Title: Tubular aggregates and cylindrical spirals have distinct immunohistochemical signatures

Running title: Analysis of tubular aggregates and cylindrical spirals

Authors: Stefen Brady[1] DPhil, Estelle G. Healy[2] MD, Qiang Gang[3] MD, Matt Parton[4] PhD, Ros Quinlivan[4] MD, Saiju Jacob[5] DPhil, Elizabeth Curtis[6] PhD, Safa Al-Sarraj[7] MD, Caroline A. Sewry[8] PhD, Michael G. Hanna[4] PhD, Henry Houlden[3] PhD, David Beeson[9] DPhil, Janice L. Holton[3] PhD

Affiliations: [1]Department of Neurology, Southmead Hospital, Bristol. [2]Department of Neuropathology, Royal Hospitals, Belfast. [3]UCLInstitute of Neurology, Queen Square, London.[4]MRC Centre for Neuromuscular Diseases, University College Hospitals, London. [5]Department of Neurology, Queen Elizabeth Hospital, Birmingham. [6]Department of Cellular Pathology, University Hospitals, Birmingham. [7]King's College London, MRC London Neurodegenerative Diseases Brain Bank, London. [8]Dubowitz Neuromuscular Centre, UCL Institute of Child Health, London. [9]Neurosciences Group, Nuffield Department of Clinical Neurosciences, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford.

Corresponding author: Dr Janice L. Holton. UCL Institute of Neurology, Queen Square, London, WC1N 3BG. Email: janice.holton@ucl.ac.uk

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Tubular aggregates and cylindrical spirals have distinct immunohistochemical signatures

Stefen Brady[1], Estelle G. Healy[2], Qiang Gang[3], Matt Parton[4], Ros Quinlivan[4], Saiju Jacob[5], Elizabeth Curtis[6], Safa Al-Sarraj[7], Caroline A. Sewry[8], Michael G. Hanna[4], Henry Houlden[3], David Beeson[9], Janice L. Holton[3]

[1]Department of Neurology, Southmead Hospital, Bristol

[2]Department of Neuropathology, Royal Hospitals, Belfast

[3]UCLInstitute of Neurology, Queen Square, London

[4]MRC Centre for Neuromuscular Diseases, University College Hospitals, London

[5]Department of Neurology, Queen Elizabeth Hospital, Birmingham

[6]Department of Cellular Pathology, University Hospitals, Birmingham

[7]King's College London, MRC London Neurodegenerative Diseases Brain Bank, London

[8]Dubowitz Neuromuscular Centre, UCL Institute of Child Health, London

[9]Neurosciences Group, Nuffield Department of Clinical Neurosciences, Weatherall Institute of Molecular Medicine, University of Oxford

ABSTRACT

Tubular aggregates and cylindrical spirals are two distinct ultrastructural abnormalities, observed on muscle biopsy, with similar histochemical staining characteristics on light microscopy. Both are found in a wide range of disorders. Recently a number of genetic mutations have been reported in conditions with tubular aggregates in skeletal muscle. It is widely accepted that TA arise from the sarcoplasmic reticulum, but the origin of cylindrical spirals has been less clearly defined. We describe the histopathological features of myopathies with tubular aggregates, including a detailed immunohistochemical analysis of congenital myasthenic syndromes with tubular aggregates due to mutations in *GFPT1* and *DPAGT1* and myopathies with cylindrical spirals. Our findings support the notion that cylindrical spirals, like tubular aggregates, derive primarily from the sarcoplasmic

reticulum, however, immunohistochemistry indicates that different molecular components of the sarcoplasmic reticulum may be involved and can be used to distinguish between these different inclusions. The immunohistochemical differences may also help to guide genetic testing.

INTRODUCTION

Tubular aggregates (TA) and cylindrical spirals (CS) are histopathological findings on muscle biopsy. (1–3) They are of unknown aetiology and have been observed in patients with varied clinical disorders. Despite marked ultrastructural differences between TA and CS, they are indistinguishable with routine histochemical stains. It is widely accepted that TA arise from the sarcoplasmic reticulum (SR) but there have been few detailed studies of CS and their sub-cellular origin is unknown.

On ultrastructural examination, TA consist of tightly packed parallel arrays of single or double walled cylinders in the sarcoplasm. These typically stain bright red with modified Gömöri trichrome, strongly positive with nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) and myoadenylate deaminase (MAD) and are negative with succinate dehydrogenase (SDH) and cytochrome c oxidase (COX). Evidence supporting the SR origin of TA comes from immunohistochemical (IHC) studies of genetically unclassified cases. (3–5) Nuclear, mitochondrial and sarcolemmal proteins have been described in TA, (6–8) suggesting that other organelles may play a role in their formation.

