Biomarkers to predict susceptibility to muscle wasting in critically ill patients.

A thesis submitted in partial fulfilment of the degree of

BACHELOR OF HEALTH AND MEDICAL SCIENCES (HONOURS)

In the Discipline of Acute Care Medicine, Adelaide Medical School

Faculty of Health Sciences

University of Adelaide

By

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June 2020

Word count: 4745 words

Abstract

Introduction: Many survivors of critical illness suffer long-term physical impairments, reflective of the significant muscle wasting that occurs during their ICU admission. It is currently unknown whether specific plasma miRNAs or proteins can predict which patients are susceptible to muscle wasting.

Aims: To measure if specific miRNAs and circulating proteins exhibit a change in their molecular profiles which relates to muscle wasting in critically ill adults.

Subjects and Methods: In this exploratory sub-study, critically ill patients were enrolled from three Australian ICUs. Blood samples and ultrasound-derived muscle size were collected at baseline (within 48-hours of enrollment), day 7, day 14 and ICU discharge. MiRNAs and protein concentrations known to relate to muscle mass or strength were assayed. Data are mean (SD) or median [IQR].

Results: Thirty-five patients were included (mean age 58 (17) years). Muscle loss from baseline to ICU discharge was significant (2.9 (1.2) to 2.4 (0.8) cm (n= 25); $p<0.001$). There were significant increases in miR-422a concentrations baseline to day 14 ($p=0.01$) and baseline to ICU discharge ($p=0.0001$). There was no change from baseline in miR-1 or miR-133a concentrations. GDF-15 increased significantly from baseline to day 7 ($p=0.0001$). No association was found between baseline miRNAs levels and muscle loss from baseline at day 7. Baseline concentrations

of GDF-15 were inversely correlated with % muscle loss at day 7 (r=-0.405). The absolute change in concentrations of MiR-422a, GDF-15 and IGF-1 showed a moderate to weak correlation with muscle loss from baseline to day 7 (r=0.312; 0.408; 0.440 respectively).

Conclusion: This study showed that plasma miRNA-422a, GDF-15 and IGF-1 may provide useful biomarkers for muscle wasting. These results improve the current understanding of muscle loss in the ICU and permits the improved targeting of certain interventions which ameliorate the advancement of further muscle loss and severe ICU-related muscle disorders.

Introduction

Critically ill patients admitted to an Intensive Care Unit (ICU) experience acute muscle wasting which increases their risk of developing long-term ICU related disorders[1] such as ICU-acquired weakness (ICU-AW)[2]. ICU-AW is associated with a prolonged duration of mechanical ventilation and affects the ability for patients to return to pre-ICU activities, limiting common aspects of daily life[3-5]. Findings from observational data reports that 69% of mechanically ventilated patients in ICU admission for greater than 48 hours, are restricted in performing daily activities one year after discharge[6]. The majority of these critically ill survivors experience early and rapid muscle wasting within 7 days of ICU admission[7], which is likely to contribute to the poor functional outcomes and impaired recovery[8] reported in this population. Common measures of muscle size (functional magnetic resonance imaging[9] and dual-energy X-ray absorptiometry[10]) cannot be easily used in an ICU setting due to patient immobility and radiation dosage. Therefore, ultrasound imaging has gained popularity as a practical and reliable tool that can quantify muscle loss at the bedside. Two prospective observational studies in critically ill adults have shown significant muscle loss when measured using ultrasonography; Parry *et al.* reported 10.3% reduction of the quadriceps muscle by day 7 of ICU admission[11], and Puthucheary *et al.* reported a 30% reduction of the rectus femoris cross sectional area by day 10[12]. Furthermore, studies in patients with traumatic brain injury[13] and acute respiratory distress syndrome[14, 15] demonstrate that patients that lose less muscle have improved long-term recovery. Hence having the ability to predict the patient's trajectory of muscle loss using a biomarker would enable the early application of suitable therapies (nutritional[16, 17], physical and occupational therapy[18] and electrostimulation[19]) to help attenuate muscle wasting, thus improving the functional recovery of these patients.

Potential biomarkers associated with muscle wasting are likely to be those involved in muscle protein turnover, either those that promote muscle protein synthesis or degradation; as well as molecules that are released into circulation during muscle degradation. Ribosomal[20, 21]and proteasomal[22]pathways are altered in response to certain factors such as immobility[23], inflammation[24], pharmaceutical drugs[25], ageing[26], and starvation[27] leading to a decrease in rates of muscle protein synthesis and increase in muscle protein breakdown. The potential role of a number of these circulating proteins and miRNA as biomarkers has been explored in other conditions in which muscle wasting occurs.

GDF-15 is a stress molecule produced in response to inflammatory and metabolic stress placed on mitochondria[28] when excessive damage or disease occurs. Plasma concentrations of GDF-15 have been shown to increase with age, are inversely associated with physical activity[29], and are higher in patients with sarcopenia or muscle atrophy[30]. An observational study in high-risk cardiovascular surgical patients compared those with established ICU-AW (n=20) and those without (n=7) reported that plasma GDF-15 concentrations were higher in patients with ICU-AW[31]. They also demonstrated that treatment of C2C12 myotubes (cell-culture) over a four day period with GDF-15 (50ng/mL) increased expression of genes relating to muscle atrophy[31] and resulted in downregulation in expression of miRNAs known to be involved in muscle homeostasis (miR-133a and miR-1)[31]. Further, a prospective longitudinal observational study by Bloch *et al.* observed pre- and post-operative (days 1, day 2) plasma samples of forty-two patients undergoing high-risk cardiothoracic surgery, reporting elevated post-operative levels in all patients[32]. Measurements were additionally preformed at day 7 and showed that sustained levels were only present in patients who developed muscle wasting, with GDF-15 concentrations returning to their baseline values in

the non-wasting group[32]. Collectively, this data suggests that GDF-15, either by down-regulating muscle promoting miRNAs or by directly causing muscle atrophy, is a key instigator of muscle wasting.

Furthermore, unpublished data from our collaborators at Imperial College, London shows that several other proteins are associated with increased or decreased muscle mass and/or strength in ICU patients following aortic surgery (*Table 1*). These include: TXNDC12 (antioxidant protein); CRLF1-CHRL1 (circulating co-receptors); Mammaglobin-B and Lipocalin-2 (regulators of steroid activity); Resistin (insulin-signaling); FSTL3 (cell signaling); and Stanniocalcin-1 (mitochondrial function). These regulators of muscle mass and strength may also play a role in muscle wasting in a heterogenous population of critically ill patients.

Table 1: Correlation between circulating proteins and muscle mass or strength.

Protein	TXNDC12	Lipocalin-2	Stanniocalcin-1	GDF-15
Correlation between concentration levels (pg/mL) and muscle loss/strength	Negative	Positive	Positive	Positive
Cross-sectional area (CSA) of the quadricep muscle or strength grip test (dynamometer)	Rectus femoris CSA loss %	Rectus femoris CSA loss %	Hand grip % difference	Rectus femoris CSA loss %

Unpublished data from our collaborators at Imperial College, London,

MiRNAs are small noncoding RNA molecules, consisting of between 18-28 nucleotides[33]. They play a major role in the post-transcriptional regulation and degradation of messenger RNA and their potential as a biomarker to predict muscle wasting has been demonstrated in other populations. In patients undergoing cardiac surgery, pre-surgical concentrations of miR-422a expression in the quadriceps muscle tissue were reported to be positively associated with both strength and the rectus femoris cross-sectional area (RF_{CSA})[34]. Furthermore, those patients with the greatest muscle loss (>10% of RF_{CSA} over 7 days) had lower concentrations of miR-422a expression[34]. MiR-422a concentrations (quadriceps muscle tissue) in stable COPD patients are reported to be elevated when compared to healthy controls. Interestingly, the same study observed plasma levels of miR-422a and reported a negative association with a loss of strength in COPD patients[34]. Plasma concentrations of two muscle-specific miRNAs (myomirs) miR-1 and miR-133a are higher in patients with stable COPD (n=103) when compared to their age-matched controls (n=25)[35]. Contrarily, plasma levels of the same miRNAs were shown to be reduced in patients with established ICU-AW[31]. These opposing results between plasma myomirs amongst stable COPD and ICU-AW patients may reflect disease severity, with the advancement of muscle wasting specific aliments (ICU-AW) greatly diminishing the size of muscle that these myomirs are released from, this being demonstrated in lower plasma levels.

Overall, increased expression of miRNA in patients with COPD, cardiac disease and ICU-AW may reflect the loss of muscle mass making circulating levels of these miRNAs' potential biomarkers for muscle wasting. While these biomarkers have been demonstrated to be associated to muscle wasting in these other states, there is limited evidence on the putative role played by these potential biomarkers in a heterogenous cohort of critically ill patients. Hence, the aim of this

thesis was to investigate relationships between miRNAs and circulating proteins and muscle wasting in general critically ill adults.

The specific aims of this thesis were to:

(1) Quantify changes in concentrations of circulating plasma miRNAs (miR-1, miR-422a and miR-133a) and circulating proteins (GDF-15, Stanniocalcin-1, CHRL1, CRLF1, Lipocalin-2, Resistin, IGF-1, FSTL3) in mechanically ventilated critically ill adults over the ICU admission.

