Non-coding RNA Research 14 (2025) 166–176

Contents lists available at ScienceDirect

Non-coding RNA Research

journal homepage: www.keaipublishing.com/en/journals/non-coding-rna-research

Original Research Article

Serum lncRNAs NEAT1, PVT1 and H19 as novel biomarkers for sarcopenia diagnosis and treatment response

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ARTICLE INFO

Keywords:
Aging
Sarcopenia
lncRNA
Exercise
Biomarkers
Serum

ABSTRACT

Sarcopenia, the loss of muscle mass and function generally associated to age, leads to increased dependence and mortality in older adults. Despite its clinical significance, unclear molecular mechanisms hinder the development of universal diagnostic and therapeutic monitoring methods. Recent research suggests long non-coding RNAs (lncRNAs) as potential biomarkers for muscle damage and sarcopenia. This study investigates the role of six specific lncRNAs as biomarkers for diagnosing and monitoring sarcopenia following physical training. For this purpose, an initial cohort of participants was divided into two experiments: Trial 1, a cross-sectional study comprising 54 sarcopenic patients and 29 robust controls, both including men and women; Trial 2, a non-randomized controlled trial, where the same sarcopenic patients from Trial 1 were divided in two groups: a Control Group (CG, n = 15); and a Trained Group (TG, n = 22). RNA was extracted from serum samples for all the participants, and the expression of 6 lncRNA (PVT1, HOTAIR, MALAT1, NEAT1, GAS5, H19), selected from the literature, was quantified by RT-PCR and compared between the different groups. Statistical evaluation uncovered four lncRNAs with significantly distinct expression in Trial 1: PVT1 (LOG2FC = 1.194), GAS5 (LOG2FC = 0.8224), NEAT1 (LOG2FC = 1.497) and H19 (LOG2FC = −0.9958) and three lncRNA significantly different between TG and CG in Trial 2 (PVT1 (LOG2FC = −1.796), MALAT1 (LOG2FC = 2.834) and H19 (LOG2FC = 1.355). Among them, NEAT 1 stands aout as promissing diagnostic marker ans PVT1 and H19 may serve as both diagnosis and treatment monitoring, altough further validation in larger cohorts is needed to confirm these results.

Peer review under the responsibility of Editorial Board of Non-coding RNA Research.
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https://doi.org/10.1016/j.ncrna.2025.06.003
Received 6 May 2025; Received in revised form 9 June 2025; Accepted 22 June 2025
Available online 23 June 2025

1. Introduction

Sarcopenia, characterized by the progressive loss of skeletal muscle mass, strength, and function, is common among older adults. It is associated with adverse aftermaths such as falls, fractures, reduced quality of life, and increased mortality [1] and the World Health Organization (WHO) has formally recognized it as a disease with distinct clinical signs from physiological ageing [2]. Despite its recognition as a significant public health concern, sarcopenia remains underdiagnosed and undertreated. Its diagnosis currently relies on the quantification of muscle mass, strength, and physical performance, but robust and accessible diagnostic biomarkers are still lacking [3].

Physical training has been proposed as a potential therapeutic strategy in frail community-dwelling older adults [4]. We and others have demonstrated that multicomponent training (MCT) programs improve functional ability in older adults who are frail or at risk of frailty by mitigating age-associated declines in muscle mass, strength, and recovery potential, as well as slowing down or preventing impairments in muscle metabolism [4–6]. Unfortunately, the lack of validated therapy-monitoring biomarkers for sarcopenia also represents a significant burden in the management of this condition.

In the field of transcriptomics, studies focus on finding small non-protein-coding RNA molecules, such as microRNAs (miRNAs), circular RNAs (circRNAs) or long non-coding RNAs (lncRNAs), that regulate gene expression at the post-transcriptional level and can mediate epigenetic mechanisms related to inflammatory ageing, muscle atrophy and mitochondrial damage [7,8] and could therefore represent attractive diagnostic and therapeutic targets. Long non-coding RNAs (lncRNAs) are a subtype of RNA molecules with a length of more than 200 nucleotides that do not translate into proteins. Years ago, they were once considered mere transcriptional noise. Nonetheless, lncRNAs are currently acknowledged for their ability to interact with DNA, RNA and proteins influencing a range of cellular activities, such as cell growth, differentiation and apoptosis [9]. Their role in numerous diseases, including cancer, cardiovascular diseases and neurodegenerative disorders, has been extensively studied [10–12]. However, their involvement in muscle disorders and the pathogenesis of sarcopenia is a relatively new area of research. Interestingly, emerging evidence suggests that lncRNAs are implicated in regulating muscle atrophy, inflammation and fibrosis, which are present in sarcopenia [13–21].

The differential expression of lncRNAs in impaired versus healthy muscle suggests that they could serve as novel biomarkers for diagnosing and monitoring sarcopenia progression [22–24]. Therefore, in this research, we sought to study a panel of new molecular biomarkers, specifically six muscle-related lncRNA (PVT1, HOTAIR, MALAT1, NEAT1, GAS5, H19), selected from the literature, for clinical use in the diagnosis and treatment efficacy monitorization of sarcopenia after an MCT program which would allow the implementation of early and individualized treatments to optimize their effectiveness and prevent or delay the consequences for the health of patients and the socioeconomic damage in their families and health systems.

2. Methods

2.1. Patient selection

This study involved volunteer participants from four healthcare centers in Zaragoza, Spain, as part of the Elder 3.0 EXERNET project [5]. Data were registered in the clinicaltrials.gov repository (reference: NCT03831841). The study protocol was approved by the ethics committee of the Hospital Fundación de Alcorcón (16/50). All participants provided written informed consent before joining the study, which received approval from the Ritsumeikan University Ethics Committee and adhered to the Declaration of Helsinki guidelines [25]. The study involved the screening and functional classification of a sample of 155 adults over 65 years of age as either frail (n = 126) or robust (n = 29) based on the Short Physical Performance Battery (SPPB) thresholds (Frail ≤10 points; Robust >10 points). The EWGSOP2 [1] operational criteria and cut-off points were applied to identify the sarcopenic patients within the screened frail cohort. In particular, in order to detect sarcopenia, low muscle mass was measured using *Grip strength* and *Chair stand test*, low performance using *Gait speed*, Time up and go 3 m (*TUG 3m*) and *SPPB* and low muscle mass in the screened frail cohort. In this regard, Bioelectrical Impedance Analysis (BIA) along with body composition analyzer (Tanita Corp, TANITA BC418MA) was applied to assess body weight in kilograms and to estimate Appendicular Skeletal Muscle (ASM) and Skeletal Muscle Mass Index (SMI) based on the equation introduced by Janssen and colleagues in 2004 [26]. When at least one of the cutoff tests was not met, each criterion was deemed positive. According to these standards, 61 frail patients were classified as non-sarcopenic and 65 as sarcopenic (Fig. 1). Nevertheless, this research exclusively utilized samples from sarcopenic frail individuals and robust controls.

