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Small non-coding RNA profiling in patients with gastrointestinal cancer

Alessio Molfino[1], Marc Beltrà[2,3], Maria Ida Amabile[4], Roberta Belli[1], Giovanni Birolo[5], Elena Belloni[6], Serena De Lucia[2], Lorena Garcia-Castillo[2], Fabio Penna[2], Giovanni Imbimbo[1], Giuseppe Nigri[6], Barbara Pardini[7,8], Paola Costelli[2]* & Maurizio Muscaritoli[1]*

[1]*Department of Translational and Precision Medicine, Sapienza University of Rome, Rome, Italy;* [2]*Department of Clinical and Biological Sciences, University of Torino, Torino, Italy;* [3]*Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology, Barcelona, Spain;* [4]*Department of Surgical Sciences, Sapienza University of Rome, Rome, Italy;* [5]*Department of Medical Sciences, University of Torino, Torino, Italy;* [6]*Department of Medical-Surgical Sciences and Translational Medicine, Sapienza University of Rome, Rome, Italy;* [7]*Italian Institute for Genomic Medicine (IIGM), Candiolo, Italy;* [8]*Candiolo Cancer Institute, FPO-IRCCS, Candiolo, Italy*

Abstract

Background Small non-coding (snc)RNAs, including microRNAs and P-element induced wimpy testis (PIWI)-interacting-RNAs (piRNAs), crucially regulate gene expression in both physiological and pathological conditions. In particular, some muscle-specific microRNAs (myomiRs) have been involved in the pathogenesis of cancer-induced muscle wasting. The aims of the present study were (i) to profile sncRNAs in both skeletal muscle and plasma of gastrointestinal cancer patients and (ii) to investigate the association among differentially expressed sncRNAs and the level of muscularity at body composition analysis.

Methods Surgical patients with gastrointestinal cancer or benign disease were recruited. Blood samples and muscle biopsies (*rectus abdominis*) were collected during surgery. Low muscularity patients were those at the lowest tertile of skeletal muscle index (SMI; CT-scan), whereas moderate/high muscularity patients were in the middle and highest SMI tertiles. SncRNAs in the muscle were assessed by RNAseq, circulating microRNAs were evaluated by qPCR.

Results Cancer patients (n = 25; 13 females, 52%) showed a mean age of 71.6 ± 11.2 years, a median body weight loss of 4.2% and a mean BMI of 27.0 ± 3.2 kg/m^2. Control group (n = 15; 9 females, 60%) showed a mean age 58.1 ± 13.9 years and a mean BMI of 28.0 ± 4.3 kg/m^2. In cancer patients, the median L3-SMI (cm^2/m^2) was 42.52 (34.42; 49.07). Males showed a median L3-SMI of 46.08 (41.17–51.79) and females a median L3-SMI of 40.77 (33.73–42.87). Moderate-high and low muscularity groups included 17 and 8 patients, respectively. As for circulating microRNAs, miR-21-5p and miR-133a-3p were up-regulated in patients compared with controls, whereas miR-15b-5p resulted down-regulated in the same comparison (about 30% of control values). Sample clustering by muscularity and sex revealed increased miR-133a-3p and miR-206 only in moderate-high muscularity males. SncRNA profiling in the muscle identified 373 microRNAs and 190 piRNAs (72.5% and 18.7% of raw reads, respectively). As for microRNAs, 10 were up-regulated, and 56 were down-regulated in cancer patients versus controls. Among the 24 dysregulated piRNAs, the majority were down-regulated, including the top two most expressed piRNAs in the muscle (piR-12790 and piR-2106). Network analysis on validated mRNA targets of down-regulated microRNAs revealed miR-15b-5p, miR-106a-5p and miR-106b-5p as main interactors of genes related to ubiquitin ligase/transferase activities.

Conclusions These results show dysregulation of both muscle microRNAs and piRNAs in cancer patients compared with controls, the former following a sex-specific pattern. Changes in circulating microRNAs are associated with the degree of muscularity rather than body weight loss.

Keywords Biomarkers; Gastrointestinal cancer; miRNAs; Muscularity; piRNAs; Skeletal muscle; Small non-coding RNAs

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**Correspondence to: Paola Costelli, Department of Clinical and Biological Sciences, University of Torino, Torino, Italy. Email: paola.costelli@unito.it;*
Maurizio Muscaritoli, Department of Translational and Precision Medicine, Sapienza University of Rome, Rome, Italy. Email: maurizio.muscaritoli@uniroma1.it
Alessio Molfino, Marc Beltrà, Paola Costelli and Maurizio Muscaritoli contributed equally to the study.

