Increased tumour burden alters skeletal muscle properties in the KPC mouse model of pancreatic cancer

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Abstract

Background  Cancer cachexia is a multifactorial wasting syndrome that is characterized by the loss of skeletal muscle mass and weakness, which compromises physical function, reduces quality of life, and ultimately can lead to mortality. Experimental models of cancer cachexia have recapitulated this skeletal muscle atrophy and consequent decline in muscle force-generating capacity. We address these issues in a novel transgenic mouse model Kras, Trp⁵³, and Pdx-¹-Cre (KPC) of pancreatic ductal adenocarcinoma using multi-parametric magnetic resonance measures.

Methods  KPC mice (n = 10) were divided equally into two groups (n = 5 per group) depending on the size of the tumour, that is, tumour size < 250 and > 250 mm³. Using multi-parametric magnetic resonance measures, we demonstrated the changes in the gastrocnemius muscle at the microstructural level. In addition, we evaluated skeletal muscle contractile function in KPC mice using an in vivo approach.

Results  Increase in tumour size resulted in decrease in gastrocnemius maximum cross-sectional area, decrease in T₂ relaxation time, increase in magnetization transfer ratio, decrease in mean diffusivity, and decrease in radial diffusivity of water across the muscle fibres. Finally, we detected significant decrease in absolute and specific force production of gastrocnemius muscle with increase in tumour size.

Conclusions  Our findings indicate that increase in tumour size may cause alterations in structural and functional parameters of skeletal muscles and that MR parameters may be used as sensitive biomarkers to non-invasively detect structural changes in cachectic muscles.

Keywords  Cachexia; Magnetic resonance imaging (MRI); Transverse relaxation time (T₂); Magnetization transfer ratio (MTR); Diffusion tensor imaging (DTI)

Introduction

Cancer cachexia is defined as continuing loss of skeletal muscle mass with or without fat mass that cannot be fully reversed by conventional nutritional measures.¹ Cachexia causes severe fatigue and reduces mobility, leading to a loss of functional independence and reduction in overall quality of life.² Furthermore, cachexia is associated with reduced efficacy of anti-neoplastic treatments as well as an increased risk of post-surgical complications.³ Pancreatic ductal adenocarcinoma (PDA) is an aggressive cancer, with a 5 year survival rate of less than 8%⁴ and a median survival rate of
only 3–6 months. Diagnosis of pancreatic cancer is usually made at later stages of the disease progression, making treatment even more challenging. In addition to inherent problems associated with the treatment of localized pancreatic tumour progression, decrease in muscle quantity and quality pose an added threat to the patients suffering from PDA. Decrease in both muscle quality and quantity is one of the major contributors to the low survival rate and is observed in more than 85% of pancreatic cancer patients.6

Most of cachexia studies involving animals have focused on tumours that have been injected subcutaneously to study cancer cachexia.7–9 One of the primary limitations of these models is their inability to metastasize.10 In addition to the subcutaneous model, an orthotopic pancreatic mouse model was developed11 and studied for cachexia.9, 10 An alternative to implanted cells and tumours is the genetically engineered mouse model of PDA that more closely resembles the clinical features and molecular progression of human pancreatic cancer from inception to invasion. Kras, Trp53, and Pdx-1-Cre (KPC) mice express physiological levels of oncogene and tumour suppressor gene mutations in pancreatic progenitor cells, resulting in the development of spontaneous progression of ductal lesions to metastatic carcinomas.12, 13

Mounting evidence suggests that cachexia results in profound histological changes in skeletal muscle, including fibrosis14 and changes in muscle fibre size15 and quality.16 Techniques such as muscle biopsies are required to monitor and document the underlying pathophysiological changes. However, this technique is both invasive and highly localized and thus may not accurately reflect the in vivo condition of the entire muscle. This problem can be circumvented by use of modern-day sensitive and specific image based non-invasive biomarkers such as ultrasound,17 computed tomography scans,18 and magnetic resonance imaging (MRI).19 Amongst all the non-invasive techniques, MRI has an advantage due to its capability of providing high-resolution images while avoiding the use of any ionizing radiations. Additionally, the strength of MRI compared with other imaging modalities resides in its potential to provide a vast array of image contrast sequences (T1, T2, magnetization transfer imaging, and diffusion-weighted imaging). These modern-day MRI techniques enable the assessment of several aspects of muscle pathophysiology. Indeed, MRI has proven to be a valuable, non-invasive tool for the quantitative and dynamic assessment of the skeletal muscle modifications in both preclinical and clinical settings.