2.2. Experiments design

After the selection and diagnosis of the patients, two distinct studies were designed.

2.2.1. Trial 1

For the cross-sectional study, from now on *Trial 1*, 83 older adults (32 men and 51 women) aged over 65 years were selected. Among them, 54 were sarcopenic patients (mean age 81.5 ± 5.9; 21 men and 33 women) and 29 robust controls (mean age 73.7 ± 5.6; 11 men and 18 women) (Fig. 1).

2.2.2. Trial 2

37 of the 50 sarcopenic patients from *Trial 1* completed *the* non-randomized controlled trial, henceforth *Trial 2*. At baseline, they were divided into 2 groups: the Control Group, henceforth CG (n = 15; mean age 82.1 ± 4.5; 3 men and 12 women); and the Trained Group, from now on TG (n = 22; mean age 80.7 ± 7.2; 9 men and 13 women) (Fig. 1). Consult Supplementary Table S1 for more details on the patients employed in both Trial 1 and 2. The distribution in trained and control group was made to enhance participation in training based on the attendees' choices and schedule Aware of the potential bias this might introduce, we confirmed by multiple student *t*-tests that there were not differences in test scores nor in the relative expression of the lncRNAs studied between sarcopenic trained and control group at baseline, to ensure group equivalency (Supplementary Fig. S1, Supplementary Table S2).

While the CG group maintained their usual lifestyle throughout the project, the TG completed a supervised multicomponent training (MCT) of 6 months. However, both groups underwent the same testing at baseline and follow-up timepoints. Additionally, throughout the project, both groups received three 1-h talks on healthy habits, aimed at engaging CG participants and avoiding potential opting-out due to multiple evaluation periods. Both groups were evaluated at two separate time points. The first evaluation (baseline) occurred prior to the commencement of the training phase (M0). The follow-up assessment took place 3 months post-baseline (M3), during the midpoint of the intervention, to assess the preliminary impacts of the training. The following tests were performed at both times: *Grip strength*, *Chair stand*, *Gait speed*, *TUG 3m* and *SPPB*, and blood collection from all patients.

2.3. Training intervention

The Elder-Fit Multicomponent Training Program has been previously published [5]. In brief, the 6-month MCT regimen comprised three supervised weekly sessions, each lasting 1 h with a warm-up (10 min), main exercises (35–40 min) and cool down (10–15 min) featuring flexibility and cognitive tasks, always separated by 48 h to prevent

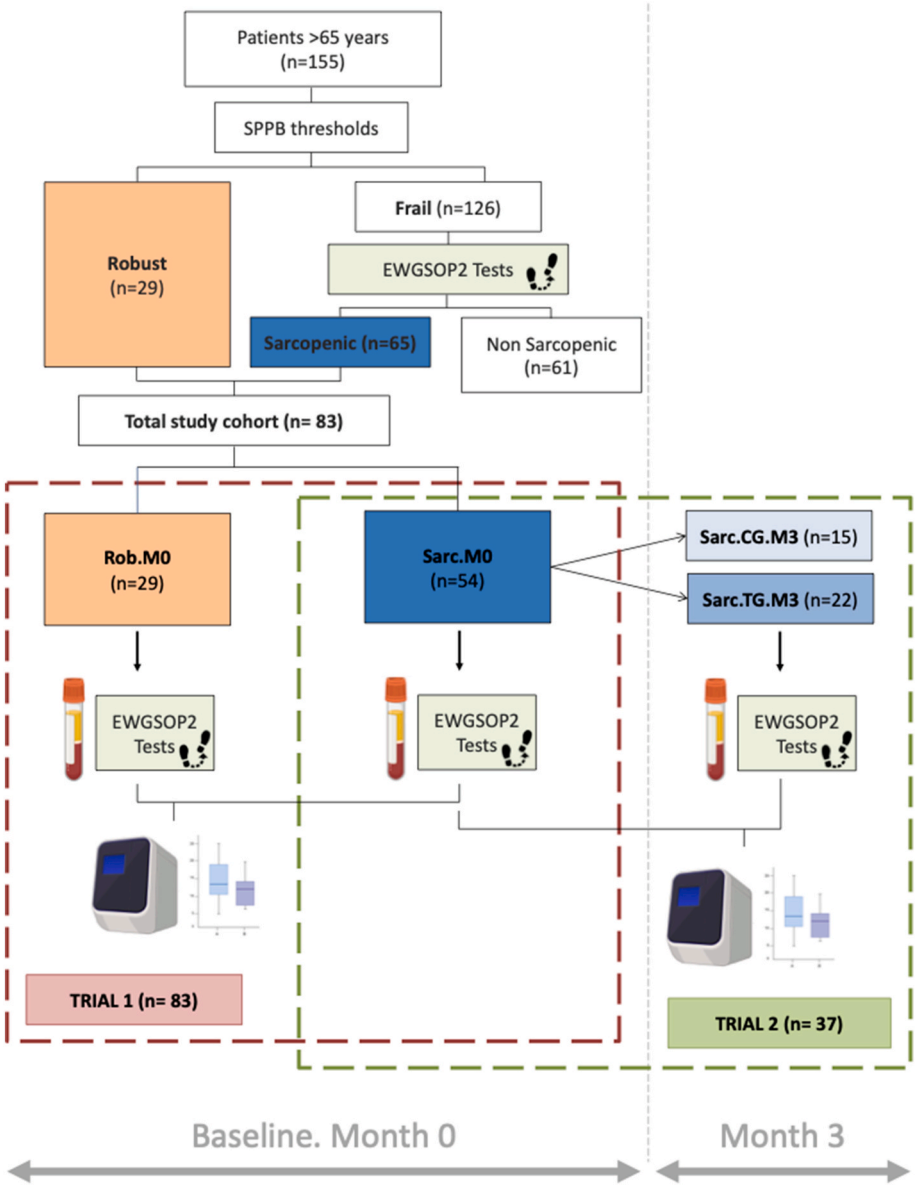

Fig. 1. Schematic workflow of the study.
SPPB: Short Physical Performance Battery; EWGSOP: European Working Group on Sarcopenia in Older People; Rob.M0: Robust controls in month 0; Sarc.M0: Sarcopenic patients in month 0; Sarc.CG.M3: Sarcopenic patients in control group in month 3; Sarc.TG.M3: Sarcopenic patients in trained group in month 3.

muscle fatigue. Weekly sessions alternated between "Strength and Functional", focusing on strength, power, balance and daily activity simulations, and "Endurance sessions", involving aerobic exercises like walking, steps, stationary cycling, and agility and coordination tasks. Experienced trainers supervised all training sessions to ensure correct technique and appropriate progression in each exercise session. The training difficulty progressed gradually throughout the MCT to ensure a proper adaptation.

2.4. Serum collection

At the beginning (M0) and in the middle (M3) of the study period, 3 mL of blood samples were obtained via venipuncture from all subjects after a minimum of 48 h of recovery since the last training session. All subjects were instructed to abstain from eating or drinking anything other than water for at least 12 h before the blood samples were taken. Blood samples were promptly centrifuged (1500 × , 15 min, 4 °C), allowing the serum to be isolated. These serum samples were preserved at −80 °C until required.