(2) Quantify changes in muscle size measured using ultrasound over the ICU admission.

(3) Determine whether baseline concentrations of miRNAs and circulating proteins predict the degree of muscle wasting.

(4) Determine if the change in miRNAs and circulating proteins levels from baseline to day 7 are associated with the degree of muscle wasting over the same time period in critically ill adults.

The hypotheses for this thesis are:

1. There will be an increase in circulating miRNA and protein concentrations from baseline to day 7, 14 and ICU discharge.
2. Muscle loss will occur over the ICU admission.
3. There will be an association between baseline concentrations of miRNAs and circulating proteins and the amount of subsequent muscle wasting from baseline to day 7.

4. There will be a relationship between the degree of change in miRNA and circulating protein concentrations and degree of muscle wasting from baseline to day 7.

Methods

This study (MUSCLE-PRO) was a multi-centre exploratory sub-study part of a 120-patient randomised controlled feasibility trial (TARGET Protein feasibility trial). The TARGET Protein feasibility trial was conducted at six sites in Australia and New Zealand and compared augmented versus standard protein delivery in critically ill adult patients with the primary aim of achieving separation in delivered protein doses between the two groups. Three of the six sites participated in this MUSCLE-PRO sub-study. The Royal Adelaide Hospital Human Research Ethics Committee approved the study (HREC Reference number: HREC/18/CALHN/658) and governance was approved at each site.

Patient recruitment

Patients that were enrolled into the TARGET Protein feasibility trial at the three participating sites were approached for consent into the MUSCLE-PRO sub-study within 48 hours of enrolment into the TARGET Protein feasibility trial. *Figure 1* is a consort diagram of the recruitment process. Study procedures were explained to the patient's Next of Kin and a signature for consent was obtained. Patients were eligible for the TARGET Protein feasibility trial and the MUSCLE-PRO sub-study if they met the following criteria:

Inclusion criteria TARGET Protein feasibility trial:

1. Aged ≥18 years.
2. Receiving invasive mechanical ventilation.
3. About to commence enteral nutrition (EN) or EN commenced within the previous 12 hours.
4. Expected to be receiving EN in ICU until at least the day after tomorrow.

Exclusion criteria TARGET Protein Feasibility trial:

1. Expected to be receiving any oral nutrition before the calendar day following randomisation.
2. Any EN or parenteral nutrition (PN) received for >12 hours in this ICU admission.
3. Previously enrolled in this study.
4. Treating clinician considers the EN goal rate (i.e. 1ml/kg of ideal body weight (IBW) per hour) to be clinically contraindicated e.g. requirement for fluid restriction.
5. Requirement for specific nutritional therapy as determined by the treating doctor or dietitian i.e. TARGET Protein protocol EN not considered to be in the best interest of the patient.
6. Death is deemed to be imminent or inevitable during this admission and either the attending doctor, patient or substitute decision maker is not committed to active treatment.
7. The patient has an underlying disease that makes survival to 90 days unlikely.

All patients admitted into the MUSCLE-PRO sub-study were additionally screened for eligibility based on the following criteria:

Inclusion criteria MUSCLE-PRO:

1. Enrolled into the TARGET Protein feasibility trial.
2. Able to obtain consent and complete baseline measures within 48 hours of enrolment into the TARGET Protein feasibility trial.

Exclusion criteria MUSCLE-PRO:

1. Patients are unable to have quadriceps measured (e.g. burns, femoral shafts, above knee amputations, prone ventilated).

Patient demographics

Patient demographic data was extracted from the TARGET Protein Feasibility trial database which included patient age, sex, pre-existing chronic co-morbidities and Acute Physiology Chronic Health Evaluation II score (APACHE II)[36], APACHE III diagnostic code, ideal/actual body weight, Body Mass Index and ICU/hospital length of stay.

Study procedures

Ultrasound images and blood samples were obtained at baseline (within 48-hours of enrollment into MUSCLE-PRO), days 7 and 14 whilst the patient remained in ICU, and ICU discharge

censored at day 28. Samples were centrifuged at 3200rpm for 10 minutes with 3-4ml of plasma pipetted into a 5.0 mL Eppendorf tubes for immediate storage in a -80°C freezer for future analysis.

Ultrasound of muscle size:

Ultrasound images of the quadriceps muscle layer thickness (QMLT) were taken by a qualified clinician with expertise in ultrasound measurement using previously described technique[37]. All bedside ultrasound measurements were taken on the right-side of the body unless the patient presented with existing barriers to measurements (e.g. burns or amputations), in which case the left-side was used. A portable 5-13 MHz and 15-6 MHz transducer was used to measure QMLT. Patients were positioned supine with their legs laid straight in the extended position. All measurements were taken (i) at the border between the lower third and upper two-thirds between the Anterior Superior Iliac Spine (ASIS) and the upper pole of the patella, and (ii) at the midpoint between the ASIS and the upper pole of the patella[37]. The transducer was held perpendicular to the skin with minimal pressure applied and still images taken in triplicate. QMLT was measured on the still image from the superficial muscle-muscle interface and via the bone-muscle interface. Three measurements were taken at the two mentioned landmarks above with the means of these three measurements used to determine a total means for each landmark. The means of these two measurements was then used to provide a total mean QMLT.

Plasma protein analysis

The eight proteins of interest were assessed using the sandwich enzyme-linked immunosorbent assay (ELISA) method. Plasma samples and reagents were brought to room temperature and

pipetted into wells with a capture antibody. The wells were washed following an incubation period. Biotinylated detection antibody was added followed by an incubation period at 37°C. Unbound biotinylated antibody was washed away with horseradish peroxidase-conjugated streptavidin being pipetted to the wells directly after. Wells were then washed again, tetramethylbenzidine substrate solution was added to develop the well colour. Incubation of plates was left at room temperature on a gentle agitator until colour intensity in sample wells had reached a contrast similar to that of our standard curve, but had not exceeded the colour intensity of the most concentrated reference well. A Stop Solution was then added to prevent further color development and samples were read at an absorbance wavelength (reference channel) of 650nm immediately on the Tecan Spark multimode microplate reader.

Plasma miRNA extraction and quantification

MiRNA extraction involved diluting the plasma with a denaturing solution. For this, we added 4ml Qiazol Lysis from the Qiagen mi-RNeasy kit to 400 µL of plasma sample. Aqueous and organic phase separation was achieved via the addition of 800µL of chloroform followed by centrifugation at 4000rpm for 25min at 4°C. 3ml of ethanol was then added to the samples and 700µL was collectively transferred to RNeasy Mini Columns and centrifuged for 15seconds at 8000rpm until all the mixture was used. Flow through was discarded with the final transfer of the RNeasy Mini Column to a new collection tube were RNA samples were eluted from the column by 50µL of RNAse free water and stored at -80°C.

Following RNA extraction, subsequent cDNA synthesis and miRNA profiling was carried out by qRT- PCR. Stem-loop reverse transcription primer method was used for the conversion of cDNA from miRNA due to its high sensitivity and specificity in recognising mature miRNA[38]. Real-time PCR was run in duplicate with the TaqMan Universal PCR Master Mix. The amplification reaction ran on the Applied Biosystems FAST 7500 Fast Real-Time PCR System. Endogenous normalisers, RNU48 and U6 were used to normalise the data for RNA extraction[39]. The plotting of the cycle threshold (Ct) values allowed us to best fit the curve using the endogenous miRNA as approximate copies from the obtained patient's plasma samples.

Outcomes

The MUSCLE-PRO sub-study outcomes included:

(1) Concentration of plasma miRNAs (miR-1, miR-422a and miR-133a) and circulating proteins (GDF-15, Stanniocalcin-1, CHRL1, CRLF1, Lipocalin-2, Resistin, IGF-1, FSTL3) at baseline, day 7, day 14 and ICU discharge in mechanically ventilated critically ill adults.

(2) Change in QMLT from baseline to day 7, day 14 and ICU discharge.

(3) Correlation between baseline concentrations of miRNAs and circulating proteins and change in QMLT from baseline to day 7.

(4) Association between change in miRNAs and circulating proteins from baseline to day 7 and change in QMLT from baseline to day 7.

Data and statistical analysis

This was an exploratory study, and thus no sample size calculation was conducted. The data is presented as mean, standard deviation (SD) for normally distributed data, and median-Interquartile Range [IQR] for non-parametrically distributed data with a 5 to 95% percentile score, unless otherwise stated. Baseline and subsequent (day 7, day 14 and ICU discharge) concentrations were compared using Wilcoxon matched pairs signed rank test. The total average QMLT between the midpoint and upper 2/3 ultrasound image was evaluated as an outcome at day 7, 14 and ICU discharge and compared to the patient's baseline thickness. A two-way ANOVA compare this outcome and a Bonferroni multiple comparison was used for post-test correction. Pearson's correlation coefficient was calculated to identify significant associations where appropriate. MiRNA levels were logarithmically transformed to produce a normal distribution and stabilise for unwanted variance[40]. Statistical analysis and graph configuration were carried out on GraphPad PRISM software (GraphPad Software 8, California, USA).

