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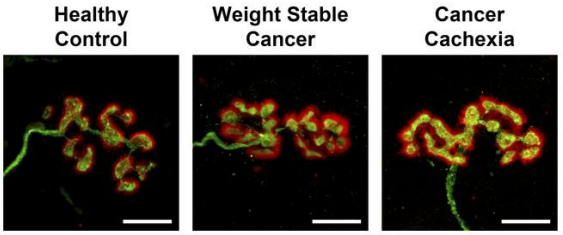
Graphical abstract



Neuromuscular junctions (NMJs) are stable in patients with cancer cachexia

Neuromuscular junctions are stable in patients with cancer cachexia

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Abstract

Cancer cachexia is a major cause of patient morbidity and mortality, with no efficacious treatment or management strategy. Despite sharing pathophysiological features with a number of neuromuscular wasting conditions, including age-related sarcopenia, the mechanisms underlying cachexia remain poorly understood. Studies of related conditions suggest that pathological targeting of the neuromuscular junction (NMJ) may play a key role in cachexia, but this has yet to be investigated in human patients. Here, high-resolution morphological analyses were undertaken on NMJs of rectus abdominis obtained from patients undergoing upper GI cancer surgery compared with controls (N=30; n=1,165 NMJs). Cancer patients included those with cachexia and weight-stable disease. Despite the low skeletal muscle index and significant muscle fibre atrophy in patients with cachexia, NMJ morphology was fully conserved. No significant differences were observed in any of the pre- and post-synaptic variables measured. We conclude that NMJs remain structurally intact in rectus abdominis in both cancer and cachexia, suggesting that denervation of skeletal muscle is not a major driver of pathogenesis. The absence of NMJ pathology is in stark contrast to related conditions, such as age-related sarcopenia, and supports the hypothesis that intrinsic changes within skeletal muscle, independent of any changes in motor neurons, represent the primary locus of neuromuscular pathology in cancer cachexia.

Introduction

Cachexia is a severe and debilitating syndrome, commonly associated with cancer and characterised by the loss of muscle, with or without corresponding loss of adipose tissue (1). Cancer cachexia is a major burden for both patients and health care systems globally, with profoundly negative impacts on the response to treatment, quality of life, and long-term survival of patients (2). At present, the consensus definition of cancer cachexia confirms loss of skeletal muscle as a key feature of the condition (3), largely mediated by pro-inflammatory cytokines and tumor-associated mediators, resulting in the activation of catabolic pathways in skeletal muscle (4). In this regard, cancer cachexia shares many of the muscle-specific and systemic inflammatory pathways common to the muscular dystrophies (5,6).

Although the majority of research to date has focused on muscle abnormalities as the major locus of pathophysiology in cachexia, many lines of evidence have implicated the neuromuscular junction (NMJ) as a critical and early mediator of neuromuscular dysfunction and breakdown. Principally, NMJ dysfunction and denervation represent a shared hallmark of several related muscle wasting conditions and neuromuscular diseases (7-9). For example, NMJ pathology is considered to represent a key early driver of neuromuscular defects in age-related sarcopenia (10-12), at least in part the result of an age-related loss of motor neurons (13). As cancer cachexia and sarcopenia share similar molecular mechanisms (14), and as cachexia is considered to be a multifactorial syndrome that includes components of both age-related sarcopenia and bed rest/reduced physical activity (15), the NMJ has similarly been implicated in the pathogenesis of cachexia. The identification of displaced mononuclei in muscle of cachectic patients and C26 tumor-bearing mice has been used to suggest the presence of denervation (16). Furthermore, a member of the ubiquitin-proteasome pathway (MuRF1) pivotal to muscle wasting in tumor-bearing mice (and other murine models of muscle wasting) is critical for maintenance of the NMJ (17). Additionally, recent mouse studies of mTOR signalling, a key regulator of protein synthesis that is suppressed by inflammatory mediators in cancer cachexia (18), have shown that muscle specific-deletion of mTOR or Raptor results in muscle fibrillation and NMJ fragmentation (19).