2.5. RNA extraction and RT-PCR

Serum samples were defrosted at 4 °C, and total RNA was extracted with the TRIzol LS reagent (10296028, Invitrogen) according to the manufacturer's protocol. RNA underwent reverse transcription with the High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems). The resultant cDNA was evaluated in triplicates via real-time polymerase chain reaction (PCR) using the Quant Studio 3 Real-Time PCR (RT-PCR) Instrument (Applied Biosystems). Tailored lncRNA primers were utilized, as specified in Supplementary Table S3. Target lncRNAs were normalized against GAPDH (glyceraldehyde 3-phosphate dehydrogenase) serving as the housekeeping gene, and the relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), employing threshold cycles (CT) from Robust patients and CG as reference samples in Trials 1 and 2, respectively. Raw lncRNA CT are showed in Supplementary Tables S4 and S5.

2.6. Statistical analysis

Results were statistically analyzed using GraphPad Prism 9 statistical software. Iterative Grubb's test was used to detect outliers which were excluded from the analysis. Parametric Student's t-test or nonparametric Mann-Whitney test was used for comparisons between two groups. To identify the effect of the disease (sarcopenic vs robust), sex and/or the age of patients in the expression of the lncRNAs two-way univariate analysis of variance (ANOVA) and Bonferroni post-hoc tests were performed. Receiver Operating Characteristic (ROC) curve analysis using the Wilson/Brown method were carried out to evaluate the diagnostic accuracy of the lncRNAs studied. The Area Under the Curve (AUC) was then estimated with a 95 % confidence interval (CI). Spearman rank correlation analyses were conducted to explore the association between the expression levels of the examined lncRNAs and the results of the primary diagnostic assessments for sarcopenia. Finally, mixed-effects models, Geisser-Greenhouse correction and Bonferroni post-hoc tests were performed to analyze the main effects of intervention in lncRNA expression during training (M0–M3). Data was confirmed to be normal, and MCAR (missing completely at random) was assumed. For comparisons in each lncRNA, the group (CG; TG) and time point (M0; M3) were included as fixed factors and participants as random factor. Data collected for both genders have been displayed together as a similar trend was noted for them and they are presented as mean ± standard error of the mean (SEM), unless stated otherwise. A significance level of 0.05 was established for the statistical analyses, and the statistical significances were categorized as follows: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

3. Results

3.1. Analysis of circulating lncRNAs identifies different expression between robust controls and sarcopenic patients

Four of the six lncRNA studied were differentially expressed in serum samples from sarcopenic patients participanting in Trial 1 (54 sarcopenic patients; 29 robust controls) and therefore became our candidate diagnostic biomarkers (Table 1).

Three of these 4 lncRNA showed higher expression and one was lower in sarcopenic patients (Fig. 2a). The upregulated lncRNAs in sarcopenic patients were PVT1 oncogene (PVT1, $p = 0.00652$; LOG2FC = 1.194), Growth arrest specific 5 (GAS5, $p = 0.00202$; LOG2FC = 0.8224) and Nuclear paraspeckle assembly transcript 1 (NEAT1, $p = 0.00055$; LOG2FC = 1.497). In terms of overall impact, NEAT1 demonstrated the most significant rise in individuals with sarcopenia (182.2 % in comparison to the robust group), trailed by PVT1 (128.7 %) and GAS5 (76.8 %). Conversely, the sole lncRNA that was downregulated in sarcopenic individuals was the Imprinted maternally expressed transcript (H19, p = 0.01427; LOG2FC = −0.9958), which was found to be 49.9 % lower in the serum of sarcopenic patients relative to the robust control group.

To assess the diagnostic potential of the candidate biomarkers for sarcopenia, ROC curve analysis was performed using data from Trial 1. As shown in Fig. 2, the AUC value of PVT1 is 0.72 (95 % CI: 0.56–0.87; Fig. 2b), GAS5's is 0.71 (95 % CI: 0.60–0.83; Fig. 2c) and NEAT1's is 0.74 (95 % CI: 0.62–0.87; Fig. 2d). Finally, H19 has an AUC value of 0.66 (95 % CI: 0.52–0.81; Fig. 2e).

To assess whether the expression levels of these lncRNAs were influenced by the participants' sex, a two-factor ANOVA test in conjunction with a Bonferroni post-hoc analysis was conducted. The results indicated no significant gender-related differences between men and women samples of Trial 1 in any of the candidate diagnostic lncRNAs (Supplementary Fig. S2, Suppementary Table S6).

Moreover, considering the disparities in average age among the vigorous and sarcopenic cohorts, we employed ANOVA analysis to verify that age did not contribute to the notable differences in any of the lncRNAs significantly expressed in the serum of sarcopenia patients compared to robust controls (Supplementary Fig. S3, Suppementary Table S7).

3.2. The expression of the candidate lncRNAs correlates with sarcopenia diagnostic test scores

With the aim of testing the power of the eight chosen lncRNA to diagnose the disease, their levels of expression were related to the EWGSOP2 sarcopenia assessments for diminished strength and reduced physical performance, namely the *Grip strength*, *Chair Stand*, *SPTB*, *Gait speed*, and *TUG 3m* evaluations in the Trial 1 group.

As detailed in Table 2, both *SPPB*, *Chair stand* and *Gait Speed* tests showed significant correlation with 3 of the 4 lncRNA candidate diagnostic biomarkers. PVT1 and GAS5 expression correlated significantly with 3 of the tests performed, followed by NEAT1 and H19, which correlated with 2 diagnostic tests. Focusing on PVT1, the *SPPB test* exhibited the most significant correlation ($r = -0.409$, $p = 0.002$), succeeded by the *Gait Speed test* ($r = -0.298$, $p = 0.020$), being this correlation negative for both tests. This means that PVT1, *SPPB* and *Gait Speed test* move in opposite directions, when the value of one variable increases, the value of the other variable tends to decrease, and vice versa. In this specific case, a rise in PVT1 expression is statistically correlated with a decrease in SPPB score and the speed the patient can walk, or, in other words, the higher the expression of PVT1, the worse the physical performance observed in patients. On the converse, PVT1 demonstrated a positive association with the *Chair Stand test* ($r = 0.286$, $p = 0.038$), meaning Both PVT1 expression and the seconds it takes the patient to stand up and sit down from a chair 5 times increases at the same time: the higher the PVT1 expression the lower the muscle strength. For GAS5, the strongest correlation was identified with the *Chair Stand test* ($r = 0.329$, $p = 0.005$), followed by the *SPPB* ($r = -0.263$, $p = 0.022$) and *Gait Speed* ($r = -0.249$, $p = 0.030$). NEAT1 showed a strong correlation with *Chair Stand* ($r = 0.409$, $p = 0.001$) and *SPPB* ($r = -0.294$, $p = 0.038$). Lastly, H19 was associated with *Gait Speed test* ($r = 0.274$, $p = 0.024$) and *TUG 3m* ($r = -0.283$, $p = 0.022$),

Table 1
List of differentially expressed lncRNA in serum samples from sarcopenia patients as compared to robust individuals of Trial 1 at baseline.