Results

This thesis represents only a partial analysis of miRNAs and circulating proteins due to limitations of time in an honours year. miR-1, miR-133a and miR-422a, Lipocalin-2, Resistin, FSTL3, Stanniocalcin-1, GDF-15, CHRL1, CRLF1 and IGF-1 have been analysed. MiR-181a, miR-499 and miR-206; circulating proteins - Mammaglobin B, Catalase, Myostatin, Carbonic anhydrase 3, TNNI2, CSRP3 and TXNDX12 are currently being analysed and will be included in the final publication.

Patient demographics and clinical outcomes

Trial recruitment occurred between April and July 2019. In total, 35 of the 70 patients enrolled in the TARGET Protein feasibility trial at three sites met the inclusion criteria for recruitment into the MUSCLE-PRO sub-study and had consent obtained within 48 hours of randomisation. The demographic data and clinical characteristics of included patients are listed in *Table 2.*

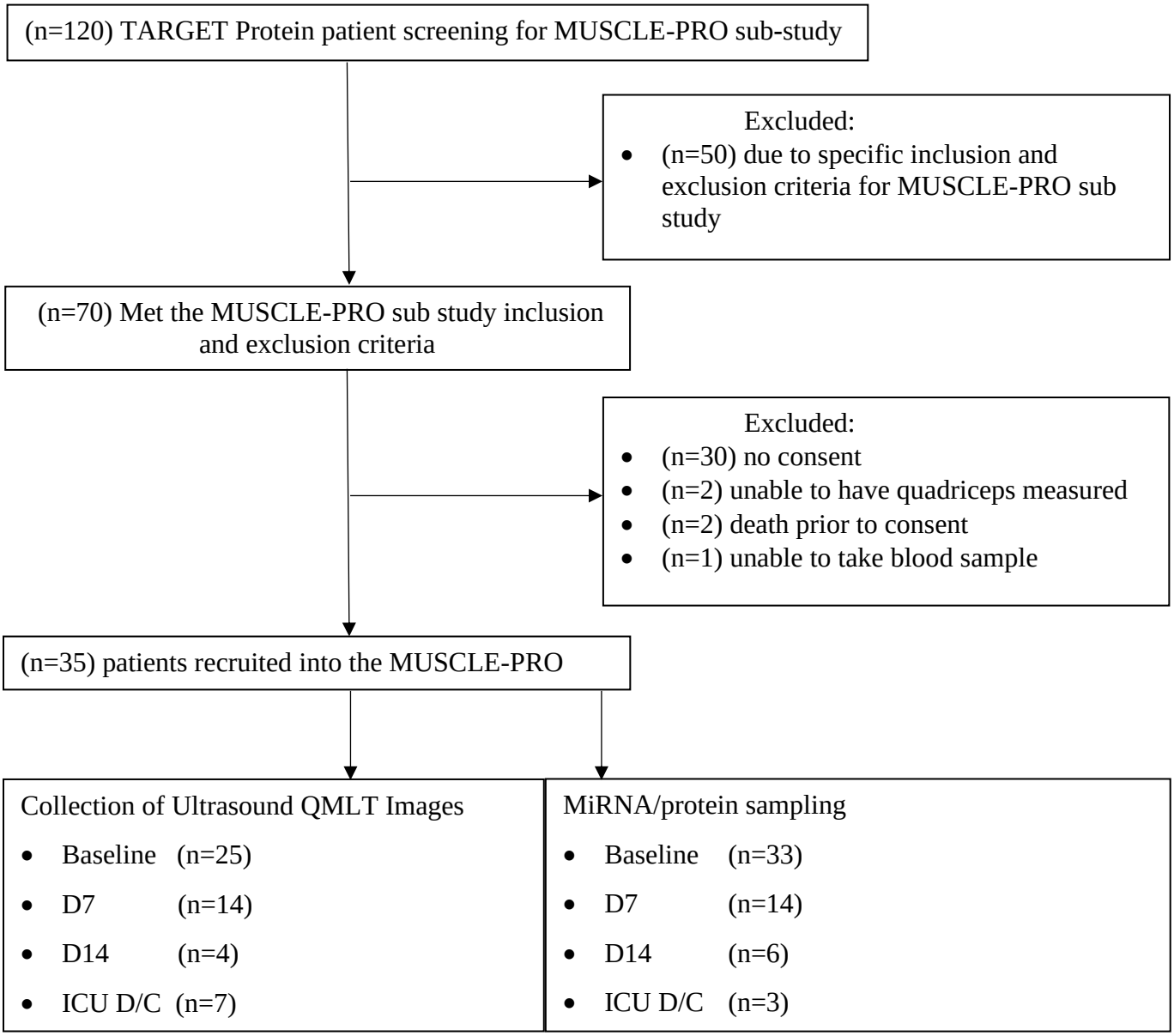
Table 2: Patient demographics and clinical characteristics

Patients	**n=35**
Age, years, mean (SD)	58 (17.77)
Sex, Male: Female	25:10
APACHE II score, median [IQR]	22 [18-27]
APACHE III admission diagnostic code, n (%)	
Trauma	3 (9%)
Respiratory	5 (15%)
Metabolic	1 (3%)
Neurological	8 (23%)
Cardiovascular	16 (47%)
Musculoskeletal/ Skin	1 (3%)
ICU LOS, days, median [IQR]	9 [4-17]
Hospital LOS, days, median [IQR]	18 [12-35]
Actual Body Weight (kg), median [IQR]	87 [80-103]
Ideal Body Weight (kg), median [IQR]	69 [58-73]
Body Mass Index (kg/m^2) from actual body weight, median [IQR]	29 [26-37]

APACHE = Acute Physiology and Chronic Health Evaluation, ICU = Intensive Care Unit,

LOS= Length of Stay, IQR = Interquartile Range

Figure 1: Consort flow diagram of patient population

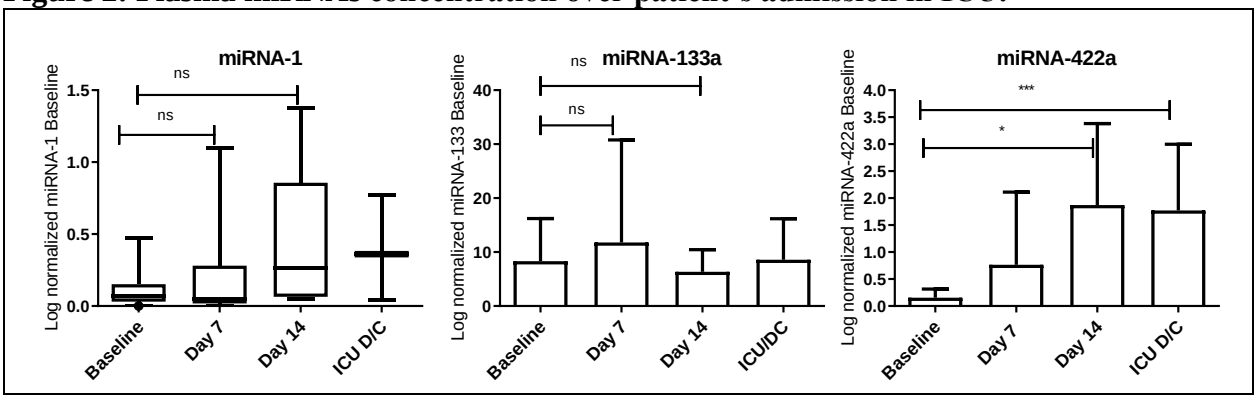


QMLT =Quadriceps Muscle Layer Thickness, D7= Day 7, D14= Day 14, ICU D/C = Intensive Care Unit Discharge

Plasma miRNA and circulating protein profiles at baseline, day 7, day 14 & ICU Discharge

Analysis of miR-422a expression showed a significant rise from baseline to day 14 (p=0.01) and ICU discharge (p=0.0001) (Figure 2). Relative levels of miR-1 and miR-133a did not significantly vary from baseline to any of the other timepoints.

Figure 2: Plasma miRNAs concentration over patient's admission in ICU.



*Data presented as box and whisker plots with median [interquartile range] and 5 to 95% percentiles. "*P <0.01, ***P <0.0001, ns - not significant. Wilcoxon matched pairs signed rank test was used for calculating change in concentration over timepoints. Baseline (n=33), day 7 (n=14), day 14 (n=6) and ICU discharge (n=3).*

Table 3: Plasma miRNA concentrations at baseline, day 7, day 14 and ICU discharge

MiRNA timepoints	**Baseline n=33**	**Day 7 n=14**	**Day 14 n=6**	**ICU discharge n=3**
miR-1	-1.16 [-1.50-0.84]	-1.32 [-1.72-0.61]	-0.41[-0.81-0.08]	-0.44[-0-92-0.27]
miR-422a	-1.04 [-1.15-0.64]	-0.73 [-1.36-0.19]	0.13 [-0.27-0.46]	0.37 [0.37-0.03]
miR-133a	0.80 [0.39-1.05]	0.55 [0.15-1.10]	0.81 [0.48-0.87]	0.87 [0.54-1.04]

Concentration levels for log expression of miRNAs reported as median [IQR] as data was not normally distributed.

