

**Title:** Reduced skeletal muscle protein balance and sarcopenia in paediatric Crohn's disease patients in remission.

**Short title:** Sarcopenia in Crohn's disease

**Authors:** Amanda Walker<sup>1</sup>, Aline Nixon<sup>1</sup>, Rafeeq Muhammed<sup>2</sup>, Kostas Tsintzas<sup>1</sup>, Sian Kirkham<sup>3</sup>, Francis Stephens<sup>4</sup>, Gordon W. Moran<sup>1,5,6</sup>.

1. Faculty of Medicine and Health Sciences, University of Nottingham, Nottingham
2. Birmingham Women's and Children's Hospital, Birmingham
3. Nottingham Children's Hospital, Nottingham University Hospitals, Nottingham
4. Sports and Health Sciences, University of Exeter, Exeter
5. Nottingham Digestive Diseases Centre, University of Nottingham, Nottingham
6. National Institute of Health Research Nottingham Biomedical Research Centre at the Nottingham University Hospitals and University of Nottingham, Nottingham

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**Abbreviations:**

Appendicular LM

ASMI: appendicular LM / height<sup>2</sup>

a-v: arterio-venous

BMC: bone mineral content

BMD: bone mineral density

BMI: body mass index

CD: Crohn's disease

CDM: male CD

CDF: female CD

ConM: male controls

ConF: female controls

COX: carbohydrate oxidation

CRP: C-reactive protein

Dom: dominant arm

EDTA: Ethylenediaminetetraacetic acid  
EGTA: Ethyleneglycol-Bis- $\beta$ -Aminoethylether Tetraacetate  
ELISA: Enzyme-linked immunosorbent assay  
FCP: faecal calprotectin  
FOX: fat oxidation  
GH: Growth hormone  
HBI: Harvey Bradshaw Index  
IBDQ: Inflammatory bowel disease questionnaire  
IBD-F: the Inflammatory Bowel Disease fatigue patient self-assessment scale  
IL-1 $\beta$ : Interleukin 1 beta  
IL-6: Interleukin 6  
IGF-1: Insulin growth factor-1  
IPAQ: international physical activity questionnaire.  
LBM: lean body mass  
LM: Lean mass  
Non-dom: non-dominant arm  
RER: respiratory exchange ratio (volume CO<sub>2</sub> expired / volume of O<sub>2</sub> inspired)  
RMR: resting metabolic rate  
SDS: standard deviation score  
TNF $\alpha$ : Tumor necrosis factor alpha  
TBM: total body mass  
TTO: time trade off valuation technique  
TEE: total energy expenditure  
VAS: visual analogue scale

**Corresponding Author:**

Gordon W. Moran

Clinical Associate Professor in Gastroenterology

NIHR Nottingham Biomedical Research Centre in Gastrointestinal and Liver Diseases, Nottingham University Hospitals NHS Trust & The University of Nottingham, Nottingham, United Kingdom

E-mail: Gordon.Moran@nottingham.ac.uk

Telephone no: +44 (0)115 9249924 ext 70608

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## **ABSTRACT**

**Background & Aims:** Sarcopenia is common in Crohn's disease (CD) and persists in remission. The aetiology is unclear. Inability to respond to anabolic stimuli (anabolic resistance) could be implicated. We aimed to investigate this association in age, gender matched children with CD and healthy controls.

**Methods:** CD patients in remission and matched healthy volunteers were recruited. Participants drank a liquid meal (Ensure plus). Arterialised hand and venous forearm blood samples were collected concurrently and brachial artery blood flow measured at baseline and every 20mins for 2hrs. Net balance of branched chain amino acids and glucose were derived, providing indices of skeletal muscle protein balance and insulin resistance. Participants had a dual energy X-ray absorptiometry scan, handgrip dynamometer test, wore a pedometer and completed a food diary. Patient-related outcome measure questionnaires were completed.

**Results:** 20 fasted, CD patients in remission (15.6y, BMI=20.6) and 9 matched healthy volunteers (16.0y, BMI=20.7) were recruited. Both groups showed a neutral BCAA balance. Male CD patients were in overall negative BCAA balance, in contrast to the positive balance of controls ( $p=0.049$ ). Male CD patients did mount an initial anabolic response to the meal, shown by increasing BCAA balance between  $t = 0$  &  $t = 20$ , but this positive balance was not sustained with BCAA balance returning to negative by  $t = 60$ . This was associated with lower appendicular muscle mass, higher levels of muscle fatigue and reduced protein intake.

**Conclusion:** The inability to sustain a positive protein balance postprandially could provide an explanation for the reduced muscle mass seen in CD patients in remission.

**Keywords:** Sarcopenia, Crohn's disease, fatigue, anabolic resistance.

## Introduction

In paediatric Crohn's disease (CD), 60% of patients present with malnutrition and weight loss<sup>1,2,3</sup>, with growth retardation and severe malnutrition apparent several years later in 6.9 and 15% of children respectively<sup>4</sup>. Many of these patients also have disproportionately lower skeletal muscle mass<sup>5,6</sup>. Not only is reduced muscle mass seen in the active disease<sup>7</sup>, but it often persists in remission<sup>8</sup>. This is important as low muscle mass has been linked to decreased muscle function<sup>9,10,11</sup>, low bone mass & density<sup>12,13</sup>, reduced physical activity<sup>13</sup>, fatigue and quality of life<sup>14</sup>.

