The Severity of Muscle Performance Deterioration in Sarcopenia Correlates With Circulating Muscle Tissue-Specific miRNAs

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Summary

Sarcopenia is defined as an age-associated loss of skeletal muscle function and muscle mass and is common in older adults. Sarcopenia as a disease is currently of interest not only to orthopedists and surgeons but also to internists, endocrinologists, rheumatologists, cardiologists, diabetologists, gynaecologists, geriatricians and paediatricians. In cooperation with the 5th Internal Medicine Clinic, we, as a unit of clinical research, aimed to describe a sarcopenic specific miRNA expression profile for disease diagnostics and classification of the severity of muscle performance deterioration. This study included a total of 80 patients (age 55-86 years) hospitalized at the V. Internal medicine clinic of LFUK and UNB with different severity of muscle performance deterioration. The study participants were evaluated and classified according to short physical performance battery score (SPPB). In this study, we investigated the role of circulating miRNAs in sarcopenia in the elderly. We hypothesized that sarcopenia effects the expression of muscle tissue-specific miRNAs (MyomiRNAs), which could be potentially reflected in the blood plasma miRNA expression profile. The expression of specific circulating miRNAs in patients with different muscle performances was analyzed. Patients' blood plasma was evaluated for the expression of myomiRNAs: miRNA-29a, miRNA-29b, miRNA-1, miRNA-133a, miRNA-133b, miRNA-206, miRNA-208b and miRNA-499, and the data were correlated with diagnostic indicators of the disease. We showed a specific sarcopenia miRNA profile that could be considered a possible biomarker for the disease. Patients with low muscle performance showed increased miRNA-1, miRNA-29a and miRNA-29b expression and decreased for the miRNA-206, miRNA-133a, miRNA-133b, miRNA-208b and miRNA-499 expression. We show that the severity of muscle performance deterioration in sarcopenia correlates with specific miRNA expression. We also propose the profile of miRNAs expression in blood plasma as a specific biomarker for sarcopenia diagnostics. Future clinical studies will be necessary to eventually naturally have to elucidate the underlined molecular mechanism responsible for specific miRNAs expression in sarcopenia pathology and progression of the disease.

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Introduction

Sarcopenia was recognized in 2016 as a disease by the World Health Organization (WHO)'s International Statistical Classification of Diseases and Related Health

Problems (ICD), however the first steps of diagnosis date to the 1980s. In 1989, Rosenberg first proposed the term "sarcopenia" (from the Greek "sarx" for flesh and "penia" for loss) to define a typical age-associated decrease in muscle mass. Today sarcopenia is defined as an age-associated loss of skeletal muscle function and muscle mass and is common in older adults (Dent *et al.* 2018, Greco *et al.* 2019). The overall prevalence of sarcopenia is estimated at approximately 6-22 % in adults aged 65 years and increases with age. It is already clear that with such an aging population, the incidence of this disease is increasing (Ethgen *et al.* 2017). Sarcopenia is associated with increased adverse outcomes including falls, nuctional decline, frailty, and mortality (Björkman *et al.* 2019). The recognition of sarcopenia as a disease has led to significant advances in research, screening and diagnosis. Sarcopenia is diagnosed based on muscle mass, muscle strength and physical performance assessments. Even though no sarcopenia treatment is currently available, early diagnosis is essential as it reduces the risk of falls, fractures, functional decline, hospitalization and mortality associated with this condition. Management of sarcopenia involves a variety of approaches (Dionyssiotis 2019). Sarcopenia also reduces the amount of metabolically active tissue, which means that it increases metabolic diseases, including cardiovascular diseases, diabetes, hypertension and hyperlipidemia, and other diseases in the elderly. This will bring a heavy economic burden to the medical service system (Biolo *et al.* 2014).