Circulating Proteins

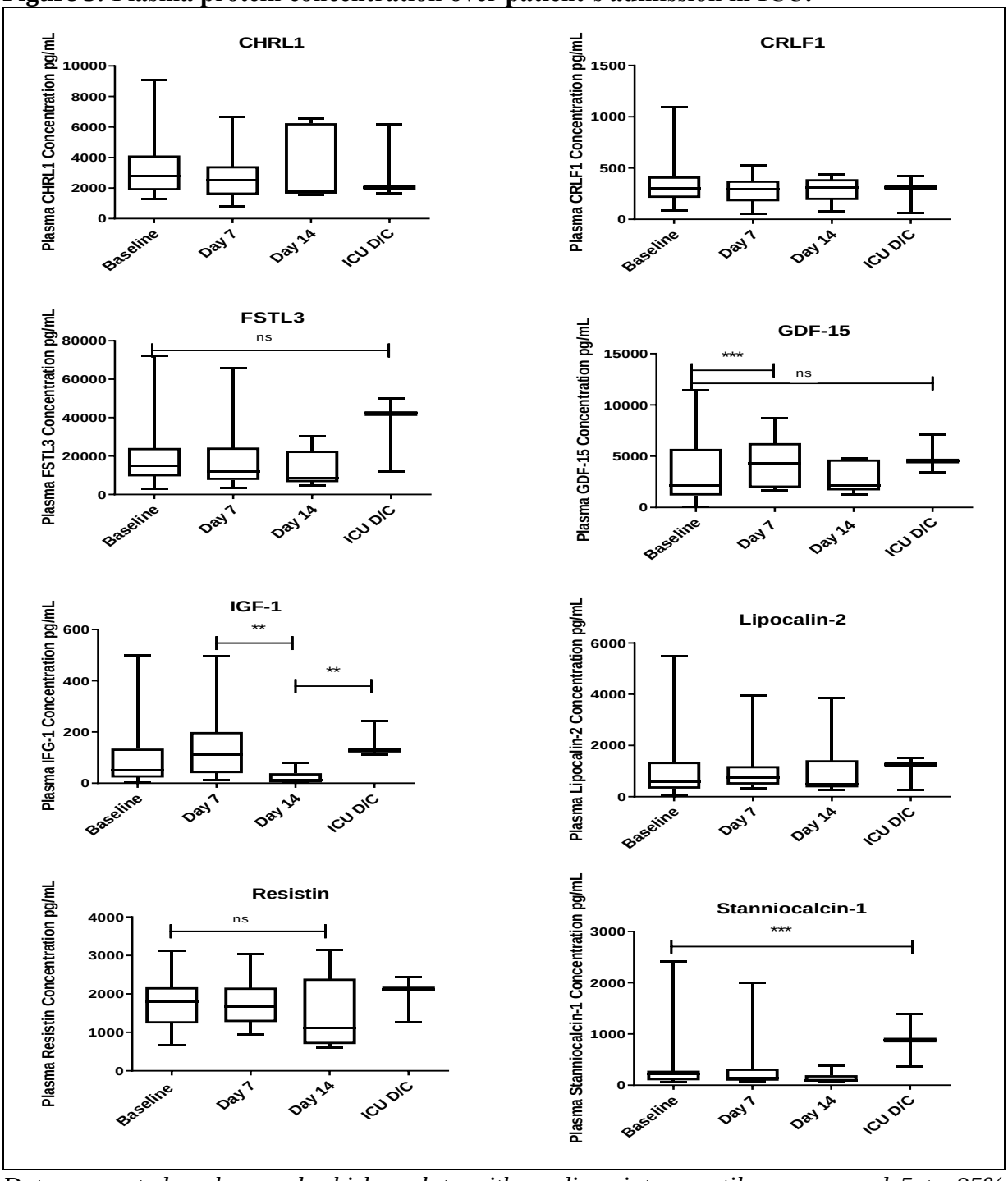
Two proteins increased in their concentrations over the ICU stay: plasma concentration of GDF-15 was significantly higher at day 7 compared to baseline ($p<0.0001$: Figure 3), as was the concentration of Stanniocalcin-1 at ICU discharge ($p<0.0001$) when compared to baseline. In contrast, IGF-1 plasma levels were significantly lower ($p<0.001$) at day 14 compared to day 7. There were no significant changes in median concentrations values for Resistin, FSTL3, CHRL1, CRLF1 and Lipocalin between time-points (baseline to ICU discharge).

Table 4 Plasma protein concentrations at baseline, day 7, day 14 and ICU discharge

Circulating proteins	**Baseline n=33**	**Day 7 n=14**	**Day 14 n=6**	**ICU D/C n=3**
GDF-15	2137 [1419-5576]	4339 [2058-6143]	2159 [1847-4064]	4534 [3997-5837]
Stannio-calcin-1	225 [102-278]	143 [91-310]	85 [78-121]	880 [624-1135]
CHRL1	2768 [1858-3995]	2527 [1649-3303]	1793 [1682-5082]	2044 [1860-4113]
CRLF1	304 [212-416]	295 [178-373]	310 [230-373]	307 [184-365]
Lipocalin-2	599 [346-1295]	747 [502-1074]	487 [407-594]	1250 [759-1390]
Resistin	1793 [1241-2123]	1677 [1340-2076]	1115 [761-1953]	2126 [1701-2287]
IGF-1	51 [23-128]	110 [45-191]	12 [1-24]	129 [119-186]
FSTL3	14961 [9858-23414]	10572 [7411-21699]	8548 [7096-17653]	42074 [27078-46037]

Concentration levels for proteins in pg/mL reported as median IQR as data was not normally distributed.

Figure 3: Plasma protein concentration over patient's admission in ICU.



*Data presented as box and whisker plots with median, interquartile ranges and 5 to 95% percentiles. **P <0.001, ***P <0.0001, ns - not significant. Wilcoxon matched pairs signed rank test was used for calculating change in concentration over timepoints. Baseline (n=33), day 7 (n=14), day 14 (n=6) and ICU discharge (n=3).*

Ultrasound measurements of quadriceps muscle layer thickness

Ultrasound scans were performed on all 35 patients at baseline 2.9(SD, 1.0); however, 10 patients only provided a baseline image with no follow-up scan obtained. Therefore, only patients with a follow-up scan from baseline (e.g. baseline to day 7, baseline to day 14 and baseline to ICU discharge) could be included in this two-point analysis. A baseline QMLT means of 2.9 (SD 1.2) was established for 25 patients with their corresponding final measurement being 2.4 (SD 0.8) (Table 5).

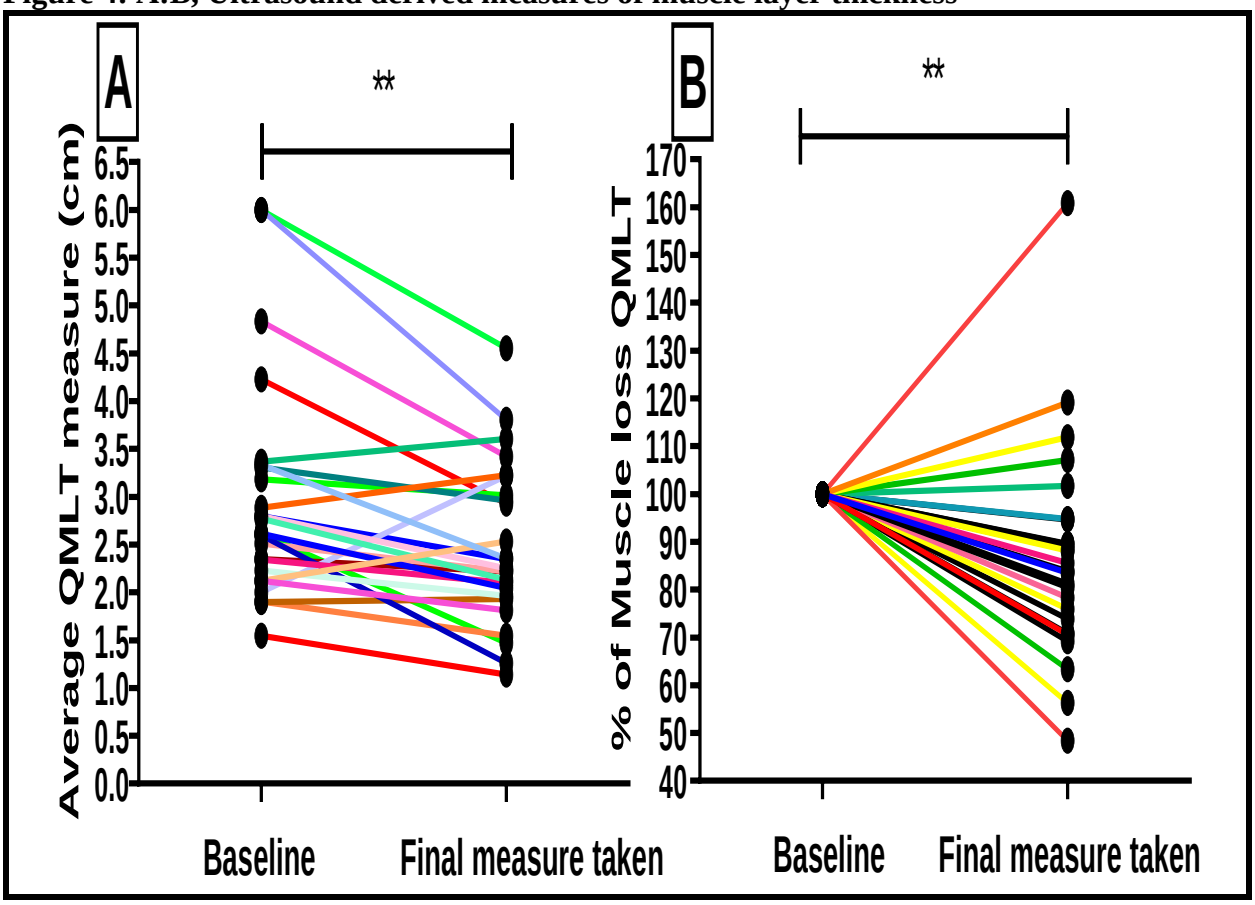
Table 5: Quadriceps Muscle Layer Thickness

