POLG-related disease

Introduction

This project aimed to further our understanding of POLG-related disease by examining the published literature on two main aspects; (1) specific clinical symptoms associated with POLG-related disease and (2) model systems of POLG-related disease. The current review was prompted by clinical assessment of a male aged 18 years (Patient F) who was seen by the clinical team in Newcastle in December 2020. Patient F harbours bi-allelic pathogenic variants; p.Gly848Ser and p.Arg309Cys, within the polymerase and exonuclease domains respectively of *POLG1*, the gene encoding the mitochondrial polymerase γ enzyme. Polymerase γ is the only enzyme responsible for replication of the mitochondrial genome and it is therefore not surprising that mutations in *POLG1* are responsible for mitochondrial DNA (mtDNA) defects; in the form of accumulation of mtDNA deletions and point mutations, or depletion of mtDNA copy number.

Following consensus agreement, specific clinical symptoms of POLG-related disease including peripheral neuropathy, myoclonus, and achalasia were selected to further our understanding of prevalence, aetiology, current treatments, and areas requiring further basic and clinical research. In addition to this, a systematic review of model systems attempting to recapitulate the clinical and molecular genetic features of POLG-related disease was conducted (MRes student; Jon Meyrick). This systematic review was registered on the international prospective register of systematic reviews (PROSPERO; York University) on 25th February 2021 (https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=234883).

1. Spinal cord involvement in mitochondrial disease

The spinal cord provides a pathway of communication between the body and the CNS and is encased in a vertebral column. It is approximately 40-50cm long and 1-1.5cm in diameter with two consecutive rows of 31 nerves roots either side of the cord. Each spinal nerve consists of dorsal root and ventral root axons which pass through a notch in the vertebrae column. The spinal nerves are divided into four groups and each segment is named after the vertebrae adjacent to where the nerves originate; cervical (C) 1-8, thoracic (T) 1-12, lumbar (L) 1-5, and sacral (S) 1-5.

The internal organisation of the spinal cord is arranged so that the interior of the cord consists of grey matter which is surrounded by white matter (Figure 1). In transverse sections, the grey matter is divided into posterior, lateral and ventral horns. The dorsal horn neurons receive sensory information that enters the spinal cord via the dorsal roots (dorsal root ganglia) of the spinal nerves. The lateral horns are present primarily in the thoracic region and contain the preganglionic visceral motor neurons that project to the parasympathetic ganglia. The ventral horns contain the cell bodies of motor neurons that send axons via the ventral roots and synapse on to striated muscles.

The primary sensory neurons are housed within the dorsal root ganglia (DRG) which are located adjacent to the spinal cord. These neurons are pseudounipolar which means that they consist of a bifuricated axon with a central and peripheral branch. The peripheral branch conveys information from the body, for example the skin, to the cells within the DRG while the central branch conveys information from the DRG to the tip of the dorsal horn of the spinal cord.

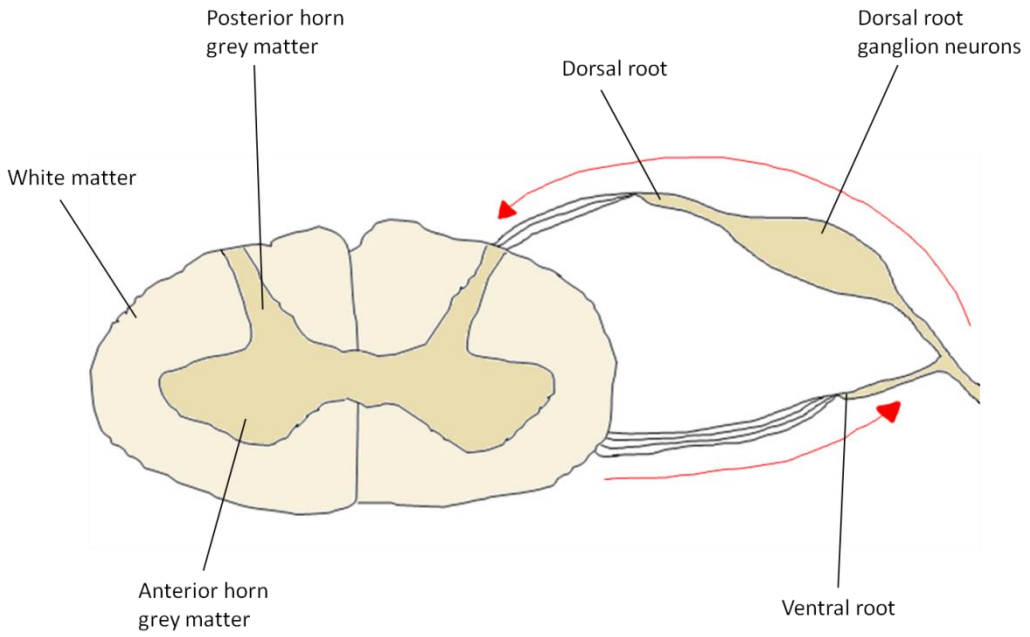

Figure 1 - The anatomy of the transverse spinal cord and dorsal root ganglia.

The spinal cord is shown in a transverse with the H-shaped grey matter (pale brown) surrounded by the white matter tracts. The dorsal root ganglia (DRG) contain large sensory neurons and is located adjacent to the spinal cord. The DRG relays sensory information through the dorsal roots into the dorsal horn of the spinal cord.

Here we review the neuroimaging, neuropathology and neurophysiology associated with biallelic *POLG* mutations, and other mitochondrial genetic defects in the context of peripheral neuropathy and spinal generated myoclonus.

2.1 Neuroimaging studies

A recent study evaluated spinal cord magnetic resonance imaging (MRI) in 119 children with genetically confirmed mitochondrial disease. Of those 119 children, 33 had undergone spinal MRI for a variety of clinical reasons relating to weakness, gait abnormalities, acute bowel and bladder symptoms, scoliosis, extremity tremor and spinal myoclonus. 19/33 patients demonstrated spinal cord lesions; 16/19 lesions were cervical and in 6 patients these extended to include thoracic levels while in 3/19 lesions were restricted to the thoracolumbar levels that extended to conus medullaris. In terms of the cross-sectional anatomy, lesions were most frequently distributed centrally around the central canal (11/19) followed by lesions involving the posterior columns (7/19). Two main imaging patterns were determined; Group A (12/19) showed involvement of the peripheral white matter with or without involvement of the central grey matter (resembling demyelinating disease), and Group B (7/19) showed lesions limited to the spinal cord grey matter (resembling spinal cord infarct or acute viral infections) (1).

Posterior column degeneration, manifesting as signal abnormalities, is a common finding on spinal MRI in patients with mitochondrial disease that are on restrictive diets or pernicious anaemia. Posterior column signal changes were in a small cohort of patients (4/6) subjected to spinal imaging. These included two patients; one harbouring pathogenic m.11778G>A variant causing a Leber's hereditary optic neuropathy (LHON)-plus phenotype and another patient harbouring a single large-scale mtDNA deletion causing Kearns-Sayre syndrome (KSS) (2). Posterior column degeneration was previously observed in neuropathological study of a patient with LHON (3).

An abnormal variant in *DARS2* leads to a spectrum of disease known as leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL). LBSL is characterized by slowly progressive cerebellar ataxia and spasticity with spinal posterior column dysfunction leading to decreased position and vibration sense in the most affected individuals. MRI of 15 patient spinal cords showed cervical spinal lesions affecting the posterior columns and lateral corticospinal tracts (4).

2.2 Neuropathological studies

2.2.1 MtDNA point mutations: m.3243A>G and m.8344A>G pathogenic variants

Severe neuronal cell loss and astrogliosis has been observed in Clarke's nucleus and the posterior horns of the cervical and thoracic spinal cord (5-7). Severe degeneration of the gracile fasciculus and posterior spinocerebellar tract, with moderate degeneration of the cuneate fasciculus and mild degeneration of the lateral and anterior corticospinal and anterior spinocerebellar tracts (7). Anterior horn cell pathology has been reported with decreased expression of complex IV subunit COX2 within this cellular population and molecular genetic evidence of high heteroplasmy for the pathogenic m.8344A>G variant within these cells (8).

2.2.2 Biallelic *POLG* mutations

The posterior columns of the spinal cord feature extensive axonal and myelin loss (axonal > myelin loss) with greater involvement of the gracile fasciculus relative to the cuneate fasciculus (Figure 2). This pattern of pathology may be described throughout all levels of the spinal cord (9, 10), while in other studies it is reported only within the cervical spinal cord (11). Evaluation of astrocytes and oligodendrocytes showed an increase density of both cellular populations in these affected areas. Quantification of neuronal cell density showed decreased neuronal density in posterior horn while neuronal density was unaffected in anterior horn. Immunohistochemistry revealed downregulation of complex I subunits NDUFB8, NDUFS3 and NDUFA9 within neurons residing in the lateral and anterior horn cells while mitochondrial mass (as judged by porin, and complex II SDHA) was high. Loss of complex I subunit expression was not always uniform throughout the neuron and some neurons showed retained expression in perinuclear and membrane regions (data not shown) (9).