The purpose of this study was two-fold: (i) to characterize atrophic changes in the skeletal muscles of KPC mice at different stages of tumour development using multi-parametric MRI and (ii) to measure skeletal muscle function in tumour-bearing mice. We hypothesized that with progressive nature of tumour development, structural and functional deficits will occur in skeletal muscles of KPC mice.

Methods

Animals

The study was conducted with the approval from the University of Washington Institutional Animal Care and Use Committee. KPC (n = 10) mice included in this study were divided equally into two groups (n = 5 per group) depending on the size of the tumour, that is, tumour size <250 and >250 mm3. All the animals were housed with unrestricted access to a balanced pellet diet and water. Mice were housed in a temperature-controlled room with a 12:12 h light : dark cycle.

Magnetic resonance imaging

In vivo MRI was performed on a 14 T NMR spectrometer (Bruker Biospin, Billerica, MA). Mice were anaesthetized using 3% isoflurane and kept anaesthetized with 1% isoflurane and a mixture of air and oxygen at 3:1 ratio by using a vaporizer. The vertical bore of the magnet was maintained at 30 °C to maintain thermo-neutrality of the animal, and respiratory rate was monitored and maintained at 50–70 breaths per minute by adjusting the anaesthetic concentration. T1-weighted images were acquired with the following parameters: field of view = 15 × 15 × 15 mm3; matrix size = 256 × 192 × 96; repetition time (TR) = 50 ms; echo time (TE) = 7 ms; number of signal averages = 2; and flip angle = 40°. The quantitative T2 measurements utilized spin-echo sequences to generate T2 maps. T2 maps were generated using a multi-slice, multi-echo sequence (TR/TE = 4 s/6–100 ms, 16 echoes with 6.3 ms spacing) with fat signal suppressed (Gaussian pulse, pulse length = 1.3 ms, bandwidth = 2100.5 Hz) at 14 T. We utilized: SI = Ae−TE/T2 to fit the T2 values to generate maps, where SI is the signal intensity and A is the amplitude. Magnetization transfer (MT) suppression ratios, or MT ratios (MTRs), were measured using the following ratio: (SI0 − SI)/SI0, where SI0 represents the tissue signal intensity with no saturation pulse applied while SI includes the saturation pulse. We utilized a gradient echo sequence (TR/TE = 939/5 ms, flip angle = 30°) with an off-resonance frequency of 5000 Hz and a saturation pulse of block pulse shape, 50 ms width, and 10 μT amplitude. Finally, diffusion-weighted images were acquired with duration of diffusion gradient pulse δ = 2.5 ms per separation between diffusion pulses Δ = 10 ms. One image was acquired with a minimum b value (50 s/mm2) and six images with maximum b value (1000 s/mm2), TR/TE = 500/17.4 ms, and number of signal averages = 1. Field of view, slice thickness/gap, and acquisition matrix were 25 × 11 × 20 mm3, 1 mm/0 mm, and 128 × 64 × 41, respectively, yielding a voxel resolution of 0.02 × 0.017 × 0.048 mm3.

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**Muscle force and fatigue measurements**

All mice were placed under anaesthesia as described above throughout the duration of in vivo stimulation and recovery. A mouse was positioned on a 37 °C heated platform with the right limb secured at the knee joint and the foot taped to a foot plate and force transducer (Aurora Scientific Inc., Ontario). Subdermal needle electrodes were placed and secured at the proximal and distal end of the gastrocnemius (GA) muscle. Muscle stimulation was delivered to the GA muscle at a titrated voltage of 20–22 V. Dynamic Muscle Control software (Aurora Scientific Inc., Ontario) was used to control electrical stimulation and to acquire force vs. time data. After adjusting knee-to-footpedal length to optimize tetanic force output (tetanic stimulus: 200 Hz stimulation frequency for 300 ms), force–frequency measurements were made by delivering stimuli once per minute at frequencies ranging from 10 to 250 Hz to test muscle contractile response to submaximal and maximal stimulation frequencies. To test the capacity of muscle to sustain force output, GA muscle was subjected to a series of repeated contractions to simulate exercise and cause fatigue. Muscle fatigue was measured by delivering tetanic stimuli (200 Hz) at 2 s intervals for 4 min and then 1 and 5 min after the final stimulus to assess recovery from fatigue.

