Circulatory microRNAs are not effective biomarkers of muscle size and function in middle-aged men

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Abstract

Loss of muscle size and strength with aging are a major cause of morbidity. Whilst, muscle size and strength are measured by imaging or fiber cross-sectional staining and exercise testing respectively, the development of circulatory biomarkers for these phenotypes would greatly simplify identification of muscle function deficits. MicroRNAs (miRNAs) are short non-coding RNAs that regulate gene translation and thereby contribute to muscle phenotype. To assess circulatory miRNAs (c-miRNAs) applicability as potential biomarkers of muscular phenotypes, fasting plasma and muscle samples were obtained from 50 middle-aged healthy men (mean ± SD: age 48.8 ± 4.5 years, BMI 26.6 ± 3.3 kg/m^2). RT-PCR of 38 miRNAs with known regulatory function within skeletal muscle identified four c-miRNAs (miR-221, -451a, -361 and -146a) related to either total body lean mass, leg lean mass and 50% thigh cross sectional area (CSA) but not strength. There was no relationship with the expression of these miRNAs in muscle. 6 miRNAs within muscle were correlated with whole body lean mass, leg lean mass and isometric knee extension torque (miR-133a, -and 146a,), 50% thigh CSA (miR-486, -208b, -133b and -208a). Only miR-23b demonstrated a relationship between tissue and circulatory expression, however only 10% of the variance was explained. miR-146a in both plasma and muscle was related to phenotype, however no relationship between plasma and muscle expression was evident. A different subset of miRNAs related to muscle phenotype in muscle compared to plasma samples suggesting that c-miRNA biomarkers of muscle phenotype are likely unrelated to muscle expressions in healthy individuals.

Keywords: miRNA, skeletal muscle, circulatory microRNAs, biomarkers, RT-PCR

Introduction

Skeletal muscle strength and size are important determinants of physical function and mobility in the elderly. Reduced muscle mass and physical function often become clinically relevant with sarcopenia onset resulting in increased falls and fractures risk and a reduced quality of life (15). In addition to declines in muscle size; changes in composition, architecture and innervation, together explain the greater observed loss of muscle strength than size (25, 27). Whilst severe phenotype impairments with age generally present in later age, it is believed declines in muscle mass may commence as early as the 5th decade of life.

Central to the determination of the complex processes that regulate muscle mass is a range of miRNA species thought to inhibit translation of specific mRNAs involved in muscle phenotype regulation. Differences in expression of pri-miRNAs, miR-1,-133 and -206 were identified in young compared to aged muscle (11). However, miR-451a, -15a and 16 were elevated in aged muscle (1, 2). These miRNAs inhibit cyclin-dependent cell cycle activity and along with miR-126 regulate angiogenesis (9, 13). miR-1, -133a, -206, -486 and let -7 family miRNAs mediate satellite cell-dependent muscle remodeling and repair (4, 10, 29). miR-23a/b inhibit atrophy via downregulation of catabolic factors, MuRF1 and Atrogin1 (30) and correlations between miR-23a and Atrogin1 miRNA expression were demonstrated *in-vivo* following an atrophic stimulus (8). Several miRNAs mentioned above and others have additionally been implicated in the regulation of atrophy (8), dystrophy (19), cancer cachexia (17) and in various aspects of muscle regulation contributing to muscle size and strength.

In an increasingly diverse range of cancers, organ system diseases including muscular dystrophy and cardiovascular diseases, analyses of circulatory miRNA (c-miRNAs) profiles have provided proxies for risk prediction, disease severity and diagnosis. While factors measured, including miRNA, within skeletal muscle are likely the best predictors of muscle

health and function, the process of skeletal muscle biopsy collection is invasive and not possible in all population groups (12). It is likely that a subgroup of c-miRNAs may be directly secreted from muscle and therefore might reflect intramuscular miRNA expression or muscle phenotype. Given that plasma is a more readily available sample matrix than muscle tissue, plasma biomarkers of muscle phenotype are an attractive prospect. As yet, two studies have identified c-miRNAs as potential biomarkers of whole body aerobic capacity following periods of aerobic training in healthy individuals (3, 24).