Patient Timepoint:	**Baseline n=35**	**Day 7 n=14**	**Day 14 n=4**	**ICU D/C n=7**	**FMT n=25**
QMLT, cm	2.9(SD, 1.0)	2.6(SD, 1.1)	2.8(SD, 1.1)	1.9(SD, 0.3)	2.4(SD, 0.8)

QMLT=Quadricep Muscle Layer Thickness is presented as mean (SD) as data was normally distributed. FMT=Final measure taken

In those patients with more than one measurement available, the change in means QMLT (cm) from baseline to the final measure conducted was significant ($p<0.001$: Figure 4A). Similarly, the decrease muscle size (17.24%) from baseline was significant for the 25 patients ($p<0.001$: Figure 4B) that provided a follow-up ultrasound image from baseline.

Figure 4: A:B, Ultrasound derived measures of muscle layer thickness

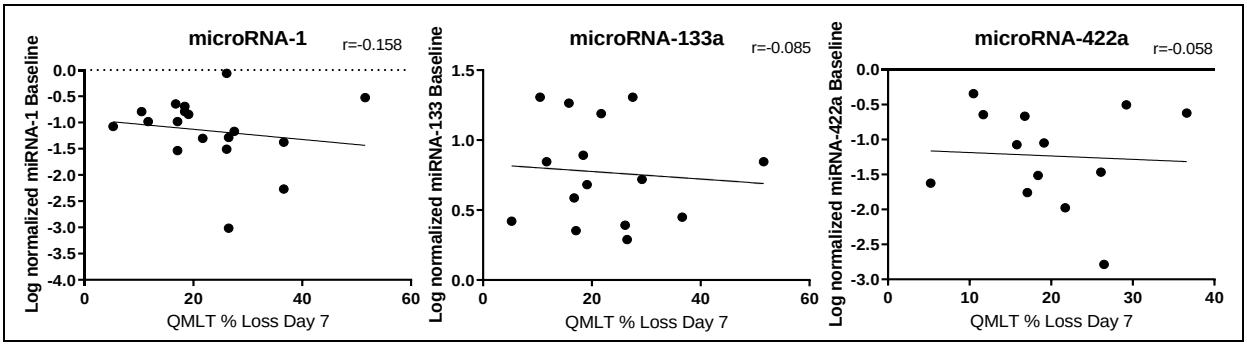


*A: Change in the ultrasound derived quadriceps muscle layer thickness from baseline to final measure (n=25). B: Percentage change from baseline to final measure in (n=25) critically ill patients. Data alpha (**P<0.001) value was calculated from a two-way ANOVA and Bonferroni multiple comparison was used for a post-test correction.*

Correlation between baseline miRNA and circulating protein concentrations and % change in quadriceps muscle layer thickness from baseline to day 7

Baseline concentrations for both myomirs, miR-1 (r= 0.158: Figure 5), and miR-133a (r= 0.085), showed no correlation with the percentage of muscle loss from baseline to day 7. Similarly, there was no correlation with baseline values for miR-422a (r= 0.058) and muscle loss from baseline to day 7.

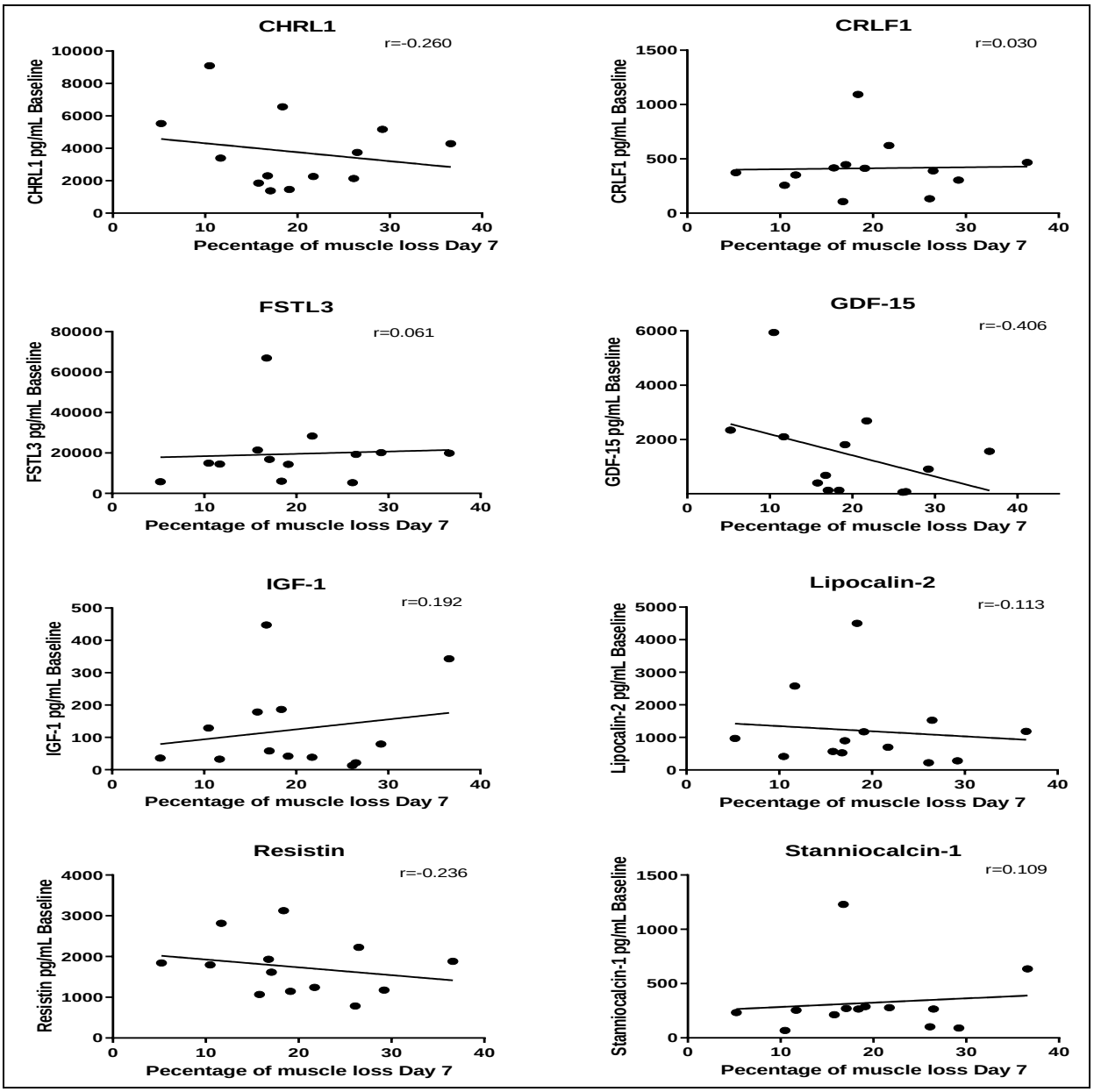
Figure 5: MiRNA levels at baseline and % change in quadriceps muscle layer thickness from baseline to day 7



The positive and negative linear correlation were determined for the patients (n = 13) microRNA expression and muscle loss at day 7. Pearson's r value was used to determine the correlational coefficient.

Baseline GDF-15 (r=-0.405), CHRL1 (r= -0.260), Resistin (r= -0.236) and Lipocalin-2 (r= -0.113) concentration were all negatively correlated with loss of muscle wasting over a 7 day period (Figure 6). No correlation was observed for baseline concentrations of Stanniocalcin-1 (r= 0.109), IGF-1 (r= 0.192), FSTL3 (r= 0.051) and CRLF1 (r= -0.030) (Figure 6) and muscle wasting.