**Magnetic resonance data analysis**

Images were converted to Digital Imaging and Communication in Medicine format on the Bruker console to measure tumour size and GA muscle cross-sectional area (CSA). GA was outlined on the axial images of the lower limb to determine maximum CSA (CSA_max). CSA_max was calculated as the mean of the consecutive three slices having the greatest CSA for GA muscle. MRI images were analysed using ImageJ software (http://rsbweb.nih.gov/ij), developed by the National Institutes of Health, to measure mean values T1, T2, MTR, and diffusivity parameters of GA muscle. Regions of interest (ROIs) were selected in the GA, and mean T1, T2, and MTR were calculated as an average of values across three slices. Additionally, mean diffusivity (MD), radial diffusivity (RD), fractional anisotropy (FA), eccentricity (e), and eigenvalues (primary eigenvalue λ1 > secondary eigenvalue λ2 > tertiary eigenvalue λ3) were determined within ROIs in GA muscle. MD and RD were calculated using [(λ1 + λ2 + λ3)/3] and [(λ2 + λ3)/2], respectively. Additionally, two anisotropic parameters, ellipsoid eccentricity (e) = sqrt([λ1 - λ3]/λ1) and FA, were calculated as previously described.

**Histological analysis**

The left GA muscle was collected and coated with optimum cutting temperature medium, frozen in isopentane cooled in liquid nitrogen, and stored at −80 °C. Frozen sections (8 μm) were obtained from the mid-belly region of GA muscle using cryostat (CM1950, Leica Biosystems Inc., Buffalo Grove, IL), and subsequent slices were stained with haematoxylin and eosin stains. Muscle fibre diameter was analysed using minimal ‘Feret’s diameter’ plugin in ImageJ software. Data from 150–200 fibres per mouse were computed and analysed.

**Statistics**

Data are presented in the text as mean ± standard deviation. Differences between the experimental groups were determined by using Student’s t-test for unpaired data. Correlation was evaluated using Pearson test (Prism 6.0, GraphPad Software). Pearson correlation coefficient (r) with Bonferroni correction was computed to assess the relationship between the tumour volume and different MR parameters. Statistical significance was accepted for P < 0.05.

**Results**

**Body weight and muscle cross-sectional area**

Prior to imaging, body weight of all the animals was recorded and documented. Mice were assigned to different groups based on the tumour size, that is, <250 and >250 mm³. This threshold value of 250 mm³ was based on our previously published results. Mice assigned to the larger tumour group (>250 mm³) had significantly higher body weight compared with the smaller tumour group (<250 mm³, 21.50 ± 0.92 g vs. >250 mm³, 25.98 ± 3.32 g; mean ± SD; Figure 1A). Tumour volume was calculated as previously described. Mice in the larger tumour group had significantly larger tumour size compared with the smaller tumour group (>250 mm³, 500.1 ± 163.4 vs. <250 mm³, 186.1 ± 79.2 mm³; Figure 1B). Additionally, CSA_max of GA muscle was not different between the tumour groups (<250 mm³, 25.1 ± 0.3 mm² vs. >250 mm³, 24.1 ± 0.8 mm²; P > 0.05; Figure 1C). However, there was a significant correlation between increase in tumour volume and decrease in CSA_max of GA muscle (r = -0.84, P < 0.05; Figure 1D).

**T1, T2, and magnetization transfer ratio changes in gastrocnemius muscle**

Quantitative T1, T2, and MTR maps were overlaid on the corresponding images and displayed in Figure 2A. T1 relaxation time of GA muscle was not different between the smaller and larger tumour groups (<250 mm³, 3146.0 ± 141.3 ms vs. >250 mm³, 3269.0 ± 34.08 ms;
Figure 1  (A) Significant difference in the body weight of KPC mice between <250 mm\(^3\) (white) and >250 mm\(^3\) (red) group. (B) Significant increase in pancreatic tumour volume in mice in a larger tumour group. (C) Significant decrease in gastrocnemius (GA) muscle CSA\(_{\text{max}}\) was seen in mice in the larger tumour group. (D) There was a significant negative correlation between increase in tumour size and GA CSA\(_{\text{max}}\) (\(r = -0.84, P < 0.01\)). *Significant difference between <250 mm\(^3\) (white) and >250 mm\(^3\) (red). Data are expressed as mean ± standard deviation. \(* P < 0.05; \** P < 0.01.