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Introduction

Muscle wasting represents a hallmark of the catabolic condition occurring in cancer patients.[1,2] Overall, body composition analysis reveals that cancer patients can be stratified according to the level of muscularity.[3] In particular, patients characterized by low muscularity very often show a poorer prognosis than those with moderate-high muscularity.[3] In this setting, several clinical factors may promote the development of muscle wasting, including the presence of anorexia, low energy and protein intakes, and reduced physical activity.[4] At the molecular level, muscle wasting is associated with enhanced protein turnover rates, mainly due to overactivation of intracellular protein breakdown. Particularly relevant in this regard are the ubiquitin-proteasome system and the acidic vacuolar proteolysis, although both caspases and calcium-dependent calpains have been proposed to play a role as well.[5] In addition, several factors, including pro-inflammatory cytokines produced by the tumour, its microenvironment and the host have been identified as contributors of muscle wasting.[6,7] Interestingly, circulating and tissue-specific factors may represent both potential biomarkers and therapeutic targets of cancer-induced muscle atrophy and weakness.

Small non-coding RNAs (sncRNAs) are increasingly recognized to contribute to a wide variety of biological processes involved in both physiology and pathology. The most studied sncRNAs are microRNAs (miRNAs), short sequences of approximately 22 nucleotides detectable in different tissues, blood included.[8] MiRNAs regulate gene expression targeting the 3′ untranslated region of mRNAs, resulting in accelerated degradation of mRNA and/or inhibition of translation. They are involved in several events such as cell proliferation, differentiation and death, DNA repair and oxidative stress response.[9] MiRNAs have been shown to be stable and able of circulating in blood, either in extracellular vesicles (EVs) or protein-bound,[10] explaining their ability to act also distally from the site of production and release.[11,12] Some miRNAs, also named myomiR, are enriched in the skeletal muscle, and their involvement in the pathogenesis of muscle wasting during cancer has been hypothesized.[13,14] In this regard, miRNAs have been suggested as potential biomarkers of cancer-induced muscle wasting. Moreover, understanding miRNA contribution to the onset and progression of cancer cachexia could be useful to design targeted therapeutic strategies.

Some studies have characterized the expression of miRNAs in the skeletal muscle of cancer patients according to their degree of cachexia using the international consensus framework.[15] Narasimhan and colleagues found up to eight miRNAs dysregulated in cachectic compared with non-cachectic patients affected by metastatic pancreatic and colorectal cancer.[16] Similarly, five miRNAs were up-regulated and 23 down-regulated in the muscle of non-small cell lung carcinoma patients with cachexia compared with healthy subjects. Of these five up-regulated miRNAs, only the expression of miR-424-3p was higher in non-cachectic patients than in controls, likely being related to the presence of the tumour.[14] Despite these observations, the relationship potentially underlying miRNA expression and the level of muscularity in cancer patients is still unclear.

Besides miRNAs, other biotypes of sncRNAs have emerged as potential drivers of disease. Among them, the P-element induced wimpy testis (PIWI)-interacting-RNAs (piRNAs) are 26- to 30-nucleotide-long molecules able to interact with the PIWI subfamily of Argonaute proteins. Accumulating evidence is positioning piRNAs as relevant epigenetic regulators, and studies have demonstrated their relevance to the progression of some diseases.[17] Some recent pieces of evidence indicate that piRNAs might be important during muscle atrophy.[18] Yet, to our knowledge, no characterization of sncRNA signatures in the skeletal muscle exists in the context of cancer.

In the present study, we aimed to (i) profile miRNAs and other sncRNAs in skeletal muscle samples and in plasma obtained from gastrointestinal (GI) cancer patients and controls and (ii) evaluate the association of differentially expressed sncRNAs with the level of muscularity.

Methods

Study population

An observational, cross-sectional study was conducted on patients with a new diagnosis of gastric, colon or pancreatic cancer and eligible for surgical tumour resection. Control group included patients undergoing surgery for benign diseases. The study was performed at the Department of Translational and Precision Medicine and at the Department of Medical and Surgical-Sciences, Sapienza University of Rome. The informed consent was obtained from all the participants. All the study procedures were performed according to the Declaration of Helsinki and received the approval of the local Ethics Committee.

