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Delving into disability in Crohn's disease: Dysregulation of molecular pathways may explain skeletal muscle loss in Crohn's disease☆

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Vitamin D;
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Abstract

Background/aims: In Crohn's disease (CD), skeletal muscle mass and function are reduced compared to healthy controls, potentially resulting in disability. Mechanisms contributing to muscle impairment, and thus potential therapeutic targets, are poorly understood. This study aimed to measure and compare skeletal muscle size and molecular targets involved in skeletal muscle growth, in CD subjects and healthy controls.
Methods: CD (n = 27) and healthy (n = 22) subjects were recruited from the IBD outpatient clinic and via local advertisement respectively. Demographics and clinical data were collected via survey and interview. Quadriceps muscle cross-sectional area was measured using peripheral quantitative CT scanning. Levels of muscle hypertrophy and atrophy signalling targets using quantitative PCR and western blotting were measured in muscle biopsies.
Results: Muscle size was 14% lower ($p = 0.055$) and a 54% lower phosphorylated:total (p:t) Akt ratio was measured in the muscle samples ($p < 0.05$), indicating an attenuated muscle hypertrophy pathway in CD compared with controls. In those with CD, a lower p:t Akt ratio (<0.97) was associated with lower serum vitamin D3, lower physical activity indices (49 vs 64 mmol/L, 1.7 vs 2.2 × 10^6 accelerometer counts respectively, each $p < 0.05$) and a trend

Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; pQCT, peripheral quantitative computed tomography.

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towards lower serum ferritin levels (128 vs 322 mg/L, p = 0.07), compared with CD subjects with normal/high p:t Akt ratios.
Conclusion: The reduced muscle mass in CD may be explained, in part, by impaired activation of muscle protein synthesis pathways, notably the IGF1–Akt pathway. Normal vitamin D levels and regular exercise may be protective in CD against this trend, though confirmatory longitudinal studies are needed.

1. Introduction

Skeletal muscle is the primary organ for movement and metabolism and, therefore, plays a key role in maintaining human health. Conversely, loss of muscle mass and function associated with chronic disease or ageing is an important predictor of future disability.[1] In patients with Crohn's disease (CD), when compared to healthy controls, skeletal muscle mass and strength are reduced[2–4] and local muscle fatigue is increased.[5] Multiple factors including poor nutrition, physical inactivity, hormonal changes, prolonged corticosteroid therapy as well as direct effects of the underlying chronic disease,[4] would appear to negatively influence the molecular targets controlling skeletal muscle mass and function. However at present the molecular mechanisms involved in skeletal muscle dysfunction in CD are not well characterized.

Maintaining skeletal muscle mass is a tightly regulated process controlled by the fine balance between muscle hypertrophy and atrophy signalling pathways. IGF-1 activates several downstream signalling cascades including Akt/mTOR/$p70^{s6k}$/S6K, Akt/mTOR/4E-BP1 and Akt/GSK3β/eIF2B that are known to stimulate muscle hypertrophy.[6] Additionally, Akt activation also inhibits the upregulation of the muscle atrophy genes, MuRF1 and atrogin-1.[7,8] CD patients have a decrease in circulating IGF-1 levels and elevated TBARS, a marker of oxidative stress.[5] As IGF-1 and oxidative stress increases and decreases Akt signalling respectively,[9] these factors may a play role in the reduction in muscle mass in CD.[2] Pro-inflammatory factors, such as TNF-α and IL-6 are also increased in CD patients[10,11] and their elevation is associated with lower muscle mass and strength in elderly populations.[12,13] Whether or not their elevation is related to a reduction in muscle mass and Akt, $p70^{s6k}$ and GSK3β protein levels in CD are unknown.

The dysregulation of the Akt/mTOR/$p70^{s6k}$/S6K, Akt/mTOR/4E-BP1 and Akt/GSK3β/eIF2B and MuRF1/atrogin-1 pathways has been observed in atrophied skeletal muscle of patients with other chronic diseases such as amyotrophic lateral sclerosis and chronic obstructive pulmonary disease, as well as in the elderly.[14–16] Presently, the regulation of these pathways in skeletal muscle of subjects with CD has not been investigated, but is a vital step in understanding the mechanisms of muscle dysfunction as a potential major contributor to the increasingly recognized long term disability incurred in CD.[17–19]

Hence, the aim of the present study was to develop an understanding of the signalling factors that may negatively impact skeletal muscle size and function in CD patients. To achieve this, cross-sectional area of the quadriceps muscle and the levels of hypertrophy and atrophy signalling targets, including Akt, $p70^{S6k}$, 4E-BP1, GSK-3β, MuRF1 and atrogin-1 in skeletal muscle biopsies were measured. Correlations were made between these variables and previously measured levels of physical activity, circulating pro-inflammatory cytokines including TNFα, IL-6, IL-17 and IFN-γ, anabolic factors such as IGF-1 and testosterone, as well as other factors putatively important in muscle health, including vitamin D and magnesium.[20,21] All measurements were performed in patients with CD and healthy age and sex matched controls.

2. Materials & methods

2.1. Ethical approval

The study was approved by the Deakin University and Eastern Health research ethics committees (on 15/06/2009). For all investigations conducted, informed consent was obtained from all participants and the study was performed in concordance with the *Declaration of Helsinki* (2008 version).

