Title: SKELETAL MUSCLE ANABOLIC AND INSULIN SENSITIVITY RESPONSES TO A MIXED MEAL IN ADULT PATIENTS WITH ACTIVE CROHN'S DISEASE

Short title: Muscle physiology in adult Crohn's Disease

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Abbreviations: ASMI: appendicular skeletal muscle index; a-v: arterio-venous**;** BCAA: branched chain amino acids; BMI: body mass index; CD: Crohn's disease; Con: controls; COX: carbohydrate oxidation; CRP: C-reactive protein; dom: dominant arm; EGTA: Ethyleneglycol-Bis-β-Aminoethylether Tetraacetate**;** FCP: faecal calprotectin; FFA: free fatty acids; FFM: fat free mass; FM: fat mass; FOX: fat oxidation; HBI: Harvey Bradshaw Index; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance; IPAQ: international physical activity questionnaire; LBM: lean body mass; LM: lean mass; non-dom: non-dominant arm; REE: resting energy expenditure; RER: respiratory exchange ratio (volume CO_2 expired / volume of O_2 inspired); THL: tetrahydrolipostatin; TNFα: Tumor necrosis factor alpha; TBM: total body mass.

ABSTRACT

Background and Aims: We have previously shown reduced protein balance in response to nutrition in paediatric Crohn's disease (CD) in remission, associated with reduced lean mass (sarcopenia) and reduced protein intake in males. We aim to compare skeletal muscle metabolic response to feeding in adult active CD and healthy volunteers.

Methods: Eight CD participants with active disease (41.3 ± 4.5 yrs; BMI 26.9 ± 1.5 kg/m^2) and eight matched healthy volunteers (Con) (41.2 ± 4.3 yrs; BMI 25.1 ± 1.1 kg/m^2) were recruited. Participants had a dual energy X-ray absorptiometry scan, handgrip dynamometer test, wore a pedometer and completed a food diary. Arterialized hand and venous forearm blood samples were collected concurrently and brachial artery blood flow measured at baseline and every 20mins for 2hrs after the ingestion of a standardized mixed liquid meal. Net balance of branched chain amino acids (BCAA), glucose and free fatty acids across the forearm were derived.

Results: No differences in muscle BCAA, glucose or FFA net balance were found between CD and Con. Neither were differences in muscle mass and function, physical activity or diet found. CD did not differ from Con in whole body insulin and lipid responses, or in energy expenditure and fuel oxidation.

Conclusions: Skeletal muscle mass, function, dietary protein intake and response to a test meal in an adult CD cohort with active disease is similar to that seen in healthy volunteers. Combining these results with our previous findings in paediatric patients suggests that age of onset and / or disease burden over time, as well as daily protein intake, may be significant in the

development of sarcopenia in CD. Longitudinal studies investigating these factors are required.

Keywords: Crohn's disease; Inflammatory Bowel Disease; Nutrition; Sarcopenia.

INTRODUCTION

The prevalence of reduced muscle mass (sarcopenia) in adult patients with Crohn's Disease (CD) has been estimated to be between 21% [1] and 61% [2]. Reduced muscle mass has been linked with reduced muscle function [3] [4], osteopenia [1], fatigue and lower quality of life [5]. However, the aetiology of reduced muscle mass in CD remains unclear.

Skeletal muscle mass is determined by the balance between muscle protein synthesis and breakdown. Reduced thigh muscle cross-sectional area in adult CD has been associated with reduced activation (phosphorylation) of key anabolic and insulin signalling proteins, such as protein kinase B (Akt) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP-1), in *vastus lateralis* muscle biopsies, suggesting a diminution of muscle protein synthesis [6]. Muscle protein breakdown signalling targets E3 ubiquitin ligases (MuRF-1 & atrogin-1) were also investigated but were found to be no different from healthy control tissue. The net effect of these signaling changes would likely be a reduction in net leg protein balance.