Sarcopenia in chronic illness

Sarcopenia is classified as primary sarcopenia when the onset is related to the aging process and secondary, which is related to comorbidities, malnutrition, or immobility. Sarcopenia comorbidities include organ failure (e.g. heart, kidneys, lungs, brain and liver), inflammatory diseases, cancer, and endocrine diseases. Malnutrition due to gastrointestinal disorders, anorexia in cases of polypharmacy, and inadequate intake due to psychosocial disorders can also result in secondary sarcopenia. Finally, inactivity as a result of postoperative deconditioning or prolonged rehabilitation can also result in activity-related secondary sarcopenia (Saggini *et al.* 2017).

Sarcopenia and microRNA

MicroRNAs (miRNA) are promising RNA molecules for targeted diagnosis and treatment in the 21st century. This group of RNAs plays a key role in a wide range of physiological and pathological processes. MiRNAs are non-coding regulatory RNA molecules of 20-30 nucleotides in length that regulate gene expression at the post-transcriptional level (Brown a Goljanek-Whysall 2015). Growing amount evidence of suggests that circulating miRNAs present in serum or plasma are remarkably stable, easily detectable and can indicate a variety of health changes. It is believed miRNAs presence in serum or plasma can be used as potential biomarkers for the diagnosis and treatment of various diseases. A growing amount of evidence supports circulating miRNAs as a potential therapeutic biomarker for sarcopenia as the expression of specific circulating miRNAs is associated with muscle loss in the elderly (Siracusa *et al.* 2018).

In this study, we investigated the role of circulating miRNAs in sarcopenia in the elderly. We analysed the expression of specific circulating miRNAs in patients with different muscle performances. We hypothesized that sarcopenia effects the expression of muscle tissue-specific miRNAs (MyomiRNAs), which could be potentially reflected in the blood plasma miRNA expression profile. We analysed the expression of myomiRNAs: miRNA-29a, miRNA-29b, miRNA-1, miRNA-133a, miRNA-133b, miRNA-206, miRNA-208b and miRNA-499 in patient's blood plasma. We correlated these expression levels with diagnostic indicators of sarcopenia and we showed a specific miRNA sarcopenia profile that could be a possible biomarker.

Materials and methods

The study has been following the proceedings of the Declaration of Helsinki and further amendments. The study protocol and the informed consent form have been approved by the ethics committee of the University Hospital in Bratislava. Study participants signed informed consent before any procedure and agreed with anonymized storage and publication of data.

Participants

This study included a total of 80 patients (age 55-86 years) hospitalized at the 5th Internal medicine clinic of LFUK and UNB with different severity of muscle performance deterioration. The study participants were evaluated and classified according to short physical performance battery score (SPPB). The short physical performance battery score is the sum of points from three tests: 1. Chair-stands (s), the time necessary for 5 chair stands without the help of hands, 2. Balance test (s), time

the patient maintains the balance having feet together, at the semi-tandem and tandem position for maximum 10 seconds in each, 3. Gait Speed, by measuring the time necessary to walk 5 meters (m/s). Participants were divided into 3 groups according to the SPPB score, evaluated at the sum of individual tests ranging from 0 (not possible) to 4 (best performance). The maximum score is 12 (Table 1). The initial clinical parameters of the patients in the individual groups are shown in Table 2.

Table 1. Division of patients into groups according to the SPPB score.

Group	SPPB score	Muscle performance
Group 1	SPPB ≤ 6	low muscle performance
Group 2	SPPB 7-9	moderate muscle performance
Group 3	SPPB > 9	high muscle performance

Table 2. Gender, age and BMI distribution of the 3 studied sarcopenia patient groups. The data is shown at mean ± SD.

Clinical parameters	male	female
I. Sarcopenia group 1 (n=31) SPPB ≤ 6		
Genger	18	13
Age (years)	70.61 ± 8.51	75.46 ± 7.68
BMI (kg/m^2)	30.16 ± 7.97	27.9 ± 3.89
II. Sarcopenia group 2 (n=17) SPPB 7-9		
Genger	6	11
Age (years)	64.33 ± 10.89	69.64 ± 6.84
BMI (kg/m^2)	26.75 ± 2.57	30.28 ± 7.32
III. Sarcopenia group 3 (n=32) SPPB > 9		
Genger	16	16
Age (years)	64.81 ± 6.44	66.56 ± 6.27
BMI (kg/m^2)	27.4 ± 3.6	27.9 ± 3.9

Plasma sampling

Venous blood was collected into silicone-coated serum tubes with enhanced silica-containing clot activator 1/2h after exercise and measurement of anthropometric parameters. At 4 °C, blood samples were centrifuged at 3,500 rpm. Within 10 minutes plasma and erythrocytes were separated. Plasma was immediately aliquoted and frozen at -80 °C until further analyses.