Although TA have been observed in inflammatory, metabolic and toxic myopathies, their presence defines a clinically heterogeneous group of disorders termed ‘tubular aggregate myopathies’ (TAM) which includes clinical phenotypes of exertional myalgia, limb girdle myopathy, periodic paralysis and congenital myasthenic syndromes (CMS). Recently, mutations in a number of genes have been described in some TAM. These include autosomal recessive (AR) mutations in *GFPT1* and *DPAGT1* which encode enzymes in the protein glycosylation pathway and cause CMS (9,10) and mutations in genes encoding proteins involved in calcium homeostasis, *STIM1* and *ORAI1* in TAM with miosis.

(11,12) Although the staining characteristics of TA are relatively uniform, differences have been reported. This variation could be accounted for by different genetic mutations.

Triggers resulting in the formation of TA are unknown. Their presence in a variety of clinical conditions may indicate that they are a non-specific response to metabolic derangements or toxic insults. It is suggested that they, like the SR, act as a calcium sink and are formed as a mitigating response to intracellular hypercalcaemia. (4) Others have proposed that they are a unique form of protein aggregation. (13)

Cylindrical spirals (CS) are a rare finding on muscle biopsy. Ultrastructurally, they appear as groups of electron dense whorled cylinders in cross section. They have been reported in a number of unrelated conditions but are most frequently described in adults with myalgia and cramps. Despite their distinctive ultrastructural appearances, CS have similar staining characteristics to TA, appearing bright red with the modified Gömöri trichrome stain and negative with SDH. Like TA, the staining characteristics of CS are not invariable and they have been described as staining weakly, or brown rather than blue in colour with NADH-TR. (14) TA and CS have been observed within the same muscle biopsy, (14,15) and in direct continuity at the ultrastructural level, (16–18) suggesting that they may have a common origin. However, detailed studies to validate these observations are lacking and the organelles from which CS are derived are undetermined. A recently published study of two Chinese siblings with myopathy and CS on muscle biopsy reported that CS may derive exclusively from the longitudinal SR (LSR). (19)

We report a comprehensive study of the histochemical, IHC and ultrastructural features of these structural inclusions in myopathies with TA, including recently described CMS cases with mutations in *GFPT1* and *DPAGT1*, non-CMS TAM cases, and myopathies with CS.

MATERIALS AND METHODS

From the Neuropathology archives at the University College London Institute of Neurology, ten cases with TA or CS were identified: non-CMS TAM (*n*=4), CMS with TA (CMS with mutations in *DPAGT1 n*=2 and *GFPT1 n*=2) and CS (*n*=2). Demographic and clinical details are presented in table 1. All patients were evaluated and followed up by specialist neuromuscular services. The presence of TA and CS was confirmed by electron microscopy (EM). Serial frozen muscle sections were stained with haematoxylin and eosin (H&E), modified Gömöri trichrome, NADH-TR, SDH, COX, combined COX/SDH, Periodic acid Schiff (PAS), PAS with diastase, Sudan black and MAD. IHC was performed for T-tubule (TT) and SR proteins: dihydropyridine receptor (DHPR), ryanodine receptor (RyR1), sarco/endoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA1) and 2 (SERCA2), and the endoplasmic reticulum membrane trafficking protein SAR1; nuclear membrane protein: lamin A/C; mitochondrial membrane protein: COX IV; and Golgi complex membrane protein: GM130. Additional IHC was performed for p62, myotilin, desmin, fast and slow myosins, caveolin-3 and dysferlin. Primary antibody binding was visualised using the Dako REAL EnVision Detection System which contains horse-radish peroxidase labelled goat anti-rabbit/mouse secondary and 3,3′-diaminobenzidine. Details of commercial antibodies and conditions are provided in supplementary table 1. IHC for each antibody was performed simultaneously on all cases with positive and negative controls, therefore, the absence and presence of staining reported reflects the true pattern. Biopsies were assessed by three independent observers (SB, EGH, JLH) blinded to the clinical details and diagnosis in each case. To identify mutations known to cause TA and new mutations, whole exome sequencing was performed on all non-CMS TAM and myopathies with CS. This did not reveal mutations in genes associated with periodic paralyses or *DPAGT1, GFPT1, ORAI1, STIM1* and *PGAM2,* and no additional candidate mutations were identified.