LncRNA	ΔCT Mean of Robust Controls	ΔCT Mean of Sarcopenic Patients	*p value*		Difference (Log2 Fold Change)	2^(−ΔΔCT) Mean of Robust Controls	2^(−ΔΔCT) Mean of Sarcopenic Patients	SD Mean of Robust Controls	SD Mean of Sarcopenic Patients
PVT1	3.637	2.443	0.00652	**	1.194	1	2.287	0.341	0.416
HOTAIR	3.598	4.3	0.12922	ns	−0.702	1	0.615	0.432	0.378
MALAT1	3.355	4.116	0.11246	ns	−0.761	1	0.59	0.38	0.337
GAS5	1.673	0.8504	0.00202	**	0.822	1	1.768	0.43	0.444
NEAT1	3.628	2.131	0.00055	***	1.497	1	2.822	0.337	0.347
H19	1.112	2.108	0.01427	*	−0.996	1	0.501	0.269	0.397

Transcription levels of PVT1, HOTAIR, MALAT1, GAS5, NEAT1 and H19 were assessed in 29 robust controls (11 men and 18 women) and 54 sarcopenic patients (15 men and 34 women) at baseline (M0: Month 0). Expression levels were evaluated by Real Time PCR and parametric Student *t*-test or nonparametric Mann-Whitney test were used to compare the groups, depending on their normality.

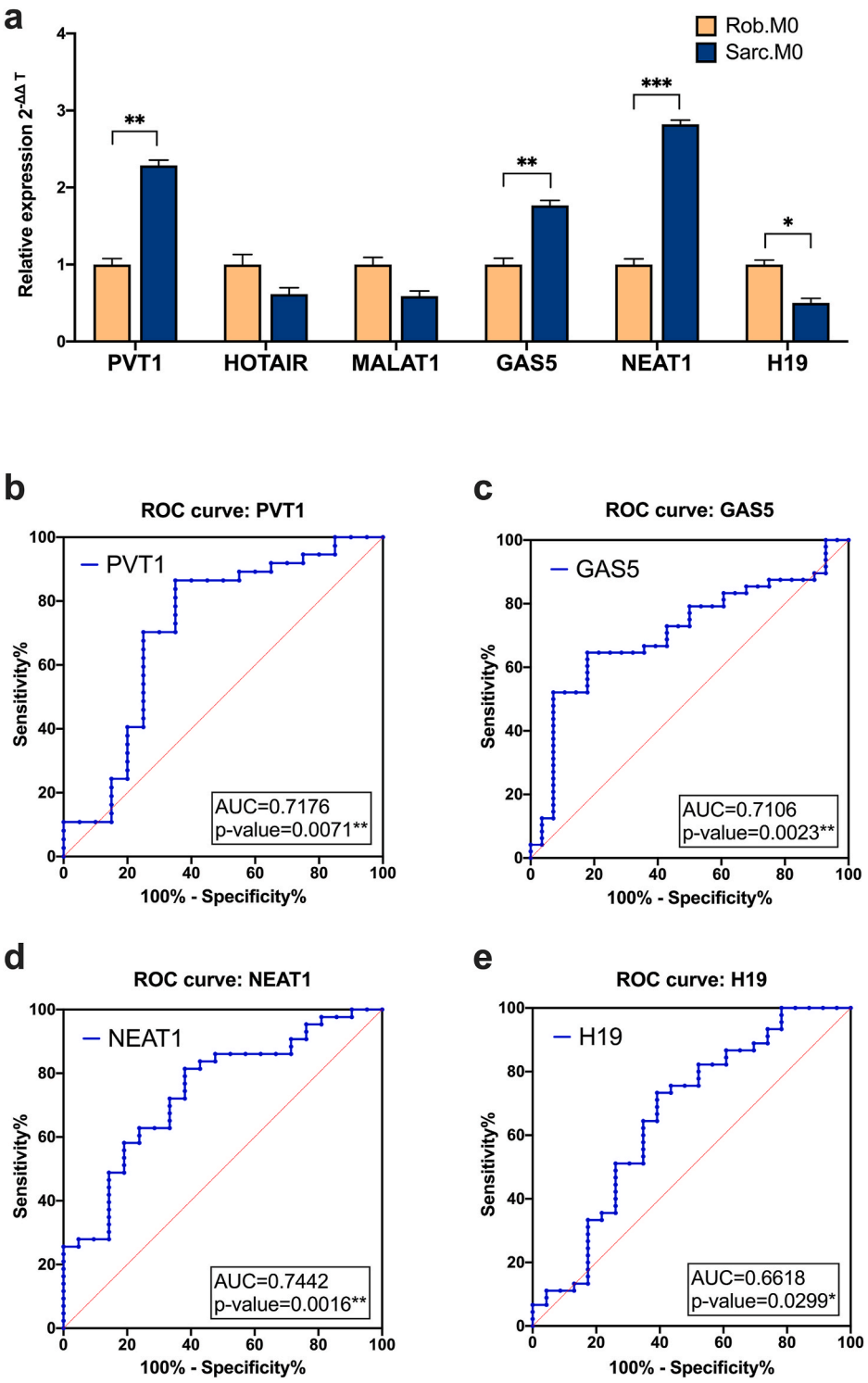

Fig. 2. Differential serum lncRNA expression in sarcopenic patients and robust controls of Trial 1 at baseline. Transcription levels of PVT1, HOTAIR, MALAT1, GAS5, NEAT1 and H19 were assessed in 28 robust controls (Rob.M0; 10 men and 18 women) and 53 sarcopenic patients (Sarc.M0; 20 men and 33 women) at baseline (month 0). (a) Expression levels were evaluated by Real Time PCR using the mean value of robust controls as reference for the second normalization in the $2^{-\Delta\Delta CT}$ method. Parametric Student *t*-test or nonparametric Mann-Whitney test were used to compare the groups, depending on their normality. ROC curve of candidate lncRNA PVT1 (b), GAS5 (c), NEAT1 (d) and H19 (e) for the diagnosis of sarcopenia.
ROC: Receiver Operating Characteristic; AUC: Area Under the Curve.

meaning the last that, when H19 is lower, the time that takes the patient to rise from a chair, walk 3 m, turn, walk back to the chair, and sit down increases.

3.3. The multicomponent training program improved physical tests results in the trained sarcopenic patients of trial 2

Intending to test the possible improvements in strength, mass and muscle performance of the participants after 3 months from the

Table 2
Correlation of the scores for the EWGSOP2 recommended sarcopenia diagnostic tests and the candidate diagnostic lncRNA.