The study aimed to identify from a cohort of 50 healthy middle-aged men, the ability of c-miRNA or intramuscular miRNAs to act as predictive biomarkers of skeletal muscle mass, size and strength. An a priori set of 38 previously established target miRNA species regulating key processes related to muscle size and function were included. The secondary aim was to determine if a relationship exists between microRNA expression of muscular and circulatory compartments in order to assess the ability of c-miRNA to function as proxies of miRNA measurements made directly from skeletal muscle.

Methods

Participants and Sample Collection

50 healthy middle-aged men (mean ± SD: age 48.8 ± 4.5 years, BMI 26.6 ± 3.3 kg/m^2) were recruited to the study. Participants underwent strength testing and muscle imaging analyses prior to participating in two separate trials (21, 23) which were registered with the Australia New Zealand Clinical Trial Registry# ACTRN12615000454572 and ACTRN12615001375549 on 11th May 2015 and 17th December 2015 respectively. Participants were sedentary to recreationally active, did not perform regular resistance exercise and were free from any metabolic, oncological or neuromuscular injury or disease. Written consent was obtained prior to study commencement which was approved by the Northern Health and Disability Ethics Committee (New Zealand), (15/NTB/154/AM01 and 14/NTA/146).

Participants were instructed not to perform any exercise or strenuous activity for >48 hours prior to the trial day to ensure sampling was not affected by previous activity. Following an an overnight fast, participants arrived at the laboratory at ~7 am for the trial day.

Imaging

As previously described , whole body dual energy x-ray absorptiometry (DXA, Lunar Prodigy, GE, Waltham, MA, USA) scans were performed with automatic segmentation by Lunar Prodigy encore 2007 Version 11.40.004 (GE, Madison, WI, USA), and tissue regions were defined by lines positioned on the image, allowing measures of total body lean mass and leg lean mass to be determined (21, 23).

Muscle cross sectional area (CSA) at 50% of femur length was assessed using a Stratec XCT 3000 peripheral quantitative computed tomograph (pQCT) with software version 6.20C

(Stratec Medizintechnik, Pforzheim, Germany). pQCT CSA measures were determined as per (21), and were analyzed by the same operator.

Muscle function

Isometric knee extensor strength was measured using a Biodex dynamometer (Shirley, New York, United States) with the knee angle set to 90º of flexion. Three maximal isometric knee extensions of 5 s each with 30 s of rest was completed. The highest values were used for analysis.

Blood sampling

A cannula (20-gauge) was inserted into an antecubital vein from which 10 mL of plasma was collected into an EDTA-vacutainer. Samples were centrifuged immediately upon collection at 4°C at 1900 g for 15 minutes. The supernatant was collected in 1.6 mL sterile tubes as 1 mL aliquots and stored at -80^{0}C.

Muscle biopsy sampling

Muscle biopsies (~100 mg) were collected at rest from the *vastus lateralis* under local anesthesia (1% Xylocaine) using a Bergstrom needle modification of manual suction. Biopsies were frozen in N_2 and stored at -80°C.

miRNA isolation

Muscle miRNA was extracted from ~20 mg of tissue using the AllPrep® DNA/RNA/miRNA Universal Kit (QIAGEN GmbH, Hilden, Germany). Plasma miRNA was isolated from 200 µL plasma as per (6). 10 pg of the exogenous spike-in cel-miR-39 and cel-miR-238 were added to samples prior to extraction and cDNA synthesis to account for variation in sample preparation.

Muscle and circulatory miRNA cDNA/RTPCR

RNA from muscle and plasma were used for cDNA synthesis using TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) and miRNA abundances were measured by RT-PCR on a QuantStudio 6 (Thermo Fisher Scientific, Carlsbad, CA, USA) using Applied Biosystems Fast Advanced Master Mix (Thermo Fisher Scientific, Carlsbad, CA, USA).

Target miRNAs described in table 1 (Thermo Fisher Scientific, Carlsbad, CA, USA), were chosen from a literature search for miRNAs suggested to regulate muscle function, myogenesis or be altered following exercise. Probes performance and CT assessment were carried out as per (6). The geometric mean of three reference miRNAs (miR-361, -191 and -186) for muscle and four for plasma (miR-191 and -186, -320a and -423) were used for normalization using miRNAs that showed least variance in the current sample set. The CT mean ± SD for each geomean was 26.28 ± 0.67 cycles in muscle and 27.06 ± 0.81 in plasma respectively. Data was analyzed using the $2^{-\Delta CT}$ method (26). For plasma samples, hemolysis was assessed using differences between miR-451a and miR-23a-3p with a CT difference >7 used as the cut off for sample hemolysis, as values lower than 7 indicate little to no hemolysis.