Skeletal muscle mass is determined by the balance between muscle protein synthesis and breakdown. Studies<sup>15,16,17,18</sup> using stable isotope infusion techniques, showed high rates of whole-body protein turnover in children with active disease, which could be reversed with conventional treatments (nutritional interventions, corticosteroids & anti-tumour necrosis factor alpha (TNF $\alpha$ ) therapy. However, whereas several weeks of treatment with elemental diet (13% energy from amino acids)<sup>17</sup>, corticosteroids<sup>17</sup>, or anti-TNF $\alpha$  therapy<sup>18</sup> reduced fasting protein breakdown, it also reduced fasting protein synthesis, resulting in no change in net protein balance in remission compared to active disease.

Protein synthesis is stimulated by amino acids, particularly the branch chain amino acids (BCAA's), and positive whole-body protein balance can be achieved via parenteral or very high intragastric (3.2 g/kg) protein doses<sup>15,18</sup> in children with active disease. A recent study reported increases in muscle cross-sectional area (CSA) after 12 weeks treatment with an elemental diet that induced remission in children with newly diagnosed CD<sup>13</sup>. However, no further improvement in muscle CSA was observed thereafter at 52 weeks<sup>13</sup>. Once the inflammatory burden is reduced and BMI has improved, patients in remission may still suffer from low muscle mass<sup>8</sup>, despite receiving apparently adequate protein nutrition<sup>15,19,20,21</sup>.

This suggests that reduced muscle mass in active disease is driven by inflammation and a sub-optimal muscle protein synthetic response to protein feeding, termed anabolic resistance<sup>22</sup>, that may persist in remission. Anabolic resistance is thought to be the main driver of muscle loss in critical illness, ageing and disuse<sup>23</sup> while CD activity and disease duration have been negatively correlated with lean mass<sup>7,24</sup> and key inflammatory markers such as TNF $\alpha$ , C-reactive protein (CRP) & interleukin (IL)-6<sup>5,24</sup>.

We hypothesized that reduced muscle mass and function in paediatric CD patients in remission would be associated with anabolic resistance of skeletal muscle to a meal. The aim of the present study is to measure skeletal muscle protein balance in a

cohort of paediatric CD patients and age- and body mass index (BMI)-matched controls in both the fasted and fed states together with a number of functional readouts to provide a holistic muscle phenotype of children with CD.

## **Methods**

### Study population

We aimed to recruit, male and female CD outpatients (age 11-18 years) from Nottingham Children's Hospital at Nottingham University Hospitals Trust and Birmingham Children's Hospital at Birmingham Women's and Children's NHS Foundation Trust. Remission was defined as a Harvey Bradshaw index (HBI) <4 and CRP <5mg/dl or faecal calprotectin (FCP) <250ug/g. Any CD-related medication apart from corticosteroids within 3 months prior to recruitment were permitted. Age, gender and BMI matched healthy volunteers were recruited through advertisements at Nottingham University Hospitals and University of Nottingham campuses, in local press, and through departmental social media posts to parents. All potential participants were given comprehensive written & verbal explanations of the study before giving informed consent (parental consent at  $\leq 15$  yrs) and were free to withdraw at any time. Participants completed a general health questionnaire and the latter underwent a short medical screening prior to participation. The study was approved by the NHS Health Research Authority, West Midlands - Coventry & Warwickshire Research Ethics Committee (15/WM/0285) on the 26<sup>th</sup> July 2016.

### Outcomes Measures

The primary outcome of this study is the forearm skeletal muscle protein net balance under fasted and fed conditions. Secondary outcomes were forearm skeletal muscle glucose and free fatty acid (FFA) net balance; forearm and whole body insulin sensitivity; resting and fed metabolic rate, daily physical activity; forearm muscle isometric strength and fatigability; appendicular lean mass (LM) and appendicular skeletal muscle index (ASMI); daily energy intake and dietary macronutrient composition; markers of active disease (IL-1, IL-6, TNF, CRP and faecal calprotectin); and quality of life measures namely UK Inflammatory bowel disease questionnaire (IBDQ) and the Inflammatory Bowel Disease fatigue (IBD-F) patient self-assessment scale.

Differences between arterialised venous and venous concentrations (a-v difference) of BCAA, glucose and FFAs, multiplied by brachial artery blood flow and corrected for forearm lean mass determined net balance of these nutrients across the forearm under fasted and fed conditions. Lean mass was measured by Dual-energy X-ray

absorptiometry (DEXA) (Luna Prodigy, GE Healthcare). The Matsuda index was used as an index of whole body insulin sensitivity<sup>25,26</sup>. This composite index takes into account fasting and fed concentrations of arterial glucose and serum insulin. The lower the index the higher the level of insulin resistance. Muscle strength measurements were standardized for muscle size, as well as for height and age<sup>27</sup>, to facilitate comparison. Appendicular LM (sum of lean mass in the limbs measured by DEXA) and ASMI were calculated to give a more precise idea of skeletal muscle mass than total lean mass alone.