2.2. Subject recruitment

Patients with Crohn's disease who had recently completed a survey in relation to a wider research programme were then consecutively, in order of survey completion date, invited to participate in the present cross-sectional study at their next visit, subject to informed consent. They were recruited from the Box Hill Hospital Inflammatory Bowel Disease Clinic and all had a confirmed diagnosis of CD according to standard criteria. Healthy volunteers were consecutively recruited via local advertisement in hospital and university publications, and the local newspaper in the same period. Exclusion criteria for the study included those aged less than 18 years or greater than 65 years, those with significant medical or psychiatric comorbidities likely to cause restrictions in functional performance and/or those who were pregnant. Also, healthy controls with first degree relatives with known IBD and those unable to give informed consent were excluded from the study. All subjects attended one study visit with an investigator prior the muscle testing in order to assess for these exclusion criteria and to ensure that they did not engage in regular high intensity exercise which may have otherwise biased the study results.

2.3. Calculation of muscle size

Muscle cross-sectional area (CSA) measurements (units of mm^2) were performed using peripheral quantitative CT (pQCT) scanning with a *Stratec XCT 3000* scanner (Stratec Medical, Pforzheim, Germany) at the level of the quadriceps muscle (at 25% of the bone's length measured from distal end of the lateral condyle of the femur). Scout views of the distal femur

were performed and scans were placed at 4% and 25% of the bone's length measured from the reference line, which was on the distal end of the lateral condyle of the femur. Slice thickness was 2.3 mm and voxel size was set at 0.3 mm with a scanning speed of 10 mm/s. Only data from 25% (the diaphysis) of the bone's total length was used for this study. The radiation dose for each subject was calculated to be 0.2 μSv per scan and per scout view.

2.4. Muscle biopsy

Muscle samples were obtained from the belly of the vastus lateralis muscle of the left leg via a percutaneous needle biopsy technique as published previously.[22] To ensure standardization between participants as much as possible, all but two (96%) of the muscle biopsies were performed in the morning between 0900 and 1100 h. However food intake was neither controlled nor documented in this study.

2.5. RNA extraction and analysis

Total RNA was extracted from 5 to 20 mg skeletal muscle samples using TRI-Reagent® Solution (Ambion Inc., Austin TX) according to the manufacturer's protocol. RNA was extracted, cDNA prepared and RT-PCR performed as published previously.[16,23] mRNA expression was measured in triplicate and normalised to total cDNA as determined using the Quant-iT OliGreen ssDNA Assay Kit (Invitrogen, Mulgrave, Australia).[24]

2.6. Protein extraction and analysis

Protein was extracted from skeletal muscle in radioimmunoprecipitation assay (RIPA) buffer (Millipore, Billerica, MD, USA) containing a protease inhibitor and phosphatase inhibitor cocktail (Sigma-Aldrich, Sydney, Australia). Samples were homogenised briefly then rotated overnight at 4 °C. The samples were then centrifuged at 13,000 rpm for 15 min at 4 °C and the supernatant was collected. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL). The protein was stored at −80 °C until further use.

Electrophoresis was performed using a 4–12% NuPAGE® Novex Bis–Tris Gel (Invitrogen, Carlsbad, CA) in NuPAGE® sodium dodecyl sulphate & 3-(*N*-morpholino)propanesulphonic acid (SDS MOPS) running buffer (Invitrogen). Protein transfer was performed in NuPAGE® Transfer Buffer (Invitrogen) with

Table 1 Characteristics of healthy controls and patients with Crohn's disease.

Variable	Control	Crohn's disease	p value
Number of subjects	22	27	–
Female sex (%)	59 [39, 77]	56 [37, 72]	1.0
Age (years)	43 [36, 49]	43 [38, 48]	1.0
Current smoker (%)	5 [0, 24]	15 [5, 33]	0.36
BMI (kg/m^2)	24 [23, 26]	25 [23, 27]	0.63
Total activity counts in 7 days (×10^6) [a]	2.1 [1.8, 2.4]	1.9 [1.6, 2.2]	0.45
Time in sedentary activity [a] (% of time worn)	70 [65, 75]	70 [66, 75]	0.86
Time in moderate–vigorous exercise (% of time worn)	4 [3, 5]	3 [2, 4]	0.14
Limited employment status (%)	9 [1, 29]	41 [25, 59]	0.02
Location (%) [b]			
L1 ileal	–	30 [16, 49]	
L2 ileocolonic	–	33 [17, 54]	
L3 colonic	–	37 [21, 56]	
L4 upper GI	–	7 [1, 25]	
P perianal	–	26 [13, 45]	
Behaviour (%) [b]			
B1 non-stricturing/penetrating	–	59 [41, 76]	
B2 stricturing	–	22 [10, 41]	
B3 penetrating	–	19 [8, 37]	
Duration IBD (years, range)	–	13 [9, 17]	
Active disease (HBI ≥ 5, %)	–	30 [16, 49]	
Prior bowel resection (%)	–	33 [17, 54]	
Current medical therapy (%)			
Oral corticosteroid	–	7 [1, 25]	
Oral aminosalicylate	–	37 [21, 56]	
Immunomodulator	–	56 [37, 72]	
Anti-TNFα	–	26 [13, 45]	
Biologic therapy naive (%)	–	59 [41, 76]	

[a] Physical activity measures as per accelerometer.
[b] As per Montreal classification.