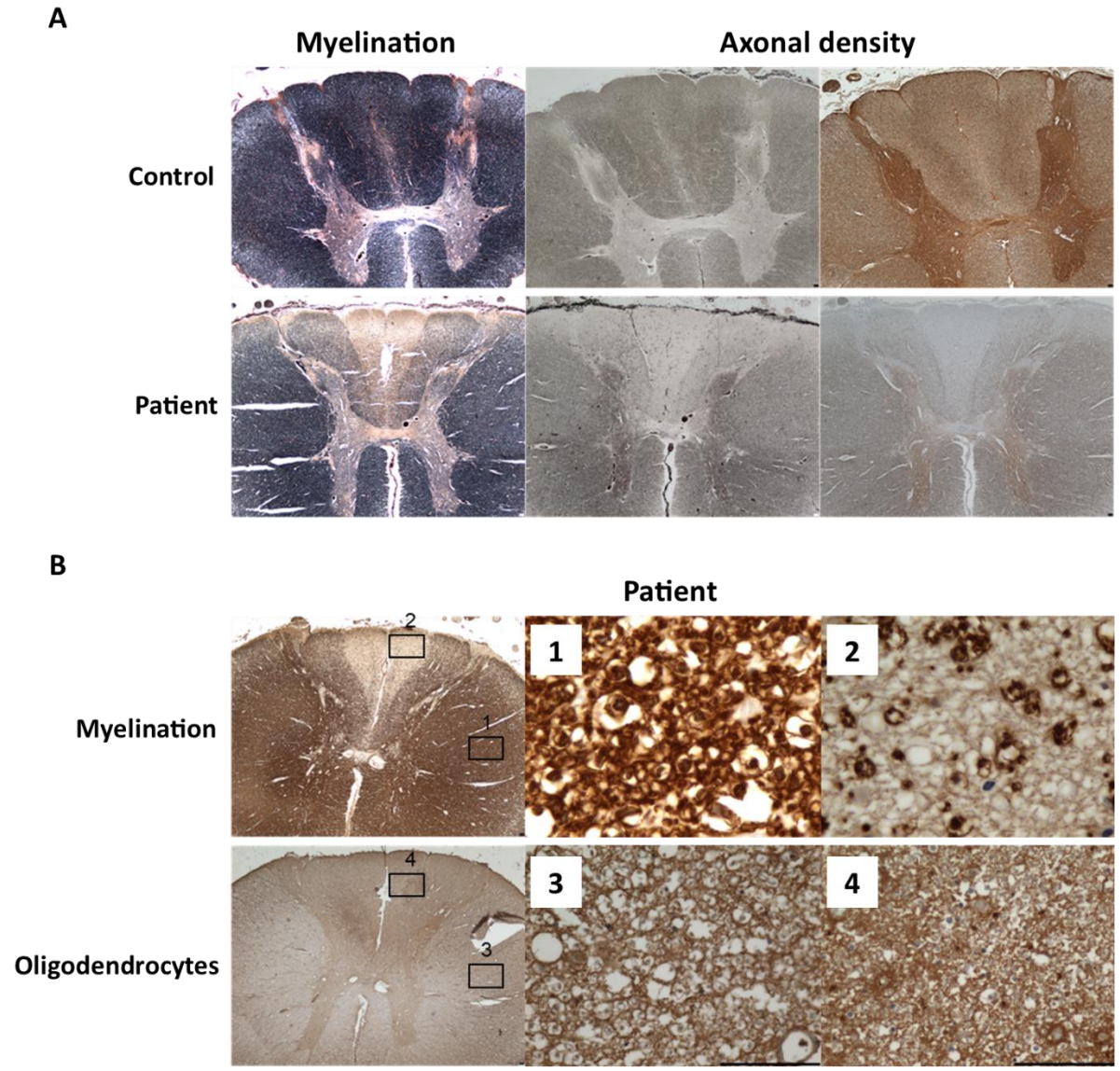

Figure 2 - Loss of myelinated axons in the dorsal columns of the spinal cord.

A. Spinal cord from a control individual shows preserved myelination of the white matter tracts (Loyez stain) and intact axonal density in white matter tracts (Bielschowsky's silver stain and phosphorylated neurofilament IHC). In contrast, spinal cord from a patient demonstrates a loss of myelin (Loyez stain) and axons (Bielschosky's silver stain and phosphorylated neurofilament IHC) from the posterior columns (fasciculus gracilis and fasciculus cuneatus).B. In patient spinal cord, there is a profound loss of myelin basic protein (MBP) from the fasciculus gracilis (and reduced density in the fasciculus cuneatus) in patient spinal cord.Panel 1 shows intact MBP surrounding axons in a normally myelinated region of the patient spinal cord (higher magnification) while panel 2 shows reduced density of MBP in the demyelinated posterior column of patient spinal cord. There is a corresponding increase in2',3'-cyclic dinucleotide 3' phosphodiesterase (CNPase) expression in demyelinated regions suggesting an increase in oligodendrocytes (panel 4) relative to CNPase expression in a normally myelinated region. Scale bar=100µm.

Intriguingly, prominent mitochondrial dysfunction was observed in the central canal of the spinal cord (Figure 3). A global absence of cytochrome *c* oxidase (COX) activity was detected using dual COX/SDH activity and a

dramatic reduction in expression of subunits comprising complexes I and IV were observed, despite high mitochondrial mass. The central canal consists of ependymal cells that function to provide a barrier between the CNS and cerebrospinal fluid (CSF) and therefore play an important role in CSF homeostasis. They also possess neural stem cell properties, similar to ependymal cells of the subventricular zone (SVZ) in the lateral ventricles of the brain (12). Though their status as a stem cell is somewhat contentious, they express a neural stem cell marker, CD133, and they are thought to be quiescent and only undergo division following injury or insult (13, 14).

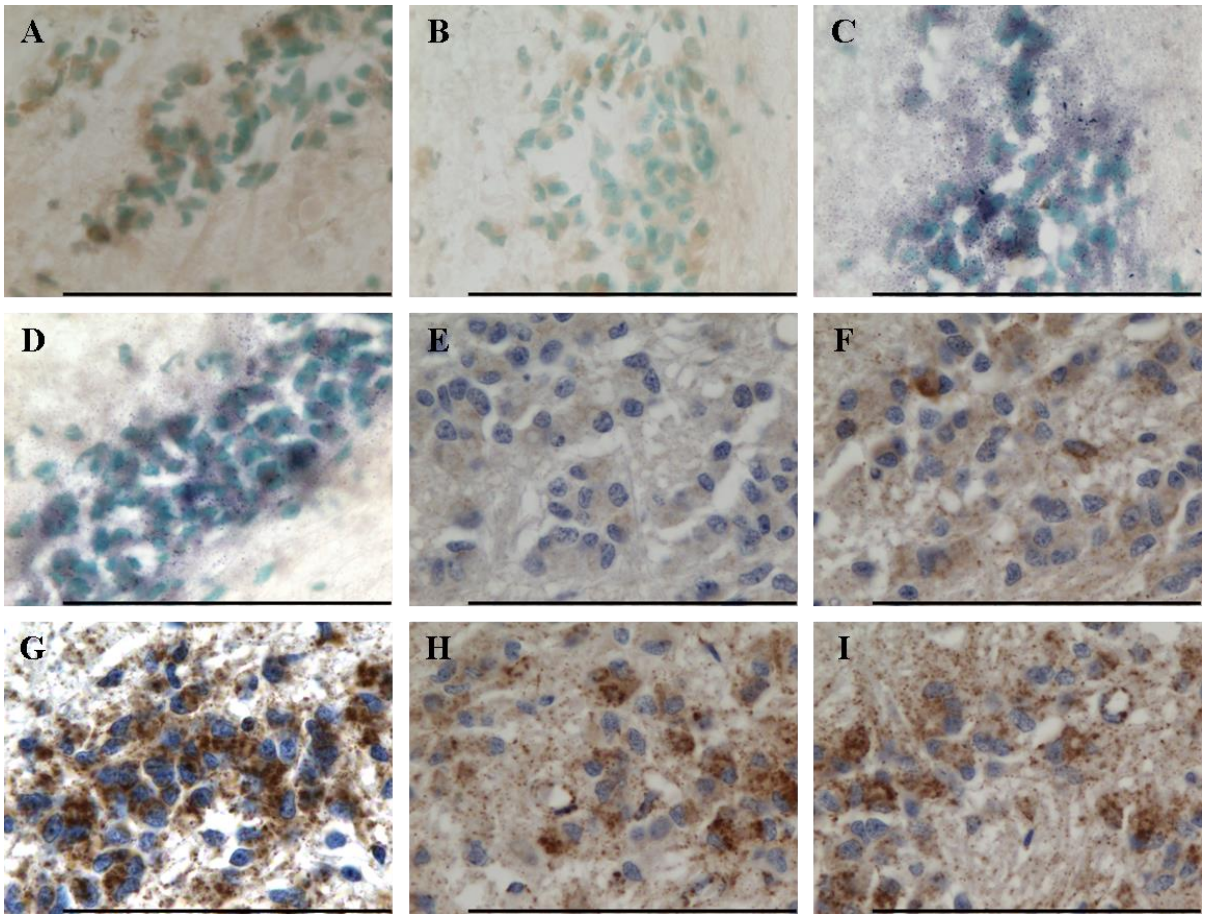

Figure 3 - Mitochondrial dysfunction is prominent spinal cord central canal in Patient 10.