Figure 6: Protein levels at baseline and % change in quadriceps muscle layer thickness from baseline to day 7

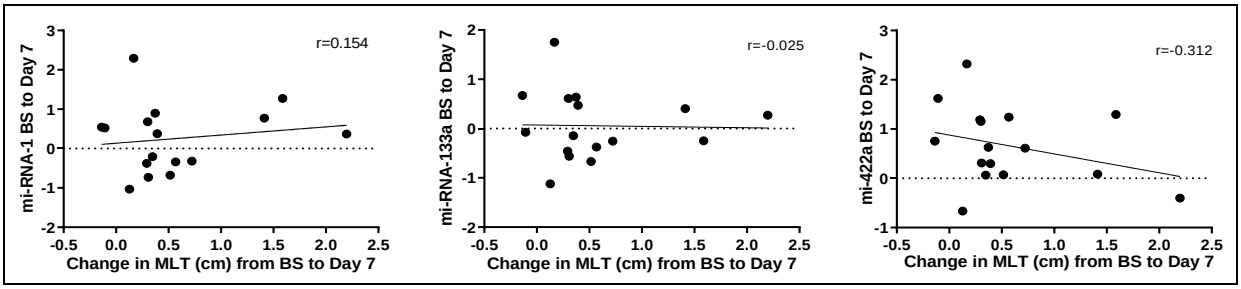


A liner positive or negative correlation was identified between the following circulating proteins concentrations and the corresponding % of muscle loss to day 7. To determine the correlation with protein concentration and muscle loss, the baseline values for patients (n=13) were assessed with the equivalent percentage in muscle loss to day 7. Pearson's r value was used to determine the correlational coefficient.

Correlation between change in miRNAs and circulating proteins concentrations and % change in quadriceps muscle layer thickness from baseline to day 7

There was no correlation between the absolute change in either miR-1 (r= 0.154: Figure 7) or miR-133a (r= 0.025), and muscle loss from baseline to day 7. However, miR-422a did show a negative correlation with muscle wasting (r= 0.312).

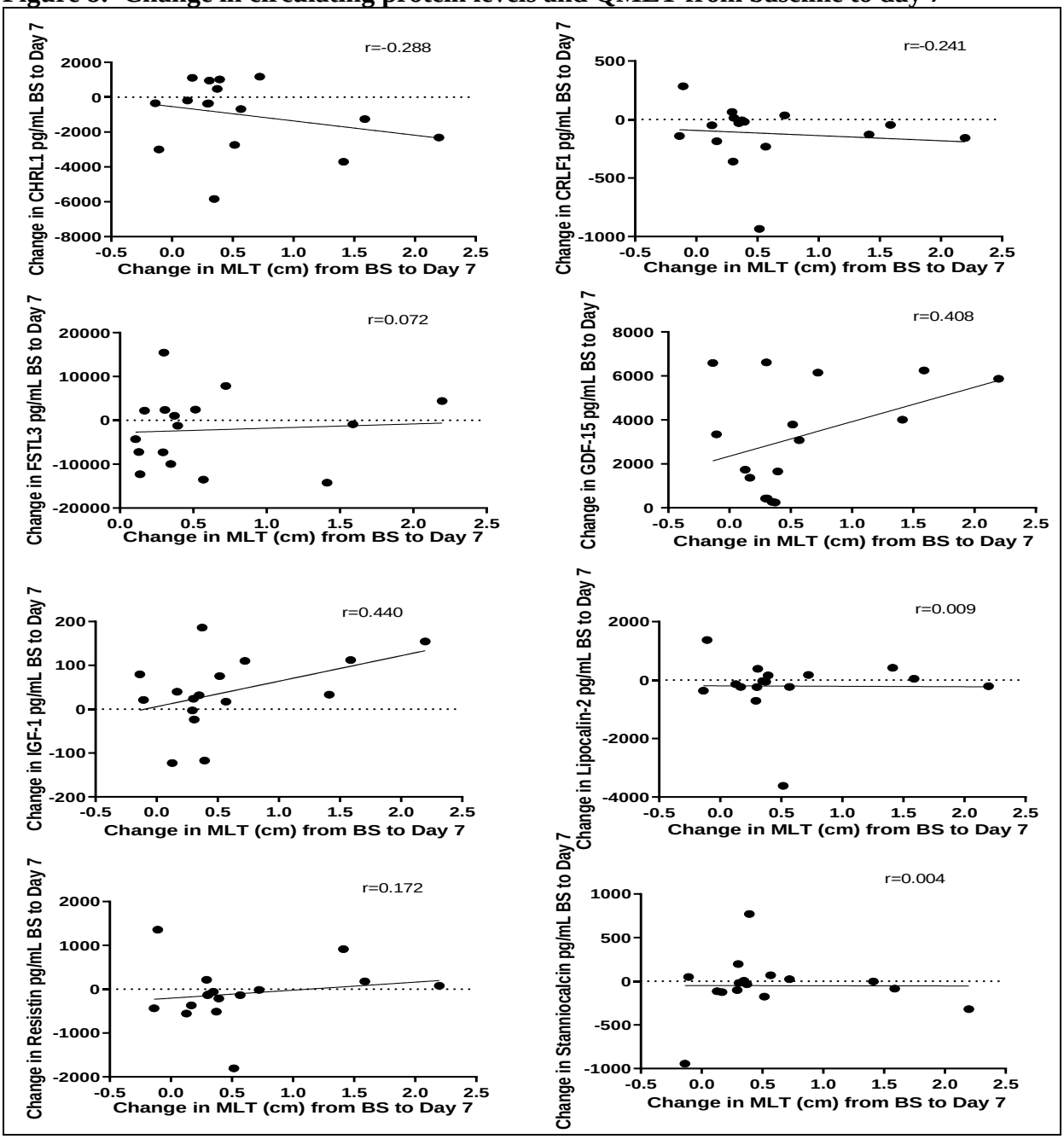
Figure 7: Change in miRNA levels and QMLT from baseline to day 7



Correlation between the patients (n=16) change in plasma miRNA levels and the corresponding change in QMLT after 1-week. Pearson's r value was used to determine the strength of this relationship. Baseline (BS).

A moderate and weak correlation was reported between change in the following protein concentrations and degree of muscle wasting from baseline to day 7: GDF-15 (r= 0.408: Figure 8); IGF-1 (r= 0.440); Resistin (r= 0.172); CRLF1 (r= 0.241) and CHRL1 (r= 0.228:). No correlation was found for the following proteins: Lipocalin-2 (r= 0.009), Stanniocalcin-1 (r= 0.004) and FSTL3 (r= 0.072).

Figure 8: Change in circulating protein levels and QMLT from baseline to day 7



Correlation between the patients (n=16) change in plasma protein levels and the corresponding change in QMLT from baseline to day 7. Pearson's r value was used to determine the strength of this relationship. Baseline (BS).

Discussion

This is the first study to our knowledge to measure change over time in plasma concentrations of miRNA and circulating proteins associated with muscle wasting in a heterogenous critically ill adult population. As expected, miR-422a concentrations increased from ICU admission to day 14 and ICU discharge, while neither miR-1 or miR-133a concentrations changed over the study period. Previous research has only assessed these mi-RNAs from muscle at a single timepoint in stable COPD patients, and revealed that miR-422a, miR-1 and miR-133a concentrations were all elevated compared to healthy controls[34, 35]. In our study we may not have demonstrated a change over time in miRNA concentrations given the baseline measurement was taken within 48hours of recruitment into this study, and thus the patients miRNA concentrations may have shown significant change prior to the baseline measurement. Additionally, no change in either myomirs could be a consequence of GDF-15 proposed role in the downregulation of muscle specific miRNAs[31], and given that our study revealed a significant rise in GDF-15 levels from baseline to day 7 provides another reason why no directional change was observed for either myomir. These results suggest future ICU studies should include analysis of miR-422a; however, the baseline time point may contribute to observed differences between studies.

Concentrations of Resistin, CHRL1, CRLF1 and Lipocalin-2 did not change over the study period; however, there was a significant rise from baseline to ICU discharge for FSTL3 and Stanniocalcin-1. GDF-15 levels were also elevated at day 7 which parallels the elevated concentrations shown in other studies with ICU-AW patients[31]. Our data suggest that IGF-1 levels are stable for the first 7 days of ICU stay and then decline. Previous reports of critically ill patients have indicated an

association between reduced plasma IGF-1[41], severe ICU-acquired paresis[42] and sepsis-induced catabolism[43] over varying timepoints. Intriguingly, Meyer *et al.* showed in murine and human skeletal myoblast cells (cell-culture) that IGF-1 could both promote or antagonise the expression of muscle promoting miRNAs[44] (miR-1, miR-133a and miR-206) indicating cellular cross-talk between proteins and miRNAs[45].

QMLT measurements (cm) as reported in this study was 2.9 (SD 1.2) at baseline for 25 patients with their final measurement recorded as 2.4 (SD 0.8). This 17.24% decrease in muscle size is consistent with previous reports showing a 30% reduction in 22 critically ill patients within 10 days of ICU admission by Parry *et al.*[11] and a 10.3% reduction reported by Puthucheary *et al.* in 28 critically ill patients at the same timepoint[12]. Findings from our study are consistent with previous papers that successfully measured the amount of muscle lost in critically ill adults[46, 47].