Figure 2B). However, \(T_2\) relaxation time of GA muscle differed significantly between the two groups (<250 mm\(^3\), 19.0 ± 1.1 ms vs. >250 mm\(^3\), 17.7 ± 0.3 ms; Figure 2C). Furthermore, MTR of GA muscle was significantly higher in the larger tumour group compared with the smaller tumour group (<250 mm\(^3\), 81.5 ± 1.5% vs. >250 mm\(^3\), 83.37 ± 0.84%; Figure 2D).

**Histogram analyses**

There is mounting body of research that has reported average MR parameters over the entire ROIs. However, calculating and comparing voxel by voxel values may prove to be more sensitive to small underlying changes in the tissue. Histogram analyses were performed on the ROIs used to calculate mean \(T_1\), \(T_2\), and MTR values (Figure 3A–3C). Histograms were then normalized by plotting the percentage of pixels remaining above the specific measurement in the x-axis, generating a cumulative histogram (Figure 3D–3F). Finally, the area under the curve with units of % pixels × parameter on x-axis was used to compare values in two different groups (Figure 3G–3I): a significant increase in \(T_1\) (<250 mm\(^3\), 121.24 ± 14.68 vs. >250 mm\(^3\), 158.26 ± 22.20) and MTR (<250 mm\(^3\), 1254 ± 28.13 vs. >250 mm\(^3\), 1344.60 ± 22.33) and decrease in \(T_2\) (<250 mm\(^3\), 830.72 ± 21.01 vs. >250 mm\(^3\), 745.42 ± 17.42).

**Diffusion changes in gastrocnemius muscle**

Diffusion parameters exhibited significant changes in GA muscle with increase in tumour size. To determine changes in diffusion parameters, we measured different parameters of GA muscle. MD of GA muscle decreased significantly in the larger tumour group (<250 mm\(^3\), 1.40 ± 0.05 × 10\(^{-3}\) mm\(^2\)/s vs. >250 mm\(^3\), 1.31 ± 0.04 × 10\(^{-3}\) mm\(^2\)/s, \(P < 0.05\); Figure...
Figure 2 (A) Representative $T_1$, $T_2$, and magnetization transfer ratio (MTR) colour maps acquired from the lower hind limbs of KPC mice having $<250$ mm$^3$ (white) and $>250$ mm$^3$ (red) tumour group. Quantitative differences in gastrocnemius muscle (A) $T_1$, (B) $T_2$, and (C) MTR between $<250$ and $>250$ mm$^3$ tumour group. Data are expressed as mean ± standard deviation. *$P < 0.05$.

4A). RD of GA muscle in the larger tumour group decreased significantly ($<250$ mm$^3$, $1.03 ± 0.03 \times 10^{-3}$ mm$^2$/s vs. $>250$ mm$^3$, $0.96 ± 0.02 \times 10^{-3}$ mm$^2$/s, $P < 0.01$; Figure 4B). There was a significant increase in both anisotropic measures, that is, FA ($<250$ mm$^3$, $0.45 ± 0.04$ vs. $>250$ mm$^3$, $0.52 ± 0.01$, $P < 0.01$; Figure 4C) and $e$ ($<250$ mm$^3$, $0.78 ± 0.02$ vs. $>250$ mm$^3$, and $0.86 ± 0.01$, $P < 0.001$; Figure 4D), with increase in tumour size. There was no significant difference in $\lambda_1$ ($<250$ mm$^3$, $1.16 ± 0.14$ vs. $>250$ mm$^3$, $2.00 ± 0.09 \times 10^{-3}$ mm$^2$/s; Figure 4E); however, a significant decrease in $\lambda_2$ ($<250$ mm$^3$, $1.21 ± 0.04 \times 10^{-3}$ mm$^2$/s vs. $>250$ mm$^3$, $1.40 ± 0.03 \times 10^{-3}$ mm$^2$/s, $P < 0.01$; Figure 4F) and $\lambda_3$ ($<250$ mm$^3$, $0.84 ± 0.03 \times 10^{-3}$ mm$^2$/s vs. $>250$ mm$^3$, $0.52 ± 0.04 \times 10^{-3}$ mm$^2$/s, $P < 0.01$; Figure 4G) was discovered in GA muscle with increase in tumour volume.

