>Δex7/8</sup> mice**

Using hearts from wild-type (WT) and CLN3<sup>Δex7/8</sup> mice, we recently undertook a systematic investigation into putative structural changes in cardiomyocytes that might be associated with the NCLs. Scanning electron microscopy was used to examine the ultrastructure of the cardiomyocytes from the WT and CLN3<sup>Δex7/8</sup> mice. Most ventricular cardiomyocytes from 6 month-old WT and CLN3<sup>Δex7/8</sup> animals showed a highly organised structure that would be expected of healthy terminally-differentiated cardiac cells, with well aligned myofibres and rows of intermyofibrillar mitochondria in-between the myofibres (Figure 1A and B). These mitochondria are essential to supply the energy to the working myocardium to keep up the regular heartbeat [78]. Sections obtained from 18 months-old WT and CLN3 mice also showed cardiomyocytes that were highly organised, similar to those observed with 6 months-old animals (Figure 1C and D). However, there were obvious regions within the cardiomyocytes from 18-month old CLN3 mice where the ultrastructure of the cells was



disrupted. Specifically, there were cellular regions with apparent empty spaces between mitochondria, and between mitochondria and myofibres (Figure 1C and D). Most cells studied previously from CLN3 model animals and from patients showed the presence of storage material (summarised in Tables 1 and 3). No storage material was obvious in cardiomyocytes from the WT animals. It also did not appear frequently in the cardiomyocytes from either 6 or 18 months-old CLN3<sup>Δex7/8</sup> mice. When storage material was observed, it was found between myofibres (Figure 1Ei, Eii and Eiv) or close to the sarcolemma (Figure 1, Eiii). Some large areas of storage material were observed (Figure 1, Eiii), but they did not resemble curvilinear or fingerprint bodies, both of which were found in human heart biopsies [53, 61].



**Figure 1: Ultrastructure of ventricular cardiomyocytes.** Cardiac tissue from WT animals at 6 (panel A) and 18 months of age (panel B), and tissue from 6 (panel C) and 18 (panel D) months-old  $CLN3^{\Delta ex7/8}$  mice. Panel E illustrated examples of storage material from 18 months-old  $CLN3^{\Delta ex7/8}$  mice. Animal husbandry and tissue collection complied with the

ARRIVE guidelines were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986. **m** - mitochondria, **dm** - deteriorated mitochondria, **my** - myofibres, \* cellular regions with apparent empty spaces between mitochondria, and between mitochondria and myofibres. Scale bar: 2  $\mu\text{m}$ .

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## Conclusions

A growing body of evidence indicates that the deteriorating heart function can be a significant co-morbidity in NCL, and that rectifying heart function, even to a modest degree, may enable patients to access a better quality of life. At present, the bulk of evidence has been linked to juvenile CLN3, although it seems likely that cardiac phenotypes will be manifest for all NCL forms. It is possible that NCL-linked cardiac pathologies, especially bradycardia, are under-reported because they may be mistaken for other complications such as epileptic seizures. To date, the underlying causes of altered cardiac function and the time-course of changes have rarely been investigated. Although some cardiac abnormalities, such as bradycardia and left ventricular hypertrophy, occur frequently in NCL patients, and were found in every patient over the age of 20 in a study in Denmark, the combination of symptoms that manifest in an individual can vary considerably. It is therefore currently difficult to describe a typical cardiac pathology for any of the NCLs. However, due to the evidence for progressive cardiac impairment in NCL, it has been recommended that heart function is examined at least once per year beyond the age of 18 years [10] so that appropriate interventions can be sought in a timely manner.

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