10% methanol using polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS), after which they were incubated overnight at 4 °C with the following primary antibodies diluted 1:1000 in 5% BSA in PBS: Akt (9272S, Cell Signalling, Danvers, MA), phosphorylated (phospho, or p)-Akt (Ser473) (9271S, Cell Signalling), GSK-3β (9315S, Cell Signalling), phospho-GSK-3α/β (Ser21/9) (9331S, Cell Signalling), P70 s6 Kinase (9202S, Cell Signalling), phospho-P70 s6 Kinase (Thr389)(9234S, Cell Signalling), atrogin-1 (AP2041; ECM Biosciences, Versailles, KY) and MuRF1 (MP3401; ECM Biosciences).

Following washing, the membranes were incubated for 1 h with a goat anti-rabbit IgG antibody labelled with an infrared-fluorescent 800 nm dye (IR-Dye 800CW, LI-COR Biosciences, Lincoln, NE) or a rabbit anti-mouse IgG antibody labelled with an infrared-fluorescent 680 nm dye (Alexa Fluor® 680, Invitrogen) diluted 1:5000 in PBS containing 50% Odyssey® blocking buffer (LI-COR Biosciences) and 0.01% SDS. After washing, the proteins were exposed and individual protein band optical densities were determined using the Odyssey® Infrared Imaging System (LI-COR Biosciences). The blots were normalised against the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein (G8795, Sigma-Aldrich).

Plasma cytokine analysis, blood and faecal testing and measurement of habitual physical activity and disease activity for these subjects have been reported previously.[5]

2.7. Statistical analyses

Normality of data was assessed using *IBM-SPSS* software version 19 (Chicago, IL, USA) which was also used for all statistical analyses. Where data was non-parametric as per the Shapiro–Wilk test, square root transformation of data was applied depending on skewness to ensure the data met criteria for a normal distribution. Hence, data comparisons were performed with parametric statistics including unpaired t-tests for comparison of means (with confidence intervals or standard error of the mean provided) and Pearson's correlation coefficients; proportions were compared with the Fisher exact test. A p value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Subject characteristics

27 subjects with CD and 22 age- and sex-matched healthy control subjects completed the study. These were the same subjects who participated in our previously published study.[5] Subject characteristics, including disease characteristics of the CD group, are represented in Table 1. Both groups were well matched for age, weight, BMI and physical activity levels.

3.2. Muscle cross-sectional area and its relationship to age and disease duration

Quadriceps muscle CSA was 14% lower (p = 0.055) in the CD group (6277 [95% CI 5649, 6905] mm^2) when compared with the healthy control group (7279 [6351, 7932] mm^2) (Fig. 1).

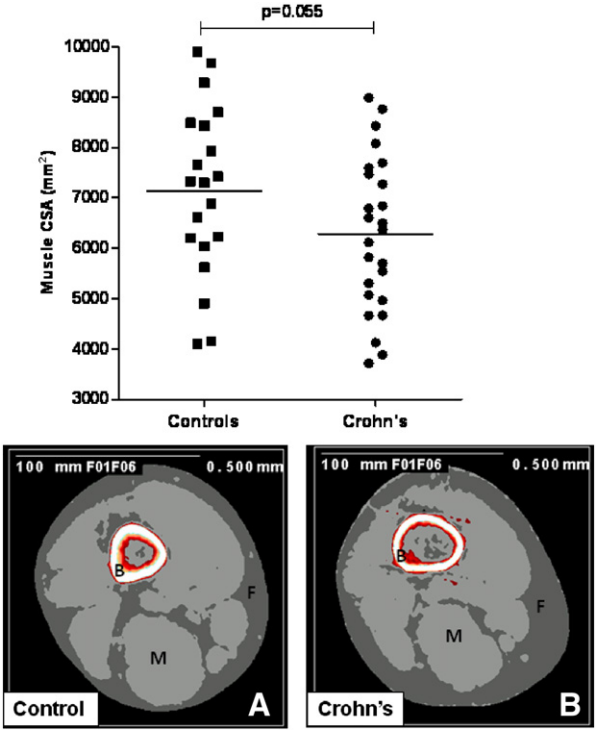

Figure 1 Scatter plot of muscle cross-sectional area (CSA) as measured by pQCT by group. A) Horizontal lines represent means, showing on average a smaller CSA in subjects with CD versus healthy controls (p = 0.055). B) Representative pQCT scans of a healthy subject (left) and a patient with CD (right). B, bone; M, muscle; F, fat.

3.3. Hypertrophy and atrophy signalling targets

With respect to hypertrophy signalling targets, CD patients had 54% lower levels of phosphorylated (active) Akt (p = 0.03), with no difference in the total amount of Akt (Fig. 2), when compared to healthy controls. Furthermore, this reflects a 54% reduced p:t Akt ratio in CD compared to controls. One subject with CD was excluded from this analysis as the measured pAkt level was zero (0).

No differences in the levels of phosphorylated $p70^{s6k}$ levels were observed between groups. However, the CD group had a 36% increase in total $p70^{s6k}$ levels (p = 0.01). This resulted in a tendency towards a 54% lower ratio of phosphorylated $p70^{s6k}$:total p^{70s6k} in the CD group (p = 0.08). Also, phosphorylated and total 4E-BP1 levels were 30% and 32% lower in CD respectively compared to controls, though the latter was not statistically significant (p = 0.04, p = 0.08 respectively), with the p:t 4E-BP1 ratio therefore similar between controls and CD (1.21 vs 1.19, p = 0.89). There were no significant differences in total or phosphorylated GSK-3β levels between the two groups (data not shown). The signalling targets outlined above are represented schematically in Fig. 4.

With respect to atrophy signalling targets, no differences in the MuRF-1 or atrogin-1 mRNA or MuRF1 protein levels were observed between groups (see Fig. 3).