A. Dual COX/SDH histochemistry in control tissue reveals intact COX activity in the central canal ependymal cells (Methyl green counterstain). B. COX histochemistry reveals low levels of COX activity in patient ependymal cells (Methyl green counterstain). C. SDH histochemistry in patient ependymal cells reveals high levels of SDH activity (Methyl green counterstain). D. Dual COX/SDH histochemistry in patient tissues reveals a mosaic pattern of activity, however most cells are COX-deficient (Methyl green counterstain). E. Lack of CI-20 expression in patient ependymal cells. F. Profound loss of CI-30 expression in patient ependymal cells. G. CII-70 expression is high suggesting an abundance of mitochondria within the ependymal cells in patient. H. Reduced COXI expression in patient ependymal cells. I. Reduced COXIV expression in patient ependymal cells. Scale bar = 100µm. Data is unpublished.

The dorsal root ganglia (DRG) are collection of sensory neurons which are located adjacent to the spinal column in pairs, in total there are 31 DRG pairs which span the entire cord. These sensory neurons are responsible for thermoception (sensing temperature), nociception (feeling pain), mechanoreception (sensing pressure) and proprioception (sensing body spatial position) and relaying this information to the CNS. DRG neurons have a pseudo-unipolar morphology since they have a single axonal process which bifurcates within the ganglion into a peripheral (distal) and a central (proximal) axonal branch. The peripheral branch sends information about touch, proprioception and vibration along the posterior column medial-lemniscal pathway (along the gracile and cuneate fasciculi) to the medulla oblongata. The central axonal branch sends information about temperature and pain to the neurons of the posterior horn.

In our study, we observed severe neuronal degeneration within the DRG characterized by significantly reduced cell density ($p=0.0093$; Figure 4), altered mitochondrial mass and OXPHOS defects in remaining neurons (Figure 5). Mitochondrial mass fluctuated between neurons with some neurons appearing almost devoid of mitochondria whilst others demonstrated accumulation of mitochondria around the periphery of the neuronal cytoplasm (this pattern is reminiscent of the 'ragged red fibres' observed in skeletal muscle biopsies from patients with mitochondrial disease). 60% of DRG neurons were COX-deficient, and levels were highest within cervical, lumbar and sacral levels relative to thoracic. Reduced expression of subunits comprising complex I and IV was observed in neurons signifying OXPHOS deficiencies. Intriguingly, this loss was not uniform across the neuronal cytoplasm since numerous cells exhibited a loss of complex I or IV with a small area of immunoreactivity within the cytoplasm. Subsequent analysis found that ubiquitin immunoreactivity was evident in a similar pattern suggesting these proteins are targeted for destruction via proteolytic degradation (unpublished observation). Molecular genetic analysis of laser captured DRG neurons reveals that the mean level of mtDNA deletion was low, 34.08% and 19.02% in COX-deficient and COX-positive neurons respectively. However, these neurons had undergone severe depletion of mtDNA ($p<0.0001$) which corroborates the immunohistochemical findings (9).

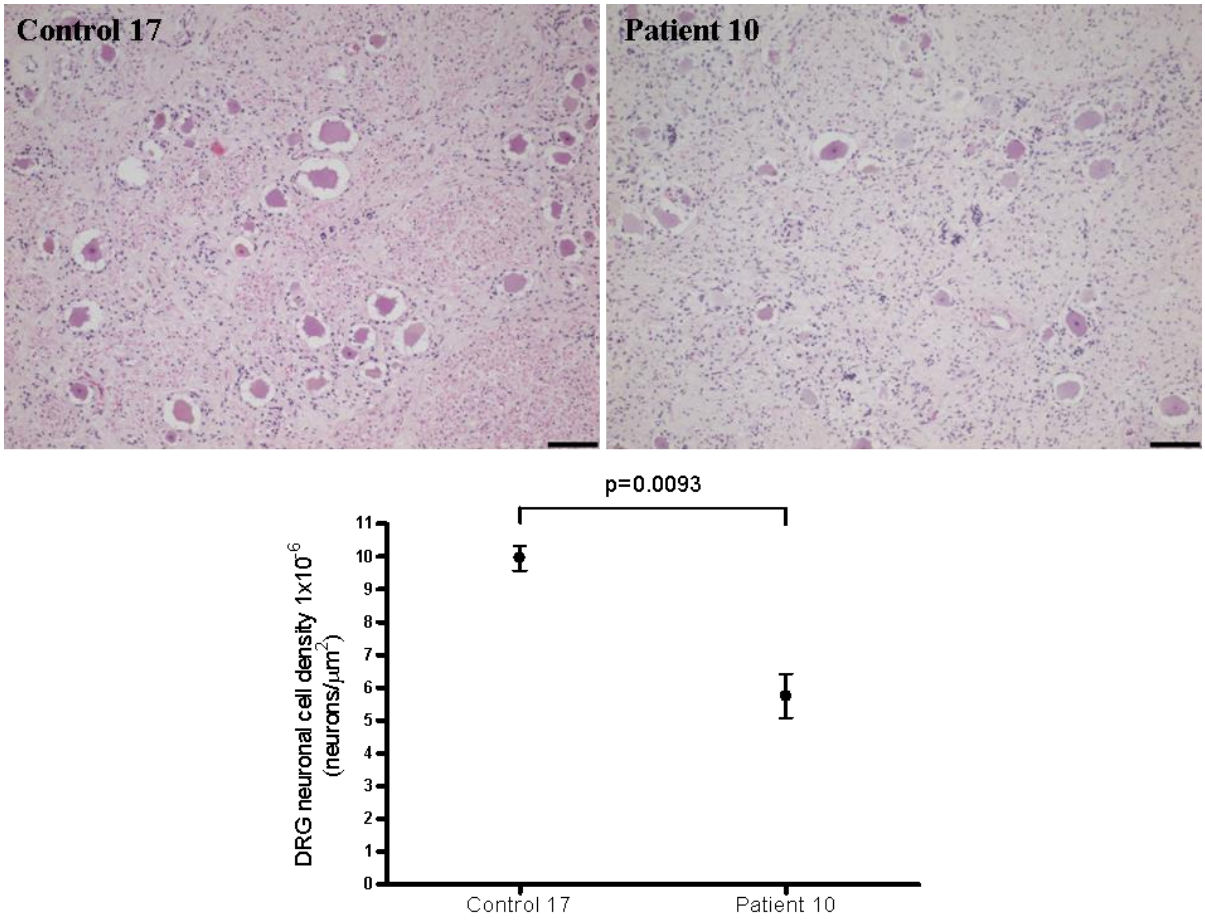

Figure 4 - Neuronal cell density in the DRG is reduced in patient tissues.

H&E staining of DRG tissues demonstrating the neuronal populations from control case 17 and Patient 10. The H&E stain provides visual confirmation of a reduced neuronal cell density in patient 10 relative to control 17. Quantitation of neuronal cell density reveals a significantly lower neuronal density in patient tissues relative to an age-matched control (Control 17). Graph shows the mean neuronal density from three separate sites ± the SEM.

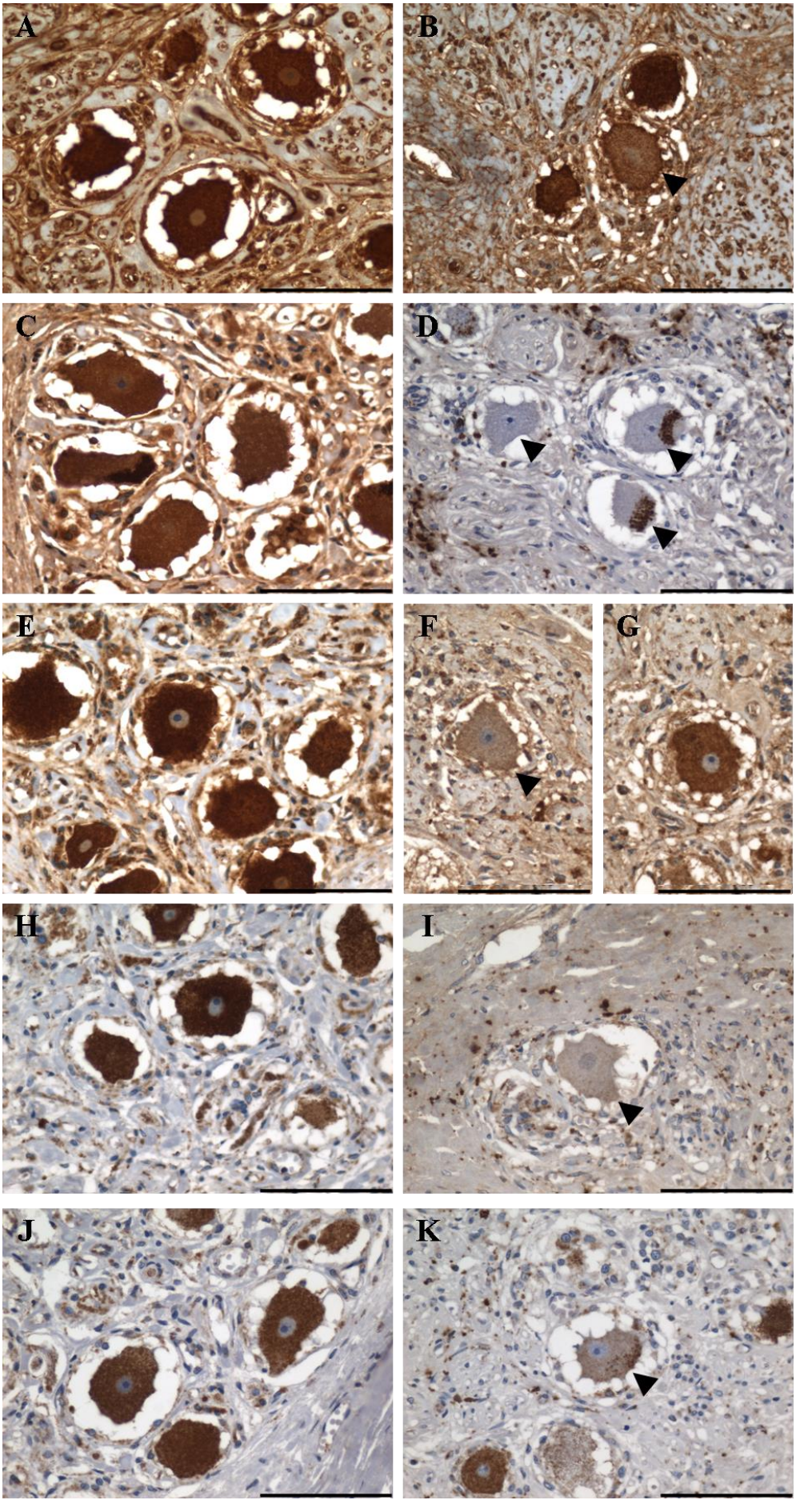
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Figure 5 - Mitochondrial dysfunction in the DRG.