In our study we found there were no correlations between miRNA-1, miRNA-133a or miRNA-422a at baseline and subsequent muscle loss. These findings are in contrast to observations made by Paul *et al.*[34] who reported that patients with higher pre-operative levels of miR-422a (from muscle biopsy) lost less muscle by day 7 ($r=0.57$, $P< 0.001$). Although no increase in miR-422a levels were observed from baseline to day 7 in our study, our findings reported significantly elevated miR-422a levels at day 14 which suggests that a longer ICU admission or a mixed cohort may cause the release of miR-422a concentrations to increase at a later timepoint. Additionally, miRNA concentrations in muscle tissue may differ to that in plasma given the function of miRNAs is primarily intracellular (occurring in muscle)[33, 48], while plasma concentrations may only represent atrophy leakage from the muscle rather than specific export[49]. Baseline concentrations

of CHRL1 and Resistin had a weak and negative correlation with muscle wasting by day 7, while no correlation was found for CRLF1, Lipocalin-2, Stanniocalcin-1 or IGF-1. Lower baseline GDF-15 concentrations were negatively correlated with increased muscle loss. A correlation with muscle strength has been reported previously; preoperative GDF-15 levels in cardiovascular and sarcopenia[50] patients have been shown to negatively correlate with handgrip strength highlighting that these early sampling points may be a biomarker for identifying future muscle wasting and consequently strength.

We found a negative correlation between change in miR-422a concentrations and muscle loss from baseline to day 7 (r=-0.312), but no correlation for myomirs miR-1 and miR-133a. This provides further evidence which suggests that miR-422a expression may be upregulated in response to muscle loss, but not miR-1 or miR-133a. These results differ to previous research in which plasma miR-1 concentrations were negatively associated with type I fibre cross sectional area (r=-0.27, p=0.027) when assessed in stable COPD patients[35]. Previous research has not specifically assessed plasma miR-1 and miR-133a concentrations with muscle wasting and given its identical role in muscle physiology in comparison to miR-1 suggests that a similar result is expected for them both[51]. No relationship for either miR-1 or miR-133a and muscle wasting (day 7) could additionally be due to elevated levels of GDF-15 (reported at day 7 in this study) affecting circulating concentrations of both myomirs[31].

A negative correlation for CRLF1and CHRL1 was observed for muscle loss from baseline to day 7, while FSTL3, Resitin CRLF1, Lipocalin-2 and Stanniocalcin-1 showed no association with muscle wasting. It is possible that these results for the above proteins represent factors involved in

the acute inflammatory response of cardiac surgery and chronic inflammation in COPD and is less likely to be observed in a mixed cohort of ICU patients. Additionally, these results could be explained by the small sample size (n=13) and perhaps underestimates the putative role of these markers in muscle wasting. Change in GDF-15 concentrations from baseline to day 7 in our study was positively correlated with muscle loss. This parallels results in other ICU patients, including those undergoing high risk cardiothoracic surgery[32] and individuals with established ICU-AW[31]. Both groups profile an increase in circulating plasma concentrations that correlates with more muscle wasting as ICU length of stay and their disease progresses relative to controls. Interestingly, healthy individuals demonstrate a similar scenario showing that when the muscle surpasses the threshold of what constitutes healthy activity, there is a significant increase in GDF-15 that is associated with decreased muscle performance and increased inflammation as seen in post-race plasma samples of endurance athletes[52]. The dualistic role of GDF-15 is also observed in response to sepsis and demonstrated by inhibition of GDF-15 which results in greater muscle loss because there is less liberation of lipid from the liver[53]. The reason for observing a negative relationship between baseline GDF-15 levels is possibly owing to better management of lipid resources within the first 48 hours (at first sample point). However, as ICU duration and disease progress there is less lipid left to release, explaining why a positive correlation was established when we accessed change over time.

Strengths and Limitations

Although prior studies have grouped patients by their diagnostic population (aortic surgery and COPD), to our knowledge this is the first time that the role of miRNA/circulating proteins in muscle wasting has been assessed in a heterogeneous ICU cohort. Additionally, we report miRNA

and circulating protein concentrations throughout the ICU admission which is another first. This exploratory approach is advantageous for improving subsequent research questions by flagging what is pertinent for future investigations.

This study had a few limitations. Firstly, the sample size was limited by the inclusion of patients from the main feasibility trial into this sub-study, resulting in a relatively small sample. In addition, complete data was not available for patients at all pre-specified timepoints given the differing length of stay of included patients, reflecting the complex nature of intensive care research. Therefore, only participants with baseline and day 7 values were analysed for muscle wasting; this diminished our sample size by 50%. As ICU patients cannot provide a plasma and QMLT test prior to admission this could underestimate the amount of muscle loss and biomarker concentrations taken at baseline. This highlights another difficulty when studying patients in the ICU ward. Additionally, the increase in muscle gain reported from ultrasound images for some patients could be explained by the variation in pressure applied between operators or the accumulation of oedema within the muscle tissue, which is common in critical illness[54]. Lastly, no data was analysed on potential confounding factors which include, delivered calorie and protein dosages and assessment of muscle strength.

A perfect prognostic model for ICU-related muscle wasting does not exist. However, our exploratory study expands upon the current literature and broadens our current understanding beyond specific diagnostic groups to a more general ICU population acknowledging that acute muscle wasting and ICUAW can affect any critically ill patient. Our data showed that a potential pattern of change emerges in both circulating miRNA and proteins (miRNA-422a, GDF-15 and

IGF-1) which could reflect key modulating factors that drive muscle wasting in critical illness. These results advance our current understanding and offer an intriguing insight into the structural and functional changes of skeletal muscle. This permits improved targeting of certain interventions which ameliorate the progression of acute muscle wasting, thus avoiding the development of more severe ICU-related muscle disorders.

Conclusion

In this study, miR-422a, GDF-15 and IGF-1 concentrations were related to muscle wasting suggesting their potential role as biomarkers during ICU admission. This data paves the way for studies evaluating their use as predictors of muscle wasting in critically ill patients.

References

1. Williams TA, Dobb G, Finn J, Webb SAR. Long-term survival from intensive care: a review. *Intensive Care Med.* 2005;31(10):1306-1315.

2. Batt J, dos Santos CC, Cameron JI, Herridge MS. Intensive care unit-acquired weakness: clinical phenotypes and molecular mechanisms. *Am J Respir Crit Care Med.* Feb 1 2013;187(3):238-246.

3. Szakmany T, Walters AM, Pugh R, Battle C, Berridge DM, Lyons RA. Risk Factors for 1-Year Mortality and Hospital Utilization Patterns in Critical Care Survivors: A Retrospective, Observational, Population-Based Data Linkage Study*. *Critical Care Medicine.* 2019;47(1):15.

4. Intiso D, Amoruso L, Zarrelli M, et al. Long-term functional outcome and health status of patients with critical illness polyneuromyopathy. *Acta Neurol Scand.* Mar 2011;123(3):211-219.

5. Hashem MD, Nallagangula A, Nalamalapu S, et al. Patient outcomes after critical illness: a systematic review of qualitative studies following hospital discharge. *Crit Care.* Oct 26 2016;20(1):345.

6. van der Schaaf M, Beelen A, Dongelmans DA, Vroom MB, Nollet F. Poor functional recovery after a critical illness: a longitudinal study. *J Rehabil Med.* Nov 2009;41(13):1041-1048.

7. Koukourikos K, Tsaloglidou A, Kourkouta L. Muscle Atrophy in Intensive Care Unit Patients. *Acta Informatica Medica.* 2014;22(6):406-410.

8. Jolley SE, Bunnell AE, Hough CL. ICU-Acquired Weakness. *Chest.* Nov 2016;150(5):1129-1140.

9. Reeves ND, Maganaris CN, Narici MV. Ultrasonographic assessment of human skeletal muscle size. *Eur J Appl Physiol.* Jan 2004;91(1):116-118.

10. Guglielmi G, Ponti F, Agostini M, Amadori M, Battista G, Bazzocchi A. The role of DXA in sarcopenia. *Aging Clin Exp Res.* Dec 2016;28(6):1047-1060.

11. Parry SM, El-Ansary D, Cartwright MS, et al. Ultrasonography in the intensive care setting can be used to detect changes in the quality and quantity of muscle and is related to muscle strength and function. *Journal of Critical Care.* 2015;30(5):1151.e1159-1151.e1114.

12. Puthucheary ZA, Rawal J, McPhail M, et al. Acute skeletal muscle wasting in critical illness. *JAMA.* Oct 16 2013;310(15):1591-1600.

13. Chapple L-AS, Deane AM, Williams LT, et al. Longitudinal changes in anthropometrics and impact on self-reported physical function after traumatic brain injury. *Critical Care and Resuscitation.* 2017;19(1):29-36.

14. Herridge MS, Cheung AM, Tansey CM, et al. One-Year Outcomes in Survivors of the Acute Respiratory Distress Syndrome. *The New England Journal of Medicine.* 2003;348(8):683-693.