MiRNA analysis
RNA isolation

Before isolation, the plasma was dissolved at room temperature. For stability, repeated freezing and thawing cycles of serum samples were minimized. Isolation was performed on the miRNeasy Serum / Plasma Advanced Kit. Control ce-miRNA-39 (miRNeasy serum/plasma Spike-In control) was added to the process. The process resulted in total RNA eluate from 200 µl of the patient's blood plasma.

Reverse RNA transcription

Commercially available miRCURY LNA RT Kit (Qiagen) was used for reverse transcription according to the manufacturer's instructions. The resulting cDNA was further analyzed by qPCR.

qPCR method

The target sequence was analyzed by qPCR miRCURY LNA SYBR® Green PCR Kits (Qiagen). The PCR reaction was performed in a StepOnePlus Real-Time PCR System (Life Technologies, USA). Table 3 lists the PCR primers used in this study.

Statistical analysis

Statistical evaluation was performed by Graphpad using one way ANOVA test and Chi test. Significant changes were considered to be those at which $p<0.05$.

Table 3. List of microRNA primers (Qiagen GmbH, Germany)

microRNA	LNA PCR primer ID	microRNA	LNA PCR primer ID
cel-miR-39-3p	YP00203952	hsa-miR-133a-3p	YP00204788
hsa-miR-1-3p	YP00204344	hsa-miR-133b	YP00206058
hsa-miR-206	YP00206073	hsa-miR-208b-3p	YP00204636
hsa-miR-29a-3p	YP00204698	hsa-miR-499-5p	YP00205935
hsa-miR-29b-3p	YP00204679		

Table 4. The values of SPPB test for 3 studied sarcopenia patient group. Each participant underwent a measurement: grip strength, balance, walking speed and chair stand test. The data is shown at mean ± SD.

Anthropometric indexes	male	female
I. Sarcopenia group 1 (n=31) SPPB ≤ 6		
Grip strength	20.24 ± 6.51	14.49 (± 3.40)
Balance (s)	22.61 (± 4.98)	17.61 (± 6.37)
Walking speed (m/s)	0.47 (± 0.24)	0.45 (± 0.17
Chair stand test (5x)	22.11 (± 8.07)	19.74 (± 7.46)
II. Sarcopenia group 2 (n=17) SPPB 7-9		
Grip strength	28.61 (± 3.89)	16.76 (± 3.26)
Balance (s)	26.5 (± 3.51)	26.36 (± 3.75)
Walking speed (m/s)	0.83 (± 0.33)	0.75 (± 0.24)
Chair stand test (5x)	15.25 (± 5.21)	14.35 (± 1.91)
III. Sarcopenia group 3 (n=32) SPPB > 9		
Grip strength	31.52 (± 9.80)	19.08 (± 5.85)
Balance (s)	29.44 (± 2.25)	29.44 (± 1.79)
Walking speed (m/s)	1.03 (± 0.23)	1.00 (± 0.29)
Chair stand test (5x)	10.75 (± 1.87)	11.82 (± 2.43)

Results

In this study, we intended to determine whether the severity of muscle performance deterioration in sarcopenia patients correlates with specific blood plasma miRNA expression profiles. The clinical characteristics for all subjects are shown in Table 1. The patients were divided into 3 groups based on SPPB score (muscle performance deterioration) determined by anthropologic parameters including grip strength, balance, walking speed and chair stand test (Table 4). Biochemical parameters of the clinical study participants were included in the analysis however we did not identify any correlation with the severity of the SPPB score (Table 5). From all the studied clinical parameters participants age alone correlated with the SPPB score. Group 1 showed the highest age (70.61 ± 8.5 male, 75.46 ± 7.68 female).