The role of the nerve-muscle interface in human cachexia is therefore of interest to both clinician and basic scientists, and relevant to our understanding of disease pathophysiology

and the development of effective treatments. Thus, cancer cachexia has been the subject of co-culture/informatics projects (20) and ongoing patient studies. Experimental evidence supporting a direct role for NMJs in human cachexia is however highly reliant on studies of animal models of related conditions (21-23). Significantly, recent data have revealed striking and unexpected differences between the cellular and molecular anatomy of human NMJs compared to those of other model organisms (24), suggesting that findings from animal models may not be directly applicable to human patients.

The present study builds upon our recent work establishing a robust protocol to facilitate the sampling and high-resolution, quantitative morphological analyses of human NMJs from patients undergoing surgery (24,25). We have adapted these protocols to facilitate a comprehensive analysis of the NMJ in patients with cancer cachexia.

Results and Discussion

To investigate the role of the NMJ in human cancer cachexia, we performed a comprehensive morphometric analysis of the NMJ in samples of rectus abdominis (RA) muscle obtained from patients undergoing surgery for upper GI cancer (Supplementary Table 1). RA was selected for two reasons: i) it is readily accessible in the majority of surgical approaches to the abdomen and therefore a well-utilized muscle for sampling and characterisation of human cancer cachexia (26); ii) nerve roots innervating RA are unlikely to be affected by radiculopathy or other common spinal pathology, rendering neurogenic remodelling an unlikely possibility.

Cachectic patients demonstrated significantly lower skeletal muscle index (SMI) by CT criteria compared with weight stable patients (Supplementary Table 1) and also demonstrated a trend towards lower subcutaneous adiposity and higher visceral adiposity. These two body composition phenomena are associated with worsened outcomes in cancer patients (27-29) further confirming the cachectic patients as a high-risk group. Two patients in the weight stable cancer group exhibited a small degree of weight loss, but were not cachectic by the consensus definition (3,30).

To confirm/validate the patient groupings based on the clinical and radiological guidelines (% weight loss and SMI, respectively) we assessed muscle fibre diameter on teased muscle fibre preparations from RA. Mean muscle fibre diameter was significantly reduced (by almost 15%) in the cachectic patients compared to both control and weight stable groups ($p < 0.0001$; Figure 1). However, there was no significant difference in mean muscle fibre diameter between control patients and those with weight stable disease ($p > 0.05$; Figure 1). These observations are in keeping with published data showing marked muscle fibre atrophy in cachexia (31,32) and support the patient group allocations based on consensus definition (3).

Given that muscle fibre atrophy would be predicted to result from and/or lead to NMJ instability based on findings from animal studies (8,9) we next performed an initial qualitative assessment of NMJs. Despite the presence of muscle fibre atrophy in cachectic patients, NMJ morphology was indistinguishable from that observed in weight stable and control patients (Figure 2). NMJs of all three cohorts were noted to display the typical 'nummular' morphology characteristic of human NMJs (24) and despite the predicted heterogeneity in form across the complete pool of NMJs (Figure 3) we found no evidence of gross pathological changes or denervation of skeletal muscle fibres.

Although initial qualitative observations suggested an absence of gross pathology at the NMJ, more subtle changes in NMJ morphology could still have been present in the cachectic patients. We therefore undertook a comprehensive NMJ-morph analysis of all patient NMJs (Figure 3; Supplementary Table 2). NMJs from RA were initially compared with our existing database of human NMJs from several lower limb muscles (24) to determine their likeness (or otherwise) to established human NMJ morphology in other muscle groups. NMJ-morph analysis revealed comparable NMJ morphology across RA and four lower limb muscles (Supplementary Figure 2).

Quantitative NMJ-morph analyses confirmed that there were no significant differences in any aspect of NMJ morphology in RA across the three patient groups (Figure 3; Supplementary Table 2). Crucially, and in stark contrast to predictions based on mechanistic animal models, there was no evidence of denervation (defined by % overlap between nerve terminal and endplate), demonstrating that this is not a major feature of pathogenesis in cachectic

patients. Similarly, there was no evidence for increased NMJ fragmentation, a classical feature of NMJ pathology found in animal models of neurodegeneration (7-9) and cardiac cachexia (33).