The study was registered with UCL/UCLH/RF Joint Research Office and granted local R&D approval (ref. no. 11/0194).

RESULTS

The histopathological, IHC and EM findings are summarised in tables 2 and 3 and figures 1 and 2.

DPAGT1 and GFPT1 CMS with TA

Four CMS cases with *DPAGT1* (n=2) and *GFPT1* (n=2) mutations were analysed. At least two muscle biopsies were performed in all four cases. The mean time between biopsies was 11.2 years (range 5-16 years). Histopathological findings appeared to be more pronounced in later biopsies. Vacuolated and necrotic fibres were increased in later biopsies in cases 2 and 3, while PAS staining was prominent in more recent biopsies in cases 2, 3 and 4. In cases 2 and 4 TA were universally small in the initial biopsies whereas in later biopsies small, medium and large TA were observed. However, the percentage of muscle fibres containing TA was no greater.

Myopathic changes, including variation in fibre size, increased numbers of internal nuclei, fibre atrophy and split fibres were present in all biopsies. Infrequent fibre necrosis and regeneration was observed in three of the four cases. There was an excess of COX negative fibres and ragged red fibres in case 2. Cytoplasmic bodies and eosinophilic nuclear inclusions were seen in cases 1 and 2 respectively. PAS staining revealed increased glycogen staining in all four cases with diastase resistant areas. Rimmed vacuoles were observed in all cases with H&E, modified Gömöri trichrome and PAS stains and contained diastase resistant material. Rimmed vacuoles were not present in the initial biopsy in two of the four cases. Modified Gömöri trichrome staining revealed large red subsarcolemmal aggregates in all four cases. These areas stained dark blue with NADH-TR but were unstained with SDH and COX. MAD staining was not available for cases 1, 3 and 4, but aggregates in case 2 were unstained. Aggregates in the *GFPT1* CMS cases 3 and 4 stained with PAS, and in the *DPAGT1* cases 1 and 2, aggregates were PAS negative. Aggregates were present in type I and II fibres in each of the four cases. On IHC staining, the aggregates were immunoreactive with antibodies to RyR1, DHPR, SERCA1 and SERCA2 in all cases. Dysferlin staining was positive in aggregates in each case except case 3. Ultrastructural analysis revealed typical TA in longitudinal and transverse sections, containing flocculent material and located in the vicinity of glycogen granules, neutral lipid

droplets and dilated mitochondria. In cases 2 and 3 only doubled walled TA (DWTA) were observed, in case 4 only single walled TA (SWTA) and both SWTA and DWTA were present in case 1. Accumulation of whorled membranous debris was seen in cases 2 and 3.

Non-CMS TAM

Five biopsies from four cases of non-CMS TAM were evaluated. Fibre size variation was observed in each case. Fibre atrophy and increased numbers of internal nuclei were seen in cases 7 and 8 respectively. Large and medium sized subsarcolemmal and centrally located aggregates were observed with H&E and modified Gömöri trichrome and stained strongly with NADH-TR and MAD but not with SDH and COX. In case 8 aggregates were stained with PAS. In all cases the aggregates were mainly present in type II fibres. Aggregates were immunoreactive with antibodies to RyR1, DHPR, SAR1 and SERCA1 in all four cases. Dysferlin staining was positive in aggregates in cases 5, 7 and 8 and positive dysferlin staining was limited to the periphery of the aggregates in case 6. Ultrastructural analysis revealed DWTA in cases 5, 6 and 7 and a mixture of DWTA, SWTA and vesicular membrane collections in case 8. TA were accompanied by small groups of large neutral lipid droplet and, increased sarcoplasmic glycogen and thickening of the capillary basal lamina was observed.

Myopathies with CS

Biopsies revealed mild variation in fibre size in each of the two cases. Increased numbers of internal nuclei, fibre atrophy and two abnormal fibres, one containing rimmed vacuoles and a single ring fibre, were noted in case 9. There was a mild excess of COX negative fibres in case 10. Large basophilic subsarcolemmal aggregates were observed on H&E staining in both cases. These areas stained red with modified Gömöri trichrome and were positive with MAD staining but were unstained with SDH and COX. In case 9 aggregates were stained with PAS, but were unstained in case 10. On NADH-TR stained sections, aggregates appeared light blue in case 9 and dark blue in case 10. The morphology and distribution of aggregates differed between the two cases. In case 9 they were circular and

appeared in type II fibres and in case 10 they were linear and present in type I and II fibres. Aggregates were immunoreactive with antibodies to SERCA1 and SAR1. Ultrastructurally, subsarcolemmal clusters of CS were associated and interspersed with accumulated glycogen.