15. Herridge MS, Tansey CM, Matté A, et al. Functional Disability 5 Years after Acute Respiratory Distress Syndrome. *The New England Journal of Medicine.* 2011;364(14):1293-1304.

16. Ferrie S, Allman-Farinelli M, Daley M, Smith K. Protein Requirements in the Critically Ill: A Randomized Controlled Trial Using Parenteral Nutrition. *JPEN. Journal of parenteral and enteral nutrition.* 2016;40(6):795.

17. Fetterplace K, Deane AM, Tierney A, et al. Targeted Full Energy and Protein Delivery in Critically Ill Patients: A Pilot Randomized Controlled Trial (FEED Trial. *Journal of Parenteral and Enteral Nutrition.* 2018;42(8):1252-1262.

18. Schweickert WD, Pohlman MC, Pohlman AS, et al. Early physical and occupational therapy in mechanically ventilated, critically ill patients: a randomised controlled trial. *Lancet.* May 30 2009;373(9678):1874-1882.

19. Meesen RLJ, Dendale P, Cuypers K, et al. Neuromuscular Electrical Stimulation As a Possible Means to Prevent Muscle Tissue Wasting in Artificially Ventilated and Sedated Patients in the Intensive Care Unit: A Pilot Study. *Neuromodulation: Technology at the Neural Interface.* 2010;13(4):315-321.

20. Figueiredo VC, McCarthy JJ. Regulation of Ribosome Biogenesis in Skeletal Muscle Hypertrophy. *Physiology (Bethesda, Md.).* 2019;34(1):30.

21. Wen Y, Alimov AP, McCarthy JJ. Ribosome Biogenesis is Necessary for Skeletal Muscle Hypertrophy. *Exercise and sport sciences reviews.* 2016;44(3):110.

22. Lecker SH, Solomon V, Mitch WE, Goldberg AL. Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. *J Nutr.* Jan 1999;129(1S Suppl):227S-237S.

23. Glover EI, Phillips SM, Oates BR, et al. Immobilization induces anabolic resistance in human myofibrillar protein synthesis with low and high dose amino acid infusion. *Journal of Physiology.* 2008;586(24):6049-6061.

24. Biolo G, Fleming RYD, Maggi SP, Nguyen TT, Herndon DN, Wolfe RR. Inverse regulation of protein turnover and amino acid transport in skeletal muscle of hypercatabolic patients. *The Journal of clinical endocrinology and metabolism.* 2002;87(7):3378.

25. Gibson JNA, Poyser NL, Morrison WL, Scrimgeour CM, Rennie MJ. Muscle protein synthesis in patients with rheumatoid arthritis: effect of chronic corticosteroid therapy on prostaglandin F 2α availability. *European Journal of Clinical Investigation.* 1991;21(4):406-412.

26. Kumar V, Selby A, Rankin D, et al. Age-related differences in the dose–response relationship of muscle protein synthesis to resistance exercise in young and old men. *Journal of Physiology.* 2009;587(1):211-217.

27. Tipton KD, Elliott TA, Cree MG, Aarsland AA, Sanford AP, Wolfe RR. Stimulation of net muscle protein synthesis by whey protein ingestion before and after exercise. *American journal of physiology. Endocrinology and metabolism.* 2007;292(1):E71.

28. Fujita Y, Taniguchi Y, Shinkai S, Tanaka M, Ito M. Secreted growth differentiation factor 15 as a potential biomarker for mitochondrial dysfunctions in aging and age-related disorders. Vol 16; 2016:17-29.

29. Semba RD, Gonzalez-Freire M, Tanaka T, et al. Elevated Plasma Growth and Differentiation Factor 15 Is Associated With Slower Gait Speed and Lower Physical Performance in Healthy Community-Dwelling Adults. *The journals of gerontology. Series A, Biological sciences and medical sciences.* 2020;75(1):175.

30. Patel MS, Lee J, Baz M, et al. Growth differentiation factor-15 is associated with muscle mass in chronic obstructive pulmonary disease and promotes muscle wasting in vivo. *Journal of Cachexia, Sarcopenia and Muscle.* 2016;7(4):436-448.

31. Bloch SA, Lee JY, Syburra T, et al. Increased expression of GDF-15 may mediate ICU-acquired weakness by down-regulating muscle microRNAs. *Thorax.* Mar 2015;70(3):219-228.

32. Bloch SAA, Lee JY, Wort SJ, Polkey MI, Kemp PR, Griffiths MJD. Sustained elevation of circulating growth and differentiation factor-15 and a dynamic imbalance in mediators of muscle homeostasis are associated with the development of acute muscle wasting following cardiac surgery. *Critical care medicine.* 2013;41(4):982.

33. Wahid F, Shehzad A, Khan T, Kim YY. MicroRNAs: Synthesis, mechanism, function, and recent clinical trials. *BBA - Molecular Cell Research.* 2010;1803(11):1231-1243.

34. Paul R, Lee J, Donaldson AV, et al. miR-422a suppresses SMAD4 protein expression and promotes resistance to muscle loss. *J Cachexia Sarcopenia Muscle.* Feb 2018;9(1):119-128.

35. Donaldson A, Natanek SA, Lewis A, et al. Increased skeletal muscle-specific microRNA in the blood of patients with COPD. *Thorax.* Dec 2013;68(12):1140-1149.

36. Giangiuliani G, Mancini A, Gui D. Validation of a severity of illness score (APACHE II) in a surgical intensive care unit. *Intensive Care Medicine.* 1989;15(8):519-522.

37. Tillquist M, Kutsogiannis DJ, Wischmeyer PE, et al. Bedside ultrasound is a practical and reliable measurement tool for assessing quadriceps muscle layer thickness. *JPEN J Parenter Enteral Nutr.* Sep 2014;38(7):886-890.

38. Chen C, Ridzon DA, Broomer AJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res.* Nov 27 2005;33(20):e179.

39. Kroh EM, Parkin RK, Mitchell PS, Tewari M. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). *Methods.* Apr 2010;50(4):298-301.

40. Wu J, Cai H, Xiang YB, et al. Intra-individual variation of miRNA expression levels in human plasma samples. *Biomarkers.* May - Jun 2018;23(4):339-346.

41. Jespersen JG, Nedergaard A, Reitelseder S, et al. Activated protein synthesis and suppressed protein breakdown signaling in skeletal muscle of critically ill patients. *PLoS One.* Mar 31 2011;6(3):e18090.

42. Sharshar T, Bastuji-Garin S, De Jonghe B, et al. Hormonal status and ICU-acquired paresis in critically ill patients. *Intensive Care Med.* Aug 2010;36(8):1318-1326.

43. Heemskerk VH, Daemen MA, Buurman WA. Insulin-like growth factor-1 (IGF-1) and growth hormone (GH) in immunity and inflammation. *Cytokine Growth Factor Rev.* Mar 1999;10(1):5-14.

44. Prakash KR, Roshan MK, Mina F, Scott B, Harvey FL. Myogenic factors that regulate expression of muscle-specific microRNAs. *Proceedings of the National Academy of Sciences.* 2006;103(23):8721.

45. Meyer SU, Thirion C, Polesskaya A, et al. TNF-α and IGF1 modify the microRNA signature in skeletal muscle cell differentiation. *Cell communication and signaling : CCS.* 2015;13(1):4.

46. Sipila S, Suominen H. Ultrasound imaging of the quadriceps muscle in elderly athletes and untrained men. *Muscle Nerve.* Jun 1991;14(6):527-533.

47. Worsley PR, Kitsell F, Samuel D, Stokes M. Validity of measuring distal vastus medialis muscle using rehabilitative ultrasound imaging versus magnetic resonance imaging. *Manual Therapy.* 2014;19(3):259-263.
48. Bartel DP. MicroRNAs: Target Recognition and Regulatory Functions. *Cell.* 2009;136(2):215-233.
49. Sannicandro AJ, Soriano-Arroquia A, Goljanek-Whysall K. Micro(RNA)-managing muscle wasting. *Journal of applied physiology (Bethesda, Md. : 1985).* 2019;127(2):619.
50. Nakajima T, Shibasaki I, Sawaguchi T, et al. Growth Differentiation Factor-15 (GDF-15) Is a Biomarker of Muscle Wasting and Renal Dysfunction in Preoperative Cardiovascular Surgery Patients. *Journal of Clinical Medicine.* 2019;8(10).
51. Chen JF, Mandel EM, Thomson JM, et al. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet.* Feb 2006;38(2):228-233.
52. Conte M, Martucci M, Mosconi G, et al. GDF15 Plasma Level Is Inversely Associated With Level of Physical Activity and Correlates With Markers of Inflammation and Muscle Weakness. *Front Immunol.* 2020;11:915.
53. Luan HH, Wang A, Hilliard BK, et al. GDF15 Is an Inflammation-Induced Central Mediator of Tissue Tolerance. *Cell.* 2019;178(5):1231-1244.e1211.
54. Puthucheary Z, Montgomery H, Moxham J, Harridge S, Hart N. Structure to function: muscle failure in critically ill patients. *J Physiol.* Dec 1 2010;588(Pt 23):4641-4648.