Inclusion criteria were age ≥18 years and capability to give the informed consent. Patients with coexisting conditions inducing malnutrition, chronic kidney diseases, infections, heart failure, liver cirrhosis, rheumatologic disorders and with clear sign of malabsorption or intestinal occlusion, as well as dysphagia, were excluded.

Blood and specimens of skeletal muscle were collected from patients who underwent abdominal surgery for cancer disease (GI cancer) or for a non-malignant conditions (controls), including hernia, gallstones or cysts. Blood was collected into K2EDTA vacutainers and centrifuged, and the resulting plasma was stored at −80°C for subsequent analysis. Muscle biopsies from the *rectus abdominis* muscle were collected during the first phases of surgery. Samples were in

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part snap-frozen in liquid nitrogen and in part included in PolyFreeze matrix (P0091, Sigma-Aldrich) inside a cryomold and then frozen in liquid nitrogen and stored at −80°C.

Nutritional, inflammatory characteristics and assessment of muscularity

In both cancer patients and controls, nutritional status was evaluated by assessing current body weight, calculating body mass index (BMI) and registering involuntary body weight loss in the previous 6 months. In fasting condition, blood samples were collected and stored in EDTA tubes, and albumin, C-reactive protein concentration and haemoglobin levels were assessed with standard automated techniques. Based on patient clinical records, data on tumour stage and histology, as well as on co-morbidities, were collected.

As for muscularity, all cancer patients underwent total body CT-scan for cancer diagnosis and staging, allowing to evaluate the level of muscularity using previously validated indexes in this setting.[S1] In brief, axial slices at the level of the third lumbar vertebrae (L3) were identified. Skeletal muscle area (SMA) was determined. This includes paraspinal muscles (erector spinae and quadratus lumborum), psoas, abdominal wall muscles, including rectus abdominis, transversus abdominis, external oblique and internal oblique muscle. Skeletal muscle index (SMI) was calculated as SMA (cm^2)/patients' height-squared (m^2). All images were analysed by Osirix Lite DICOM Viewer (version 11.0.3, Geneva, Switzerland).

Patients were divided into two groups according to sex-specific tertiles of SMI, as previously described[S2,S3]; in particular, low muscularity patients were identified as those at the lowest tertile of SMI, whereas the moderate/high muscularity patients were those falling in the middle and highest tertiles of SMI.

RNA isolation and quantification

RNA from muscle biopsies was isolated using the standard phenol-chloroform method. Briefly, approximately 100 mg of pulverized muscle were resuspended in 1 mL of TRI Reagent. After a quick centrifuge at 12 000× *g*, 200 μL of chloroform were added to the supernatant, mixed vigorously and centrifuged at 12 000× *g* for 15 min. The RNA in the aqueous part was precipitated with 2-propanol and 70% ethanol, dried at room temperature, resuspended in sterile water, incubated at 55°C for 10 min and stored at −80°C until use. The RNA quality and quantity were verified according to the MIQE guidelines (http://miqe.gene-quantification.info/). The RNA concentration was quantified with Qubit(R) microRNA Assay Kit (Invitrogen) with Qubit fluorometer 4.0 (Invitrogen). As for plasma samples, 250 μL aliquots were centrifuged at 16 000× *g* for 15 min at 4°C to remove cell debris, and total RNA was isolated using the miRneasy Serum/Plasma Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. RNA quantification was performed using the Multiskan Sky spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Finally, circulating MVs were isolated from in 1 mL of plasma and RNA was extracted as previously described.[19]

Quantification of circulating miRNAs by real-time quantitative PCR (RT-qPCR)

Mature miRNAs from plasma samples were retrotranscribed from 10 ng of total RNA using the TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Waltham, MA, USA) following the manufacturer's protocol. Transcript levels were determined by quantitative real-time PCR using the Step-One Real-Time PCR System (Applied Biosystems) and TaqMan Fast Advanced Mater Mix (Applied Biosystems) following manufacturer's instructions. Quantification was performed using specific TaqMan miRNA Assay probes (A25576, Applied Biosystems; Table S1): hsa-miR-15b-3p, hsa-miR-21-5p, hsa-miR-29a-3p, hsa-miR-29b-3p, hsa-miR-133a-3p, hsa-miR-206 and hsa-miR-486-5p. The hsa-miR-16-5p was used as endogenous control after having verified the stability of its expression.