To our knowledge, only one study in adult CD has measured whole body protein balance in the fed state [7]. Findings in that study are in accordance with those in paediatric CD patients [8] [9] showing increased protein turnover in active disease, which was positively correlated with disease severity but whole body protein net balance did not improve with a reduction in disease activity. Further, changes in protein turnover in these studies could be related to changes in other tissues, such as the gut, as measurements were only performed at the whole body level. Indeed we have previously shown that paediatric CD patients in clinical remission have a reduced protein balance

versus healthy controls in response to a mixed meal, as measured across the forearm muscles [10]. This was associated with reduced fat free mass (FFM) z-scores, suggesting reduced muscle mass in paediatric CD in remission could be due to a reduced muscle protein synthetic response to dietary protein termed ‘anabolic resistance’ [11]. Indeed, male CD participants in particular had an overall negative protein balance, lower appendicular lean mass, higher levels of muscle fatigue and lower protein intake than controls. However, whether anabolic resistance is also implicated in reduced muscle mass in adult CD patients is not known. Insulin cell-signaling pathways are also linked to protein synthetic and proteolytic cascades and as such, measurement of insulin sensitivity may provide further insight into the mechanisms involved in the development of anabolic resistance in CD.

We aimed to measure skeletal muscle protein balance (as an index of anabolic resistance) and net glucose uptake (as an index of insulin resistance) across the forearm muscles in response to a physiological mixed-meal stimulus in a cohort of adult CD patients in active disease and in age-, sex- and BMI-matched controls. It was hypothesized that anabolic resistance will be associated with reduced lean mass in adult CD and that these patients will also be insulin resistant due to active disease.

MATERIALS AND METHODS

Subjects

We recruited male and female CD outpatients with active disease (aged 18-75) from Nottingham University Hospitals Trust. Disease activity was defined through objective markers of inflammation: faecal calprotectin (FCP) of >250 µg/g or C-reactive protein (CRP) of >5 g/dL or recent ileocolonoscopy, computed tomography [CT] or magnetic resonance [MR] enterography showing active disease. CD symptoms, measured with a Harvey-Bradshaw Index [HBI] score, were recorded at inclusion. Any CD-related medication apart from corticosteroids within 3 months prior to recruitment were permitted. Age-, sex- and BMI-matched healthy volunteers were recruited through advertisements on the Nottingham University and Nottingham University Hospitals Trust campuses. All potential participants were given comprehensive written and verbal explanations of the study before giving written informed consent and were free to withdraw at any time. Participants completed a general health questionnaire and underwent a short medical screening prior to participation. The study was approved by the Health Research Authority (15/WM/0285) on the 26th July 2016 with the study conforming to the recognised standards of the Declaration of Helsinki.

Outcome Measures

The primary outcome of this study was to compare forearm skeletal muscle branched chain amino acids (BCAA) net balance under fasted and fed conditions between CD and HV participants. Secondary outcomes were; forearm skeletal muscle glucose net balance (as an index of skeletal muscle

insulin sensitivity) and whole body insulin sensitivity; forearm skeletal muscle free fatty acids (FFA) net balance; serum triacylglycerol (TAG); forearm muscle isometric strength and fatigability; appendicular lean mass (LM) and appendicular skeletal muscle index (ASMI); resting fasted and postprandial metabolic rate, daily physical activity; and daily energy intake and dietary macronutrient composition.

Differences between arterialized-venous and deep-venous concentrations (a-v difference) of BCAA and glucose, multiplied by brachial artery blood flow and corrected for forearm lean mass, were used to determine the net balance of these nutrients across the forearm under fasted (postabsorptive) and fed (postprandial) conditions. Positive values indicated net tissue uptake, whereas negative values indicated net release of nutrients. The following equation was used for a given nutrient (N):

Net balance of N (μmol/min/100g of forearm lean mass) = {Blood flow (ml/min) x ([N]arterialised – [N]venous (mmol/L or μmol/L)}/1000 (not for glucose)/ {lean mass (g) x 100}.