TA and CS in every case did not stain with antibodies for lamin A/C, COX IV, GM130, p62, myotilin, desmin, fast and slow myosins and Caveolin-3.

DISCUSSION

We describe the histopathological findings of inclusions in non-CMS TAM, CMS with TA associated with mutations in *DPAGT1* and *GFPT1* and myopathies with CS. Our results confirm that TA have characteristics suggesting they arise from the SR as reported in previous studies. We also describe the IHC characteristics of genetically confirmed cases of CMS with TA. TA and CS could not be clearly differentiated on routine tinctoral stains and enzyme histochemistry, and TA in non-CMS TAM and CMS cases could not be distinguished at the ultrastructural level. Our IHC findings support the hypothesis that CS, like TA, originate from the SR. IHC results were characteristic for each of the three groups we studied. The differences observed may permit the use of a panel of IHC to distinguish these three categories of inclusion without the need for ultrastructural examination.

We found that routine stains and histochemistry did not distinguish between TA in TAM and CS but there were differences between the TA observed in CMS cases and TAM. In the CMS cases, TA were unstained with H&E. In contrast, TA were strongly basophilic with H&E staining in non-CMS TAM cases. A similar finding has been reported in other cases with TA on muscle biopsy. (20) This observation has not been reported in previously published descriptions of patients with either *GFPT1* or *DPAGT1* CMS. (9,10,21–23) Only one of these publications, describing a single case, indicates that TA stained with H&E. (21) However, these reports do not focus on the histopathological findings. Consequently, at this time, it is difficult to know whether this absence of H&E staining is a consistent finding, and therefore of diagnostic use. *GFPT1* and *DPAGT1* encode enzymes involved in

cellular glycosylation. The resulting deficient or defective protein glycosylation caused could account for the altered uptake of oxidised haematein by TA in CMS cases and explain the absent or reduced basophilic staining observed. Reports of variations in the tinctorial staining characteristics of TA by ourselves and others, may result from the combined analysis of cases with different clinical features, based solely on the unifying feature of TA on muscle biopsy. The presence of rimmed vacuoles and TA is suggestive of CMS with TA. However, in our four CMS cases, rimmed vacuoles were not always present in the initial biopsy, limiting the diagnostic utility of this observation.

The main role of the SR is regulation of muscle contraction through calcium uptake, storage and release. It accomplishes this through interaction with sarcolemmal T-tubules (TT). The TT interface with enlargements of the SR network called terminal cisternae (TC). A TT and two associated TC are defined as a triad. The membrane of the TC facing the TT is known as the junctional SR (JSR) and is dedicated to calcium release. The TC are continuous with the LSR which is specialised for calcium uptake. (24,25) The locations of different proteins in the T-tubule and SR are summarised in table 4. In each of the three groups we studied there was IHC expression of SR proteins, confirming the composition of TA and CS. In addition, the combination of proteins present was characteristic for each group. TA in CMS cases were immunoreactive for TT, TC/JSR and LSR proteins: DHPR, RyR1 and SERCA1 and SERCA2 respectively. TA in non-CMS TAM were immunoreactive for T-tubule, TC/JSR, LSR and ER exit sites proteins: DHPR, RYR1, SERCA1 and SAR1 respectively. CS were immunoreactive for LSR and ER exit sites proteins SERCA1 and SAR1, but not for proteins present in the triad, namely DHPR and RyR1. The plasma membrane protein dysferlin showed negative staining of CS, but was positive in TA in three out of four CMS TAM cases and all of the TAM cases. In the latter group, one case showed peripheral staining of the aggregates only. It has been demonstrated that dysferlin associates with TT. (26–28) TA in our study have shown consistent positive staining for the TT protein DHPR, with associated variable dysferlin staining, which may indicate partial incorporation of sarcolemmal components into their structure. No cases showed positive staining for the dysferlin binding partner Caveolin-3. Distinct from TA, CS were exclusively

positive for other SR components related to calcium uptake and ER exit sites (SERCA1 and SAR1) and negative for triad and sarcolemmal proteins. This may indicate that CS incorporate different proteins from distinct subcellular locations of the SR. Finally, a pattern of peripheral IHC staining in TA was reported previously by Bohm *et al.* with antibodies to RyR1 and STIM1 in TAM with mutations in the ER calcium sensing protein *STIM1*. (11) In our cases, aggregates stained homogenously with RyR1 IHC and no mutations in *STIM1* were identified. Overall, our findings are largely in agreement with other IHC studies of TAM, however, it is difficult to directly compare studies of TAM as the diagnoses are often unknown. (3,5) The consistent and individual pattern of TA and CS staining found in each group of cases we analysed strongly suggests that aetiology influences the pathological findings. In addition, we believe that the IHC differences observed implies that these structures arise from different parts of the SR. Alternatively, it is possible that differences may be due to alterations in the protein composition of SR through protein misfolding or excess protein production.