A. Uniform mitochondrial distribution throughout control DRG neurons (Porin IHC). B. Variable mitochondrial density in patient DRG neurons, with pale (arrow) and dark neurons evident (porin IHC). C. Intact CI-15 expression throughout control neurons. D. Global marked loss of CI-15 expression and unusual sub-cellular localisation in patient DRG neurons. E. CII-70 expression is strong and uniform throughout control neurons. F. and G. Variable CII-70 expression in patient DRG neurons, this reflects porin IHC. H. Control DRG neurons demonstrate high COXI expression. I. Absence of COX staining in patient DRG neurons (arrowhead). J. COXIV demonstrates uniform expression in control DRG neurons. K. COXIV staining is dramatically reduced in patient DRG neurons.

2.3 Clinical symptoms associated with spinal cord: peripheral neuropathies and myoclonus

2.3.1 Peripheral neuropathy

The term peripheral neuropathy refers to dysfunction of the peripheral nervous system which comprises a vast array of nerves which permit communication between the central nervous system (CNS; brain and spinal cord) and all other parts of the body. Neuropathic symptoms are typically severe and progressive leading to severe disability in affect individuals. Symptoms can be divided into sensory and/or motor; sensory symptoms include tingling, pins and needles, numbness, tightness, burning, pain and sensory ataxia while motor symptoms include muscle cramps, stiffness, weakness and wasting.

Neurophysiological examination plays an important role in the diagnosis of peripheral neuropathy and helps distinguish the types of neuropathy. Typically, two types of neuropathy may predominate and are referred to as axonal or demyelinating depending on the inferred pathophysiology. In the case of polyneuropathy, a combined axonal and demyelinating type of neuropathy is described. Nerve conduction studies measure both the motor and sensory nerve conduction velocities (MNCV and SNCV) and reveal how well action potentials are maintained along axons. A low conduction velocity might be indicative of demyelination. The amplitudes of compound muscle action potentials (CMAPs) and sensory nerve action potentials (SNAPs) enable evaluation of action potential propagation along an axon. Small CMAPs and SNAPs might be indicative of axonal dysfunction. A third type of neuropathy can exist which is due to neuronopathy rather than degeneration of peripheral nerves. Neuronopathies are caused by degeneration of the neuronal cell bodies that reside either in the anterior horn cell population (motor neuronpathies) or the DRG (sensory neuronopathies).

Pathogenic *POLG* variants are frequently associated with peripheral neuropathy and often this forms part of the major clinical features (15). This is certainly the case in ataxia neuropathy spectrum (ANS) disorders which

comprise an overlapping group of disorders which are variably referred to as spinocerebellar ataxia epilepsy (SCAE), myoclonic epilepsy myopathy sensory ataxia (MEMSA), sensory ataxia neuropathy with dysarthria and ophthalmoparesis (SANDO) and mitochondrial recessive ataxia syndrome (MIRAS). Peripheral neuropathy associated with POLG-related disease has a reported prevalence of 42.2% and 53% in an Italian (16) and Norwegian cohort respectively (17). Patients with neuropathy have an increased prevalence of ataxia, hearing loss, muscle weakness and muscle wasting and there are some questions around whether neuropathy causes or exacerbates the neuromuscular symptoms (16). Peripheral neuropathy is most common in patients aged between 12-40 years and correlates with a longer disease duration (17). Neurophysiology typically reveals neuropathy in POLG-related disease is consistent with axonal or mixed, mainly sensory polyneuropathy or sensory neuronopathy and is associated with pain in more than 1/3 of patients (9, 16, 18). A neurophysiology study of eleven patients with POLG-related disease showed both sensory and motor abnormalities in the majority of cases (8/11). Of these patients, over half have greater involvement of the sensory neurons relative to motor components. Indeed, the neurophysiology results of 3/11 patients are consistent with a sensory neuronopathy (9). Neuropathy is thought be a major contributory factor to the high prevalence of chronic pain among patients with mitochondrial disease (19). Although chronic pain did not show a reduction in quality of life in these patients, this study focused on a small sample size so may be underrepresenting the impact on patients.

Our understanding of the pathophysiology of peripheral neuropathy in POLG-related disease is incomplete and consequently there are no effective disease-modifying therapies. Current treatment of neuropathies is limited to symptom management and can take the form of pharmacotherapy for alleviating nerve pain (including amitriptyline and gabapentin), physiotherapy to improve muscle weakness, and nutritional supplementation for those patients with poor nutrition owing to swallowing difficulties and gastrointestinal dysmotility. Nutritional supplementation may be relevant since certain vitamin deficiencies, including thiamine or vitamin B deficiency, may contribute to neuropathy.

Although neurophysiology data and limited neuropathological studies provide strong evidence that POLG-related neuropathy is a sensory neuronopathy further work is required to understand the pathophysiology of neuropathy in these patients. Given the lack of biomarkers and inability to biopsy the dorsal root ganglia (sensory neurons), future work should focus on post mortem investigations of the sensory neurons, and their

projections to the spinal cord and clinical assessment of patients to understand the natural history of sensory neuronopathy. Recently a sensory ataxia rating scale (SEARS) has been developed to capture the severity of sensory neuronopathies and could prove useful in multicentre studies for treatment trials (20).

2.3.2 Myoclonus

Myoclonus is a hyperkinetic movement disorder which is characterised by sudden, brief involuntary movements associated with bursts of muscular activity (positive myoclonus) or sudden interruption of muscle contraction (negative myoclonus). Myoclonus can be present in conjunction with epilepsy (myoclonic epilepsy) or without epilepsy. Myoclonus can be classified according to the presumed anatomic source of myoclonus; cortical, subcortical, spinal or peripheral myoclonus. The most common type of myoclonus is cortical myoclonus which mainly affects the distal upper limbs and face. The pathophysiological mechanisms underpinning cortical myoclonus are thought to arise from a loss of intercortical inhibition and therefore increase pyramidal neuron hyperexcitability in pre- or post-central cortex. Neurophysiological analysis, including electroencephalogram and electromyography, is crucial to providing insight into the underlying pathophysiology basis of myoclonus.

The focus of this review is to provide an understanding the aetiology of spinal myoclonus and whether this is part of the myoclonus phenotype observed in POLG-related disease. Myoclonus of a spinal origin can be caused by trauma, spondylitis, tumours, infection, myelitis or ischaemia and generally shows a lack of EEG correlate. There are two types of spinal myoclonus; spinal segmental myoclonus (SSM) and propriospinal myoclonus (PSM). In SSM, the main clinical features include jerks involving one or to limbs that may be rhythmic or semi-rhythmic and are generally not stimulus sensitive. Neuropathological finding reveal sparing of the large anterior horn cells (lower motor neurons) in contrast to considerable loss of the small and medium sized interneurons (21).The pathophysiology is thought to arise from a loss of inhibition of the spinal interneurons contributing to hyperexcitation of the anterior horn cells (22). In PSM, the main clinical features include slow truncal jerking with flexion more common than extension and it is stimulus sensitive but with a longer latency than cortical myoclonus. The pathophysiology is postulated to be due to defects in the propriospinal pathways (which have not yet been proven in humans). A psychogenic aetiology has also been proposed. Although both types of spinal myoclonus are often refractory to pharmacological treatment, clonazepam is sometimes useful and other anticonvulsants including tetrabenazine, carbamazepine,

piracetam, and levetiracetam have been tried. There is evidence that botulinum toxin injections have some efficacy in treating neck, shoulder and lower extremity myoclonus (23).