Statistical analysis

Stepwise linear regressions were used to examine the relationships between measures of phenotype including total body and leg lean mass, isometric knee extension strength, age and 50% thigh muscle CSA as dependent variables with miRNAs of interest as independent variables (IBM SPSS Version 23 (IBM Corp. USA)). Alpha was set as <0.05. Graphs show the miRNAs with the strongest relationship to each phenotype measure. Independent R^2 for these miRNAs are expressed in their respective graphs.

Results

Plasma miRNAs related to phenotype

No relationships were observed between any measures of muscle phenotype and chronological age so all analysis was conducted on the full cohort without age adjustment. Linear regression of plasma miRNA expression indicated a positive relationship between age and c-miR-146a (R^2 = 0.0938 and p=0.038) (Figure 1A). Total body lean mass was significantly negatively correlated with c-miR-451a expression (R^2 = 0.193 and p=0.002) (Figure 1B). c-miR-451a was best negatively correlated with leg lean mass (Figure 1C) and together with miR-146a which demonstrated a positive relationship, predicted ~25% of subject variability (R^2 = 0.252 and p=0.002) (Table 2) while 50% thigh muscle cross sectional area was most strongly negatively correlated with c-miR-361 (Figure 1D), together with c-miR-222 which was positively related with thigh CSA, predicted ~40% of the observed participant variance(R^2 = 0.392 and p<0.001) (Figure 1D). Isometric knee extension strength showed no relationship to abundance of any of the c-miRNAs analyzed in the present study. Beta and p-values for multiple miRNA independently correlated with a single phenotype measure are presented in Table 2.

Muscle miRNAs related to phenotype

Linear regression analyses indicated a significant positive relationship between muscle let-7d-5p expression and age (R^2 = 0.175 and p=0.0047) (Figure 2A). Lean mass was best found to positively correlate with miR-146a (Figure 2B) and together with miR-133a which demonstrated a negative relationship, predicted ~34% of participant variability. miR-133a was found to best correlate negatively lean leg mass (Figure 2C) and together with miR-146 which had a positive correlation, explained ~33% of the observed participant variance. 50% thigh muscle CSA was positively associated with miR-486 and -208a but negatively related

with miR-208b and -133b. The combined expression of these miRNAs explained roughly ~51% of subject variance ($R^2 = 0.509$ and $p<0.001$) of which, miR-486 was the most strongly related to this measure (positively) (Figure 2D). Isometric knee extension strength was found to best correlate negatively with miR-133a and (Figure 2E) together with miR-146a which demonstrated a positive relationship explained ~20% participant variability ($R^2 = 0.203$ and $p=0.002$) Beta values and p-value for independent c-miRNAs significantly correlated with phenotype are presented in Table 2.

Muscle miRNA to plasma miRNA abundance correlates

Only miR-23b showed a significant relationship between fasted, non-exercised muscle and plasma miRNA abundances ($R^2 =0.102$ and $p =0.030$).

Discussion

The current cohort of middle-aged men showed a ~1.7 fold range in total body lean mass, a ~1.9 fold range in leg lean mass, ~3.3 fold range in 50% thigh CSA and ~3.3 fold spread in peak knee extensor torque. The chronological age of the participants (38-57 years) did not affect any of the muscle phenotype measures and therefore other factors would be responsible for the observed variance in phenotype.

c-miRNAs related to phenotype

c-miR-222 was positively related with thigh muscle CSA. However, as c-miR-222 expression predicts less than 25% of the observed phenotype variance, it has limited utility as a biomarker. c-miR-451a was negatively related to total body lean mass and leg lean mass and c-miR-361 was negatively related with thigh CSA. Previously, miR-451a was shown to be elevated in aged muscle of non-human primates (20). There was however, no relationship to participant age in the current cohort of men. Given the disconnect between muscle and circulatory miRNA content, it is not surprising that abundances of c-miR-451a were unrelated to participant age.