Alongside analyses of denervation and NMJ fragmentation, our NMJ-morph analysis confirmed no statistically-significant changes in any of the other morphological variables investigated (Figure 3; Supplementary Table 2); similar axon diameters (~1µm) were observed across all 3 groups with no evidence of axonal swelling, neurofilament accumulation or polyneuronal innervation (indicative of denervation/reinnervation processes). Thus, neither gross nor subtle perturbations at the NMJ were observed in cachectic patients. However, the relative contribution of muscle regeneration and myopathic changes still requires definitive demonstration in human cachexia patients.

It should be noted that cachectic patients in the current study represent the more extreme end of the clinical diagnostic definition criteria, having both weight loss and low CT muscularity. However, our study only enrolled patients who were eligible for surgery with potentially curative intent. It is not possible, therefore, to draw conclusions concerning a possible late disruption of the NMJ in palliative cancer patients with refractory cachexia and severe functional impairment.

Whilst RA proved to be an excellent muscle for the current study, our findings differed from those observed in human age-related sarcopenia and in animal models of muscle wasting, both situations where weight-bearing muscles are usually assessed experimentally. It remains possible therefore that skeletal muscle with different functional and/or biochemical properties may respond differently in cachexia. Equally, the observed differences between human patients and animal models may reflect anticipated differences in cachexia pathophysiology between the two. Human cachexia is proposed to be a multifactorial condition in which diverse drivers of muscle wasting all contribute to varying degrees in individual patients and tumor types (15). In comparison, in vivo tumor-bearing models may demonstrate accelerated wasting, which lacks the complexity and heterogeneity of the human condition. This supposition is supported by previous studies that have demonstrated

little overlap in gene expression profiles between muscle biopsies from human cancer patients and equivalent animal models (34).

All patients received intravenous atracurium besilate during anesthesia, which competitively displaces acetylcholine from its receptors. Its half-life is 17-21 minutes, but whether it has longer-lasting effects on the form or function of the NMJ is not known (35). Importantly therefore, samples from the current study were compared with lower limb samples from patients who had received spinal anesthesia only. No differences were observed, suggesting that the choice of anesthetic was unlikely to have had any significant impact on the morphology of the NMJ.

In summary, we report that the human NMJ retains full structural integrity in both cachexia and weight stable cancer. This suggests that denervation of skeletal muscle and/or NMJ disruption are not major drivers of disease pathogenesis in cancer cachexia and that cancer cachexia represents a unique neuromuscular condition that needs to be differentiated from related conditions, including age-related sarcopenia. This observation supports the hypothesis that intrinsic changes within skeletal muscle, independent of any changes in motor neurons, represent the primary locus of pathology in cachexia. Since the NMJ remains intact in patients with cancer cachexia, promotion of muscle hypertrophy using exercise and neural stimulation should remain a viable therapeutic intervention for future clinical trials.

Methods

Patient recruitment

Patients with a confirmed diagnosis of gastrointestinal (GI) cancer suitable for surgical resection with curative intent were recruited from the regional multi-disciplinary team meeting (n = 20; Supplementary Table 1) and comprised patients with both cancer cachexia (n = 10) and weight stable disease (n = 10) based on consensus definition (Fearon et al. 2011). Suitable age-matched control patients undergoing a range of elective abdominal procedures (e.g. repair of aortic aneurysm or donor nephrectomy) were also recruited (n = 10; Supplementary Table 1). All patients were at least 18 years of age and provided written informed consent (inclusion criteria). Patients with a previous history of malignancy were not eligible for the control cohort (exclusion criteria).

Body composition analysis

See supplement.