2.3.2.1 Myoclonus in mitochondrial disease

An Italian study found myoclonus affected 3.6% (39/1,086) patients with mitochondrial disease (24). Myoclonus is often reported as a prominent clinical feature in Myoclonic Epilepsy with Ragged Red Fibres (MERRF), which is associated with pathogenic mtDNA mutations, the most frequent being the m.8344A>G variant in the transfer RNA lysine ($tRNA^{Lys}$). However, in this study only 29.2% of patients with clinically-defined MERRF actually had myoclonus present at disease onset. In these patients, myoclonus was associated with cerebellar ataxia, epileptic seizures, hearing loss and cognitive impairment. Myoclonus may also be an important part of the clinical presentation in other mitochondrial disorders. POLG-related disease is an important cause of myoclonus and this will be discussed separately.

A recent study of adult mitochondrial disease in a large Italian cohort comprising 764 patients identified that 13.7% of patients were affected by a movement disorder. Of these patients, 21.9% harboured pathogenic POLG variants and 16.2% harboured the pathogenic m.8344A>G variant. Movement disorders were further classified and 12.3% (13/105) of patients exhibited myoclonus which was mainly associated with the pathogenic m.8344A>G variant (10/13). Myoclonus was described as segmental in 1/13. Levetiracetam was the most common option for treatment of myoclonus (25).

1.3.2.2 Myoclonus In POLG-related disease

Myoclonus is frequently reported in patients with POLG-related disease and particularly in the context of myoclonic seizures (Speccio et al 2020). A recent cohort study of 41 paediatric patients with confirmed biallelic *POLG* mutations identified 11 patients with myoclonus, which was described as either epileptic or non-epileptic and at least two patients experiencing myoclonic jerks during sleep (26). In a large series of 155 patients with POLG-related disease, 74% were affected by myoclonic seizures with 37% of these patients exhibiting symptoms at disease onset (17).

2.4 Future direction

Since treatment for myoclonus, particularly myoclonic seizures, in POLG-related disease remains ineffective using current anticonvulsants and given the high prevalence of myoclonus in these patients, further research is imperative to understand how myoclonus is generated. Clinical studies utilizing quantitative neuroimaging, neurophysiology and clinical symptoms would provide further insights into the contribution of spinal cord dysfunction to the constellation of clinical symptoms in these patients. In conjunction, neuropathological studies using genetically and clinically well-characterised human post mortem tissues to provide insights into pathology and potential mechanisms. It would be particularly important to explore the OXPHOS deficiencies observed in both anterior and dorsal horn cell populations, and to quantify the neuronal cell population densities in order to delineate selective neuronal vulnerabilities. Since human tissues typically show end-stage pathology, we hope to develop pre-clinical studies utilising transgenic mice (such as the interneuron-specific mitochondrial transcription factor A (TFAM) transgenic mouse model (PVCre TFAM-/-) to better understand mechanisms and also provide a platform from which we can develop novel therapeutic strategies. The possibility of exploring spinal cord circuitry using *in vivo* electrophysiology on acute slice preparations in conjunction with pharmacological manipulations (either of mitochondrial function or neurotransmitters e.g. glycine) would be particularly interesting.

2. Achalasia

1.1 Idiopathic Achalasia

Idiopathic achalasia is a rare disorder that affects between 10-26 individuals per 100,000 and manifests as an obstruction to the passage of food from the oesophagus to the stomach (27-30). At the junction between the oesophagus and stomach resides a muscular ring known as the lower oesophageal sphincter (LOS) which opens to allow food to pass from the oesophagus into the stomach and constricts to prevent reflux of gastric contents (Figure 6). In achalasia, this muscular ring fails to open properly (or at all) and results in stasis of ingested foods. Affected individuals present with dysphagia, regurgitation of food, weight loss, and less commonly, chest pain and aspiration pneumonia (31).

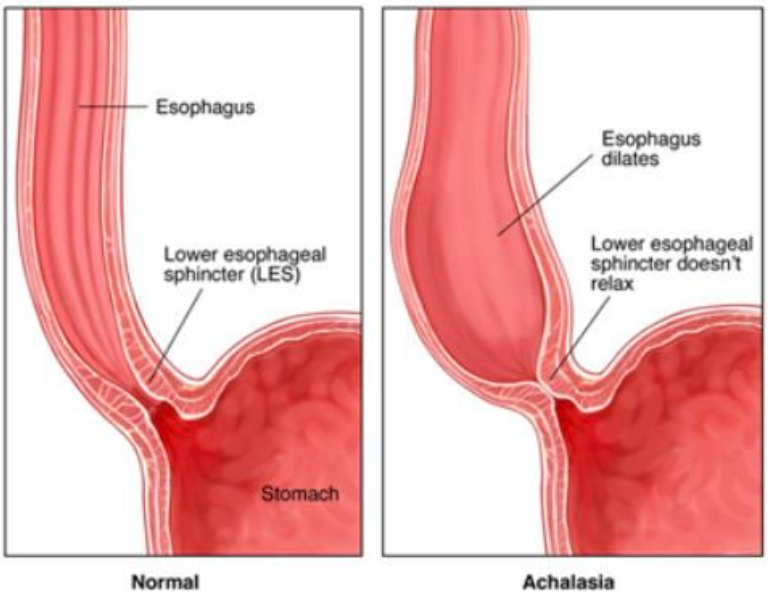


Figure 6 – The lower oesophageal sphincter (LOS) in normal circumstances and achalasia.

Under normal conditions, the muscles of the lower oesophageal sphincter (LOS) briefly open during swallowing to allow the passage of food and then close to prevent reflux of gastric contents. In achalasia, the LOS remains tightly constricted and prevents food from entering the stomach. Over time, this contributes to dilation of the oesophagus.

The diagnostic hallmark of achalasia is incomplete relaxation of the LOS which is reflected by an increased integrative relaxation pressure in the absence of peristalsis. Diagnosis can be achieved using high resolution manometry (HRM) which is recognised as the gold standard diagnostic method. This involves using a catheter with pressure sensors spaced 1cm apart and this is positioned to span the hypopharynx to the stomach and then pressures generation along the entire length of the oesophagus can be measured simultaneously. Sophisticated software processes the pressure output by using interpolation to generate oesphageal pressure topography (EPT) plots that represent oesophageal motility and sphincter function on colour-coded, pressure-space-time plots (Figure 7). Another diagnostic method is the Barium swallow test during which a contrast agent, Barium, is swallowed and a series of X-rays are taken of the oesophagus. Typically, the X-rays show a classic 'Bird's beak' appearance of the LOS due to oesophageal distention.

HRM has led to the subclassification of achalasia based on contractility patterns known as the Chicago Classification v4.0 (CCv4.0; Figure 7) (32). In type I achalasia (referred to as classic achalasia) there is minimal contractility of the oesophageal body, while type II achalasia is defined by intermittent periods of pan-oesophageal pressurisation and type III achalasia (referred to as spastic achalasia) features premature or

spastic oesophageal contractions. It is thought that subclassification of achalasia can guide treatment with type II achalasia having the best response to treatment, followed by type I achalasia and type III achalasia being the most challenging to treat (33).

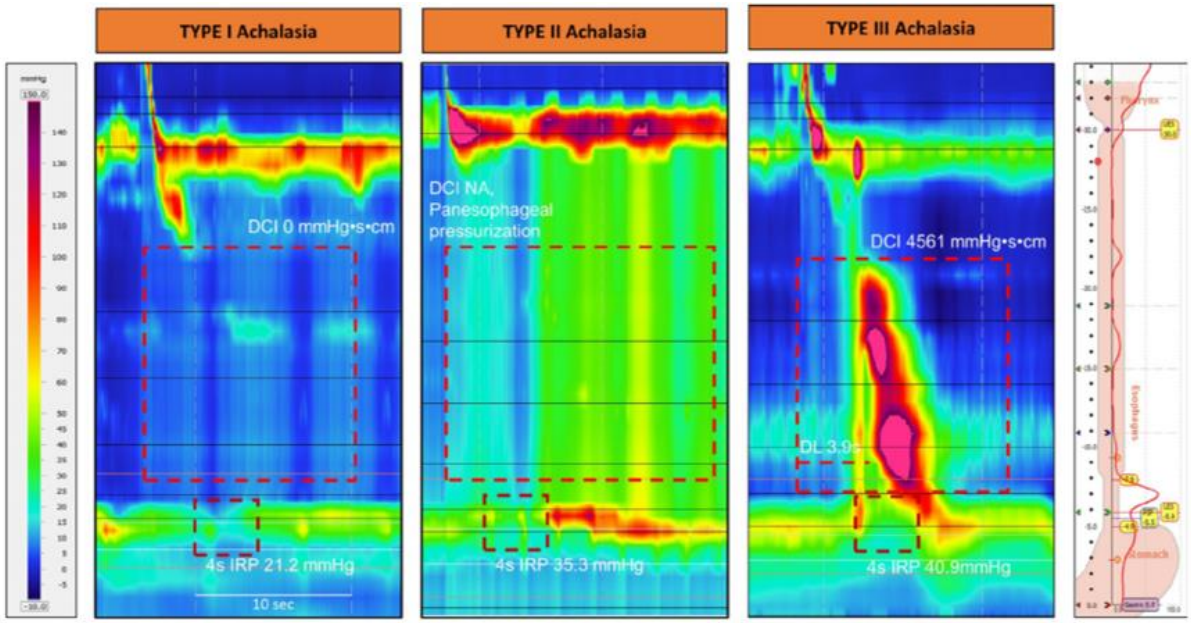


Figure 7 – Achalasia subtypes as defined by HRM.