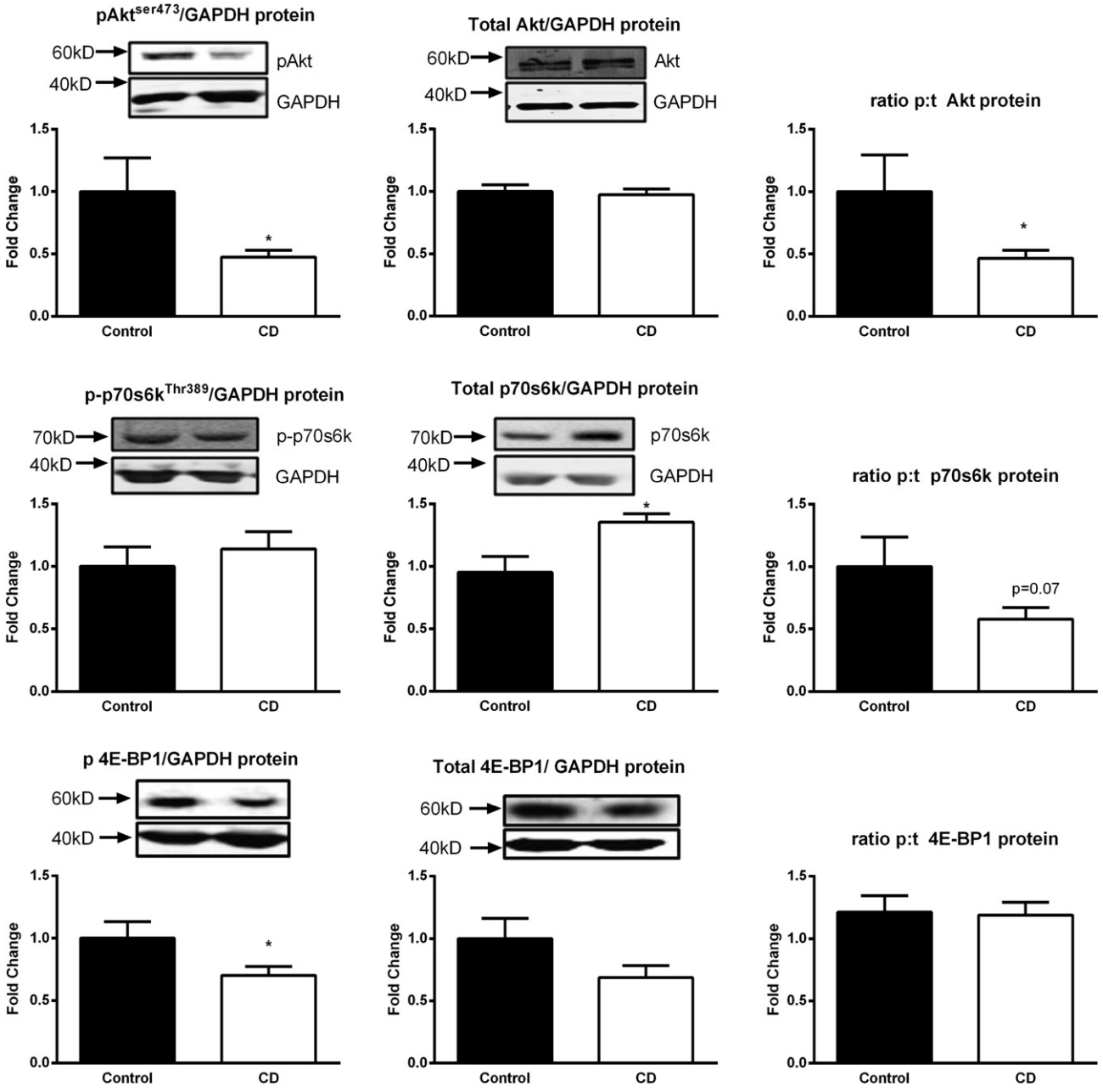

Figure 2 Phosphoproteins involved in muscle protein synthesis (hypertrophy), typically reduced in CD compared with healthy control subjects. Insets are representative western blot images of the proteins measured for the control and CD subjects. *$p < 0.05$.

3.4. Comparing factors in those with low versus normal phosphorylated:total Akt ratios (CD only)

In a previous study using these subjects, levels of circulating pro-inflammatory cytokines including TNFα, IL-6, IL-17 and IFN-γ, anabolic factors such as IGF-1 and testosterone as well as vitamin D and magnesium were measured.[5] Serum IGF-1 levels were lower while oxidative stress was higher in CD patients. Also, low serum vitamin D, IGF-1 and magnesium, and higher IL-6 levels were associated with increased muscle fatigue in CD patients.[5] Therefore in this present study, we evaluated if any of these factors were associated with a higher or lower p:t Akt ratio in skeletal muscle in subjects with CD. Using a p:t Akt ratio cutoff of 0.97 (the mean for the CD group) within the CD subjects only, a number of differences in relevant factors were noted (Table 2). Those with a lower p:t Akt ratio had significantly lower vitamin D3 levels and lower habitual physical activity according to accelerometry compared to those with normal ratios. Furthermore, there were non-significant trends to lower serum ferritin levels, higher plasma IL-6 and higher serum IGF1 levels in the low p:t Akt ratio group.