miR-146a, was elevated in muscle of individuals with increased total body lean mass, leg lean mass, increased 50% thigh CSA and knee extensor strength. However, c-miR-146a was found to positively relate with age and lean leg mass. Oxidative stress is thought to increase with age and the relationship between c-miR-146a and age is consistent with other reports (28) and may reflect oxidative stress. The current analyses are limited however due to miRNAs being promiscuous molecules capable of being transcribed, released and functioning in multiple tissue types making it impossible to isolate whether the observed c-miRNAs trends are indeed reflective of muscle regulation or whether they are driven by miRNA expression in non-muscle tissues. It is also possible that miRNA may play only an indirect

secondary role in muscle regulation or that these miRNAs are simply the product of metabolism and are biomarkers without regulatory function.

Intramuscular miRNAs related to phenotype.

Of the eight previously identified canonical myomiRs in muscle, miR-133a, -133b, -208a, -208b and -486 were all related to measures of muscle size and function. miR-133a was negatively related with whole body muscle mass, leg muscle mass and isometric knee extensor strength. miR-486 and 208a were positively related with 50% thigh CSA whilst miR-133b and -208b demonstrate a negative relationship with the same measure. miR-208a/b are involved in regulating muscle fiber type expression and therefore it seems reasonable that these miRNAs would be reflective of muscle size measures (9, 14). miR-133a and -486 are crucial regulators of satellite cell dependent muscle repair and remodeling via inhibition of PAX7 (4, 10, 29), this presents a plausible mechanism for the observed relationship with muscle phenotype. These data are consistent with our previous demonstration of miR-133a's negative but non-significant trend with both muscle strength and CSA ($p=0.078$ and $p=0.101$ respectively) in a similar cohort of middle-aged men via miRNA sequencing analyses (22). Likewise in overload induced plantaris hypertrophy in rats following surgical soleus and gastrocnemius, miR-133a was found to negatively correlate with muscle mass (16). Additionally, lower miR-133a expression was evident in powerlifters compared to healthy controls (5). Together these findings suggest a consistent negative relationship between miR-133a expression and skeletal muscle size across multiple studies and models.

The observed cross-sectional relationships fit well with previous longitudinal intervention studies. However, predictors of age and lean mass within muscle only explained ~33% of the observed variance, and ~50% of thigh CSA. The present study is potentially limited by the lack of inclusion of a dystrophic, sarcopenic or elderly group. The findings suggest a clear

potential for the utility of intramuscular miRNA content when explaining thigh muscle phenotype and identify key miRNA targets for future mechanistic studies of muscle phenotype regulation.

Applicability of c-miRNAs as phenotype biomarkers

Of the 38 miRNAs assessed, only miR-23b expression demonstrated a weak relationship between plasma and muscle samples. Only miR-146a abundance related to phenotype in both circulation and muscle, but no relationship was evident between sample matrices ($R^2 = 0.011$ and $p=0.493$). Additionally, the inability of any c-miRNA to explain variance in muscle strength further highlights the limited capacity for c-miRNA abundances to act as effective proxies of muscle miRNA expression. To date, most studies that report relationships between muscle phenotype and miRNAs expression involve disease states or periods of exercise training. The model employed by the present study of fasted and rested sampling in men free from major chronic or acute illness and showing large variations in muscle strength allowed for muscle phenotype *per se* to be isolated from possible confounders such as age, cachexia or organ system disease. The present findings indicate that the measured c-miRNAs selected due to their enrichment in muscle, role in myogenesis or exercise responsiveness cannot be used, in our cohort of middle-aged men, as proxy for measurements made directly from sampling muscle tissue or to function as biomarkers of muscle strength. Independently the best c-miRNA predictor of muscle phenotype was miR-451 which predicted ~20% of the subject variance in whole body lean mass, however, no c-miRNA effectively predicted muscle strength. The best muscle miRNA predictor of phenotype was miR-486 which also predicted ~20% of variance in 50% thigh muscle CSA. These data suggest that plasma miRNA are stronger predictors of whole body muscle mass compared to muscle miRNA predictors which better relate to the muscle size and function of the muscle which they are isolated from. Additionally this does not preclude the possibility that these and other non

analysed c-miRNAs could become important predictors of muscle function and disease progression in conditions such as myopathies, dystrophies or cancer cachexia.