Type 1 achalasia: integrated relaxation pressure (IRP) is elevated with failed peristalsis (distal contractile integral (DCI <100mmHg-s-cm), and without panoesophageal pressurisation. Type II achalasia: IRP is elevated with failed peristalsis and panoesophageal pressurisation. Type III achalasia: IRP is elevated with a normal DCI, and a reduced distal latency (taken from).

1.2 Physiology of the lower oesophageal sphincter

The terminal 2-4cm of oesophagus generally constitutes the lower oesophageal sphincter (LOS) and it is composed of circular and longitudinal muscle layers. The LOS is innervated by both sympathetic and parasympathetic nerve fibres. The sympathetic nervous system is thought to exert only a minor effect on LOS function. The vagus nerve mediates parasympathetic innervation with efferents arising in the dorsal motor nucleus (DMN) and synapsing on to the myenteric (Auerbach's) plexus of the enteric nervous system. The parasympathetic innervation contributes to inhibitory and excitatory neurons with excitatory nerves arising from rostral DMN and inhibitory nerves from caudal DMN. The excitatory neurons are cholinergic and communicate using a neurotransmitter, acetylcholine (Ach) while the inhibitory neurons are predominantly

nitrergic and communicate using nitric oxide (NO), with some contribution from vasoactive inhibitory peptide (VIP), purine structures and carbon monoxide.

1.3 Pathophysiology

Evidence from histopathology studies of idiopathic achalasia suggest the initial pathological insult is mild inflammation within the myenteric plexus, which progressively worsens and coincides with loss a specific subset of myenteric ganglion cells; nitrergic inhibitory interneurons, and fibrosis. As the disease progresses, there is a global loss of myenteric ganglion cells comprising both inhibitory and excitatory neurons (Figure 8). A cohort of patients with achalasia who underwent oesophagectomy reveal a complete absence of myenteric ganglion cells in 64% and a marked reduction in 36% of samples. There was also a marked, T-cell inflammatory infiltrate of the myenteric plexus with fibrosis that inversely correlated with the number of preserved ganglia (34).

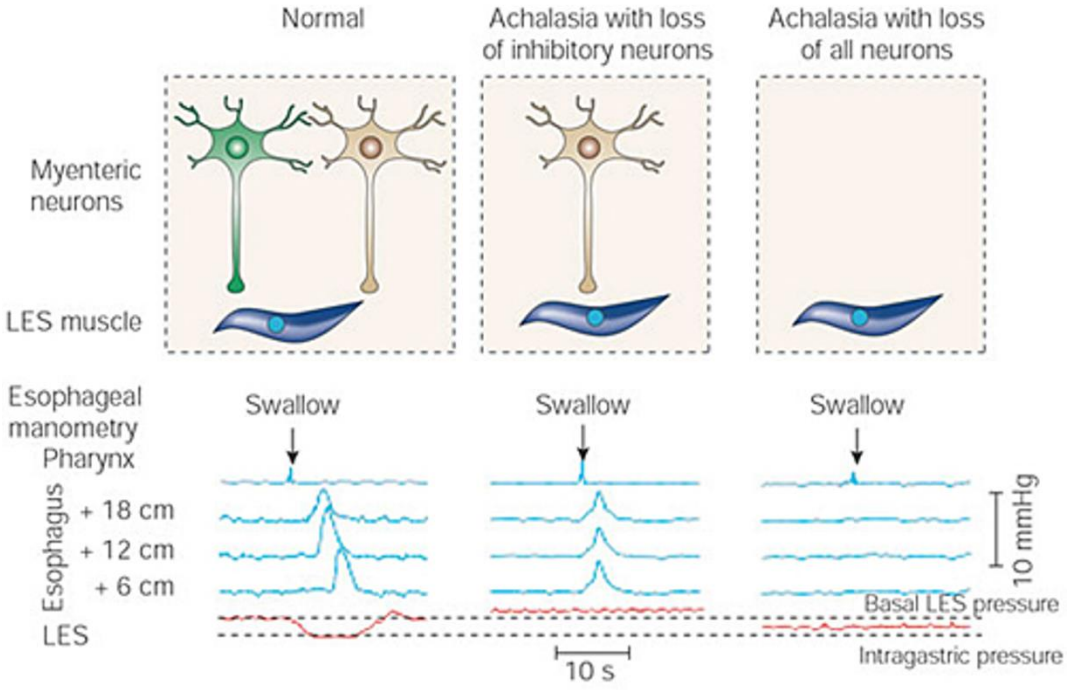


Figure 8 – Pathophysiology of achalasia.

In the normal condition where excitatory, cholinergic (Ach) motor neurons innervate the smooth muscle cells of the LOS and contribute to the genesis of basal pressure of the LOS. Inhibitory, nitric oxide (NO) neurons also act on the LOS to produce the relaxation that accompanies a swallow.
Achalasia resulting from the loss of inhibitory neurons. In this situation, the absence of NO interneurons results in an elevation in the basal LOS pressure and absence of swallow induced relaxation of the LOS. Oesophageal aperistalsis is defined by simultaneous oesophageal body contractions.

Achalasia with complete loss of myenteric neurons. Here the basal LOS pressire is below normal owing to the absent excitatory neurons, and swallow-induced relaxation is absent owing to the lack of inhibitory neurons. Esophageal aperistalsis is defined by the absence of esophageal body contractions. (Taken from Hirano et al.)

1.4 Causes

Although the precise aetiology of achalasia remains undetermined, there is evidence to suggest that an initial insult such as an autoimmune reaction to a viral infection, such as Herpes Simplex virus, in genetically predisposed individual may be important. A viral infection results in cell-mediated and immune-mediated attack on the neurons residing within the myenteric plexus. It has been speculated that the different manometrically defined subclasses may be associated with certain immunotoxicity patterns. In type III (spastic) achalasia, there is no demonstrable neuron loss from the myenteric plexus however there is impaired inhibitory neuronal function. While a progressive plexopathy is observed in type II achalasia which progressive to type I achalasia with cytotoxic immune response leading to agangliosis (35).

Secondary achalasia may be a prominent feature in other disorders with symptoms that share the manometrical and radiological features of idiopathic achalasia. These secondary forms provide important insights into the pathophysiological processes underlying idiopathic achalasia. One such disorder is Chaga's disease – the result of an infection with a parasite, *trypanosome cruzi*, endemic to Central and South America and Mexico and transmitted via blood-sucking triatomine insects (36). The oesophagus is most commonly affected and manifests with achalasia in 7-10% of chronically infected individuals. Antibodies directed at M2 Muscarinic acetylcholine receptors within the myenteric plexus have been demonstrated in patients with Chagas' disease and achalasia (37).

There are limited studies reporting achalasia in patients with spinal cord injuries (SCI). A recent study of 25 adult patients with chronic SCI (> 1 year) demonstrates an association with oesophageal dysmotility. Patients were divided into 2 groups; 12 patients with paraplegia (injury between T4-T12) and 13 with tetraplegia (injury between C5-C7) in comparison with 14 able bodied controls. 84% of the cohort had at least one motility disorder; 12% with type II achalasia, 4% with type III achalasia, 20% with oesophageal-gastric junction outflow obstruction, 4% with hypercontractile oesophagus and 48% with other peristaltic abnormalities. Additionally, patients self-reported difficulty swallowing followed by regurgitation of food (38). This raises the possibility of

primary pathology arising in the spinal cord contributing to LOS constriction likely due to impaired sympathetic innervation of the LOS.

1.5 Treatment

Current treatment strategies aim to achieve symptomatic control of achalasia by reducing the contractility of the LOS and improving patient quality of life. There is also a need to prevent progression to end stage achalasia and prevent complications. Treatment options include pharmacological, endoscopic and surgical however endoscopic treatments are the preferred choice (39).

There is no convincing evidence that smooth muscle relaxants (e.g. calcium blockers, phosphodiesterase inhibitors or nitrates) provide symptomatic relief in adults with achalasia. They also cause side effects and are therefore not recommended for use (39).

Endoscopically targeted botulinum toxin (BTX) injections provide short-term effective symptomatic management of achalasia. BTX is a potent inhibitor of acetylcholine (Ach). ACh is a neurotransmitter which is released by cholinergic neurons at the LOS and causes smooth muscle contraction. By blocking Ach, BTX causes partial paralysis of the LOS muscles and allows the LOS to open. It is a low risk procedure but the benefits are temporary with most patients requiring further treatment within 1 year (40).

Endoscopic pneumatic dilation is a widely used, effective nonsurgical treatment (41, 42). Pneumatic dilation involves positioning a pressurised balloon at the LOS to cause disruption of the muscle fibres. Progressive dilation using balloon sizes 30, 35 and 40 mm diameter. A complication is transmural perforation which is thought to occur in <1% of cases.

Heller myotomy is performed by laparoscopically incising the distal 4-6cm of oesophageal musculature and extends 1-2cm onto the gastric cardia. *Per oral* endoscopic myotomy (POEM) is a relatively new and minimally invasive technique which is performed by endoscopy. It involves a mucosal incision made longitudinally to allow the endoscope into the submucosal space. A submucosal tunnel is created to allow passage of the endoscope toward the gastro-oesophageal junction (GOJ). Once the GOJ is identified, the endoscope is passed 2-3cm beyond it. A selective myotomy of the inner circular muscle bundles is performed 6cm above the GOJ and extending 2-3cm beyond the GOJ. Closure of the mucosal incision completes the procedure (43, 44).