Finally, there were no significant differences in any of the above hypertrophy or atrophy markers or ratios (including p:t Akt ratio) in terms of Crohn's disease activity (i.e., active vs inactive), other disease characteristics or treatment variables

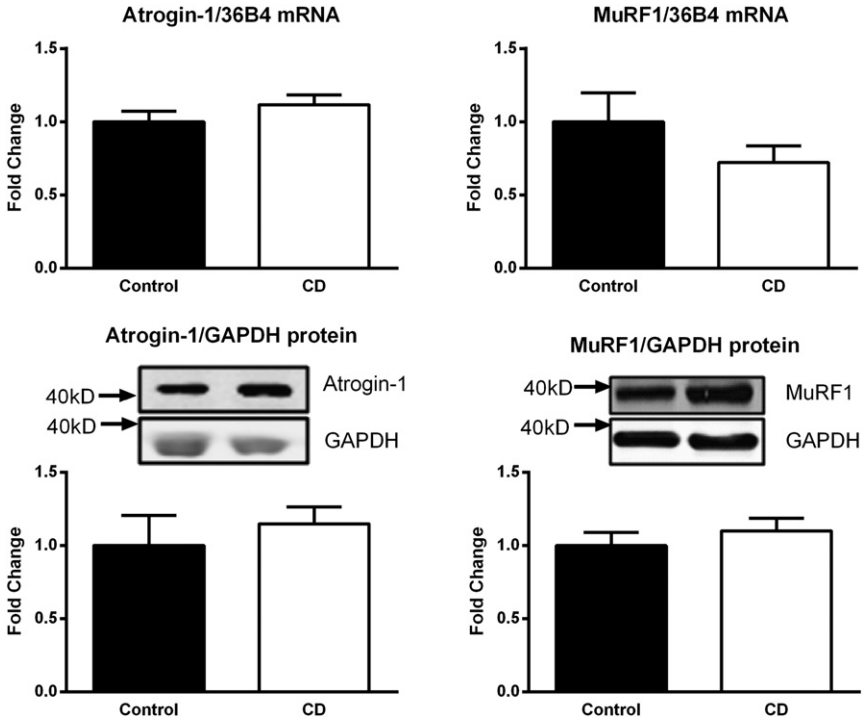

Figure 3 Genes/proteins of the muscle atrophy pathway (apart from MuRF1 mRNA, levels of atrophy markers were on average higher in subjects with CD compared to healthy controls, but differences were non-significant).

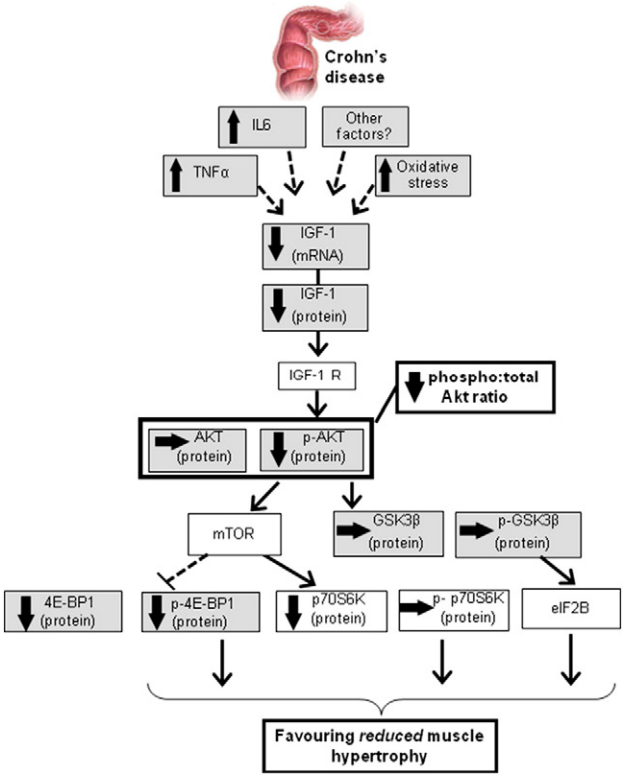

Figure 4 Diagram representing the attenuated muscle hypertrophy pathway as reflected by signalling factor levels in CD determined in this study, along with those in similar chronic disease models elsewhere.[6,16,23] Arrows shown represent increased (↑) decreased (↓) or similar (→) levels of markers tested in CD, compared with controls.

(such as concurrent anti-TNF and/or immunomodulator versus not in each case).

4. Discussion

The aim of the present study was to better understand the signalling factors that may negatively impact skeletal muscle size and function in patients with CD. Quadriceps muscle CSA was a mean of 14% lower in patients with CD compared to that of matched healthy controls, which, while just failing to reach statistical significance, supports previous findings of a lower muscle mass in CD.[2,3]

To better understand the molecular factors potentially involved in this reduced muscle CSA, we measured expression levels of key components of the hypertrophy stimulating IGF-1/Akt pathway and levels of atrogin-1 and MuRF1 proteins involved in muscle atrophy. As represented in Fig. 2, a reduced level of phosphorylated Akt, without changes in total Akt, was observed in CD patients compared to controls. As the levels of phosphorylated Akt are indicative of its activity, this observation suggests a reduced capacity of CD patients for active muscle protein synthesis.[7,23,25] Furthermore, both phosphorylated and total 4E-BP1 and phosphorylated $p70^{S6k}$ levels were generally lower in CD than control subjects; as both are downstream targets of activated Akt, this is again suggestive of reduced protein synthesis. Conversely, the increase in total $p70^{S6k}$ protein content in CD may be a failed compensatory attempt to maintain skeletal muscle protein synthesis and attenuate muscle wasting, in lieu of reduced Akt activation. Hence in CD there are multiple perturbations in the intracellular signalling pathways that activate muscle protein synthesis and hypertrophy. There were no differences in the expression

Table 2 Differences in selected laboratory and physical activity variables in those subjects with CD, grouped by 1) low and 2) high phosphorylated:total Akt ratios measured in skeletal muscle.