In each group, we analyzed the relative expression of miRNA-1, miRNA-29a, miRNA-29b, miRNA-133a, miRNA-133b, miRNA-206, miRNA-208b and miRNA-499 standardized to ce-miRNA-39. The results are shown in Fig. 1.

We showed increased miRNA-1 expression in group 1, patients with the lowest muscle performance, however, the difference was not significant. We observed a significant increase in miRNA-1 expression only between groups 2 and 3. However, we observed a decrease in miRNA-206 expression in the group of patients with the weakest muscle performance (Fig. 1A). The expression of miRNA-133a and miRNA-133b decreased in the group of patients with low muscle performance (Fig. 1B). In the miRNA-29 family, we did not observe any significant changes in either miRNA-29a expression or miRNA-29b (Fig. 1C). The expression of miRNA-208b and miRNA-499 decreased in patients with the lowest muscle output (Fig. 1D).

Table 5. Biochemical parameters of all the studied sarcopenia patient group. The data is shown at mean ± SD.

| Biochemical parameters	male	female
I. Sarcopenia group 1 (n=31) SPPB ≤ 6		
Hemoglobin	118.94 (± 17.18)	120.05 (± 21.31)
Bilirubin	17.20 (± 12.85)	10.99 (± 7.21)
Urea	8.64 (± 5.55)	7.80 (± 5.05)
Kreatinin	110.28 (± 61.47)	125.01 (± 107.86)
CRP	49.02 (± 77.98)	15.12 (± 25.40)
D-vitamin	16.23 (± 9.56)	17.31 (± 7.00)
II. Sarcopenia group 2 (n=17) SPPB 7-9		
Hemoglobin	109 (± 21.50)	121.67 (± 17.17)
Bilirubin	47.07 (± 70.10)	21.03 (± 23.14)
Urea	11.00 (± 7.51)	10.13 (± 15.95)
Kreatinin	121.93 (± 68.91)	77.56 (± 19.81)
CRP	17.472 (±23.36)	8.61 (± 10.02)
D-vitamin	15.18 (±8.31)	17.75 (± 2.43)
III. Sarcopenia group 3 (n=32) SPPB > 9		
Hemoglobin	123.45 (± 29.20)	116.5 (± 15.73)
Bilirubin	144.06 (± 466.31)	15.24 (± 24.03)
Urea	5.66 (± 1.67)	4.70 (± 2.97)
Kreatinin	89.81 (± 27.57)	76.58 (± 16.84)
CRP	37.57 (± 67.06)	30.44 (± 35.17)
D-vitamin	19.91 (± 9.44)	17.65 (± 9.37)

Discussion

Aging of the musculoskeletal system is a common health problem today. Sarcopenia as a disease is currently of interest not only to orthopedists and surgeons but also to internists, endocrinologists, rheumatologists, cardiologists, diabetologists, gynecologists, geriatricians and pediatricians. Many unanswered questions give us room to examine more deeply the issue of this disease in terms of proper diagnosis and treatment, as well as clinical complications. In cooperation with the 5th Internal Medicine Clinic, we, as a unit of clinical research, aimed to describe a sarcopenic specific miRNA expression profile for disease diagnostics and classification of the severity of muscle performance deterioration. We examined blood plasma patient samples for expression of specific myomiRNA expression. We chose blood plasma analysis as it is generally preferred for diagnostic purposes, while an easily obtainable material in the clinical setting. Compared with muscle biopsy it is a significantly less invasive procedure for the patient.