### Experimental protocol

Participants reported to the laboratory at 0800, following an overnight fast, having abstained from strenuous exercise for the previous 48 hours. On arrival their body composition was assessed by DEXA. Body mass, whole body, and regional body composition and body mass index were calculated. Subsequently, participants were asked to rest in a supine position on a bed while a cannula was inserted in a retrograde fashion into a superficial vein on the dorsal surface of the dominant hand. This hand was kept in a hand-warming unit (air temperature 55°C) to arterialize the venous drainage of the hand. A second cannula was placed in an antecubital vein in the non-dominant forearm<sup>28</sup>. Both these cannulas were used for blood sampling. After baseline blood samples, and measurements of brachial artery blood flow (in the non-dominant arm), as measured by Doppler ultrasound (Toshiba Aplio 300) and indirect calorimetry (Cosmed, Italy), participants ingested a 220ml bottle of Ensure plus nutrition shake (t = 0). This meal provided 330kcal, consisting of 30% of energy as fat (11g), 53% of energy as carbohydrates (44g), and 17% of energy as protein (14g).

Arterialized-venous (2 ml) and venous (2 ml) blood were obtained from the heated hand vein and antecubital vein along with brachial artery blood flow measurements at t = 0 and every 20 minutes thereafter for 2 hours, so that forearm muscle net balance of amino acids, glucose and fatty acids (FFA) could be calculated in the fasted and fed states. At t = 100 a final indirect calorimetry was performed providing energy expenditure (TEE), respiratory exchange ratio (RER) and carbohydrate and fat oxidation rates (COX & FOX respectively) to compare with fasting levels.

At the end of the 2-hour postprandial period an assessment of forearm muscle function was made. Participants performed 12 maximal static voluntary contractions using a dynamometer (MIE medical research Ltd. UK), with both dominant and non-dominant arms. The peak contraction was taken as maximal handgrip isometric

strength (kg). Level of fatigue was derived from the difference in peak strength and strength measured at the end of 12 maximal contractions (mean of the last 3).

#### Blood metabolite, cytokine & hormone analysis

Blood glucose levels were measured using Yellow Springs Instrument Analyzer, YSI, 2300 STAT PLUS. Plasma separated from Ethyleneglycol-Bis-( $\beta$ -Aminoethylether)Tetraacetate (EGTA) treated blood was analysed for FFA by colorimetric kit (NEFA C, Wako), and branched chain amino acid (BCAA) concentrations by spectrophotometric assay based on the method proposed by Beckett et al.<sup>29</sup>. Serum separated from arterialised blood was analysed for insulin concentration by Enzyme-linked immunosorbent assay (ELISA) (DRG diagnostics, Germany). CRP was measured by ELISA at the Department of Clinical Chemistry, Queen's Medical Centre, Nottingham, either as part of the initial screening process or subsequently in baseline blood samples. Inflammatory cytokines TNF $\alpha$ ; IL-6; IL-1 $\beta$ , and bioavailable testosterone, were measured in baseline arterialized plasma samples by colorimetric ELISA (R&D systems, Minneapolis, US) according to manufacturer's instructions.

#### Assessment of physical activity & habitual dietary intake

Step counts measured using a pedometer (Omron, Kyoto, Japan) for 3-days in advance of the study visit and self-reported levels of physical activity, using short form International Physical Activity Questionnaire (IPAQ)<sup>30</sup> were used to assess physical activity levels. Routine energy intake was measured using a 3-day paper-based food diary completed by participants in the days preceding their study visit. This was subsequently analysed using Nutritics software (Dublin, Ireland).

#### Patient-related outcome measures (PROMS)

The Inflammatory bowel disease questionnaire (IBDQ)<sup>14</sup> and the Inflammatory Bowel Disease fatigue (IBD-F) patient self-assessment scale<sup>31</sup> were completed by CD participants only.

#### Statistical analyses

A power calculation for this work was not possible, as anabolic resistance has not been previously assessed in a paediatric cohort, although small cohorts have been previously used when assessing muscle size in CD<sup>13</sup>. Our primary analyses was a comparison between CD and Con with a sub-analyses undertaken by gender. The parametric or non-parametric nature of the data was determined with a Shapiro-Wilk

test. Data are presented as mean  $\pm$  standard error of the mean (SEM) throughout. Parameters have been examined using t-tests or where time is also a variable, two-way analysis of variance (condition  $\times$  time) with Sidak's multiple comparisons if required. Where data were not normally distributed, these were analysed using a Mann-Whitney test. Area under the curve (AUC) has also been calculated to illustrate glucose net uptake in response to feeding. P value of  $<0.05$  was considered significant. Data analysis was carried out with Prism software V.7.0 (La Jolla, San Diego, US).

## **Results**

### Subject characteristics

20 CD subjects in remission ( $15.6y \pm 0.5$ , BMI  $20.6 \pm 0.9$ ) were recruited (Table 1). Mean number of years since diagnosis was  $4.3 \pm 0.6$ . Nine healthy control (Con) subjects ( $16.0y \pm 0.6$ , BMI  $20.7 \pm 0.6$ ) were recruited & well matched to the CD patients. All CD participants were in remission with an HBI of  $< 4$ , CRP of  $<5\text{mg/dl}$  and FCP of  $132 \pm 41\text{ug/g}$ . It was not possible to cannulate 2 of the female CD participants so for mechanistic studies CD  $n=18$ .