Library preparation for small RNA-sequencing (sRNA-Seq)

Small RNA (sRNA) transcripts were converted into barcoded cDNA libraries using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (Protocol E7330, New England BioLabs Inc., USA; New England BioLabs Inc., USA) as previously described.[S4-S6] For each sample, approximately 100 ng of RNA was used as starting material to prepare libraries. Each library was prepared with a unique indexed primer so that the libraries could all be pooled into one sequencing lane. Each pool of 24–28 libraries was loaded on a single read sequencing flow cell and sequenced on an Illumina NextSeq500 (75 cycles sequencing-by-synthesis; Illumina Inc., USA).

After library prep PCR amplification, the cDNA constructs were purified with the QIAQuick PCR Purification Kit (Qiagen, Hilden, Germany) as described in the NEBNext Multiplex Small RNA Library Prep Protocol E7330. We performed a size selection of the pooled cDNA constructs using Novex Tris-borate-EDTA (TBE) gels (Invitrogen) (6%) and following the procedure of gel electrophoresis running and purification of the construct described in the Illumina TruSeq small RNA library prep protocol. The 140-nt and 150-nt bands correspond to adapter-ligated constructs derived from RNA fragments of 21 to 30 nt (mainly miRNAs and piRNAs but also other sRNAs

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of similar size). Final quality checks of size, purity and concentration for the sequences in the DNA libraries were performed with Bioanalyzer 2100 run performed with a high-sensitivity DNA kit (Agilent Technologies, Germany) and Qubit DNA high-sensitivity assay kit (Invitrogen). Raw sequencing data were deposited on Gene Expression Omnibus with the identifier GSE210546 (token for reviewer access: ctsnkeggrbyhpyt).

Analysis of sRNA-seq data

Raw reads were preprocessed and converted to a count matrix by the pipeline described in Sabo et al. (2022).[S6] Briefly, after adapter clipping, reads were mapped to a sncRNA reference, and they were assigned to the best matching reference sequence. For miRNAs and piRNAs, detected reads had to cover at least 70% of the reference sequence, while for longer sncRNAs, this was not required, thus accepting also fragments of the reference sncRNAs. Finally, sncRNAs without at least 20 reads in at least half of the samples were dropped from the final matrix. Differential expression analysis was performed with DESeq2 package for R with the sample pool, age and sex of the patients as confounders. sncRNAs were considered significantly associated with a condition or a trend if their p-value was below the 0.05 threshold after adjustment for multiple testing by false discovery rate (FDR). miRNA target genes were retrieved by miRWalk 3.0 database.[S7] EnrichR was used for gene ontology (GO) and pathway enrichment analyses.[S8,S9]

Statistical analysis

Data representation and statistical tests were performed with Prism (version 7, GraphPad) software, unless differently stated. The normality of distributions was evaluated by the Shapiro–Wilk test. The significance of the differences among groups was evaluated by appropriate two-sided statistical tests indicated in figure legends. Receiver-operating characteristic (ROC) analysis was performed using the Wilson/Brown method with a confidence interval of 95%.

Results

Patient characteristics and muscularity assessment

The study population included a total 40 participants, being 25 GI cancer patients and 15 controls who underwent abdominal surgery for cancer disease or for a non-malignant condition. Among GI cancer patients, 9 had colorectal, 7 gastric and 9 pancreatic tumours (stage I-II, n = 16 and stage III-IV, n = 9). GI cancer patients (12 males, 48%) presented with a mean age of 71.6 ± 11.2 years, a median body weight loss of 4.2% (3.3; 6.5) and a mean BMI of 27.0 ± 3.22 kg/m^2 (Table 1). Control group (6 males, 40%) showed a mean age 58.1 ± 13.9 years and a mean BMI of 28.0 ± 4.3 kg/m^2 (Table 1). Among control group, none of the participants reported involuntary body weight loss in the previous 6 months.