Lean mass (LM) 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 [16,17], with a lower index indicating a lower level of insulin sensitivity:

Matsuda Index = $10000 / (\sqrt{} G_0 * I_0 * G_{mean} * I_{mean})$

Where G_0 = fasting plasma glucose (mg/dl), I_0 = fasting serum insulin mIU/L, G_{mean} = mean plasma glucose concentration during MTT (mg/dl) 0-120mins and I_{mean} = serum insulin concentration during MTT (mIU/L) 0-120mins.

Muscle strength measurements were standardized for muscle size. Appendicular LM (sum of lean mass in the limbs measured by DEXA) and ASMI (appendicular lean mass (kg) / height $(m)^2$) were calculated to provide more precise indices of skeletal muscle mass than total lean mass alone [18].

Indirect calorimetry was used to measure fasted and postprandial oxygen consumption and carbon dioxide expiration in order to calculate: resting energy expenditure (REE); substrate (carbohydrate and fat) oxidation and; respiratory exchange ratio (RER). Peronnet and Massicote's 1991 equations [19] were used to calculate substrate oxidation:

Rate of fat oxidation (FOX) (g/min) = $1.695\ VO_2 - 1.701\ VCO_2$

Rate of carbohydrate oxidation (COX) (g/min) = $4.585\ VCO_2 - 3.226\ VO_2$

Where volume of carbon dioxide (VCO_2) and volume of oxygen (VO_2) inputs are in litres per minute (l/min).

Experimental protocol

Participants reported to the laboratory at 0800, following an overnight fast, having abstained from strenuous exercise and alcohol for the previous 48 hours. On arrival, their body composition was assessed by DEXA. Subsequently, participants were asked to rest in a semi-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 [20]. A second cannula was placed in a deep antecubital vein in the non-dominant forearm [21]. After collections of baseline blood samples from both cannulas, and measurements of brachial artery blood flow (in the non-

dominant arm) using Doppler ultrasound (Toshiba Aplio 300), and resting energy expenditure by indirect calorimetry (Cosmed, Italy) for 20 min, all participants ingested a 220ml bottle of Ensure plus nutrition shake (Abbott Nutrition, Maidenhead, UK) (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). This meal composition mirrors dietary recommendations and specifically the protein content equates to 24% of the daily protein requirements of the CD subjects (0.75g/kg bw).

Arterialized-venous (2 ml) and venous (2 ml) blood samples were obtained concurrently from the heated hand vein and deep 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 BCAA, glucose and FFA could be calculated in the fasted and fed states. At t = 100 min a final indirect calorimetry measurement was performed for 20 min to allow calculations of REE and RER to compare with fasting levels.

Following the 2h postprandial period, an assessment of forearm muscle function was undertaken. Participants were familiarised with the protocol during screening and the same trained operator both gave instructions and took measurements from all participants [22]. Participants performed 12 maximal static (isometric) 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), usually achieved within the first 2-3 contractions. Level of fatigue was derived from the difference in maximal strength and strength measured at the end of 12 maximal contractions (mean of the last 3) [23].

Blood metabolite analysis

Blood glucose levels were measured using Yellow Springs Instrument Analyzer, YSI, 2300 STAT PLUS. Plasma separated from Ethyleneglycol-Bis-(β-Aminoethylether) Tetraacetate (EGTA) treated blood was analysed for BCAA (sum of leucine, isoleucine and valine) concentrations by spectrophometric assay [24]. Plasma samples, further treated with tetrahydrolipostatin (THL), were analysed for FFA by colorimetric kit (NEFA C, Wako). Serum separated from arterialised-venous blood was analysed for insulin concentration by radioimmunoassay (Milipore, Human insulin specific kit) and TAG concentrations by colormetric enzymatic assay (ABX Pentra Triglycerides CP, Horiba, Kyoto, Japan). CRP was measured by ELISA at the Department of Clinical Chemistry, Queen's Medical Centre, Nottingham University Hospitals, as part of the initial screening process or in baseline blood samples.

Assessment of habitual physical activity and 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) [25] were used to assess habitual physical activity levels. Habitual energy intake was measured using a 3-day paper-based food diary completed by participants in the days preceding their study visit (across 1 weekend day and 2 week-days).