### Muscle physiology: protein, glucose & lipid metabolism

Arterialized plasma BCAA (A BCAA) peaked at  $t = 40$  in response to feeding (time  $p<0.0001$ ) (Fig 2A). Over the entire study period both Con and CD showed a neutral BCAA balance (Fig 2B). CDM had lower levels of BCAA than ConM at baseline & post feeding (condition  $p=0.027$ ), while CDF did not differ from ConF. CDM were in overall negative BCAA balance, in contrast to the positive balance of ConM ( $p=0.049$ ). CDM did mount an initial anabolic response to the meal, shown by increasing BCAA balance between  $t = 0$  &  $t = 20$  (Fig 2C), but this positive balance was not sustained with BCAA balance returning to negative by  $t = 60$ . ConM remained in positive balance throughout the postprandial period ( $p=0.049$ ).

Glucose a-v difference across the forearm increased in response to feeding in all groups and was not different between CD & Con (Fig 3A). Blood flow increased during the second hour of the study (time  $p<0.001$ ) (Fig 3B) in all groups except in CDF where it was significantly lower than ConF (interaction  $p=0.007$ ). There were no differences in glucose net uptake between any of the groups and therefore no differences in skeletal muscle insulin sensitivity (Fig 3C).

Arterialized plasma FFA concentrations were suppressed following feeding in all groups and reached a steady state, significantly lower than fasting levels by  $t = 60$ , time  $p<0.0001$  (Fig 4A). FFA concentrations were similar between CD and Con.

CDF had higher levels of FFA at baseline and at  $t = 20$  than ConF (interaction  $p=0.011$ , condition  $p=0.018$ ) while CDM were in line with ConM. FFA a-v difference did not differ between groups, it decreased with feeding but recovered thereafter and was close to neutral by the end of the 2-hour study (time  $p=0.01$ , Fig 4B). FFA net balance reduced post-feeding in all groups with a nadir at  $t = 20$ , before returning to baseline levels by  $t = 60$  and neutral by  $t = 120$  (Fig 4C). There were no differences between groups in FFA net balance.

#### Body composition & muscle function

Male CD (CDM) had a lower height for age when compared to male Con (ConM) (41<sup>st</sup> vs 81<sup>st</sup> centile respectively,  $p=0.014$ ). No differences between CD and Con were observed in LBM, appendicular LM, ASMI and BMD (Table 2). In CDM, appendicular LM was 24% lower than in ConM ( $p=0.034$ ), but no differences were observed for LBM, ASMI and BMC. All other body composition measures were comparable between groups. No difference in dominant arm fatigue was observed in CD vs Con but CDM fatigued significantly more than ConM ( $p=0.014$ ).

#### Whole body physiology: insulin sensitivity, energy expenditure & fuel oxidation

Arterialized plasma glucose & serum insulin levels increased in all groups post-feeding ( $t = 20$ ) and peaked at  $t = 40$ , time  $p<0.0001$  (Fig 1A & B). Glucose concentrations then dropped back, stabilizing above fasting levels by  $t = 60$ , whereas insulin continued to decline until  $t = 120$ . CD response to feeding in terms of these parameters and Matsuda index (Fig 1C) was no different to Con and therefore no whole body insulin resistance was detected. TEE, RER and COX increased post-feeding ( $t = 0$  vs  $t = 20-120$ ) in CD & Con (all  $p<0.05$ ) (Table 3). There were no differences between CD & Con.

#### Cytokine analyses

All CD participants were in remission with an HBI of  $< 4$ , CRP of  $<5\text{mg/dl}$  and FCP of  $132\pm 41\text{ug/g}$ . Similarly, no differences in TNF $\alpha$ , IL-1 and IL-6 were observed between CD and Con groups.

#### Testosterone levels

Testosterone levels were significantly higher in CD ( $15.8\pm 4.4\text{ ng/ml}$ ) when compared to Con ( $4.6\pm 1.3\text{ng/ml}$ ,  $p=0.031$ ). This difference was not observed in the gender sub-analyses.

### Activity & diet

Neither activity levels nor total energy intake differed between groups (Table 3). However, protein intake was lower in CDM ( $p=0.026$ ) with  $75 \pm 5\text{g/kg/day}$  reported in CDM and  $105 \pm 15\text{g/kg/day}$  reported in ConM.

### PROMS

IBDQ scores were on average 5.7 suggesting quality of life is moderately affected by CD. IBD-fatigue mean scores were: 6.2 for section 1, indicating moderate fatigue, and 16.7 for section 2, showing fatigue had a moderate effect on daily activities. Both were significantly greater for females than males (9.0 vs 3.0  $p=0.007$  & 30.4 vs 5.6  $p=0.018$ ). Table 2.

### **Discussion**

We hypothesised that the reduced muscle mass in CD may be driven through anabolic resistance<sup>22</sup>, that may persist even when in clinical remission.

We have shown that paediatric CD patients in remission may have a reduced skeletal muscle protein balance in response to feeding compared with controls, despite similar circulating amino acid response. This was most significant in CDM, who remarkably had an overall negative protein balance in response to 14g of protein, and was associated with lower appendicular LM, higher levels of muscle fatigue and reduced protein intake.