In cancer patients, at CT-scan, the median L3-SMI (cm^2/m^2) was 42.52 (34.42; 49.07). Males showed a median L3-SMI of 46.08 (41.17; 51.79) and females a median L3-SMI of 40.77 (33.73; 42.87). According to the sex-specific tertile of L3-SMI (identified cutoff values: 34.42 for women and 42.86 for men), moderate-high muscularity group (above the L3-SMI cutoffs) included 17 patients (six pancreatic, six gastric and five colorectal) and low muscularity group (below the L3-SMI cutoffs) included eight patients (three pancreatic, one gastric and four colorectal).

Table 1 Characteristics of the study population.

Participants (n = 40)	Cancer patients (n = 25)	Controls (n = 15)	P-value
Female, n (%)	13 (52)	9 (60)	0.622
Age, year	71.6 ± 11.2	58.1 ± 13.9	0.002
Body mass index, kg/m^2	27.0 ± 3.2	28.0 ± 4.3	0.451
Weight loss (% in 6 months)	4.2 (3.3; 6.5)	0.00 (0.00; 0.00)	<0.001
C-reactive protein, mg/dL	1.42 (0.28; 3.23)	0.27 (0.19; 0.50)	0.052
Haemoglobin, g/dL	11.27 ± 2.44	14 ± 2	0.001
Albumin, g/dL	3.2 (3; 3.5)	4.00 (3.8; 4)	< 0.001
Creatinine, mg/dL	0.83 (0.73; 1.25)	0.79 (0.71; 0.93)	0.384
L3-SMI, cm^2/m^2	42.52 (34.42; 49.07)	/	
Co-morbidities			
Diabetes, n (%)	7 (28)	4 (27)	
Hypertension, n (%)	15 (60)	9 (60)	
Dyslipidaemia, n (%)	7 (28)	7 (47)	
Type of cancer			
Pancreas, n (%)	9 (36)		
Colorectal, n (%)	9 (36)		
Stomach, n (%)	7 (28)		

Median (interquartile range) is shown for non-normally distributed variables (weight loss, C-reactive Protein, albumin, creatinine and L3-SMI).

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Plasma miRNAs in cancer patients and controls

Selected circulating miRNAs (miR-15b-5p, miR-21-5p, miR-29a-3p, and miR-29b-3p, miR-133a-3p, miR-206 and miR-486-5p) were assessed in 24 out of 25 cancer patients and in 14 out of 15 controls after the exclusion of one blood sample for each group due to specimen deterioration. The selection was based on previous reports involving those miRNAs in muscle homeostasis.[10] In particular, miR-133a-3p, miR-206 and miR-486-5p belong to muscle-specific miRNAs (myomiRs).[13] Similarly, miR-21-5p and miR-29b-3p were shown to contribute to myofiber atrophy.[20,S10]

Plasma levels of miR-15b-5p were reduced in cancer patients versus controls, whereas those of miR-21-5p were increased in the same comparison (Figure 1A). According to ROC analysis, both miR-15b-5p and miR-21-5p were sensible predictors of cancer diagnosis (Figure 1B). When GI cancer patients were stratified according to muscularity, the moderate-high group only showed a significant increase of miR-21-5p levels, associated with a trend to reduced miR-15b-5p (Figure 1C). The circulating levels of miR-29a-3p and miR-29b-3p were comparable between controls and cancer patients, irrespective of muscularity (Figure 1A,B). None of the above-mentioned miRNAs were able to distinguish between high-moderate muscularity and low muscularity groups (Supporting Information, Figure S1).

As for myomiRs, only miR-133a-3p levels were altered, being higher in plasma samples of GI cancer patients compared with controls (Figure 1A) and served as a sensible biomarker to discriminate controls and GI cancer patients (Figure 1B). When clustered according to muscularity, miR-133a-3p presented high expression in the moderate-high group (Figure 1C) and presented a high sensibility in distinguishing moderate-high muscularity patients from low muscularity ones (Figure 1D). Similarly, miR-486-5p as well was a good predictor of muscularity among cancer patients (Figure 1D). When samples were clustered by both muscularity and sex, increased levels of miR-133a-3p and miR-206 were observed only in males belonging to the moderate-high group, whereas no differences on miR-486-5p levels were observed after stratification for sex (Figure 1E). The levels of the studied myomiRs were similar among all groups in female patients (Figure 1E).