Statistical analyses

The primary analysis was a between-group analysis with further post-hoc analyses undertaken if the primary comparison was significant. If data were normally distributed, as assessed by a D'Agostino-Pearson omnibus normality

test, potential differences between CD and Con were examined using unpaired t-tests or where time was also a variable, two-way analysis of variance (experimental group x sampling time). Where data were not normally distributed potential differences between CD and Con were tested using the Mann-Whitney test. This was the case for the CRP responses only. Total area under the curve (tAUC) was used for glucose and BCAA responses, in order to estimate the overall postprandial net balance, thus indicating net uptake or efflux of these metabolites across the entire postprandial period. P value of <0.05 was considered significant. Data are presented as mean ± standard error of the mean (SEM) and analysis was undertaken with Prism software V.7.0 (La Jolla, San Diego, US).

RESULTS

Subject characteristics

Eight CD participants (41.3 ± 4.5 yrs; BMI 26.9 ± 1.5 kg/m^2; female n=4) were recruited (Table 1). Mean number of years since diagnosis was 14.5 ± 4.6 and mean age at diagnosis was 27 ± 4 yrs. Only one participant was diagnosed prior to the age of 18. See supplementary table for individual CD participant details including pharmacological therapy and disease history. All endoscopic or imaging investigations were undertaken as part of the patients' standard care.

Eight healthy age-, BMI- and sex- matched control (Con) participants (41.2 ± 4.3 years; BMI 25.1 ± 1.1 kg/m^2; female n=4) were recruited (Table 1).

Table 1 Subject characteristics of Con and CD patients

	Con	CD	p
	n=8	n=8	
Age (yrs)	41.2 ± 4.3	41.3 ± 4.5	n.s.
Height (m)	1.8 ± 0.03	1.7 ± 0.03	n.s
Weight (kg)	78.3 ± 3.3	78.5 ± 6.0	n.s
BMI (kg/m^2)	25.1 ± 1.1	26.9 ± 1.5	n.s
Diagnosis (yrs)	n/a	14.5 ± 4.6	n/a
HBI	n/a	4 ± 2	n/a
FCP (µg/g)	n/a	749 ± 275 [b]	n/a
CRP (mg/dl)	2.5 (0-8.8)	2.5 (0-18.5)	n.s

All values are means ± SEM except for CRP where values are median and interquartile range. There were no significant differences between groups. CD=Crohn's disease, Con=control, HBI=Harvey Bradshaw Index, FCP=faecal calprotectin, CRP=C-reactive protein, [b]= reduced n number (CD n=5).

Skeletal muscle responses

Skeletal muscle anabolic sensitivity

Arterialized plasma BCAA concentrations did not differ between Con and CD (Figure 1A). As expected, feeding increased BCAA net balance across the forearm compared to postabsorptive state (0.006 vs. -0.04 μmol/min/100gforearm lean body mass, respectively; p=0.023). However, neither BCAA net balance over time (Figure 1B) or when expressed as tAUC (Figure 1C) were different between Con and CD.

Skeletal muscle insulin sensitivity

No differences in glucose a-v difference between CD vs. Con (Figure 2A) were observed (interaction effect p=0.068). Blood flow increased over time (p=0.007) but there were no significant differences between groups (Figure 2B). Net glucose uptake, whether over time or expressed as tAUC, was not different between CD and Con (Figures 2C & D).

Skeletal muscle lipid metabolism

Circulating FFA concentrations, a-v difference and net balance were similar between CD and Con (Figures 3A-C).

Body composition and muscle function

There were no significant differences in body composition between groups (Table 2). Neither were there differences between Con and CD in muscle function. On average subjects fatigued to a similar level in both arms (c.17%) and also had similar levels of strength.