Forearm amino acid balance is a surrogate for muscle protein anabolism, and CD were able to mount a comparable initial positive amino acid response to feeding to that of Con. Assuming all of the amino acids taken up were incorporated into the muscle protein pool then this would suggest that CD are not completely anabolically resistant as hypothesised. However, muscle protein synthesis is highest at around 90 minutes post feeding<sup>32</sup> and the overall negative net balance suggests that there simply would not be enough amino acids available in the muscle to achieve a comparable protein synthetic response to control. Thus, it would appear that adolescent males with CD require more protein per meal compared to age matched controls in order to maintain a positive protein balance and muscle mass.

To our knowledge this is the first study to have investigated skeletal muscle protein balance in paediatric CD. In the most comparable study<sup>18</sup>, adolescents with mild disease did achieve a whole-body positive postprandial protein balance, but this was not compared to age-matched controls and therefore could not conclude if the response to feeding was adequate.

Findings of reduced muscle mass in paediatric CD in remission are congruent with the majority of the literature<sup>7,5,8,6,33</sup>: with no gender differences<sup>7</sup>; greater effects in boys<sup>6,33</sup>; and greater effects in girls<sup>5</sup>, having all been reported. It is perhaps not surprising that an earlier snapshot<sup>5</sup> (mean age 12.7yrs) showed greater effects in females, as males reach puberty later and put on significantly more muscle during puberty. Further, a whole body, rather than limb specific measure of LM, may not have shown up LM deficits due to the mesenteric hypertrophy associated with CD. In support, in the current study, CDM had significantly reduced appendicular LM ( $p=0.034$ ) but only a trend towards a reduction in LBM.

Reduced muscle function has been found in adult CD in remission<sup>9,11</sup> and in paediatric patients (mixed group: mild disease & remission)<sup>10</sup>. Further the most common symptom reported in adult remission is fatigue<sup>34</sup>. PROMS have been correlated with objectively measured levels of fatigue<sup>35</sup>, whereas we found CDF to report higher levels of fatigue than CDM ( $p=0.007$ ) despite finding similar levels in terms of physical tests. However, this may simply reflect the multi-faceted nature of fatigue<sup>36</sup> and reporting of higher levels of fatigue by females has been found in previous studies<sup>34</sup>.

To our knowledge lower protein intake in adolescent males with CD has not been previously reported<sup>37</sup>. We have previously reported a lower protein intake in adult CD with active disease when compared to age-, BMI- and gender matched HV with an associated disordered eating behaviour<sup>38</sup>. Similar studies in a paediatric cohort are unavailable. Rather, studies have found intake in CD to be comparable with controls<sup>15</sup> and in accordance with<sup>19</sup>, or in excess of protein intake recommendations<sup>20,21</sup>. However CDM still consumed more protein than recommended and their consumption was in line with National Diet and Nutrition Survey (NDNS) 2014-16<sup>39</sup> average values (CDM: 75g & NDNS males aged 11-18: 73g). So despite a significant difference in protein intake between ConM & CDM ( $p=0.026$ ), there appears to be no lack of protein in CDM diet. In the past, higher protein recommendations for populations at risk of age-related muscle loss have been criticized on the basis of the 'muscle full' hypothesis<sup>40,32</sup>, whereby simply increasing the amount of amino acids entering the muscle will not further stimulate protein synthesis. But it is now argued that the plateauing of protein synthesis in response to increasing amino acid availability ignores the fact that amino acids also suppress protein breakdown<sup>41</sup>, and as such a linear relationship between amino acid availability and protein balance holds true above the current recommended intake levels. As a result it has been proposed that protein intake for the elderly should be increased to 1.2-1.5g/kgbw/day in the case of those with chronic illness<sup>42</sup>. In support

Machado et al.<sup>19</sup> recently supplemented the diet of a group of adult CD patients, mostly in remission, who were already eating recommended levels of protein, with 25% extra protein and LM was increased after 16 weeks. The observation of a lower net protein balance in the face of adequate amino acid availability, and 1.3 g/kgbw/day dietary intake, would suggest an impaired anabolic response in CDM. Reduced levels of physical activity and sedentary behaviour are also associated with anabolic resistance<sup>22</sup> and so could be implicated in maintaining low muscle mass in remission. Interestingly, patients in remission have been shown to have lower levels of activity than healthy peers<sup>10</sup>. It is unclear whether this is a cause or consequence of low muscle mass in CD. We detected no reduction in physical activity in CD but there was a very high level of inter-subject variation in all groups. As exercise has been shown to be safe<sup>43</sup>, effective<sup>44</sup>, beneficial for quality of life<sup>43,45</sup>, and, importantly, overcome anabolic resistance, it is suggested that activity levels should be considered when giving lifestyle advice to patients.

Insulin inhibits protein breakdown and has a permissive effect in stimulating protein synthesis via Akt/mTOR<sup>46,47</sup>. Insulin resistance has been found in CD patients with active disease<sup>48</sup>, and TNF $\alpha$  has been shown to reduce insulin sensitivity in humans<sup>49</sup>. However, once disease activity is under control, in accordance with our study, adult patients in remission have been shown to be as insulin sensitive as controls<sup>50</sup>. Moreover, higher basal metabolic rate per FFM ratio and diet-induced thermogenesis has regularly been reported in CD, which could explain lower body mass. Indeed, a weak negative association has been observed between REE per kg FFM<sup>0.52</sup> and the Paediatric Crohn's Disease Activity Index<sup>51</sup>. However, there is little data in paediatric CD in remission, and the current study demonstrates that REE is similar between CD and Con.