Variable (units)	Phosphorylated:total Akt ratio		p value
	<0.97	>0.97	
	n = 13 [a]	n = 13	
CRP (mg/L)	3.2 [1.5, 5.4]	3.6 [1.0, 6.2]	0.57
Faecal calprotectin (μg/g)	308 [147, 470]	308 [123, 493]	1.0
Haemoglobin (g/L)	140 [133, 146]	136 [125, 146]	0.40
Ferritin (mg/L)	128 [65, 191]	322 [116, 527]	0.07
Vitamin D3 (mmol/L)	**49** [40, 59]	**64** [52, 77]	**0.02**
Albumin (g/L)	41 [39, 43]	40 [38, 42]	0.41
TNFα (pg/mL)	3.6 [2.0, 5.1]	4.3 [2.1, 6.5]	0.67
IL-6 (pg/mL)	1.8 [0, 3.7]	0.3 [0, 0.5]	0.07
IGF_1 (nmol/L)	21 [17, 25]	15 [9, 20]	0.08
TBARS (MDA μM)	4.9 [3.5, 5.8]	4.4 [4.0, 4.8]	0.46
Total activity counts in 7 days ($\times 10^6$) [b]	**1.7** [1.1, 2.2]	**2.2** [1.8, 2.6]	**0.009**
Time in sedentary activity (%) [b]	68 [62, 73]	63 [57, 69]	0.21
Time in mod–vigorous exercise (%) [b]	**2.0** [1.3, 4.0]	**4.2** [2.6, 5.8]	**0.02**

Means with 95% confidence intervals and unpaired t-tests shown for each variable by group. Statistically significant group differences in bold.
[a] One patient recorded zero for phosphorylated Akt thus was excluded from this analysis.
[b] Physical activity measures as per accelerometer worn over 7 days.

of activated or total forms of GSK-3β between the CD or control subjects. The reduction in Akt activity, without changes in the downstream targets $p70^{S6k}$ and GSK-3β in CD subjects, is similar to what has been observed in other human disease models with muscle wasting such as amyotrophic lateral sclerosis (ALS)[14] and chronic obstructive pulmonary disease (COPD).[15] Measurement of mRNA and protein levels of the atrophy related targets MuRF1 and atrogin-1 did not show any differences between groups. This is in contrast to other human chronic disease conditions such as ALS and COPD, which present both reductions in Akt and increases in these atrophy targets. Therefore, it appears that the net loss of muscle mass in the CD patients observed in the present study is more likely due to an impaired muscle protein synthesis (reduced Akt activity) as opposed to increased protein degradation (no change in atrogin-1 or MuRF1).

The obvious question is what mechanisms underlie the likely reduction in muscle mass in CD. CD subjects and their controls did not significantly differ in terms of haematinics, gross nutritional and micronutrient markers, or most endocrine markers such as testosterone, TSH or free T4 levels. IGF-1 is a potent activator of muscle protein synthesis and muscle hypertrophy via its activation of Akt signalling.[6,9] We have shown previously that these CD patients have lower serum IGF-1 concentrations when compared to controls[5], which is consistent with previous studies.[26,27] We have also observed a tendency for an increase in plasma IL-6 and IL-10 ($p = 0.08$) in these CD patients[5] and this may have contributed to reduced Akt activation observed in the present study. Previous studies have shown that exposure to IL-6 interferes with the anabolic activities of the GH/IGF-1 pathway,[27–30] attenuates Akt activity[31] and results in muscle wasting.[29] Elevated serum TBARS was observed in these CD patients,[5] indicating a higher degree of lipid peroxidation or oxidative stress, a factor implicated in muscle dysfunction and sarcopenia.[32–34] As oxidative stress is increased in periods of active disease,[35] oxidative damage may be perpetual and permanent in CD.[35,36] This may also play a role in attenuating Akt activity as observed previously.[37]

Plasma TNFα levels were lower in the CD subjects compared to controls, perhaps reflecting the effects of concurrent immunomodulator and anti-TNF therapies (55% and 25% of the CD group respectively) in this group. As TNFα is known to increase the atrophy genes, atrogin-1 and MuRF1, in muscle cells,[38] the lack of difference in these targets between the CD patients and the control subjects may have been influenced by the medication taken by the CD patients, though there was no discernible differences in these factors in those on and not on anti-TNF therapies.

Within the CD group, we elucidated multiple factors associated with a lower p:t Akt ratio which, pending further evaluation, may provide potential avenues for restoring muscle protein synthesis through this Akt-dependent pathway in those otherwise at risk of muscle loss. Of particular clinical interest is that a lower vitamin D3 level was found in those with a lower phospho:total Akt ratio and similarly, albeit non-significantly, a lower ferritin level. Finally, those with a lower p:t Akt ratio demonstrated lower overall physical activity indices than those with a normal ratio, suggesting that regular exercise may be protective against loss of muscle via this pathway, as is intuitive from a broader physiological sense. Nevertheless whether the higher physical activity is the cause or effect of modulating this muscle pathway cannot be ascertained here. Conversely, the degree of systemic (i.e. serum CRP) or mucosal inflammation (i.e. faecal calprotectin) did not differ between the low and high ratio groups suggesting that treatment for CD may not be enough to correct this adverse muscle molecular signalling.