Tissue sampling

Tissue sampling was performed under general anesthesia at the start of the surgical procedure. If patients underwent neoadjuvant chemotherapy, surgery was performed 4 to 6 weeks following cessation of treatment. Biopsies of rectus abdominis (RA) muscle were obtained following initial opening of the abdomen. RA is a well-characterised tissue for the study of human cancer cachexia, for which the results of previous studies have been reviewed recently (26). Evident changes of wasting, including fibre atrophy, have been robustly and repeatedly observed in several studies (32). RA has an 'in-series' architecture, with motor endplate bands located throughout its length (36). To ensure successful sampling, a longitudinal strip of muscle lying between two contiguous tendinous intersections was obtained using sharp dissection (approx. 0.5 x 0.5 x 2.0 cm). Samples were immediately fixed in 4% paraformaldehyde (PFA) for approximately 2 hours, followed by washing and storage in 1xPBS.

Tissue processing and NMJ immunohistochemistry

See supplement.

Confocal imaging & NMJ-morph analysis

See supplement.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 Software. Results are expressed as mean (± SEM). Group comparison of normally distributed data (based on D'Agostino-Pearson test, $P > 0.05$) was performed by one-way ANOVA and Tukey's post hoc test; non-parametric data was analysed by Kruskal-Wallis test and Dunn's post hoc test. $P < 0.05$ was considered to be significant.

Study approval

Ethics approval for tissue sampling was granted by the NHS Lothian Ethics Committee (IRAS 190214) in accordance with the Helsinki Declaration.

Author Contributions

All authors designed the experiments and drafted the manuscript. IB, JF, RJES, RAJ and THG performed the studies and analyzed the data. Authorship order was agreed according to the time and effort committed to the project.