Table 2 Body composition and muscle function

	Con	CD	P
	n=8	n=8	
Body composition			
FM (kg)	24.5 ± 3.3	28.6 ± 3.5 [c]	n.s.
BMC (kg)	3.2 ± 0.2	2.9 ± 0.2	n.s.
BMD (g/cm^3)	1.3 ± 0.05	1.2 ± 0.04	n.s.
LBM (kg)	50.6 ± 4.0	49.5 ± 4.1	n.s.
LBM/TM (%)	65 ± 4	61 ± 3	n.s.
Appendicular LM (kg)	24.5 ± 2.3	23.7 ± 2.4	n.s.
ASMI (kg/m^2)	7.7 ± 0.5	8.0 ± 0.7	n.s.
Muscle function			
Fatigue (%) D	17 ± 4	15 ± 3	n.s.
Fatigue (%) ND	22 ± 5	13 ± 2	n.s.
Strength D (kg/kgforearmLM)	29.2 ± 2.5	24.0 ± 2.4 [c]	n.s.
Strength ND (kg/kgforearmLM)	27.5 ± 2.3	24.6 ± 2.1 [c]	n.s.

All values are means ± SEM. There were no significant differences between groups. CD=Crohn's disease, Con=Control, FM=fat mass, BMC=bone mineral content, BMD=bone mineral density, LBM=lean body mass, TM = total body mass, LM=lean mass, ASMI= appendicular skeletal muscle index, D=dominant arm, ND=non-dominant arm, [c] = n=7.

Whole body responses

Insulin sensitivity

No difference was found between arterialized blood glucose levels in Con and CD (Figure 4A). Similarly, no differences were detected between groups in serum insulin response (Figure 4B) or in Matsuda Index (Figure 4C).

Lipids

There were no differences between groups in serum TAG, which remained relatively constant t=0-100 min at c. 1mmol/L (Figure 4D).

Energy expenditure

There were no differences between groups in REE, RER, COX and FOX.

Table 3 Energy expenditure

	Con	CD
	n=8	n=7
REE – fasted (kJ/hr/kgLBM)	5.8 ± 0.2	5.9 ± 0.3
REE – fed (kJ/hr/kgLBM)	6.4 ± 0.3***	6.5 ± 0.4***
RER – fasted	0.77 ± 0.01	0.81 ± 0.03
RER – fed	0.80 ± 0.02	0.80 ± 0.02
COX – fasted (mg/min/kgLBM)	1.5 ± 0.3	1.9 ± 0.5
COX – fed (mg/min/kgLBM)	2.4 ± 0.4	2.3 ± 0.4
FOX – fasted (mg/min/kgLBM)	1.9 ± 0.1	1.7 ± 0.3
FOX – fed (mg/min/kgLBM)	1.8 ± 0.2	1.8 ± 0.2

All values are means ± SEM. There were no significant differences between groups (n.s.) but significant differences between fed and fasted states are marked ***=p<0.001. CD=Crohn's disease, Con=Control, REE=resting energy expenditure, RER=respiratory exchange ratio, COX=carbohydrate oxidation, FOX=fat oxidation.

Physical activity and diet

Activity levels did not significantly differ between Con and CD whether assessed by pedometer or IPAQ (Table 4). There were no differences in energy intake between groups or in any of the 3 main nutrients. Protein intake exceeded current dietary guidelines of 0.75g / kg bw / day, by 24% in Con and by 41% in CD (Table 4).

Table 4: Physical activity and energy intake

	Con	CD	p
	n=8	n=7	
Physical activity			
Pedometer (000's steps/day)	10.0 ± 1.6	7.5 ± 0.9	n.s.
IPAQ (total 000's MET-mins/wk)	7.0 ± 3.2	7.8 ± 3.5	n.s.
Energy intake			
Energy intake (MJ/day)	7.5 ± 1.2	7.3 ± 0.7	n.s.
Carbohydrate intake (MJ/day)	3.6 ± 0.6	3.3 ± 0.4	n.s.
Fat intake (MJ/day)	2.6 ± 0.5	2.6 ± 0.3	n.s.
Protein intake (MJ/day)	1.2 ± 0.2	1.4 ± 0.1	n.s.
Protein intake (g/day)	73 ± 9	83 ± 9	n.s.
Recommended protein intake (g/day)	59 ± 3	59 ± 4	n.s.