**Correlation between different magnetic resonance parameters**

There was a significant negative correlation between tumour volume and GA $T_2$ ($r = -0.73$, $P < 0.05$; Figure 5A). $\text{CSA}_{\text{max}}$ was positively correlated to GA $T_2$ ($r = 0.64$, $P < 0.05$; Figure 5B), whereas MTR (%) and FA were negatively correlated to GA $T_2$ ($r = -0.71$, $P < 0.05$, Figure 5C, and $r = -0.78$, $P < 0.05$, Figure 5D), respectively. Finally, GA $T_2$ was moderately but positively correlated to RD ($r = 0.51$; Figure 5E) and $\lambda_3$ ($r = 0.65$, $P < 0.05$; Figure 5F).

**Muscle force measurements**

To determine whether cachexia is associated with contractile dysfunction, in vivo contractile measurements were performed. GA muscle from both the groups exhibited similar force-generating capacity between stimulation frequencies of 10–200 Hz (Figure 6A). However, mice with larger tumour demonstrated significant reduction in maximal tetanic force output capacity ($<250$ mm$^3$, $360.0 ± 44.3$ mN vs. $>250$ mm$^3$, $188.0 ± 84.5$ mN, $P < 0.01$; Figure 6B), and specific force-generating capacity (maximal tetanic force output normalized to $\text{CSA}_{\text{max}}$) ($<250$ mm$^3$, $14.38 ± 1.58$ mN/mm$^2$ vs. $>250$ mm$^3$, $7.70 ± 3.13$ mN/mm$^2$, $P < 0.01$; Figure 6C) of GA muscle decreased with increased in tumour size. Finally, there was no difference between groups in relative force produced during and after a 2 min fatiguing protocol. At the end of the exercise protocol, GA muscle from the smaller tumour group and larger tumour group exhibited similar normalized
force-generating capacity (<250 mm$^3$, 20.6 ± 3.4% vs. >250 mm$^3$, 22.4 ± 5.9%; Figure 6D). Finally, force output was similar between groups at 1 and 5 min after exercise, indicating full recovery from fatigue (Figure 6E).

**Histological measurements and correlation with magnetic resonance measures**

Muscle cryosections from GA muscle were stained with haematoxylin and eosin stains (Figure 7A and 7B) and were quantified for muscle fibre size by calculating minimal Feret’s diameter (Figure 7C). GA muscle from greater tumour mass demonstrated significantly reduced mean Feret’s diameter, demonstrating decrease in muscle fibre size (<250 mm$^3$, 109 ± 1.6 μm vs. >250 mm$^3$, 71.5 ± 1.7 μm). Furthermore, histogram analysis revealed shift towards smaller Feret’s diameter with increase in tumour volume (Figure 7D). Finally, a strong correlation ($r = 0.85$, $P < 0.05$; Figure 7E) was demonstrated between histological (mean minimal Feret’s diameter) and MRI (tertiary eigenvalue, $\lambda_3$) measurement.
Figure 4 Comparison of diffusion tensor imaging parameters of gastrocnemius (GA) muscle between <250 mm$^3$ (white) and >250 mm$^3$ (red). (A) Mean diffusivity, (B) radial diffusivity, (C) fractional anisotropy, and (D) eccentricity were significantly different in GA muscles of the smaller tumour group compared with larger tumour group mice. $\lambda_1$ was not different between the groups; however, $\lambda_2$ and $\lambda_3$ demonstrated significant differences between the smaller and larger tumour groups. Data are expressed as mean ± standard deviation. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Figure 5 Relationship between gastrocnemius (GA) $T_2$ and various magnetic resonance parameters. There was a significant positive correlation between GA $T_2$ and (B, F) CSA$_{max}$ and $\lambda_3$ values and significant negative correlation between GA $T_2$ and (A) tumour volume, (C) magnetization transfer ratio (MTR) (%), (D) fractional anisotropy, and (E) radial diffusivity (RD).
Discussion

Cachexia, a devastating catabolic condition, is currently defined as an unintentional weight loss of >5% over a 6 month period, or a body mass index <20 kg/m² with ongoing weight loss of >2%. Non-invasive biomarkers that may help in identifying pre-cachectic patients who will progress to cachexia and refractory cachexia are an urgent and unmet requirement. Our study was designed to evaluate multiple MR parameters to elucidate differences in GA muscle in KPC mice. In addition, we evaluated the contractile properties of GA muscle in KPC mice. We found that there were statistical differences in the mean transverse relaxation time (T₂), magnetization ratio (MTR), diffusion parameters, and contractile functions of GA muscle.