In this study, we characterized and analyzed plasma samples from hospitalized patients diagnosed with sarcopenia. Multiple studies suggest that impaired expression of myogenic miRNAs may result in the development of various pathological conditions of muscle apparatus. The study participants were divided into 3 groups based on the SPPB score value and in each of them we examined the expression of myomiRNAs as miRNA-1, miRNA-206, miRNA-29a, miRNA-29b, miRNA-133a, miRNA-133b, miRNA-208 and miRNA-499. The analysis showed a specific miRNA expression profile for each of the 3 studied groups. Our data suggest a miRNA expression correlation with muscle performance deterioration in sarcopenia and indicate miRNA role in the disease progression.

MiRNA-133a and miRNA-133b regulate the basic processes of myogenesis, including differentiation, regeneration, and determination of the fate of myoblast satellite cells (Cui *et al.* 2019, Iannone *et al.* 2020). Its overexpression has been shown to occur during
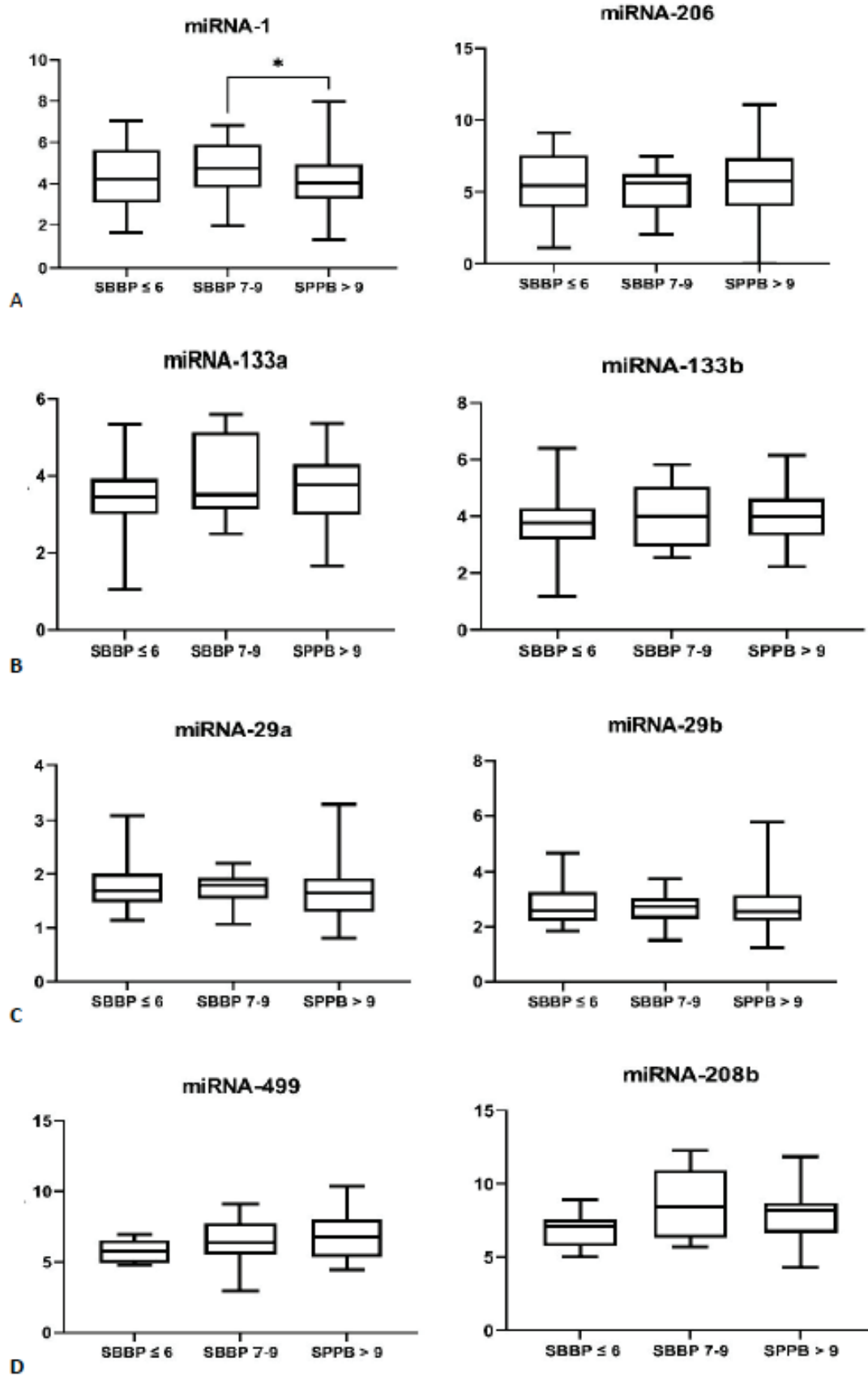
Fig. 1. A) The expression of miRNA-1 and miRNA-206 in individual groups of patients with different muscle performance deterioration standardized to ce-miRNA-39. **B**) The expression of miRNA-133a and miRNA-133b in individual groups of patients with different muscle performance deterioration standardized to ce-miRNA-39. **C**) The expression of miRNA-29a and miRNA-29b in individual groups of patients with different muscle performance deterioration standardized to ce-miRNA-39. **D**) The expression of miRNA-208b and miRNA-499 in individual groups of patients with different muscle performance deterioration standardized to ce-miRNA-39.