Most miRNAs are abundant in multiple tissues for example, miR-451a is highly expressed in skeletal muscle, erythrocytes, thyroid, spleen, liver and brain (18). The lack of tissue specificity for all but an extremely limited set of miRNAs make it impossible to identify the tissue source of c-miRNAs and therefore limits the ability to make inferences about the mechanistic functions from c-miRNAs analyses. Emerging evidence suggests most c-miRNAs are riboprotein bound with a small proportion being associated with exosomes that contain a miRNA profile unique to both muscle and plasma (7). Taken together with the present study it appears that circulating and exosomal miRNA levels are regulated by the cell state within secreting tissues rather than simple changes in tissue concentrations. Future studies ought to characterize miRNA content within muscle specific exosomes which may better identify circulatory miRNA biomarkers for muscle size and strength.

Conclusions

The current findings agree with previous work suggesting relationships between expression of the myomiRs particularly miR-133a and miR-146a and muscle phenotype. There was no evidence that the selected subset of c-miRNAs are related to their intramuscular expression. c-miRNAs showed a similar ability to intramuscular miRNA to predict whole body muscle mass however, only intramuscular miRNA showed relationships with local muscle size and strength. The identified miRNAs should be investigated further a potential mechanistic regulators of muscle phenotype however, given that the strongest individual miRNA accounted for ~20% of the variance in muscle phenotype and the strongest combination of miRNAs accounted for ~50% of the variance in muscle phenotype the clinical utility of these miRNAs as biomarkers of muscle size and function in healthy men is currently limited.

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Contributions

The study was designed by CJM, DC-S, SDP and RFD. Performed experiments: RFD and NZ. Sample Collection: RFD and CJM Analysed data: RFD. Critically evaluated and contributed to the manuscript: RFD, NZ, SDP, CJM, and DC-S. CJM is responsible for the final content of the manuscript.