Variation in muscle T₂, T₂, and magnetization transfer ratio parameters

Increased serum levels of pro-inflammatory cytokines, including interleukin 6, tumour necrosis factor-α, matrix

Figure 6 Differences in skeletal muscle function tests performed on gastrocnemius (GA) muscle between <250 mm³ (white) and >250 mm³ (red). *P < 0.05; **P < 0.01. (A) The fatigue resistance of GA muscle in KPC mice was determined in vivo. (B) Full force recovery was observed at 1 and 5 min after exercise. (C) KPC mice in both groups exhibited similar tetanic force outputs across a range of stimulation frequencies (10–250 Hz). However, significant differences between groups were observed in (D) absolute and (E) specific force-generating capacity.
metalloproteinases, and transforming growth factor-β, play pivotal role in tumour-induced muscle wasting and collagen deposition. These factors inflict a cumulative effect on skeletal muscles, thereby leading to cachectic conditions. Current state of the art MR parameters can be applied to non-invasively characterize tumours that induce cachexia. In the present study, we have demonstrated decrease in muscle T2 and an increase in GA muscle MTR with increase in tumour burden in KPC mice. T2 is a quantitative measure of tissue biophysical properties leading to signal contrast on MRI. Changes in muscle T2 have previously been used to monitor underlying pathophysiological processes. For example, studies have demonstrated that muscle T2 is altered when there is inflammation, oedema, or fibrotic tissue accumulation. Moreover, MR parameters such as T2 and T2* in infarcted/fibrotic cardiac tissue have demonstrated that MR parameters are linearly related to collagen content and tissue fibrosis based on staining for collagen content. In our study, we have demonstrated that with increase in tumour burden, T2 of skeletal muscles decreases. Moreover, a strong negative correlation between tumour size and GA muscle T2 has been demonstrated. Additionally, MT-MRI provides unique image contrast for characterization of tissues beyond conventional T1 and T2 weighting for a variety of different clinical applications. MTR is described as the interaction of tissue water protons in different macromolecular environment and is a measure of the efficiency of magnetization transfer between bound and adjacent mobile protons. The effects of MTR have been studied extensively in the diseases of the central nervous system. When tissue is damaged, there are fewer hydrogen atoms bound to macromolecules, which leads to decreased magnetization transfer. Whereas when there is an increase in macromolecules in the intracellular space such as collagen tissue, an increased number of hydrogen atoms bind to these macromolecules, thereby increasing magnetization transfer. Similar to central nervous system, skeletal muscle tissue also displays MT effect. In the present study, we have demonstrated that an increase in tumour volume leads to increase in GA muscle MTR (%). A mounting body of evidence suggests that increase in fibrotic or collagen tissue deposition in the tumour leads to decrease in T2 and increase in MTR (%). Similarly, a significant negative correlation was demonstrated between GA MTR (%) and GA T2, further corroborating our initial hypothesis that with increase in tumour volume, the skeletal muscles demonstrate increase in collagenous tissue deposition.

**Diffusion changes in cachectic muscles**

Despite the high sensitivity of T1, T2, and MTR sequences, they fail to provide comprehensive information regarding the muscle architecture. Magnetic resonance diffusion tensor