Current evidence suggest it is a safe and effective treatment with low rate of serious adverse events however there is no long-term data available to date (39).

Future experimental treatments could include vagal nerve stimulation to improve physiological function of the oesophagus through electrical pacing of the vagus nerve (45). This has been investigated in an opposum model of achalasia in which banding of the gastro-oesophageal junction prevents relaxation of the LOS during swallowing. Three groups of animals were used; group 1 subjected to sham surgery, group 2 subjected to a loose banding and group 2 subjected to tighter banding. Six weeks post-surgery, achalasia was confirmed by radiology and manometry and treatment in the form of bilateral electrical stimulation of the vagus nerve was performed using a constant current nerve stimulator. Stimulus values were modulated over a wide range (frequency 1 to 20Hz, a pulse width of 0.1-5 msec, and train duration of 0.1-10 sec) until peristaltic activity was demonstrated. Group 2 showed a significant increase in the amplitude of contractions ($p<0.001$) and return of peristaltic activity in 49% of swallows before band removal. After band removal, all contractions were peristaltic. In contrast, while group 3 demonstrated a significant increase in the amplitude of contractions ($p<0.0001$), there was no return of propagative peristalsis before band removal. Following band removal, 44% of contractions were progressive in the smooth portion of the oesophagus. The authors conclude that electrical stimulation of the vagus nerve improved the force of oesophageal contractions irrespective of the severity of disease however peristaltic activity only returned to normal in the early variety of achalasia (46).

Idiopathic achalasia represents an attractive target disease for enteric neural stem cell (ENSC) therapy for a number of reasons; 1. There is a clear understanding of the neuronal deficit. 2. Neuronal degeneration is localised. 2. Neuronal stem cells can be delivered to the target region endoscopically (47). In theory, enteric nerve stem cell replacement could restore the missing neuronal nitric oxide synthase (nNOS) and vasoactive intestinal peptide (VIP)-expressing inhibitory interneurons. This would improve oesophageal and LOS function in patients with oesophageal achalasia. This concept has been explored in preclinical studies using mice reviewed in (47, 48). Since the loss of nitric oxide-expressing neurons are a major feature of human enteric neuropathies, including achalasia, development of a neuron nitric oxide knockout (nNOS-/-) mouse model provide an opportunity to explore ENSC therapies. The nNOS-/- mouse model phenotypically demonstrates slow colonic transit owing to a complete loss of nNOS neurons in the colon. Transplantation of donor ENSC lead to formation of trans-colonic engraftment of transplanted cells within the distal colon (up to 42mm away

from the transplant site). Transplantation of ENSC resulted in development of nNOS+ neurons and the restoration of nitrergic responses. This also led to unexpected increases in interstitial cells of Cajal (ICC) number suggestive that transplanted ENSC can lead to non-cell autonomous changes in the cellular environment. These combined effects led to the rescue of impaired colonic motility (49). Recent work has developed an *ex vivo* organotypic method to model the temporal integration of ENSC into the murine gut wall (50).

A major challenge associated with ENSC therapy is the preservation of disease-causing mutations in autologously sourced cells. Recent advances in gene therapy, such as CRISPR/Cas9 technology may provide an elegant mechanism to correct disease causing mutations, and such approaches have recently been explored in Hirchsprung's disease (HSCR); a congenital condition resulting from a failure of the enteric nervous system to develop resulting in agangliosis of the distal colon with subsequent bowel obstruction. Induced pluripotent stem cells (iPSCs)-derived enteric neural crest cells from patients with HSCR due to *RET* mutations were subjected to CRISPR/Cas9 to correct the genetic defect. In addition to correcting the mutation, the enteric neural crest cell showed migration and differentiation capacity *in vitro* (51).

1.6 Achalasia in POLG-related disease

Gastrointestinal (GI) symptoms are relatively common in POLG-related disease with a recent cohort study indicating GI symptoms affected 63% of patients (17). Specific GI symptoms included feeding difficulties (52%), vomiting (28%), chronic diarrhoea (4%) and liver involvement (64%) and were typically observed in patients with early onset disease. Achalasia in POLG-related disease is infrequently reported. The most recent report is from an Italian study that describes a patient (Patient 7) harbouring biallelic pathogenic POLG variants; p.Arg309His and p.Gly1051Arg. This patient presented in infancy with epilepsy, developmental delay, severe peripheral neuropathy, ataxia and severe gastrointestinal impairment with achalasia. The same pathogenic variants were identified in his sister, who was also reported to be affected by neuropathy, ataxia and achalasia (52). A study by Chemlinsky and colleagues described achalasia in two paediatric patients who were known to have multiple mitochondrial DNA deletions in a skeletal muscle biopsy however no nuclear genetic defects were reported. The first patient (Patient 1) presented with gastroesophageal reflux at 6 months of age, and a diagnosis of achalasia was made at 3 years of age. Treatment involved surgical resection of the distal two-thirds of oesophagus which revealed loss of neurons from the myenteric plexus. At age 13 years, the patient

developed progressive gait problems, tremor, dysarthria and fluctuating limb paresthesia and pain. The second patient (Patient 2) was diagnosed with achalasia aged 7 years following deceleration of weight gain (53).

1.1.7 Achalasia associated with other mitochondrial diseases

A recent United Mitochondrial Disease Foundation externally-led patient focused drug development meeting identified two out of ninety-five patients expressed achalasia as a symptom that impacts on daily life (2019 UMDF meeting; EL-PFDD-VOP-Report_Online-Survey-Data_Appendix-1.pdf (umdf.org)). A recent publication describes achalasia in a pregnant patient with mitochondrial disease due to an unknown genetic cause however deletion of mitochondrial DNA was found but not followed up. She developed intestinal obstruction aged 9 years and she underwent surgery which failed to show any obstruction and aged 11 years received a diagnosis of chronic pseudo-intestinal obstruction. From the age of 14 years she developed daily dysphagia, while consuming solid and liquid food, and experienced weight loss (5kg). At age 15 years, 90cm of jejunum was resected due to acute intestinal obstruction and she became dystrophic. At age 29 years, esophagography reveals achalasia, gastroptosis (abnormal downward displacement of the stomach), and delayed gastric and duodenum emptying time. At age 30 years she underwent treatment with peroral endoscopic myotomy (POEM) to open the LOS (54).

1.7 Pathophysiological mechanisms in mitochondrial disease

Human studies

Evidence from pathological studies in patients with Alpers' disease due to pathogenic POLG variants confirmed the presence of abnormal mitochondria harbouring electron dense material and displacing cristae in samples from the gastrointestinal tract. Abnormal mitochondria were specifically localised to 40% of the nitrergic myenteric ganglion cell population throughout the gastrointestinal tract (Figure 9) (55). This work suggests that pathogenic *POLG* variants may contribute to mitochondrial dysfunction accumulating within the myenteric neurons which might be important in the emergence of gastrointestinal dysmotility and achalasia in these patients. Further pathological studies are necessary to expand on these findings.