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Disclosure Statement

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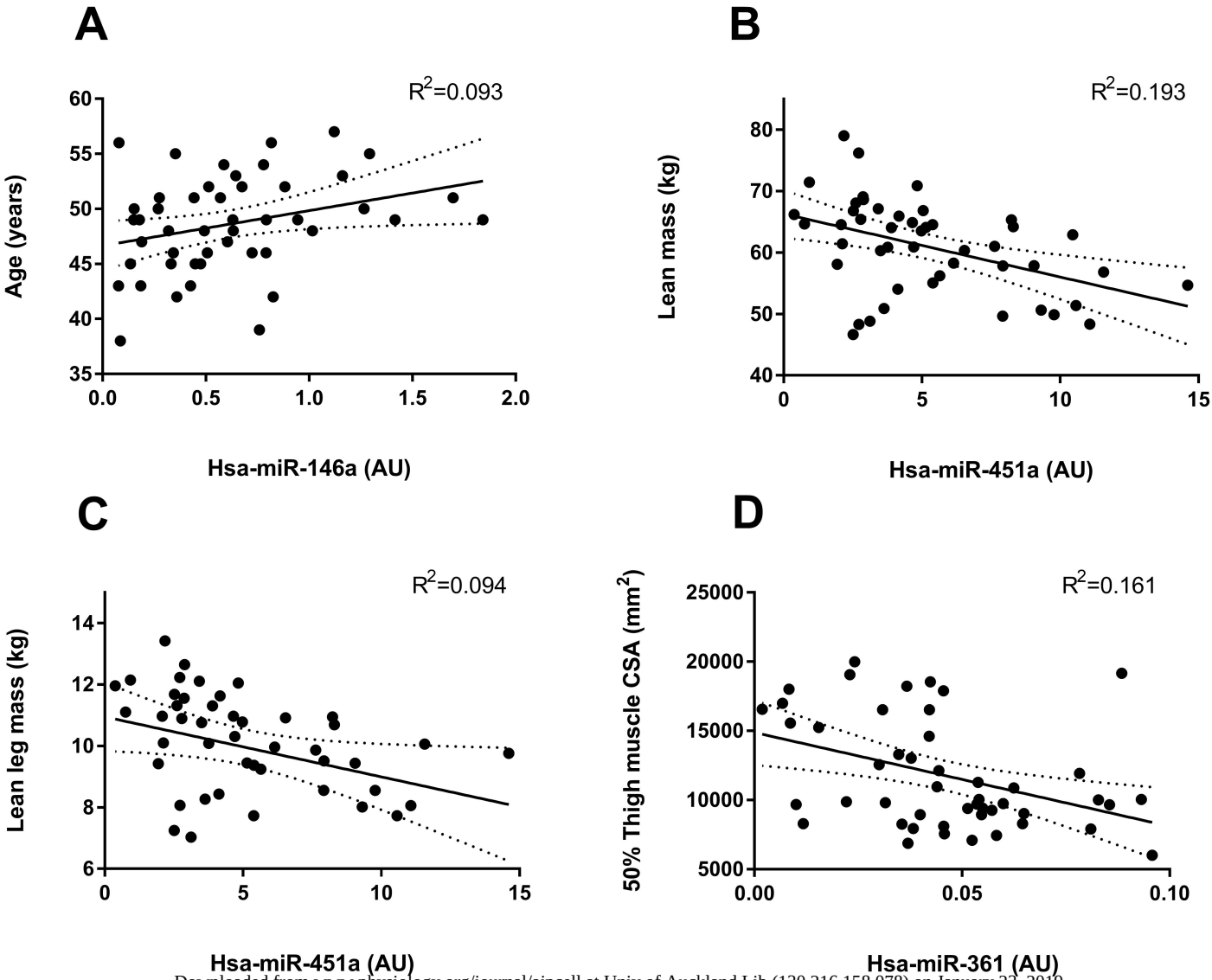
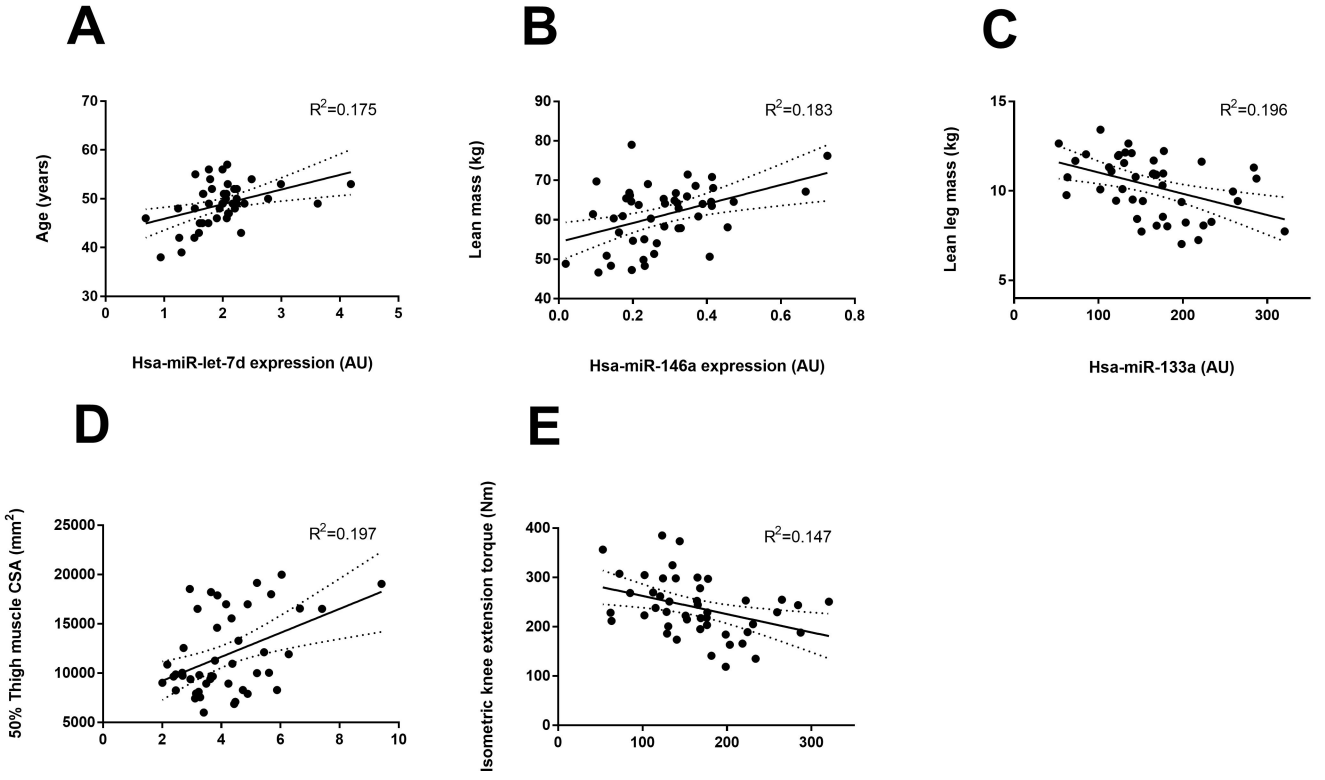
400 **Figure legends**