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Figure 7 Haematoxylin and eosin stains of gastrocnemius (GA) muscle from KPC mice with (A) < 250 mm³ and (B) > 250 mm³ tumour showing decrease in muscle fibre size and fibrotic tissue accumulation (demonstrated by black arrows). (C) Quantitative measure of muscle fibre size ‘mean minimal Feret’s diameter’ demonstrating significant differences in GA muscle. (D) Correlation between histological measure ‘mean minimal Feret’s diameter’ and magnetic resonance diffusion parameter ‘λ3’. Data are expressed as mean ± standard deviation. *P < 0.05, **P < 0.01, and ***P < 0.001. Scale bar = 100 μm (A and B). Original magnification, ×20 (A and B).
imaging, on the other hand, allows investigation of the skeletal muscle architecture by assessing the diffusion of water molecules in different directions.\textsuperscript{51} It has been hypothesized that due to the fibrillar structure of skeletal muscle, water diffusion is greater along fibre orientation than in other directions ($\lambda_1 > \lambda_2 > \lambda_3$).\textsuperscript{20} This underlying theory has been exploited and used to investigate microstructure of skeletal muscles.\textsuperscript{52, 53} Additionally, a recent study by Esposito et al.\textsuperscript{54} demonstrated changes in diffusivity parameters with skeletal muscle injury and regeneration. Similarly, in this study, we have demonstrated that diffusion parameters of skeletal muscles are sensitive to underlying pathology in yet another model, that is, cachexia. We observed a decrease in secondary ($\lambda_2$) and tertiary ($\lambda_3$) eigenvalues in the cachexia model. A possible explanation for decrease in these parameters is the reduced muscle fibre size and increase in extracellular space.\textsuperscript{55} Uncontrolled synthesis of extracellular matrix has been shown to increase in fibrotic tissue accumulation.\textsuperscript{56} An increased body of evidence suggests that in advanced stages of cachexia, there is accumulation of fibrotic tissue within the extracellular space.\textsuperscript{35, 57} Indeed, accumulation of fibrotic tissue in the extracellular space has been shown to affect $\lambda_2$.\textsuperscript{58, 59} Our findings are in line with these previous observations, suggesting decrease in $\lambda_2$ of skeletal muscles with increase in tumour burden. Previous studies have demonstrated changes in MD and $\lambda_3$ in a model of ischaemia\textsuperscript{59} and denervated muscles.\textsuperscript{60} Additionally, both anisotropic measures, e and FA, increased significantly in muscles of mice having larger tumour, suggesting atrophy of muscle fibres. Decrease in fibre CSA in cachetic mouse animal models has been reported by a number of studies.\textsuperscript{31} MRI and histological results from our study corroborate these previously published results.

**Contractile deficits in muscles of KPC mice**

Several groups have recapitulated skeletal muscle weakness in experimental models of cancer cachexia and have demonstrated decrease in maximal tetanic force in the extensor digitorum longus and tibialis anterior muscles.\textsuperscript{61–63} These muscles are commonly studied during cancer cachexia because cachexia is known to affect fast-twitch (glycolytic) muscles to a greater extent than slow-twitch (oxidative) muscles.\textsuperscript{31, 64–66} Furthermore, previous data suggest that both absolute force and specific force decrease during cancer cachexia.\textsuperscript{57, 67} Similarly, in our study, we observed a significant decrease in both absolute force and specific force in mice having larger tumour. Additionally, we did not observe any difference in contraction-induced fatigue in GA muscle between both the groups of KPC mice.

Our study has a few limitations that need to be acknowledged. First, there are a multitude of underlying factors that co-exist during the disease progression. For example, in advanced stages of cachexia, a number of underlying processes are occurring simultaneously such as (i) muscle fibres tend to atrophy; (ii) due to increased activity of pro-inflammatory cytokines, there may be significant changes in extracellular space; and (iii) due to disruption in the dystrophin–glycoprotein complex, muscle fibres may be more prone to injury. All these factors can potentially change in MR parameters. Second, we did not correlate MR findings with more nuanced histological measures for pro-inflammatory cytokines and absolute histological measures, that is, fibrotic tissue accumulation. However, recent studies have shown that MR parameters such as $T_2$,\textsuperscript{39} MTR,\textsuperscript{68} and diffusion parameters\textsuperscript{59} correlate well with histological measures. Furthermore, previous studies have used $b$ values ranging from 400 to 1000 s/mm\textsuperscript{2},\textsuperscript{21, 59} which is similar to the $b$ values used in our study. Additionally, there is a concern that signal-to-noise ratio (SNR) values <10 could lead to significant bias in estimation of eigenvalues. However, SNR in our study was 18, which is close to previously documented SNR values.\textsuperscript{38, 60} In this study, we were not able to use advanced diffusion parameters to measure the cell structure and diffusion, which includes incorporation of diffusion time ranging from 80 to 200 ms.\textsuperscript{69} Finally, future studies looking at these features and corroboration with histological measurements are warranted.

**Conclusions**

This study provides several insights into a novel cachexia mouse model using MRI as a non-invasive biomarker. We believe that MRI may provide valuable information regarding the underlying changes associated with skeletal muscle during cachexia. Finally, detailed characterization of imaging parameters and the events occurring in the tissue would help in interpreting the imaging data easier and more straightforward in preclinical settings and be ultimately translated to clinical settings.

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Conflict of interest

None declared.

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