The association of reduced protein balance with reduced skeletal muscle mass and function in CD suggests we may now have provided an explanation as to why patients in remission fail to gain muscle mass. CD may not be able to re-build muscle mass because they cannot maintain a positive protein balance in response to feeding. The difference in BCAA balance post-feeding between CDM and ConM of 0.85 $\mu$ mol/min could translate into kilograms of difference in muscle mass over time. The actual difference in appendicular LM between CDM and ConM was on average 6.6kg. Moreover Bryant et al.<sup>52</sup> reported that 20% of their young male IBD cohort (31y) had significantly reduced muscle mass (vs cohort average of 12%) raising the possibility that these LM deficits persist into adulthood. A longitudinal study would be needed to investigate this.

Furthermore, considering why we have seen this effect in males, and not females, may give indications as to why CDM in remission are in negative protein balance despite feeding. The possible role of dietary protein has already been discussed. Additionally there are clear differences between the genders in terms of their growth trajectories. Growth curves depicting the increase in muscle mass throughout adolescence<sup>53,54</sup> show not only the total accrual of muscle by males to be significantly greater than females but also that, at the average age of participants in this study, males are still laying down significant amounts of muscle, while the female rate of accrual is plateauing. It could be therefore that CDM are unable to keep up with such high pubertal demand. As testosterone is the key driver behind sexual dimorphism in muscle mass and a potent anabolic agent we measured plasma testosterone levels but intriguingly found testosterone to be raised in CD. This contrasts with previous studies that have reported both reduced androgens and delayed maturation in CD<sup>55,56,57</sup>. We measured bioavailable testosterone and although not apparent from the methods it is likely that previous studies measured total testosterone thus potentially explaining the contrasting findings. We could speculate that testosterone resistance may be at play, perhaps another facet of anabolic resistance, which is masked in the cases where total testosterone levels are measured. Alternatively, other hormones known to stimulate protein synthesis such as insulin growth factor-1 (IGF-1) could be involved. Indeed the growth hormone (GH)-IGF-1 hormonal axis has been previously noted to be impaired in CD<sup>5</sup>. Further, more general growth retardation e.g. low height for age is widely reported<sup>58,1,59,4,60</sup> and claimed to be more severe in males<sup>59,56,57</sup>. In accordance CDM in our study had significantly reduced height for age versus ConM. Moreover Gupta et al.<sup>56</sup> showed that IGF-1 levels were lower in males than females despite no difference in inflammatory markers or BMI, suggesting that this difference could explain why males tend to exhibit greater growth retardation than females.

The main limitation of this study has proved to be sample size. We are however confident in the conclusion that male CD have reduced protein balance, associated with lower appendicular LM, higher levels of muscle fatigue and reduced protein intake as an interim calculation of sample size showed the number of CDM (n=11) to be sufficient.

A key strength was our use of the a-v balance technique, which afforded the temporal resolution to demonstrate that CDM are not completely anabolically resistant. The discovery that CD can respond to anabolic stimuli has important implications for treatment.

In conclusion, we have shown that reduced muscle mass and function in CD patients is associated with a reduced protein balance in response to feeding, thus providing a possible explanation for the persistence of low muscle mass in remission. These effects appeared to be significant in male rather than female paediatric CD. This may be due to insufficient protein consumption and/or higher pubertal demand in males in this age group. Interventional studies are now needed to test dietary, exercise and pharmaceutical interventions designed to increase protein balance and restore muscle mass to healthy levels in patients in remission. Indeed, muscle contraction is known to overcome anabolic resistance. If this problem can indeed be solved, the positive effects on muscle function and fatigue, bone development and therefore quality of life in paediatric CD would be substantial.

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**Figure 1. Indicators of whole body insulin sensitivity in Crohn's disease vs. healthy controls: arterialized plasma glucose concentrations (A), serum insulin (B) & Matsuda index (C).** For total group (CD, Crohn's patients = purple) and split by gender (CDM, male CD = blue & CDF, female CD = green). Values are means  $\pm$  SEM. Significant differences are marked.

**Figure 2. Indicators of protein metabolism: arterialized plasma BCAA concentrations (A), mean BCAA net balance across the forearm standardized for forearm lean mass (B) & BCAA net balance across the forearm, over time, standardized for forearm lean mass (C).** For total group (CD, Crohn's patients = purple) and split by gender (CDM, male CD = blue & CDF, female CD = green). Values are means  $\pm$  SEM. Significant differences ( $p < 0.05$ ) & trends ( $p < 0.1$ ) are marked.

**Figure 3. Skeletal muscle insulin sensitivity: glucose arterio-venous difference across the forearm (A), brachial artery blood flow (B) & AUC glucose net uptake across the forearm standardized for forearm lean mass (C).** For total group (CD, Crohn's patients = purple) and split by gender (CDM, male CD = blue & CDF, female CD = green). Values are means  $\pm$  SEM. Significant differences ( $p < 0.05$ ) & trends ( $p < 0.1$ ) are marked.