401 **Figure 1: Strongest c-miRNA related to participant** (**A**) Age, (**B**) lean mass, (**C**) 50%
402 thigh CSA and (**D**) lean leg mass. miRNAs are plotted as $2^{-\Delta CT}$ on the x-axis. The solid line
403 represents the line of best fit as determined by linear regression with 95% confidence
404 intervals.

405

406 **Figure 2: Strongest muscle miRNAs related to participant** (**A**) Age, (**B**) lean mass, (**C**) leg
407 lean mass, (**D**) 50% thigh CSA and (**E**) isometric knee extension torque. miRNAs included in
408 each model plotted as $2^{-\Delta CT}$ on the x-axis. The solid line represents the line of best fit as
409 determined by linear regression with 95% confidence intervals.





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hsa-miR-361-5p	478056_mir
hsa-miR-126-3p	477887_mir
hsa-miR-191-5p	477952_mir
hsa-miR-145-5p	477916_mir
hsa-miR-101-3p	477863_mir
hsa-miR-149-5p	477917_mir
hsa-miR-146a-5p	478399_mir
hsa-miR-499a-3p	477916_mir
hsa-miR-26a-5p	477995_mir
hsa-miR-29b-3p	478369_mir
hsa-miR-486-5p	478128_mir
hsa-miR-451a	477968_mir
hsa-miR-208b-3p	477806_mir
hsa-miR-208a-3p	477819_mir
hsa-miR-206	477968_mir
hsa-miR-133b	480871_mir
hsa-miR-133a-3p	478511_mir
hsa-miR-1-3p	477820_mir
hsa-miR-222-3p	477982_mir
hsa-miR-221-3p	477981_mir
hsa-miR-98-5p	478590_mir
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hsa-miR-378a-5p	478076_mir
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hsa-miR-21-5p	477975_mir
hsa-miR-30b-5p	478007_mir
hsa-miR-148b-3p	477806_mir
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hsa-miR-16-5p	477860_mir
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hsa-miR-99a-5p	478519_mir
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hsa-miR-100-5p	478224_mir
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hsa-let-7b-5p	478576_mir
hsa-let-7c-5p	478577_mir
hsa-let-7d-5p	478439_mir
hsa-let-7e-5p	478579_mir
hsa-let-7g-5p	478580_mir
hsa-miR-423-5p	478090_mir
hsa-miR-186-5p	477940_mir
hsa-miR-320a-5p	478594_mir

cel-miR-39-3p	478293_mir
cel-miR-238	478292_mir

Table 1. miRNAs. miR classification, catalogue and order identification number.

	β	P-value
Lean mass		
miR-146a	0.421	0.003
miR-133a	-0.406	0.003
Lean leg mass		
c-miR-146a	0.226	0.049
c-miR-451a	-0.395	0.006
miR-133a	-0.461	0.001
miR-146a	0.344	0.014
50% Thigh muscle area (mm2)		
c-miR-222	0.482	0.019
c-miR-361	-0.473	0.005
miR-486	0.555	<0.001
miR-208b	-0.543	0.001
miR-133b	-0.287	0.021
miR-208a	0.315	0.037
Isometric knee Extension		
miR-133a	-0.331	0.026
miR-146a	0.299	0.044

Table 2: Regression model included c-miRNAs and intramuscular miRNAs that correlate with phenotype. (Independent β and p-values). c-miR indicates circulatory miRNAs, miR indicates muscle miRNAs.