All values are means ± SEM. n.s. = no significant differences between groups and a trend ($p<0.1$) is marked. CD=Crohn's disease, Con=Control, IPAQ=international physical activity questionnaire. N numbers are as specified in column headers.

DISCUSSION

The primary aim of the current study was to ascertain if muscle anabolic resistance to an oral mixed meal was associated with reduced skeletal muscle mass in adults with active CD. Secondly, to examine whether anabolic resistance was associated with insulin resistance. The major findings from this study indicated no differences in anabolic response or muscle insulin sensitivity between CD and Con. Furthermore, there were no differences between groups in body composition (including indices of muscle mass) or muscle function. This is the first study to investigate the anabolic response to a meal in adult CD and compare it to that of a healthy population.

The variability in reported prevalence of low muscle mass (sarcopenia) in the adult CD population is high, ranging from 21% [1] to c.60% [2 26]. This is likely a result of the heterogeneity of the study populations (disease duration and severity and pharmacological treatment) and the inconsistent definition of reduced muscle mass or sarcopenia used.

Although originally restricted to the elderly, sarcopenia is now used more generally in the clinical literature to indicate reduced muscle mass and function [27]. In terms of its measurement, there are a plethora of ways of defining it. In some cases it may be reduced muscle mass of one or two standard deviations (SD) below a population mean [2,18,26]. Whereas others have incorporated measures of function, including handgrip strength [4,28]. Measurement of reduced muscle mass itself may also be made by a range of techniques and assessed at a whole body or more specific level e.g.

appendicular LM or ASMI. Due to these inconsistent definitions, a large range of prevalence in the CD population would be expected.

Pharmacological history may also impact on the prevalence of reduced muscle mass and seven of eight CD patients in the current study were being treated or had been exposed to anti-TNFα therapy. Infliximab has been shown to improve volume of the quadriceps femoris muscle in adults after 3 infusions (16 weeks), although there was no further improvement after 4 infusions (25 weeks) [29]. Based on a definition of reduced appendicular lean mass of >1 SD below the sex-specific control mean, 38% of CD subjects (2/4 female and 1/4 male) in the current study did have reduced muscle mass. All of these individuals had a negative mean BCAA balance: -0.053, -0.011 and -0.011 μmol/min/100g forearm LM (vs. CD average of 0.014 μmol/min/100g forearm LM). However, the low prevalence of reduced muscle mass in CD in the current study, in conjunction with the large variation in muscle mass and small sample size meant that no overall difference was found between Con and CD.

Similarly, no differences in muscle strength or fatigue in the upper limbs were found between CD and Con in the present study. Reduced muscle strength in CD was observed previously in CD patients with reduced LM [4] and without [5]. Similar to our findings, the reduced strength found by Wiroth et al. [5] was only in the lower limbs and not in the upper limbs. The high prevalence of anti-TNFα medication in the current study may also have contributed to these findings. In the study investigating the effect of infliximab on muscle mass and function, muscle strength was also increased after 16 weeks with some

further gains at 25 weeks [29]. But it is not known whether these improvements normalised these indices as no comparison with controls or reference data was made. The median anti-TNFα exposure in our CD cohort was 8 weeks.

Postprandial insulin resistance was not detected in CD in this study either at the whole-body level (Matsuda Index) or in terms of skeletal muscle (glucose uptake across the forearm). Skeletal muscle insulin sensitivity in adults with CD has never been investigated. Higher fasting concentrations of insulin have previously been found in active CD contributing to reduced postabsorptive whole body insulin sensitivity as measured by the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) [12] [30]. However, although significantly different from Con, HOMA-IR was still within the normal range in CD in both of these studies (i.e. <2.5 [31]). Furthermore HOMA-IR is an index of fasting insulin sensitivity and largely reflects the insulin sensitivity of the liver rather than peripheral tissues.