The main limitation of this study is that the cross-sectional, observational study design is unable to determine causality. Furthermore, given the time-consuming and invasive nature of the study, only small sample sizes of patients with CD and

healthy controls were recruited. The strengths of the study, however, include the prospective recruitment of consecutive CD patients and healthy controls with strict inclusion criteria so as to minimize bias. In addition, the use of objective measurements of muscle CSA with pQCT and the robust matching of controls with the CD subjects in terms of multiple demographic characteristics, adds credence to the study findings.

In summary, our findings support the previous observations that patients with CD exhibit reduced muscle size compared with well matched healthy controls. But delving further at the molecular level, we have demonstrated that subjects with CD exhibited a lower phosphorylated:total Akt ratio, denoting a system with attenuated capacity for muscle protein synthesis, often resulting in a net loss of muscle mass over time. The loss of muscle mass appears to be multifactorial, with potential mediators including elevated levels of proinflammatory cytokines, reduced IGF-1 levels, and the long term effects of CD-related oxidative stress. Although purely observational work, this study's novel, objective approaches in examining muscle dysfunction in CD have elucidated multiple potential therapeutic targets which now require further longitudinal, interventional-based study, with the ultimate goal of maximizing physical function, and minimizing disability, in patients with Crohn's disease.

Author contributions

DR van Langenberg was involved in the study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, performed all statistical analyses, obtained funding and coordinated the study. P Della Gatta was involved in data acquisition, analysis and interpretation of the data and provided administrative and technical support. B Hill and E Zacharewicz were involved in data acquisition, analysis and interpretation of the data and provided technical support. PR Gibson was involved in the study concept and design, analysis and interpretation of the data, provided administrative, material and technical support, obtained funding and supervised the study. AP Russell was involved in the study concept and design, analysis and interpretation of the data, drafting of the manuscript, provided administrative, material and technical support and supervised the study. All authors above were involved in critical revision of the manuscript and approved the final version submitted.

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Potential competing interests

None.

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References

1. Stucki G, Bruhlmann P, Stucki S, Michel BA. Isometric muscle strength is an indicator of self-reported physical functional disability in patients with rheumatoid arthritis. *Br J Rheumatol* 1998;**37**:643–8.
2. Schneider SM, Al-Jaouni R, Filippi J, Wiroth JB, Zeanandin G, Arab K, et al. Sarcopenia is prevalent in patients with Crohn's disease in clinical remission. *Inflamm Bowel Dis* 2008;**14**:1562–8.
3. Wiroth JB, Filippi J, Schneider SM, Al-Jaouni R, Horvais N, Gavarry O, et al. Muscle performance in patients with Crohn's disease in clinical remission. *Inflamm Bowel Dis* 2005;**11**:296–303.
4. Geerling BJ, Badart-Smook A, Stockbrugger RW, Brummer RJ. Comprehensive nutritional status in patients with long-standing Crohn disease currently in remission. *Am J Clin Nutr* 1998;**67**:919–26.
5. van Langenberg DR, Della Gatta P, Hill B, Zacharewicz E, Gibson PR, Russell AP. Objectively measured muscle fatigue in Crohn's disease: correlation with self-reported fatigue and associated factors for clinical application. *J Crohns Colitis* 2014;**8**:137–46.
6. Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, et al. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol* 2001;**3**:1009–13.
7. Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, et al. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell* 2004;**14**:395–403.
8. Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, et al. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 2001;**294**:1704–8.
9. Latres E, Amini AR, Amini AA, Griffiths J, Martin FJ, Wei Y, et al. Insulin-like growth factor-1 (IGF-1) inversely regulates atrophy-induced genes via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway. *J Biol Chem* 2005;**280**:2737–44.
10. Reimund JM, Wittersheim C, Dumont S, Muller CD, Baumann R, Poindron P, et al. Mucosal inflammatory cytokine production by intestinal biopsies in patients with ulcerative colitis and Crohn's disease. *J Clin Immunol* 1996;**16**:144–50.
11. Cominelli F. Cytokine-based therapies for Crohn's disease—new paradigms. *New Engl J Med* 2004;**351**:2045–8.
12. Visser M, Pahor M, Taaffe DR, Goodpaster BH, Simonsick EM, Newman AB, et al. Relationship of interleukin-6 and tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women: the Health ABC Study. *J Gerontol Biol Sci Med Sci* 2002;**57**:M326–32.
13. Schaap LA, Pluijm SM, Deeg DJ, Visser M. Inflammatory markers and loss of muscle mass (sarcopenia) and strength. *Am J Med* 2006;**119**:526 e9-17.
14. Leger B, Vergani L, Soraru G, Hespel P, Derave W, Gobelet C, et al. Human skeletal muscle atrophy in amyotrophic lateral sclerosis reveals a reduction in Akt and an increase in atrogin-1. *FASEB J* 2006;**20**:583–5.
15. Doucet M, Russell AP, Leger B, Debigare R, Joanisse DR, Caron MA, et al. Muscle atrophy and hypertrophy signaling in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2007;**176**:261–9.
16. Leger B, Derave W, De Bock K, Hespel P, Russell AP. Human sarcopenia reveals an increase in SOCS-3 and myostatin and a reduced efficiency of Akt phosphorylation. *Rejuvenation Res* 2008;**11**:163–175B.
17. Iwase H. Determination of vitamin K1 in emulsified nutritional supplements by solid-phase extraction and high-performance liquid chromatography with postcolumn reduction on a platinum catalyst and fluorescence detection. *J Chromatogr* 2000;**881**:261–6.