**Figure 4. Skeletal muscle FFA metabolism: arterialized plasma FFA concentrations (A), FFA arterio-venous difference across the forearm (B) & FFA net balance across the forearm over time (C).** For total group (CD, Crohn's patients = purple) and split by gender (CDM, male CD = blue & CDF, female CD = green). Values are means  $\pm$  SEM. Significant differences ( $p < 0.05$ ) & trends ( $p < 0.1$ ) are marked.

**Table 1. Subject characteristics of healthy controls and Crohn's disease patients in remission.** Statistically significant differences between groups (control vs. CD, ConM vs. CDM or ConF vs. CDF) are marked \*= $p < 0.05$ .

**Table 2. Body composition, muscle function & patient-related outcome measures (PROMS).** Statistically significant differences between groups (control vs. CD, ConM vs. CDM, ConF vs. CDF or for PROMS CDM vs. CDF) are marked \*= $p < 0.05$  \*\* $p < 0.01$  \*\*\* $p < 0.001$ .

**Table 3. Energy expenditure and energy intake.** Statistically significant differences between groups (control vs. CD, ConM vs. CDM or ConF vs. CDF) are marked \* =  $p < 0.01$ .

	Control	CD	ConM	CDM	ConF	CDF
	n=9	n=20	n=5	n=11	n=4	n=9
Age (yrs)	16.0 ± 0.6	15.6 ± 0.5	16.1 ± 1.0	15.9 ± 0.7	15.9 ± 0.9	15.2 ± 0.7
Height (m)	1.71 ± 0.05	1.66 ± 0.02	1.79 ± 0.1	1.68 ± 0.03	1.60 ± 0.03	1.63 ± 0.03
Height for age (centile)	63.6 ± 11.0	50.7 ± 6.6	81.0 ± 8.6	40.7 ± 8.8*	41.8 ± 17.8	62.8 ± 8.7
Weight (kg)	60.7 ± 3.7	56.8 ± 2.7	65.9 ± 5.5	57.9 ± 3.7	54.3 ± 2.5	55.4 ± 4.1
BMI (kg/m <sup>2</sup> )	20.7 ± 0.6	20.6 ± 0.9	20.3 ± 0.9	20.3 ± 1	21.2 ± 1.0	20.8 ± 1.5
Years since diagnosis	n/a	4.3 ± 0.6	n/a	4.2 ± 0.8	n/a	4.3 ± 0.9
HBI	n/a	1.5 ± 0.4	n/a	0.8 ± 0.2	n/a	2.2 ± 0.9
FCP (µg/g)	n/a	132 ± 41	n/a	118 ± 64	n/a	146 ± 55
CRP (mg/dl)	<5 ± 0	<5 ± 0.4	<5 ± 0	<5 ± 0.6	<5 ± 0	<5 ± 0.6
IL-1β (pg/ml)	303 ± 92	1397 ± 540	396 ± 135	1420 ± 715	187 ± 110	1355 ± 878
IL-6 (pg/ml)	166 ± 52	236 ± 113	244 ± 78	316 ± 170	70 ± 13	92 ± 62
TNFα (pg/ml)	72 ± 36	484 ± 145	122 ± 63	519 ± 200	22 ± 22	420 ± 208
Testosterone (ng/ml)	4.6 ± 1.3	15.8 ± 4.4*	7.0 ± 1.6	15.0 ± 4.1	1.6 ± 0.2	17.1 ± 10.3

Table 1

	Control	CD	ConM	CDM	ConF	CDF
	n=9	n=20 <sup>w</sup> / n=18 <sup>v</sup>	n=5	n=11	n=4	n=9 <sup>w</sup> / n=7 <sup>v</sup>
Body composition <sup>w</sup>						
FM (kg)	12.3 ± 1.7	16.1 ± 2.0	9.7 ± 1.4	12.9 ± 2.3	15.5 ± 2.9	20.1 ± 3.1
BMC (kg)	2.5 ± 0.2	2.2 ± 0.1	2.8 ± 0.3	2.2 ± 0.1	2.1 ± 0.1	2.1 ± 0.1
BMD (g/cm <sup>2</sup> )	1.14 ± 0.02	1.07 ± 0.02	1.16 ±0.04	1.06 ± 0.03	1.12 ± 0.01	1.08 ± 0.03
LBM (kg)	46.0 ± 4.5	39.3 ± 1.5	53.4 ± 6.4	42.8 ± 1.9	36.6 ± 1.2	35.0 ± 1.5
LBM/TBM (%)	75 ± 3	70 ± 2	80 ± 4	75 ± 3	68 ± 4	65 ± 3
Appendicular LM (kg)	22.9 ± 2.6	18.7 ± 0.8	27.2 ± 3.6	20.6 ± 1.0*	17.5 ± 0.8	16.4 ± 0.8
ASMI (kg/m <sup>2</sup> )	7.6 ± 0.4	6.8 ± 0.2	8.2 ± 0.7	7.3 ± 0.2	6.8 ± 0.3	6.1 ± 0.2
Muscle function <sup>v</sup>						
Fatigue (%) dom	18 ± 3	26 ± 2	14 ± 2	25 ± 2*	24 ± 4	28 ± 5
Fatigue (%) non-dom	21 ± 4	23 ± 2	22 ± 6	26 ± 3	20 ± 5	17 ± 4
Strength dom (kg/kgforearmLM)	23.8 ± 1.3	25.6 ± 1.5	24.5 ± 1.6	25.4 ± 1.9	23.0 ± 2.2	25.8 ± 2.8
Strength non-dom (kg/kgforearmLM)	23.9 ± 1.1	24.3 ± 1.4	24.8 ± 1.6	24.4 ± 1.8	22.8 ± 1.4	24.2 ± 2.5
Age dependent SDS dom	-1.41 ± 0.35	-1.50 ± 0.22	-0.92 ± 0.33	-1.60 ± 0.27	-2.03 ± 0.55	-1.36 ± 0.40
Age dependent SDS non-dom	-1.62 ± 0.31	-1.90 ± 0.21	-1.04 ± 0.33	-1.88 ± 0.26 <sup>§</sup>	-2.34 ± 0.27	-1.94 ± 0.37
Height dependent SDS dom	-1.13 ± 0.35	-0.93 ± 0.34	-0.98 ± 0.47	-0.73 ± 0.41	-1.31 ± 0.59	-1.23 ± 0.62
Height dependent SDS non-dom	-1.36 ± 0.33	-1.37 ± 0.36	-1.11 ± 0.46	-1.04 ± 0.45	-1.68 ± 0.51	-1.90 ± 0.60
PROMS <sup>w</sup>						
IBDQ (mean)	-	5.7 ± 0.3	-	6.2 ± 0.2	-	5.2 ± 0.5 <sup>§</sup>
IBD - Fatigue	-		-		-	
Section 1	-	6.2 ± 1.0	-	3.8 ± 0.8	-	9.0 ± 1.6**
Section 2	-	16.7 ± 5.4	-	5.6 ± 2.4		30.4 ± 10.2*