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Figures & Legends

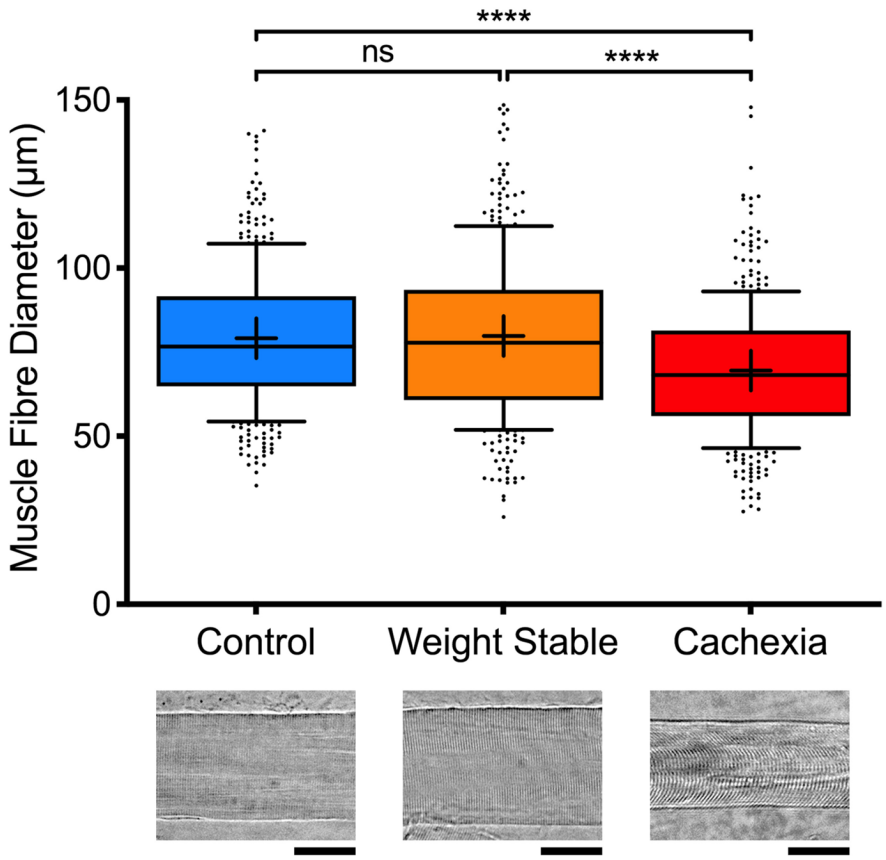

Figure 1. Atrophy of skeletal muscle fibres in cancer cachexia. Upper panel shows a box and whisker plot of muscle fibre diameters (MFD) in control (n = 10 patients), weight stable (n = 10) and cachectic (n = 10) patients. Bottom panels are representative micrographs of single, teased muscle fibres from control (left), weight stable (middle) and cachectic (right) patients. Scale bars = 50µm. Cachectic patients had significantly reduced muscle fibre diameters compared with weight stable and control cases (control n = 388, weight stable n = 362, cachexia n = 400 muscle fibres). The box contains the mean (+) and median (line) muscle fibre diameters for the group and encloses the central 90% of the data; the whiskers represent the SEM; outlying data points are shown beyond the whiskers. One-way ANOVA paired with a Tukey's post-hoc test (****$P < 0.0001$). Individual p-values are in Supplementary Table 2.

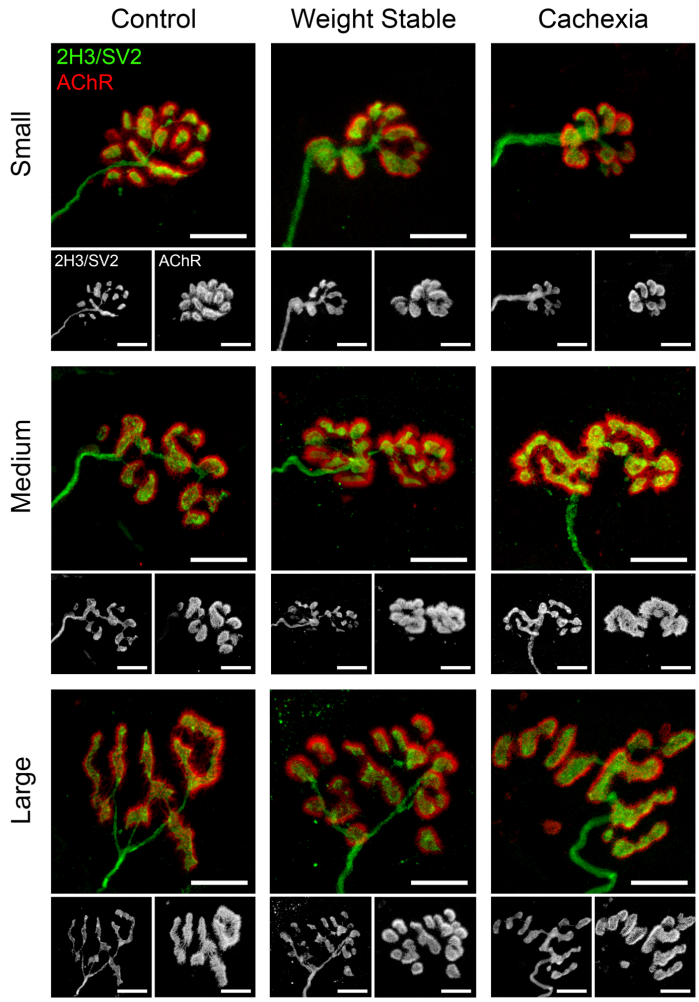

Figure 2. Conservation of NMJ morphology in cancer cachexia. Confocal micrographs of representative small, medium and large-sized NMJs from RA in the three patient groups. Despite heterogeneity in size and shape of individual NMJs, overall morphology was conserved across all groups, with no evidence of NMJ pathology in either the cachexia or weight stable groups. Axon and nerve terminals in green (2H3/SV2) and AChRs of the motor endplate in red (α-BTX). Scale bars = 10 µm.