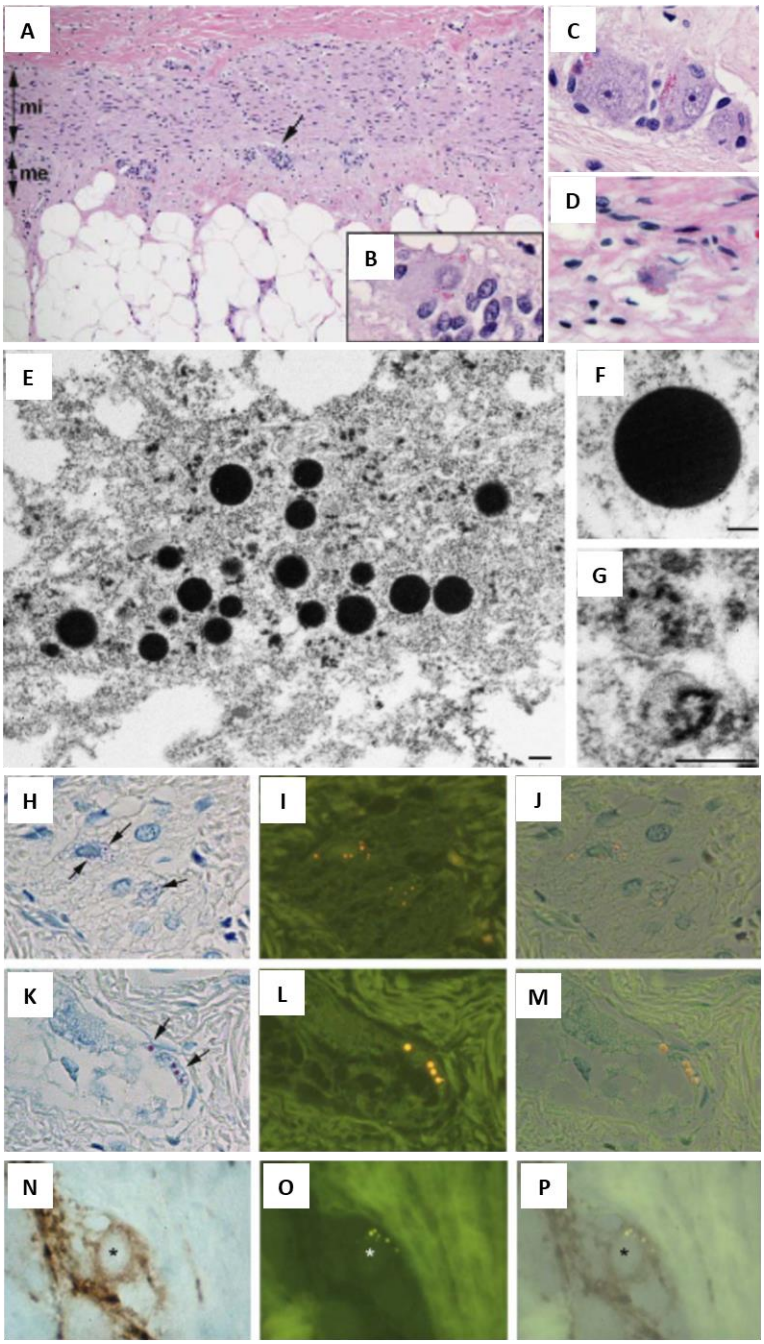


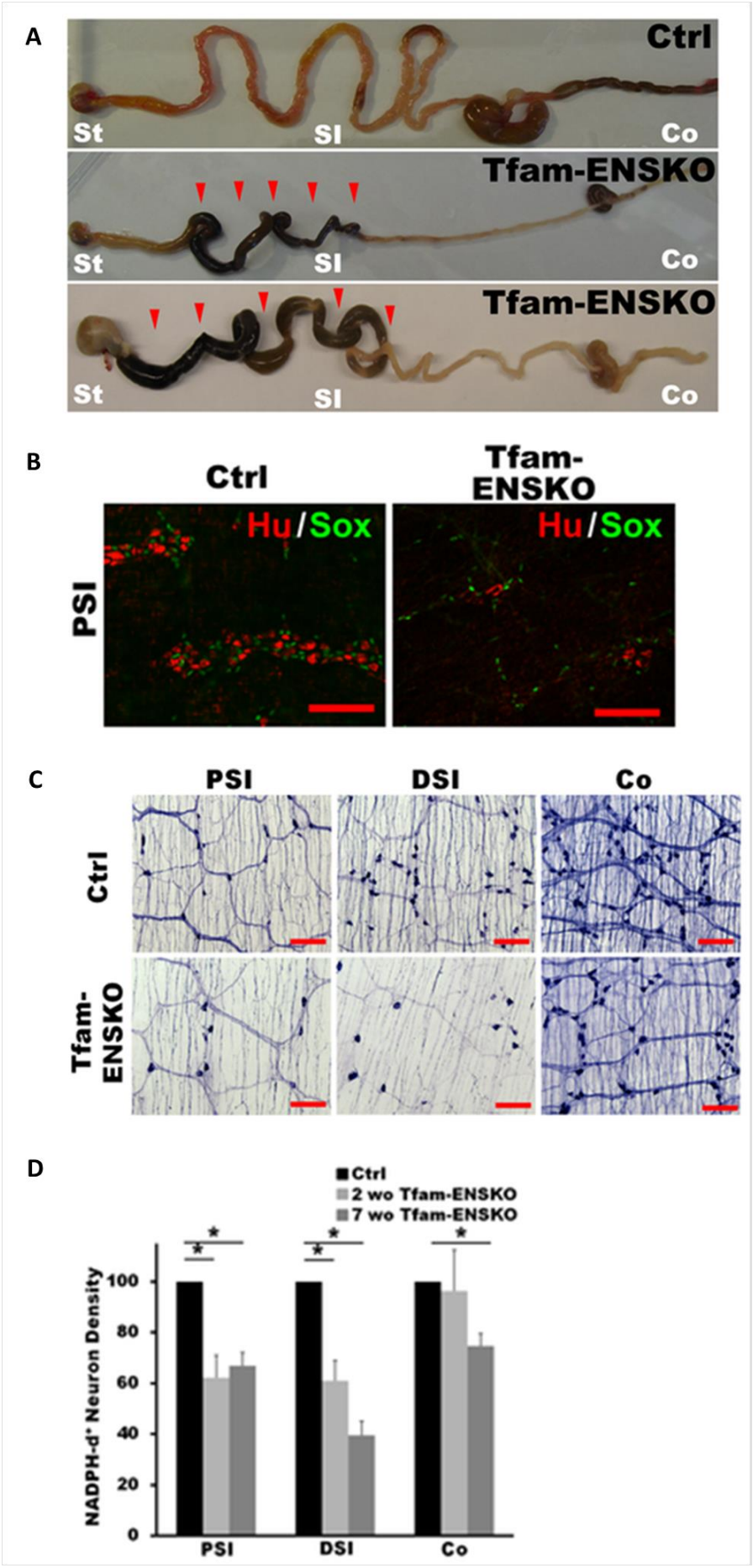
Figure 9 - Mitochondrial pathology in myenteric ganglion cells in patients with Alpers' disease.

A. Patient small intestine shows severe muscular atrophy and fibrosis of the muscularis externa (me) with preservation of the muscularis interna (mi). Myenteric ganglia (arrowhead) include a subpopulation of neurons with enlarged mitochondria. B., C. and D. show myenteric neurons containing enlarged mitochondria which appear as pink intracytoplasmic eosinophilic granules. E. Electron microscopy (EM) reveals the abnormal ultrastructure of mitochondria within myenteric neurons. Mitochondria appear as electron-dense spherical inclusions that correlate to the distribution and size of eosinophilic granules. F. EM reveals that many mitochondria are homogeneous electron dense bodies that lack cristae. G. Few neurons show early changes with electron-dense inclusions in mitochondria with discernable membrane and cristae. H. and K. show neurons stained with toluidine blue to show magenta cytoplasmic inclusions (arrows) while I. and L. show intense orange-yellow fluorescent granules. J. and M. show that magenta cytoplasmic inclusions colocalise with orange-yellow fluorescence in the same granules (Rhodamine B staining). N. cytoplasmic nNOS-specific

immunoperoxidase staining outlines the nucleus of an inhibitory neuron (asterix). O. and P. Rhodamine B staining shows that this inhibitory interneuron contains yellow immunofluorescent granules in its cytoplasm.

Mouse models

Recently, a novel transgenic mouse model has been developed by selectively inducing mitochondrial defects in enteric neurons and glia. This shows selective downregulation of the gene encoding mitochondrial transcription factor A (*TFAM*) in enteric neurons and glia (determined by Cre recombinase expression in CNP cells). These mice, referred to as TFAM-ENSKO, exhibited poor growth at 4 weeks with abdominal distention at 6-8 weeks of age and died prematurely by 12 weeks. The underlying pathology revealed massive dilation within the proximal small bowel along with relative contraction of the distal smooth bowl (Figure 10A). The enteric nervous system developed normally however mice aged 7 weeks showed a 68% reduction in myenteric neuronal cell density in the proximal smooth intestine and 41% reduction of neurons in the distal smooth intestine with a parallel loss of glial cells (Figure 10B). The neuronal population density was maintained within the colon. NADPH diaphorase staining confirms that there is an early and differential loss of nitric oxide inhibitory neurons in conjunction with severe axonal degeneration at 7 weeks (Figure 10C and D). This might alter the balance between inhibitory and excitatory inputs which are important for the rhythmic coordination of constriction and relaxation required for gut motility (56). Although achalasia has not been reported in this model, it has mechanistic potential to determine what might be happening at the level of the LOS which would have implication for preclinical studies and treatment pipelines.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NADPH-d+ Neuron Density
100
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20
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Figure 10 – Gastrointestinal pathology in the enteric nervous system TFAM knockout (TFAM-ENSKO) mice.

A. The gastrointestinal tract of a 11-12 week old control and TFAM-ENSKO mice depicts their typical appearance. Arrowheads incate regions of dilation and accumulation of luminal contents. St = stomach, SI = small intestine, C0 = colon. B. Representative images of neurons (red) and glia (green) in the myenteric plexus of a 7 week old control and TFAM-ENSKO mice. Loss of both neurons and glia are evident in TFAM-ENSKO mice at this age. Scale bar = 100um. C. Representative images of NADPH-d stained myenteric plexus show inhibitory neurons in the proximal SI and distal SI in 7 week old TFAM-ENSKO mice. Scale bar = 150um. D. Quantitative analysis of NADPH-d neuron density in Tfam-ENSKO relative to control littermates at 2 and 7 weeks of age.

1.8 Future direction

Combined clinical and histopathological studies will facilitate the translation of basic science into effective clinical management of patients. From a clinical perspective, it is important to consider achalasia in the context of patients who might present to clinic with reflux which may actually be regurgitation. Clinical investigation using the gold standard diagnostic techniques, such as HRM, would be important to diagnose achalasia and its subtype which will be used to inform treatment. Further understanding of the pathophysiological mechanisms of achalasia associated with mitochondrial disease should be provided by histological investigation of human post-mortem and resected oesophageal samples. These would provide important insights into the potential mechanisms involved and any similarities to pathological changes occurring in idiopathic achalasia. In parallel to this, investigation of mitochondrial dysfunction within enteric nervous system in transgenic mouse models would provide further mechanistic insights and phenotypic correlations. It would also provide a preclinical model in which novel treatments could be explored.

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