Tumor necrosis factor alpha (TNFα), which is often elevated in active disease, has been shown to reduce skeletal muscle insulin sensitivity by inhibiting phosphorylation of Akt [13]. Although previous studies have shown no effect of anti-TNFα therapies on insulin sensitivity [32] [33], a more recent study demonstrated a reduction in fasting insulin concentrations and improvement in HOMA-IR after 6 months [34]. Hyperinsulinaemia and reduced insulin sensitivity have also been found in response to an oral glucose tolerance test in CD [12]. However, none of the subjects in that study were being treated with anti-TNFα medication, whereas the majority in the present study (7/8 patients) had been exposed to such biological therapies. Moreover, the level of insulin

sensitivity is likely to be not just related to disease activity and pharmacological treatment but to a multitude of other factors including physical fitness and diet. Thus, not only the different ways of measuring of insulin sensitivity and high prevalence of anti-TNFα medication but similar diet and activity levels in CD subjects in the present study may explain why this cohort was not found to be insulin resistant when compared to control.

We have recently shown a link between an attenuated anabolic response to a mixed meal and reduced muscle mass in paediatric CD patients in remission [14]. It might therefore have been expected to find such an association in adults who were in active disease. Indeed, all the adults in the current study had active symptoms and objective markers of inflammation. Whereas the paediatric cohort previously studied were in remission.

There are many possible reasons why no difference in anabolic response was found between Con and CD in the current study. We hypothesized that a reduced anabolic response would be associated with reduced muscle mass and hence it is understandable that the relative parity of muscle mass in the current study groups precluded a diverse anabolic response to the meal. The difference in findings between this study in adults with active CD, and our previous findings in paediatric CD in remission [14] where male CD had reduced muscle mass versus control, may imply a negative BCAA balance is linked to an attenuated muscle mass in a paediatric group where muscle mass is on an upward trajectory and possibly linked to decreased protein intake. Indeed, the only subject in the current study diagnosed as a teenager did have reduced

appendicular lean mass versus control (> 1SD below the control mean) and negative forearm balance in response to the mixed meal.

It is also possible that the nutritional stimulus provided was not sufficient to elicit a response based on the observation that tAUC BCAA net balance was effectively neutral in both groups. Interestingly, we observed increased (p=0.023) BCAA net balance across the forearm in response to feeding when compared to postabsorptive state for all participants suggesting 14g was sufficient stimulus to elicit an anabolic response to the meal. In support of this notion, the 14g protein provided is equivalent to 24% of CD daily requirement based on 0.75g / kgbw (59g) and 17% of actual CD intake (83g). This compares well with the 12g protein eaten at breakfast by adults aged 19-64 (in a sample taken from NDNS, 2008–2014 n=3619) and equivalent to 17% of daily intake (72g +/- 23) [35]. Moreover, using a large amount of protein as an experimental stimulus has the potential to mask any potential differences [36] [37] [38]. It is therefore considered important that a relatively small dose of protein, which is comparable to real life, is used in order to detect any anabolic resistance.

Other key limitations of this study were the small sample size and the relatively heterogeneous nature of the CD cohort recruited. Inadequate power and a diverse disease burden might have impacted on the primary outcome. Nevertheless the aim of this initial study was more hypothesis-generating and to help design future definitive studies. Also potentially limiting was the lack of difference in muscle mass between the study groups, which may in turn have

been the result of the relatively high habitual levels of dietary protein intake in the CD group.

Conclusions

No difference in anabolic response, insulin sensitivity, body composition or muscle function was found between CD with active disease and Con in the present study. Yet the absence of differences does not preclude a link between these factors and further investigation is required. Linking these findings in adult CD patients with active disease, but normal muscle mass and dietary protein intake, to the negative forearm protein balance in a male paediatric group with attenuated muscle mass and decreased protein intake might imply that the age of onset and/or habitual levels of protein intake may have a major role in the aetiology of sarcopenia in CD rather than merely the presence of active disease. Collectively, these findings indicate that longitudinal studies investigating the effect of disease activity and burden, age of onset, and nutritional intake are warranted to try and deconstruct the link between muscle mass, fatigue and dietary protein intake.