18. Iwase H. Determination of vitamin D2 in emulsified nutritional supplements by solid-phase extraction and column-switching high-performance liquid chromatography with UV detection. *J Chromatogr* 2000;**881**:189–96.
19. Ciarcia G, Paolucci M, Guerriero G, Cozzolino G, Abrescia P. Determination of vitamin E in eggs and during the larval development of the sea bass, *Dicentrarchus labrax* by high performance liquid chromatography. *Biofactors* 2000;**11**: 19–21.
20. Visser M, Deeg DJ, Lips P. Low vitamin D and high parathyroid hormone levels as determinants of loss of muscle strength and muscle mass (sarcopenia): the Longitudinal Aging Study Amsterdam. *J Clin Endocrinol Metab* 2003;**88**:5766–72.
21. Dominguez LJ, Barbagallo M, Lauretani F, Bandinelli S, Bos A, Corsi AM, et al. Magnesium and muscle performance in older persons: the InCHIANTI study. *Am J Clin Nutr* 2006;**84**:419–26.
22. Russell AP, Wadley G, Hesselink MK, Schaart G, Lo S, Leger B, et al. UCP3 protein expression is lower in type I, IIa and IIx muscle fiber types of endurance-trained compared to untrained subjects. *Eur J Physiol* 2003;**445**:563–9.
23. Leger B, Cartoni R, Praz M, Lamon S, Deriaz O, Crettenand A, et al. Akt signalling through GSK-3beta, mTOR and Foxo1 is involved in human skeletal muscle hypertrophy and atrophy. *J Physiol* 2006;**576**:923–33.
24. Cannata DJ, Ireland Z, Dickinson H, Snow RJ, Russell AP, West JM, et al. Maternal creatine supplementation from mid-pregnancy protects the newborn spiny mouse diaphragm from intrapartum hypoxia-induced damage. *Pediatr Res* 2010;**68**:393–8.
25. Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, et al. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* 2004;**117**:399–412.
26. Alempijevic T, Jovanovic I, Popovic D, Kovacevic N, Milutinovic AS, Krstic M. IGF-I as a marker of disease activity and nutritional status in patients with inflammatory bowel disease. *Indian J Gastroenterol* 2008;**27**:247–8.
27. Street ME, de'Angelis G, Camacho-Hubner C, Giovannelli G, Ziveri MA, Bacchini PL, et al. Relationships between serum IGF-1, IGFBP-2, interleukin-1beta and interleukin-6 in inflammatory bowel disease. *Horm Res* 2004;**61**:159–64.
28. Bodell PW, Kodesh E, Haddad F, Zaldivar FP, Cooper DM, Adams GR. Skeletal muscle growth in young rats is inhibited by chronic exposure to IL-6 but preserved by concurrent voluntary endurance exercise. *J Appl Physiol* 2009;**106**:443–53.
29. Haddad F, Zaldivar F, Cooper DM, Adams GR. IL-6-induced skeletal muscle atrophy. *J Appl Physiol* 2005;**98**:911–7.
30. Lazarus DD, Moldawer LL, Lowry SF. Insulin-like growth factor-1 activity is inhibited by interleukin-1 alpha, tumor necrosis factor-alpha, and interleukin-6. *Lymphokine Cytokine Res* 1993;**12**:219–23.
31. Zhang L, Du J, Hu Z, Han G, Delafontaine P, Garcia G, et al. IL-6 and serum amyloid A synergy mediates angiotensin II-induced muscle wasting. *J Am Soc Nephrol* 2009;**20**:604–12.
32. Semba RD, Ferrucci L, Sun K, Walston J, Varadhan R, Guralnik JM, et al. Oxidative stress and severe walking disability among older women. *Am J Med* 2007;**120**:1084–9.
33. Howard C, Ferrucci L, Sun K, Fried LP, Walston J, Varadhan R, et al. Oxidative protein damage is associated with poor grip strength among older women living in the community. *J Appl Physiol* 2007;**103**:17–20.
34. Capel F, Rimbert V, Lioger D, Diot A, Rousset P, Mirand PP, et al. Due to reverse electron transfer, mitochondrial H_2O_2 release increases with age in human vastus lateralis muscle although oxidative capacity is preserved. *Mech Ageing Dev* 2005;**126**: 505–11.
35. Beltran B, Nos P, Dasi F, Iborra M, Bastida G, Martinez M, et al. Mitochondrial dysfunction, persistent oxidative damage, and catalase inhibition in immune cells of naive and treated Crohn's disease. *Inflamm Bowel Dis* 2010;**16**:76–86.
36. Boirivant M, Marini M, Di Felice G, Pronio AM, Montesani C, Tersigni R, et al. Lamina propria T cells in Crohn's disease and other gastrointestinal inflammation show defective CD2 pathway-induced apoptosis. *Gastroenterology* 1999;**116**:557–65.
37. Russell ST, Eley HL, Wyke SM, Tisdale MJ. Involvement of phosphoinositide 3-kinase and Akt in the induction of muscle protein degradation by proteolysis-inducing factor. *Biochem J* 2008;**409**:751–9.
38. Foletta VC, Prior MJ, Stupka N, Carey K, Segal DH, Jones S, et al. NDRG2, a novel regulator of myoblast proliferation, is regulated by anabolic and catabolic factors. *J Physiol* 2009;**587**:1619–34.