Recently, tumour-derived microvesicles (MVs) were shown to delay myoblast differentiation and to impair mitochondrial respiration in C2C12 myotubes. Such a pattern was likely associated with specific miRNAs and other sncRNAs contained into MVs.[19,20] Along this line, the changes in circulating miRNAs observed in GI cancer patients could result, at least partially, from modulations of MV content. To clarify this point, sRNA-Seq was performed on MVs isolated from the plasma of GI cancer patients and controls. The results showed that MVs content, highly enriched with miRNAs and other sncRNA biotypes, was comparable between cancer patients and controls (Supporting Information, Table S3).

Dysregulation of sncRNAs in the skeletal muscle of male GI cancer patients

A median of 13 million (minimum 6.6 and maximum of 39 million) reads were obtained from skeletal tissues of GI cancer patients (n = 25) and controls (n = 15). Of these reads, a median of 60% were correctly mapped to the reference sncRNA sequences. One sample in the GI cancer group was excluded due to suboptimal sequencing quality results. Using a minimum read cut-off of 20 normalized counts, 373 miRNAs and 190 piRNAs were identified, accounting for 72.54% and 18.7% of raw reads, respectively (Figure 2A). Principal component analysis (PCA) showed poor clustering among the two groups when mapping PC1 versus PC2 but better group separation in PC2 versus PC3 (Supporting Information, Figure S2). A total of 66 miRNAs (10 up-regulated and 56 down-regulated) resulted differently expressed in GI cancer patients in comparison to controls (Figure 2B, Supporting Information, Table S4). Among the down-regulated miRNAs, 10 presented a log2(fold change) lower than −2, with miR-223 and miR-15b being represented by both 3p and 5p mature sequences (Figure 2C). Similarly to miRNA expression pattern, among the dysregulated piRNAs (24), the majority (19) were down-regulated, including the top two most expressed piRNAs in the skeletal muscle: piR-12790 and piR-2106 (Figure 2D,E, Supporting Information, Table S4). Finally, most of the small nucleolar RNA (snoRNAs) significantly dysregulated were up-regulated (19 out of 27) in the muscle of cancer patients versus controls (Figure 3F,G, Supporting Information, Table S4).

Differences in miRNA expression levels related to sex were previously reported.[S11-S13] Considering these observations, sncRNA profiles were also analysed separately in males and females. Interestingly, 24 miRNAs, 6 piRNAs and 4 snoRNAs resulted differently expressed in male GI cancer patients when compared with controls, most of all being down-regulated (Supporting Information, Table S5), whereas no changes in sncRNA profile were observed in female patients (Supporting Information, Table S6).

Gene ontology analysis on validated miRNA targets reveals enrichment of ubiquitin-related pathways

In order to identify potential implications of the modulated miRNAs in the skeletal muscle of GI cancer patients, gene ontology (GO) and pathway enrichment analyses were conducted on validated gene targets of down-regulated miRNAs