myogenesis (Horak *et al.* 2016), while its downregulation causes muscle cell weakening, a hallmark of sarcopenia. Sarcopenic muscle also shows a low regenerative capacity and impaired differentiation potential (Sousa-Victor and Muñoz-Cánoves 2016). This miRNA family play an important role in the modulation of stem cell function, and activity, muscle homeostasis, and are implicated in various neuromuscular diseases. Specifically, Chen *et al.* showed that loss of miRNA in muscle leads to a reduction in muscle cell number, and results in mild atrophy and impaired regenerative capacity. Only recently, several studies showed that miR-1, miR-206, miR-133a and miRNA-133b could be potential biomarkers in muscle regeneration. Increased expression of the listed myomiRNAs promotes muscle cell differentiation and regeneration (Chen *et al.* 2006, Kim *et al.* 2006). Our data support the hypothesis that downregulation of miR-133b (part of the miRNA-133 family) may contribute to a reduction in the myogenic and regenerative capacity of muscle cells in patients with sarcopenia., discussed previously in multiple studies (Yu *et al.* 2014, Yanai *et al.* 2020). We also showed the similar expression pattern in miRNA-206.

Van Rooij *et al.* (2009) studied the expression of muscle-specific miRNAs, specifically miRNA-208a, miRNA-208b and miRNA-499, in multiple research reports. All three myomiRNAs are transcripts of three myosin genes. He discovered that miR-208a is cardiac muscle-specific and is essential for the regulation of miR-208b, which is also found in cardiac muscle but is highly specific for skeletal muscle as well as miRNA-499. Their expression in skeletal muscle is limited to slow muscle fibers and also plays a key role in muscle fiber displacement and muscle growth promotion (van Rooij *et al.* 2009). In our study, we observed a decrease in expression of myomiRNA, miRNA-208b and miRNA-499 in patients with low muscle performance. This finding suggests an inability to grow muscle mass in patients with sarcopenia, which demonstrates a decrease in SPPB value.

Only a few studies have previously addressed the expression of miRNA-29a and miRNA-29b in people with sarcopenia. The study by Hu *et al.* in 2014 indicated an increase in miRNA-29 expression at an older age, providing a potential mechanism for aging-induced sarcopenia (Hu *et al.* 2014). In our study, however, we did not observe any significant difference in miRNA-29 expression between patient groups.

It should be noted that very few studies have previously studied a relationship between changes in plasma/serum expression and muscle miRNAs in sarcopenia. Only a limited number of studies have considered the miRNA role in disease progression or diagnostics. We show that the severity of muscle performance deterioration in sarcopenia correlates with specific miRNA expression. We also propose the profile of miRNAs expression in blood plasma as a specific biomarker for sarcopenia diagnostics. Future clinical studies will naturally have to elucidate the underlined molecular mechanism responsible for specific miRNAs expression in sarcopenia pathology and the progression of the disease.

Conflict of Interest

There is no conflict of interest.

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