Table 2

	Control	CD	ConM	CDM	ConF	CDF
	n=9	n=20 <sup>w</sup> / n=18 <sup>v</sup>	n=5	n=11	n=4	n=9 <sup>w</sup> / n=7 <sup>v</sup>
Energy expenditure						
Indirect calorimetry <sup>v</sup>						
TEE - fasted (kJ/hr/kgLBM)	6.1 ± 0.3	5.9 ± 0.4	5.7 ± 0.4	6.3 ± 0.3	6.6 ± 0.4	5.2 ± 0.9
TEE - post-feeding (kJ/hr/kgLBM)	6.5 ± 0.3 <sup>T</sup>	6.0 ± 0.4 <sup>T</sup>	6.1 ± 0.5	6.3 ± 0.2	7.0 ± 0.3 <sup>T</sup>	5.7 ± 1.0 <sup>T</sup>
RER - fasted	0.81 ± 0.02	0.76 ± 0.05	0.81 ± 0.03	0.78 ± 0.01	0.81 ± 0.05	0.73 ± 0.13
RER - post-feeding	0.87 ± 0.02 <sup>T</sup>	0.80 ± 0.05 <sup>T</sup>	0.85 ± 0.03 <sup>T</sup>	0.86 ± 0.02 <sup>T</sup>	0.88 ± 0.02	0.70 ± 0.12
COX - fasted (mg/min/kgLBM)	2.4 ± 0.6	2.2 ± 0.4	2.2 ± 0.5	1.9 ± 0.3	2.7 ± 1.2	2.6 ± 0.9
COX - post-feeding (mg/min/kgLBM)	3.9 ± 0.4 <sup>T</sup>	3.1 ± 0.4 <sup>T</sup>	3.3 ± 0.5 <sup>TT</sup>	3.7 ± 0.5 <sup>TT</sup>	4.7 ± 0.5	2.2 ± 0.6
FOX - fasted (mg/min/kgLBM)	1.6 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	1.9 ± 0.2	1.7 ± 0.4	1.2 ± 0.4
FOX - post-feeding (mg/min/kgLBM)	1.2 ± 0.2 <sup>ST</sup>	1.3 ± 0.2 <sup>ST</sup>	1.3 ± 0.3 <sup>T</sup>	1.2 ± 0.2 <sup>T</sup>	1.1 ± 0.2	1.6 ± 0.4
Activity <sup>w</sup>						
Pedometer (no.steps/day)	8056 ± 849	7831 ± 725	8286 ± 1488	6979 ± 870	7767 ± 766	9002 ± 1176
IPAQ (total MET- mins/wk)	6196 ± 2158	4443 ± 994	5654 ± 2984	4022 ± 1557	7099 ± 3622	4910 ± 1270
Energy intake						
Food diaries <sup>v</sup>						
Energy intake (kJ/day)	9124 ± 1081	8333 ± 512	10547 ± 1669	8381 ± 624	7345 ± 687	8258 ± 944
Carbohydrate intake (kJ/day)	4299 ± 595	4099 ± 355	4901 ± 935	4162 ± 544	3547 ± 570	4001 ± 372
Fat intake (kJ/day)	3294 ± 490	2884 ± 182	3863 ± 803	2946 ± 161	2584 ± 255	2787 ± 415
Protein intake (kJ/day)	1531 ± 173	1350 ± 93	1784 ± 255	1273 ± 81*	1214 ± 108	1469 ± 204
Protein intake (g/kgbodyweight/day)	90 ± 10	79 ± 5	105 ± 15	75 ± 5*	71 ± 6	86 ± 12
Recommended protein intake (g/day)	46 ± 3	43 ± 2	49 ± 4	43 ± 3	41 ± 2	44 ± 3

Table 3