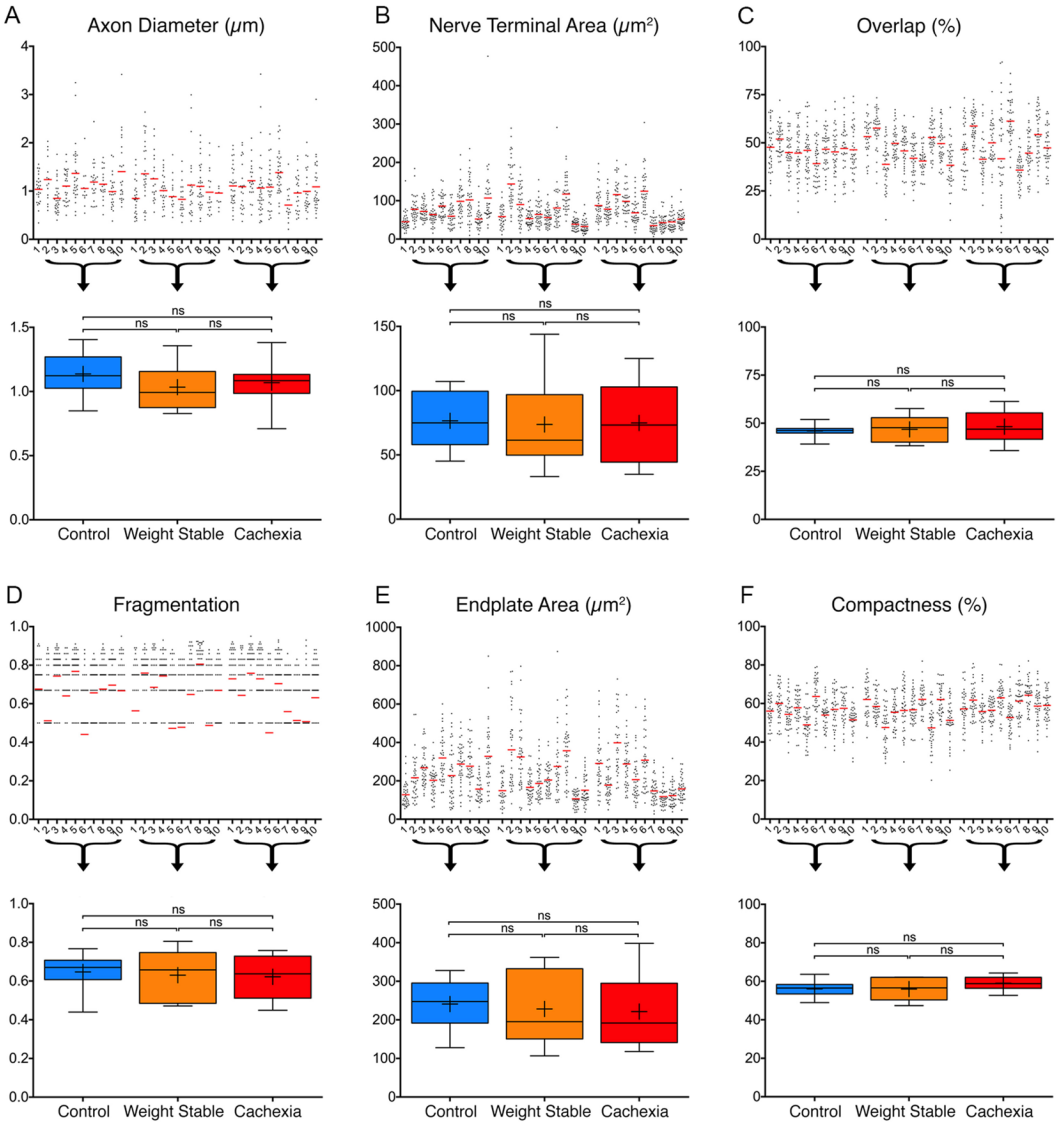

Figure 3. Structural integrity of the NMJ in cancer cachexia. Morphometric analysis using NMJ-morph revealed that NMJ morphology is conserved in both cachexia and weight stable disease. Data presented as a pair of charts (scatterplot, above; box & whisker plot, below) for key NMJ variables, including measurements of pre- and post-synaptic architecture and axon diameter. Scatterplots depict the ~40 individual NMJs (data points) for the 10 patients (1 to 10) in each group; the mean NMJ value is given by the red line; the observed heterogeneity is a normal feature of human NMJ morphology. Box and whisker plots constructed using the mean patient data in each group (10 patients; control NMJs n = 387, weight stable NMJs n = 386, cachexia NMJs n = 392). The box contains the mean (+) and median (line) values for each NMJ variable and encloses the central 90% of the data; the whiskers represent the SEM. One-way ANOVA paired with a Tukey's post-hoc test.