	PVT1	HOATIR	MALAT1	GAS5	NEAT1	H19
SPPB (points)	−0.4091**	0.1285	0.1512	−0.2628*	−0.2938*	0.2370
Chair Stand (s)	0.2861*	−0.1261	−0.1437	0.3287**	0.4085**	−0.2378
Grip Strength (kg)	−0.1326	0.2968	−0.0033	−0.0422	−0.1131	0.2346
Gait Speed (m/s)	−0.2979*	0.2797	0.1607	−0.2492*	−0.2203	0.2744*
TUG 3m (s)	0.3315	−0.3165	−0.0834	0.2761	0.2232	−0.2828*

Note: Data indicate Spearman-rho correlation coefficients in the Trial 1 cohort (n = 54 for sarcopenic patients; n = 29 for robust control). SPPB: Short Physical Performance Battery; TUG: Time Up and Go 3 m.

beginning of the physical training, both trained (n = 22; Sarc.TG.M3) and untrained (n = 15; Sarc.CG.M3) patients of Trial 2 underwent the tests and measures proposed by the EWGSOP2, namely *SPPB*, *Chair stand*, *Grip strength*, *Gait speed* and Time up and go 3 m (*TUG 3m*) test and ASM (Appendicular Skeletal Muscle) and SMI (Skeletal Muscle Mass Index) measures. In most tests, improvements were observed in the treated group as compared to the control group (Fig. 3, Table 3).

In terms of muscle strength, in the *Chair stand* test (the time that takes the patient to stand up and sit 5 times) scores in treated participants were shorter in trained vs. untrained participants ($p = 0.0263$*; log2 FC = −0.877) and the measure of *Grip strength* was significantly higher in the trained patients ($p = 0.0441$*; log2 FC = 8.207), which represents a 44.9 % increase in strength over controls. In muscle mass measurements, both ASM ($p = 0.0048$**; log2 FC = 7.071) and SMI ($p = 0.0089$**; log2 FC = 1.849) measurements increased significantly with physical exercise, showing a 52 % increase in muscle mass, compared to their non-exercised equals. Finally, regarding muscle performance, the time the trained patients took to stand up and go 3 m was shorter ($p = 0.0057$**; log2 FC = −2.162), reflecting a 21.3 % improvement in physical and muscular performance. In *SPPB* and *Gait speed* tests, no significant results were observed ($p = 0.3310$; log2 FC = −0.796 and $p = 0.3130$; log2 FC = −0.028 respectively), when comparing Sarc.TG.M3 and Sarc.CG.M3.

Table 3
EWGSOP2 diagnostic tests scores in trained and control sarcopenic patients of Trial 2.

LncRNA	Mean of Sarc.CG.M3	Mean of Sarc.TG.M3	*p value*		Difference (Log2 Fold Change)
SPPB (point score)	9.2500	10.0455	0.33101	ns	0.796
Chair Stand (s)	5.0167	4.1400	0.02626	*	−0.877
Grip Strength (kg)	18.2933	26.5000	0.04415	*	8.207
Gait Speed (m/s)	0.3225	0.3505	0.31298	ns	0.028
TUG 3m (s)	10.1371	7.9750	0.00567	**	−2.162
ASM (kg)	13.3590	20.4300	0.00478	**	7.071
SMI (kg/height^2)	5.8470	7.6964	0.00885	**	1.849

Note: Depending on their normality, student *t*-test or nonparametric Mann-Whitney test were used to compare the groups of the Trial 2 cohort: 15 sarcopenic non trained patients (Sarc.CG.M3; 3 men and 12 women) and 22 sarcopenic trained patients (Sarc.TG.M3; 9 men and 13 women) at month 3 of the training period.
SPPB: Short Physical Performance Battery; TUG: Time Up and Go 3 m; ASM: Appendicular Skeletal Muscle; SMI: Skeletal Muscle Mass Index.

3.4. LncRNA expression changed during multicomponent training program

Subsequently, the expression of the six studied lncRNA was assessed in the serum samples of the Trial 2 cohort taken at month 3 of the training program.

Changes in lncRNA expression are shown in Fig. 4. At month 3, when Control Group RT-PCR values were compared with Trained Group

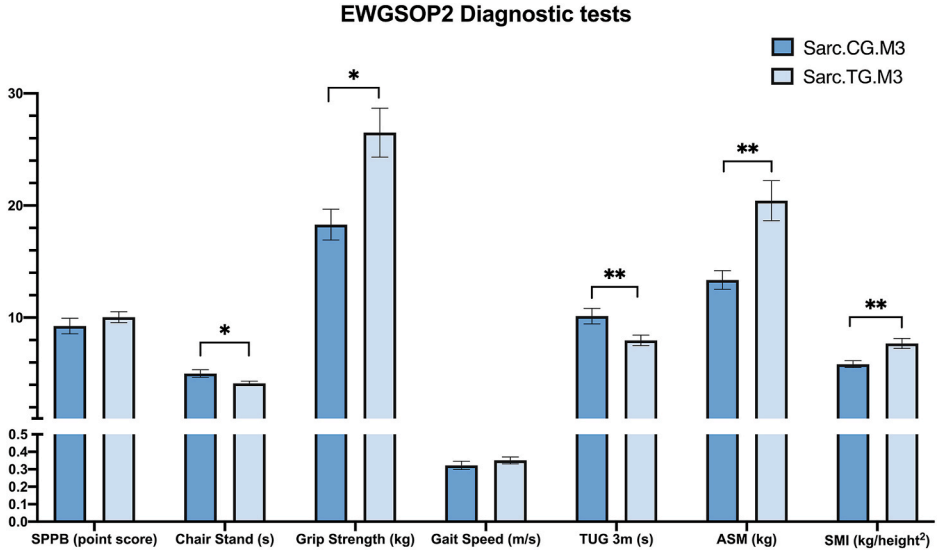

Fig. 3. Bar graph of EWGSOP2 diagnostic tests scores in trained and control sarcopenic patients of Trial 2. The physical diagnostic test proposed by EWGSOP2 in 2019 were evaluated in 15 sarcopenic non trained patients (Sarc.CG.M3; 3 men and 12 women) and 22 sarcopenic trained patients (Sarc.TG.M3; 9 men and 13 women) at month 3 of the training period. Parametric Student *t*-test or nonparametric Mann-Whitney test were used to compare the groups, depending on their normality.
SPPB: Short Physical Performance Battery; TUG: Time Up and Go 3 m; ASM: Appendicular Skeletal Muscle; SMI: Skeletal Muscle Mass Index.