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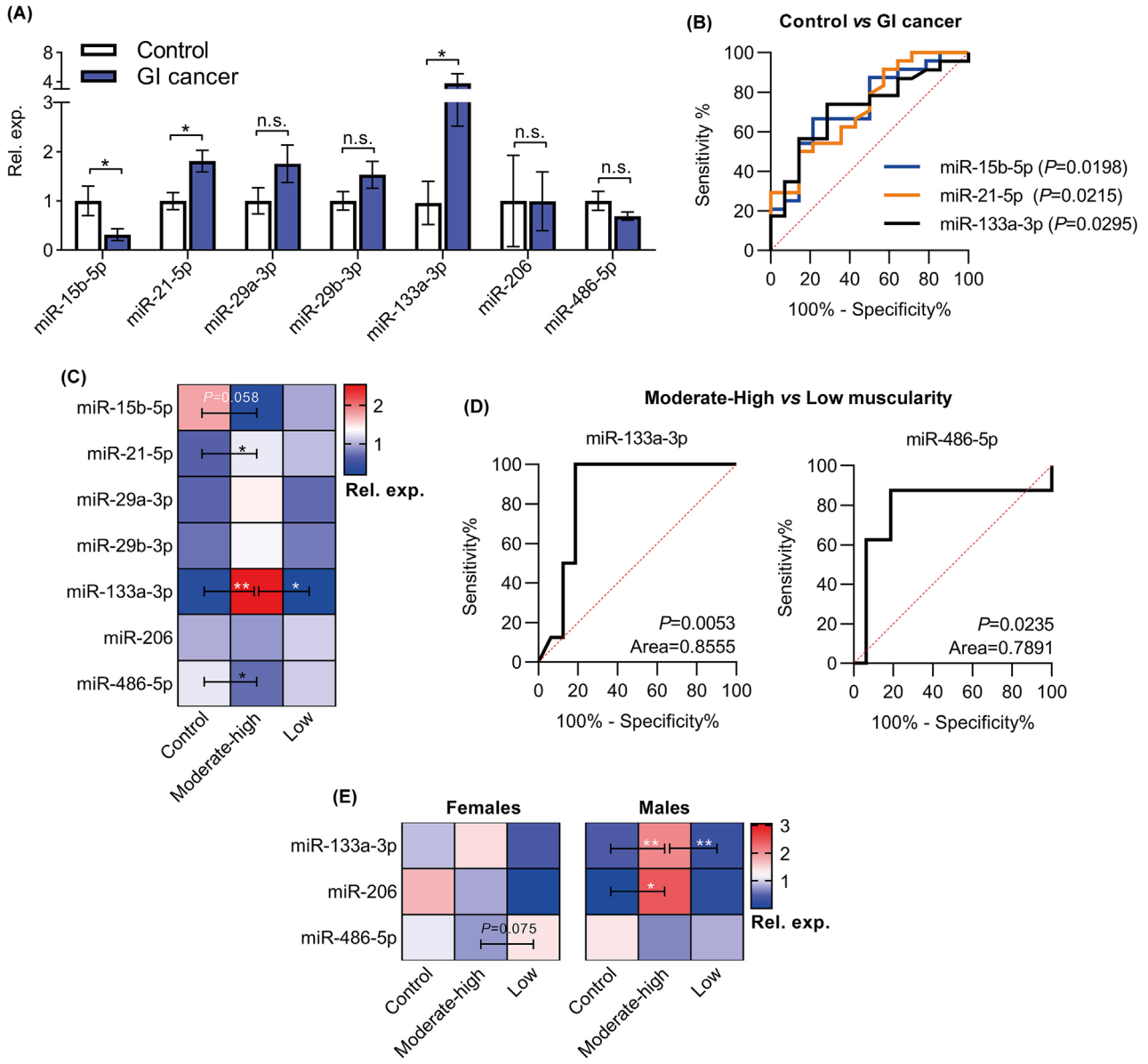

Figure 1 The levels of selected plasma circulating miRNAs are altered in GI cancer patients according to muscularity and sex. (A) Relative expression (mean ± SD) of plasma miRNAs assessed by RT-qPCR (control n = 13–14, GI cancer n = 20–24). (B) ROC curves of single miRNAs for the prediction of control or GI cancer patients. (C) Heatmap displaying the relative abundancy (mean) of miRNAs according to muscularity (control n = 13–14, moderate-high n = 13–18, low n = 7–8). (D) ROC curves of single miRNAs for the prediction of muscularity degree in cancer patients. (E) Heatmap displaying the relative abundancy (mean) of miRNAs according to both muscularity and sex (females: Control n = 8, moderate-high n = 7–9, low n = 4; males: Control n = 6, moderate-high n = 6–7, low n = 3–4). Statistical analysis: (A) Mann–Whitney test: *$P < 0.05$. (B, D) Wilson/Brown method with a confidence interval of 95%. (C, E) One-way ANOVA + Tukey's test (normal distribution) or Kruskal–Wallis + Dunn's test (non-normal distribution): *$P < 0.05$, **$P < 0.01$.

with a log2(fold change) lower than −1 (accounting for 52 miRNAs) using the EnrichR platform. GO analysis by molecular function retrieved an enrichment in 62 different pathways. Processes with less than 20 targeted genes were excluded. Among the remaining 33 pathways, four entries were related to ubiquitin-ligase and transferase activity (Supporting Information, Table S7). To further select the most relevant miRNAs that regulate genes involved in ubiquitin metabolism, miRNA-mRNA validated interactions included in the 'ubiquitin protein ligase binding' (GO:0031625) and 'ubiquitin-protein transferase activity' (GO:0004842) entries were merged and plotted as an interaction network (Figure 3). Network analysis revealed miR-106b-5p, miR-15b-5p and miR-106a-5p as central interactors with mRNAs related to ubiquitin ligase/transferase activities, interacting with 17, 12 and 10 different targets, respectively (Figure 3). The retrieved net-