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Statement of Authorship: Dr Gordon W. Moran is the guarantor of the article. Gordon W. Moran and Francis B. Stephens designed the research. Amanda Davies and Aline Nixon conducted the study and acquired the data.

Kostas Tsintzas, Francis B. Stephens and Gordon W. Moran provided study supervision. Amanda Davies, Kostas Tsintzas, Francis B. Stephens and Gordon W Moran analyzed and interpreted the data. Amanda Davies and Gordon W. Moran drafted the article. All authors critically revised the article for important intellectual content and **approved the final version of the article, including the authorship list.**

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Figure legends

Figure 1 Indicators of protein metabolism: arterialized plasma BCAA concentrations (A), BCAA net balance across the forearm standardized for forearm lean mass over time (B) and tAUC (C).

Values are means ± SEM. CD=Crohn's disease, Con=control, tAUC=total area under the curve. Results of 2-way or 1-way ANOVA are shown: v = visit effect, t = time effect and v x t = interaction effect.

Figure 2 Skeletal muscle insulin sensitivity: glucose arterio-venous difference across the forearm (A), brachial artery blood flow (B), glucose net uptake across the forearm standardized for forearm lean mass over time (C) and tAUC (D).

Values are means ± SEM. CD=Crohn's disease, Con=control, BCAA=branched chain amino acids, tAUC=total area under the curve. Results of 2-way or 1-way ANOVA are shown: v = visit effect, t = time effect and v x t = interaction effect.

Figure 3 Skeletal muscle lipid metabolism: arterialized plasma FFA concentrations (A), FFA arterio-venous difference across the forearm (B) and FFA net balance across the forearm (C).

Values are means ± SEM. CD=Crohn's disease, Con=control. Results of 2-way or 1-way ANOVA are: v= visit, t= time & v x t= interaction effect.

Figure 4 Indicators of whole-body insulin sensitivity and lipid response: arterialized blood glucose concentrations (A), serum insulin (B), Matsuda index (C) and arterialized serum TAG concentrations (D).

Values are means ± SEM. CD=Crohn's disease, Con=control. Results of 2-way or 1-way ANOVA are shown: v = visit effect, t = time effect and v x t = interaction effect. Matsuda index is an index of whole-body postprandial insulin sensitivity where the lower the index the higher the level of insulin resistance.

Supplementary Table: Disease history by adult CD subject

CD subject	Gender	Disease Duration (years)	Surgical history	HBI	Treatment at inclusion	Disease Phenotype	CRP	FCP	Endoscopy or MRE
1	F	10	None	13	Nil	A2L3B1	<5	n/a	Active TI disease
2	F	45	R hemicolectomy, transverse colectomy, defunctioning loop ileostomy	4	Infliximab	A2L3B3	<5	329	SES-CD = 10
3	F	1	None	1	Adalimumab	A3L2B2	14	1659	SES-CD = 10
4	M	16	R hemicolectomy, ileocolic resection	0	Nil	A1L3B3p	20	340	SES-CD = 5
5	M	10	Small bowel resection	0	Infliximab, mercaptopurine	A2L3B2	<5	>1800	Multi-focal stricturing small bowel CD
6	F	9	R hemicolectomy	5	Mercaptopurine	A2L3B1	<5	742	n/a
7	M	15	small bowel resections x 2	1	Nil	A2L3B2	5	566	Multifocal stricturing small bowel CD
8	M	10	None	5	Mercaptopurine	A2L3B3	20	298	n/a

Detailed individual demographics including data relating to sex, disease duration (years), surgical history, clinical symptoms as measured through Harvey Bradshaw Index (HBI), concomitant therapy at inclusion, disease location as per the Montreal Classification, C-reactive protein (CRP) in mg/dl and faecal calprotectin (FCP) in ug/g at inclusion, and recent ileocolonoscopy or magnetic resonance enterography (MRE) detailing disease activity.