TA were initially proposed to be of mitochondrial origin. (29) The close relationship between the SR and other organelles, and studies in humans and animals has led to the suggestion that other intracellular structures such as the nucleus and Golgi complex play a role in TA formation. (6,7) Other proteins found in TA include the sarcolemmal protein dysferlin and heat-shock proteins. (8) We found no evidence of nuclear, mitochondrial or Golgi complex proteins to support the involvement of these organelles in the formation of TA or CS. This is in agreement with Cheveisser *et al.* who found no evidence of mitochondrial involvement in TA and the presence of the nuclear protein emerin staining in only one of six cases they examined. (5) They suggested that its presence may have occurred accidentally as TA are formed from the ER or through pathological mislocalisation. Recently a pathogenic mutation in *SERAC1*, a mitochondrial gene involved with lipid remodelling at mitochondrial associated ER membranes, has been reported in an encephalopathic patient with TA on muscle biopsy. (30) The authors hypothesised that TA were caused by abnormal aggregation of ER constituents as a result of inefficient lipid remodelling.

Our findings support those of previous studies, confirming that TA arise from the SR and substantiate the hypothesis that CS originate from the SR. We did not find evidence to support the involvement of other organelles in the formation of TA or CS. Routine stains and histochemistry did not differentiate between CS and TA, however, the presence of TA in a muscle biopsy which are not apparent on H&E stained sections may, like rimmed vacuoles, be indicative of CMS. IHC results for TT, SR and ER exit site proteins were consistent and unique in each group of cases we studied. If this is confirmed in further studies, IHC could be used to distinguish between these three categories and may have a role to guiding genetic testing. The main limitation of our study is the small number of cases analysed, however, the rarity of TA and CS means that this is a major obstacle faced by all researchers in this area. A recent report of immunohistochemical studies in two siblings with CS, compared with two TAM cases, is in agreement with our findings, showing similar staining signatures for these different aggregates. (19) We conclude that these findings may indicate distinct mechanisms of formation for TA and CS, resulting in the incorporation of different elements of the SR.

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Figure 1. Serial biopsy sections (A,B) showing subsarcolemmal tubular aggregates (TA) in tubular aggregate myopathy (case 8). TA staining is basophilic with H&E (arrow in A) and red with modified

Gömöri trichrome (arrow in B) and TA are confirmed with electron microscopy (C). Serial sections (D,E) from case 9 showing subsarcolemmal and internal cylindrical spirals have similar staining characteristics to TA with H&E (arrow in D) and modified Gömöri trichrome (arrow in E) and are confirmed by electron microscopy (F). Serial sections from *GFPT1* congenital myasthenic syndrome with TA (case 3) reveal that TA do not show basophilic staining with H&E (arrow in G). Their presence is observed with red modified Gömöri trichrome staining (arrow in H) and confirmed by electron microscopy (I).

Scale bar represents 100 µm in (A), (B), (D), (E), (G), and (H) and 500nm in (C), (F) and (I).

Figure 2. Subsarcolemmal tubular aggregates (TA) are unstained with H&E (arrow in A) and are negative with SAR1 immunohistochemistry (arrow in J) but show positive staining, indicated by arrows, for RyR1 (D), DHPR (G), SERCA1 (M) and SERCA2 (P) in *DPAGT1*-congenital myasthenic syndrome (CMS) (case 1). TA in non-CMS tubular aggregate myopathy (case 7) are readily observed with H&E (arrow in B) and are immunoreactive, shown by arrows, for RyR1 (E), DHPR (H), SAR1 (K) and SERCA1 (N) but not SERCA2 (Q). Myopathy with cylindrical spirals (CS) (case 10) shows the presence of CS on H&E (arrow in C). CS do not stain with RyR1 (arrows in F) and DHPR (arrows in I) and SERCA2 (R) but strong staining is observed with SAR1 (arrows in L) and SERCA1 (arrows in O).

Scale bar in represents 200 µm in (B), (E), (H), (K), and (N), and 100 µm in (A), (C), (D), (F), (G), (I), (J), (L), (M), (O), (P), (Q), and (R).