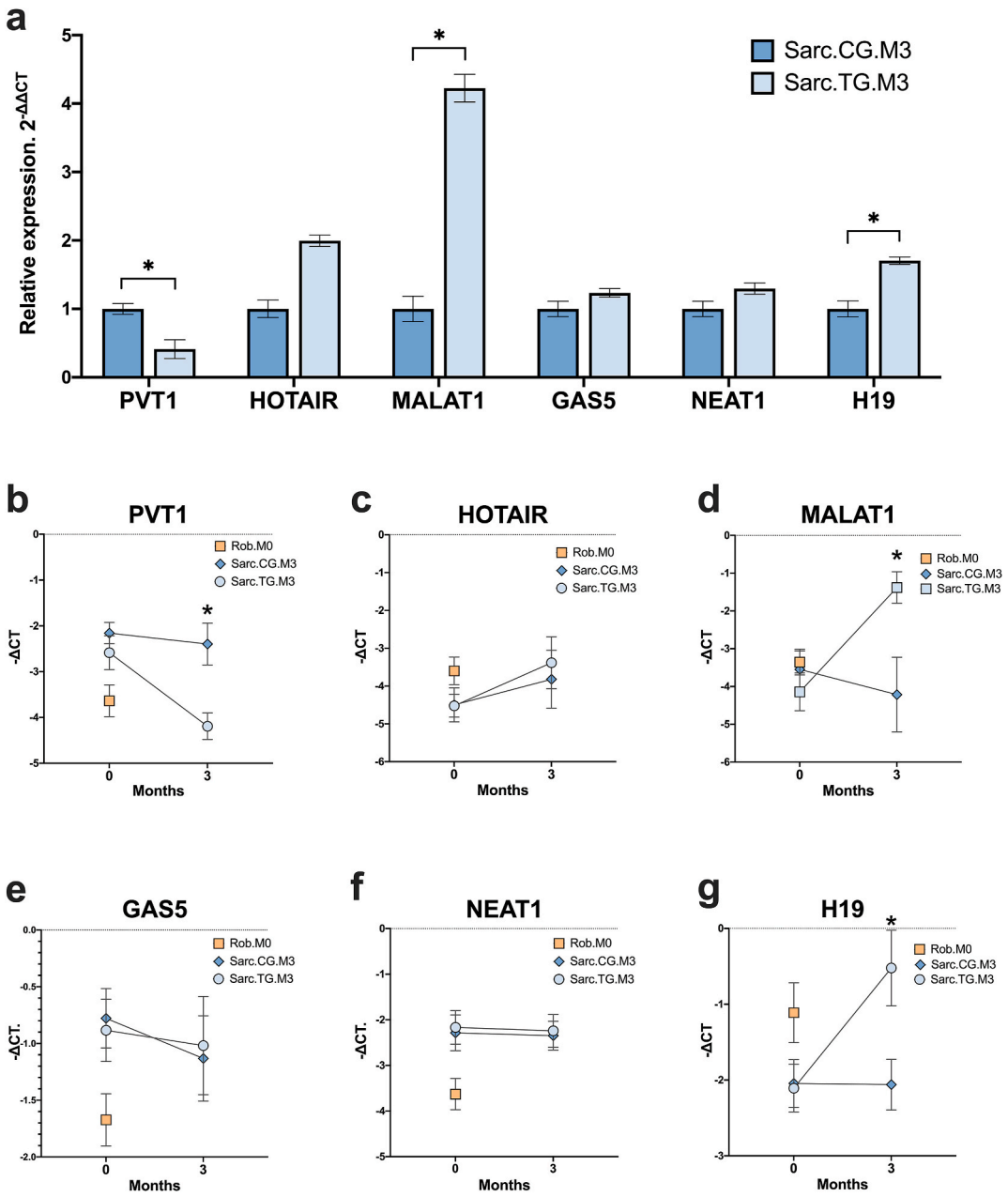

Fig. 4. Differential serum lncRNA expression in trained and control sarcopenic patients of Trial 2. Transcription levels of PVT1, HOTAIR, MALAT1, GAS5, NEAT1 and H19 were assessed in 15 sarcopenic non trained patients (Sarc.CG.M3; 3 men and 12 women) and 22 sarcopenic trained patients (Sarc.TG.M3; 9 men and 13 women) at month 3 of the training period. (a) Expression levels were evaluated by Real Time PCR using the mean value of sarcopenic non trained patients as reference for the second normalization in the $2^{-\Delta\Delta CT}$ method. Parametric Student *t*-test or nonparametric Mann-Whitney test were used to compare the groups, depending on their normality. For PVT1 (b), HOTAIR (c), MALAT1 (d), GAS5 (e), NEAT1 (f) and H19 (g) mixed effect models was performed in order to analyze the effect of the physical training and time on patients matched values.

values (Sarc.CG.M3-Sarc.TG.M3), the expression of three lncRNa studied (PVT1, MALAT1 and H19) had significantly changed during the training program and therefore became our candidate diagnostic biomarkers. Specifically, when compared with control group at month 3, two of these 3 lncRNA showed higher expression (MALAT1 ($p = 0.0191$; log2 FC = 2.834) and H19 ($p = 0.0404$; log2 FC = 1.355)) and one was lower in sarcopenic trained patients (PVT1 ($p = 0.0190$; log2 FC = −1.796)) (Fig. 4a–Table 4). In all three cases, during physical exercise (month 3) lncRNA expression in the sarcopenic Control Group (Sarc.CG.M3) remains very similar to that of the total sarcopenic group at baseline (Sarc.M0), however, the lncRNA expression in the sarcopenic Trained Group (Sarc.TG.M3) increases or decreases depending on each biomolecule, approaching the expression levels of the robust group at time 0 (Rob.M0) (Fig. 4b–d, g).

Conversely, no significant differences were found in the expression of HOTAIR, NEAT1 or GAS5 between Sarc.CG.M3 and Sarc.TG.M3 (Fig. 4a) and their expressions with the exercise remain constant in both groups as those of the total sarcopenic group at time 0 (Fig. 4c–e, f).

We perform mixed effect models for each of the six lncRNA studied to jointly analyze the effect of the physical training, time, and the possible interaction between both on patients' matched values. In this analysis, we found significant results, again, in PVT1, MALAT1 and H19 (Table 4). Specifically, for PVT1, the effect of the factor "Group" (trained vs untrained controls) ($p = 0.0173$*), "Time" (Month 0 vs Month 3) ($p = 0.0093$**) and the interaction between the two ($p = 0.0411$*) are significant. For MALAT1, just the interaction between the factors "Group"

Table 4
Differential serum lncRNA expression in trained and control sarcopenic patients of Trial 2.

LncRNA	ΔCT Mean of Sarc.CG.M3	ΔCT Mean of Sarc.TG.M3	*p value*		Difference (Log2 Fold Change)	2^(−ΔΔCT) Mean of Sarc.CG.M3	2^(−ΔΔCT) Mean of Sarc.TG.M3	SD Mean of Sarc.CG.M3	SD Mean of Sarc.TG.M3
PVT1	2.397	4.193	0.018961	*	−1.796	1	0.412	0.291	0.389
HOTAIR	3.82	3.384	0.713207	ns	1.4366	1	1.995	0.283	0.240
MALAT1	4.215	1.381	0.019098	*	2.834	1	4.225	0.321	0.493
GAS5	1.132	1.019	0.859842	ns	0.1138	1	1.234	0.390	0.271
NEAT1	2.348	2.241	0.839911	ns	0.1069	1	1.297	0.389	0.347
H19	2.165	0.7857	0.023489	*	1.379	1	1.705	0.419	0.224

Transcription levels of PVT1, HOTAIR, MALAT1, GAS5, NEAT1 and H19 were assessed in 15 sarcopenic non trained patients (Sarc.CG.M3; 3 men and 12 women) and 22 sarcopenic trained patients (Sarc.TG.M3; 9 men and 13 women) at month 3 of the training period. Expression levels were evaluated by Real Time PCR using the mean value of sarcopenic non trained patients as reference for the second normalization in the $2^{-\Delta\Delta CT}$ method. Parametric Student *t*-test or nonparametric Mann-Whitney test were used to compare the groups, depending on their normality.

and "Time" results to be significant ($p = 0.0140$*). Finally, in H19 lncRNA, both "Group" and interaction Time-Group are significant ($p = 0.0482$*, $p = 0.0378$, respectively) (Table 5). These results confirm that the expression of these lncRNAs changes depending on whether or not the individual has received physical training for several months.

4. Discussion

Sarcopenia is a progressive and generalized disease of the skeletal muscle characterized by the loss of muscle strength, mass and function generally associated with ageing. This condition ranks among the foremost health challenges faced by the elderly, owing to its widespread incidence and significant clinical and economic consequences [27]. However, its diagnosis remains challenging due to its complex and multifactorial nature. The gradual ageing of the population will lead to a significant rise in the count of sarcopenic patients in the coming years, turning sarcopenia into an escalating worldwide health concern [28]. Consequently, early diagnosis and a comprehensive and individualized approach to management are essential. To achieve this goal and taking into account its multiple origins, the evaluation and validation of a panel comprising various circulating or tissue mediators might be the best approach to detect and track sarcopenia in elderly individuals, as well as to incorporate particular biochemical analyses into the clinical evaluation of the condition. This is an active field of study and, for instance, our group has recently proposed two proteins contained in circulating extracellular vesicles with high diagnostic value that could be of great use in the identification of the disease [25]. However, they need further validation in a larger cohort and in different populations, and, therefore, have not yet been incorporated into clinical practice. The main finding of our study is that we detected biomarkers that may be useful in solving this problem.

Skeletal muscle is a highly dynamic tissue, capable of regenerating and adapting to various stimuli or physiological conditions, such as exercise, injury and ageing [6,29,30]. Recent investigations report that long non-coding RNAs (lncRNAs) are crucial in regulating muscle development, maintenance and regeneration [13] and may be critical players in the pathogenesis of sarcopenia [31]. For instance, specific lncRNAs such as NEAT1 [16] and MALAT1 [32] have been shown to influence muscle differentiation and growth by modulating the expression of key myogenic factors. These findings suggest that lncRNAs are crucial for maintaining muscle homeostasis and that their dysregulation could contribute to muscle-wasting conditions like sarcopenia. Moreover, lncRNAs are highly tissue-specific and can be released into circulation, e.g. within extracellular vesicles [33], and/or can be find free in body fluids such as blood or saliva, offering a non-invasive approach for disease assessment [34].

This study provides preliminary insights into the serum concentrations of 6 lncRNA previously related to muscle function in sarcopenia human serum. Among them, four exhibited different expression levels in sarcopenic patients in comparison with robust individuals (PVT1, GAS5, NEAT1, H19) and could be used as diagnostic biomarkers in the disease. Indeed, the expression of this lncRNA correlated with the majority of the scores from the tests recommended by EWGSOP2 to evaluate muscle strength and function and to diagnose sarcopenia. This highlights its potential clinical applicability as the proposed lncRNA biomarkers could complement existing diagnostic tools for sarcopenia, such as the aforementioned physical tests and measurements. A multi-dimensional diagnostic approach, integrating molecular biomarkers like NEAT1, PVT1, and H19 with physical performance and imaging data, could improve diagnostic sensitivity and specificity, enable earlier detection, and allow for more personalized monitoring of disease progression and treatment response. Additionally, our analysis revealed that these biomarkers are neither gender- nor age-specific, further emphasizing their effectiveness as diagnostic markers for both men and women. In this sense future research should further explore sex- and age-specific variations in lncRNA expression for the development and monitoring of therapies tailored to each patient and his or her individual needs.

However, several limitations must be acknowledged. Most notably, the relatively small sample size might restrict the statistical power of the analyses and may limit the generalizability of the findings. Another important limitation of this study lies in the technical challenges associated with the PCR amplification of cell-free circulating lncRNAs in serum samples. Cell-free lncRNAs in serum often exist at low concentrations and are sensitive to degradation by exonucleases, which can compromise both the efficiency and reproducibility of their detection [35]. These constraints underscore the exploratory nature of the study and highlight the need for further investigations before firm conclusions can be drawn or clinical applications considered. In this sense, standard practices guidelines and operating procedures, as well as replication and validation in larger and diverse cohorts, are essential for

Table 5
Results of the mixed effect models was performed on lncRNA studied.

Fixed effects (type III)	PVT1		HOTAIR		MALAT1		GAS5		NEAT1		H19	
	p value		*p* value		*p* value		*p* value		*p* value		*p* value	
Time	0.01727	*	0.30168	ns	0.11590	ns	0.49623	ns	0.69108	ns	0.04815	*
Group	0.00930	**	0.78809	ns	0.09407	ns	0.98543	ns	0.76309	ns	0.08820	ns
Time x Group	0.04113	*	0.56817	ns	0.01401	*	0.76017	ns	0.85687	ns	0.03784	*

Linear mixed models were performed to analyze the main effects of intervention in lncRNA expression during training (M0–M3). For comparisons in each lncRNA, Group (CG; TG) and Time (M0; M3) were included as fixed factors and participants as random factors.

implementation and integration into clinical workflows.

On the other hand, understanding their roles in muscle atrophy, inflammation and fibrosis could open new avenues for diagnosing and treating this age-related condition. Muscle atrophy, characterized by reduced muscle fiber size, is a hallmark of sarcopenia. Interestingly, several studies have confirmed that the expression of NEAT1 and PVT1, the two best candidates as a diagnostic biomarker according to our results, was increased during muscle atrophy [10,20,36–38]. Specifically, NEAT1 has been found to be upregulated in several muscle disorders, particularly those involving muscle degeneration, inflammation, and stress responses. A research conducted in 2020 revealed notable variations in the expression levels of Neat1 across various models of muscle wasting, being in all cases upregulated compared to control [36]. Moreover, Wei et al. observed that the upregulation of NEAT1 suppressed myocardiocyte proliferation and promoted and aggravated its apoptosis by downregulating miR-129-5p. Conversely, the suppression of NEAT1 reduced cell apoptosis and mitigated the growth inhibition caused by hypoxia [37]. Regarding PVT1, Alessio et al. conducted an extensive study in 2019 where they characterized the function of PVT1 in skeletal muscle and demonstrated the involvement of PVT1 in the modulation of muscle atrophy [20]. They demonstrated that PVT1 was up-regulated during muscle atrophy which affected apoptosis, autophagy and myofiber size in vivo *and* impacted mitochondrial morphology and respiration. Moreover, they evidenced that when PVT1 was down-expressed in C2C12 muscle cells the decline in myofiber mas was minimized and the mitochondria were protected against deterioration. They observed that, after PVT1 down-expression, c-Myc, a protein that plays an important role in the regulation of gene expression and multiplication in human cells, is unbalanced and consequently the anti-apoptotic protein Bcl-2 is up-regulated [20]. Similar findings by Zhang et al., demonstrated that knocking down PVT1 lncRNA reduced apoptosis in vascular smooth muscle cells and diminished serum pro-inflammatory cytokines in a mouse model of abdominal aortic aneurysm [38]. Inversely, the overexpression of PVT1 in vascular smooth muscle cell significantly enhanced apoptosis [38]. Our results show an increase in the expression of serum PVT1 in sarcopenic patients respect to robust controls, which could be compatible with an increased state of muscle atrophy in sarcopenic patients. With physical training, PVT1 expression decreased to levels similar to those of non-trained robust participants, whereas no changes in PVT1 are observed in untrained sarcopenic patients. Notably, physical activity and exercise are widely recognized as effective strategies to combat muscle aging [5,39]. They have been proven to reduce age-related declines in muscle mass, strength and regenerative ability, while also helping to slow down or prevent disruptions in muscle metabolism [4,6]. The MCT completed by our participants has demonstrated success in enhancing muscle mass, strength, and overall performance (Fig. 3) which is in line with the reduction in lncRNA PVT1 expression possibly reflecting attenuation of muscle atrophy in these patients.

Chronic low-grade inflammation that accompanies aging, often referred to as “inflammaging” is another key contributor to the degeneration of muscle tissue and sarcopenia [40]. A recent review unveiled that a significant variety of lncRNAs played roles in the intricate web of regulatory pathways that influence numerous facets of muscle aging such as, the modulation of myoblast proliferation, differentiation and regeneration with aging [41]. LncRNA H19, which is highly expressed in developing and regenerating muscle [42], has been shown to play a protective role against muscle loss due to inflammation [43]. Specifically, H19 enhances the differentiation and regeneration of skeletal muscle by interacting with microRNAs, such as miR-675, and influences the expression of genes involved in muscle growth and atrophy [21,44]. In aging muscle, reduced expression of H19 contributes to impaired muscle regeneration and increased susceptibility to inflammation-driven muscle wasting [45,46]. These studies would explain the decreased expression of H19 in sarcopenic patients with respect to robust controls obtained since, its dysregulation during aging have been proven to amplify the effects of inflammaging, further accelerating muscle loss and promoting catabolic processes in muscle tissue, which are mechanisms involved in the pathogenesis of sarcopenia. Regarding H19 and exercise, Yue et al. demonstrated in 2023 that H19 played a key role in maintaining slow muscle fiber types and exercise endurance [47]. Furthermore, they noticed that muscle-specific inhibition of H19 in tibialis anterior and gastrocnemius muscles in vivo induced fiber type transformation and leaded to decreased oxidative metabolism and antioxidant capacity in muscle [47]. In our study, the physical training increased H19 expression levels. In this line, the increased H19 expression observed after MCT physical training might reflect an stimulation of muscle regeneration and myoblast growth.

To sum up, dysregulation of lncRNAs could enhance fibrotic remodeling in aging muscle, further contributing to the decline in muscle quality observed in sarcopenia. Moreover, aberrant expression of these lncRNAs may exacerbate inflammatory signaling pathways, contributing to muscle loss and fibrosis in sarcopenia. As research progresses, lncRNAs may become integral to personalized medicine approaches, offering novel strategies to preserve muscle mass and function in the aging population, which MCT protocol yields the most significant benefits for frailty in older adults. While the potential of lncRNAs in sarcopenia is promising, several challenges remain regarding its technical management or clinical application. The precise mechanisms by which lncRNAs influence muscle biology, and sarcopenia need further elucidation. Future research should focus on identifying the specific roles of the lncRNAs discovered in the muscle and exploring their interactions with other molecular pathways involved in muscle ageing.

5. Conclusions

This study contributes to the growing evidence suggesting a relevant role for lncRNAs in muscle metabolism and ageing-related conditions, and it identifies several candidates with potential as biomarkers for sarcopenia. In particular, NEAT1 may serve as a diagnostic marker, while PVT1 and H19 show promise as both diagnostic and treatment-monitoring indicators. NEAT1 and PVT1 were found to be upregulated in the serum of sarcopenic patients compared to robust controls, whereas H19 was downregulated. Additionally, the expression of PVT1 and H19 appeared to be influenced by physical exercise, suggesting their possible utility in tracking therapeutic response. However, further validation in larger, independent cohorts is needed to confirm these findings and clarify their clinical applicability.

CRediT authorship contribution statement

Paula Aparicio: Writing – original draft, Methodology, Conceptualization, Validation, Formal analysis. **Tresa López-Royo:** Writing – review & editing, Methodology. **David Navarrete-Villanueva:** Writing – review & editing, Methodology. **Alba María Gómez Cabello:** Methodology. **Marcela González-Gross:** Writing – review & editing. **Ignacio Ara:** Writing – review & editing. **Germán Vicente-Rodríguez:** Conceptualization, Writing – review & editing, Funding acquisition, Supervision. **Rosario Osta:** Supervision, Writing – review & editing, Conceptualization, Funding acquisition. **Raquel Manzano:** Supervision, Conceptualization, Writing – review & editing, Funding acquisition.

Funding acknowledgements

This research was supported by the Ministerio de Ciencia e Innovación (PID2022-140911OB-I00) granted to R.M, Ministerio de Economía, Industria y Competitividad” (DEP2016-78309-R), University of Zaragoza (UZ2021-BIO-05), “Centro Universitario de la Defensa de Zaragoza” (UZCUD2017-BIO-01), PI21/00372, and Fondo Europeo de Desarrollo Regional (FEDER) “Una manera de hacer Europa” from the European Union, Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED, CB18/05/0037), CIBER

-Consorcio Centro de Investigación Biomédica en Red- (CB16/10/00477) and (CB12/03/30038). This work has been co-financed by the Spanish Ministry of Science and Innovation with funds from the European Union NextGenerationEU, from the Recovery, Transformation and Resilience Plan (PRTR-C17.I1). P.A. was supported by Departamento de Industria e Innovación from Gobierno de Aragón and Fondo Social Europeo.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rosario Osta reports financial support and administrative support were provided by Center for Networked Biomedical Research on Neurodegenerative Diseases. Paula Aparicio reports financial support and administrative support were provided by Government of Aragon Department of Science Technology and University. Raquel Manzano reports financial support and administrative support were provided by Spain Ministry of Science and Innovation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2025.06.003.

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