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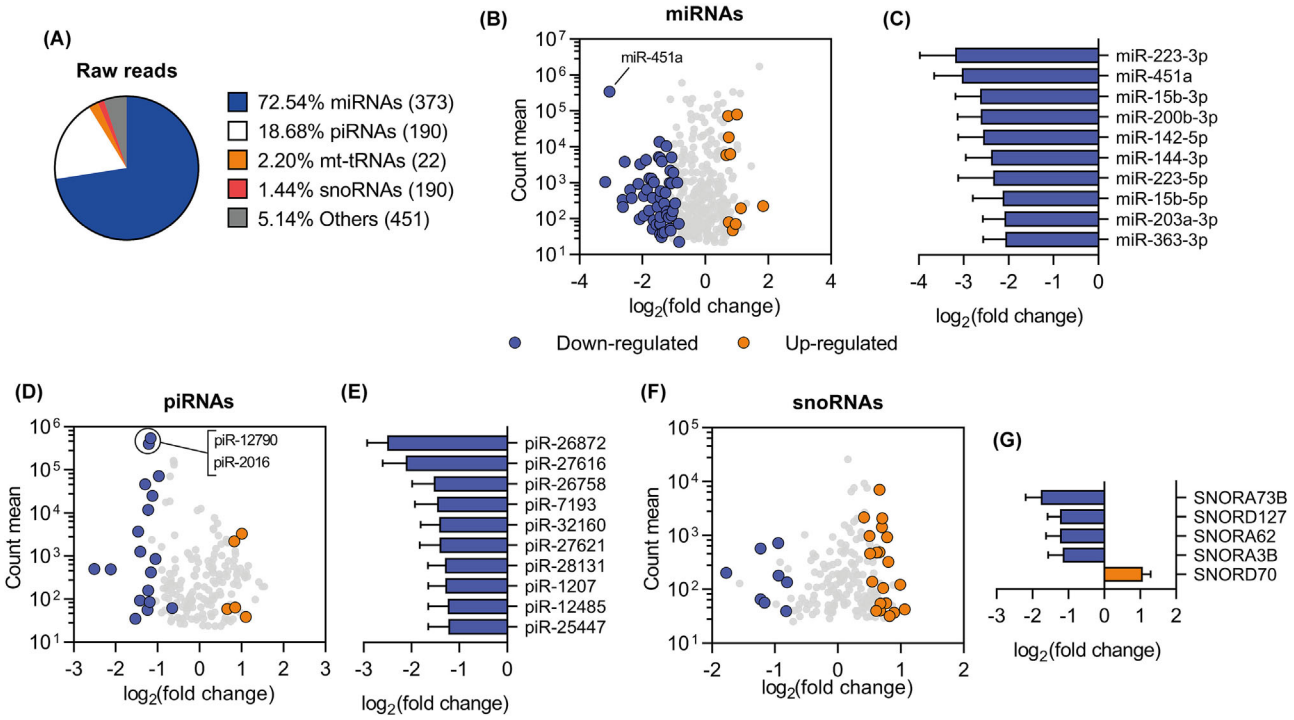

Figure 2 MiRNAs and other sncRNAs signature in the skeletal muscle of GI cancer patients versus controls. (A) Total amount of raw reads and molecular diversity (in parentheses) according to sncRNA biotypes. (B) Dot chart representing sequenced miRNAs according to up-regulation (orange), down-regulation (blue) and non-alterations (grey) in GI cancer patients compared to the control group. Dots are plotted according to count mean and log2(fold change). (C) Down-regulated miRNAs with log2(fold change) lower of −2 in GI cancer patients (mean ± SD). (D) Dot chart representing sequenced piRNAs according to up-regulation (orange), down-regulation (blue) and non-alterations (grey) in GI cancer patients versus controls. Dots are plotted according to count mean and log2(fold change). (E) Top 10 dysregulated piRNAs according to highest absolute log2(fold change) in GI cancer patients (mean ± SD). (F) Dot chart representing sequenced snoRNAs according to up-regulation (orange), down-regulation (blue) and non-alterations (grey) in GI cancer patients versus controls. Dots are plotted according to count mean and log2(fold change). (G) Dysregulated snoRNAs with absolute log2(fold change) over 1 in GI cancer patients versus controls (mean ± SD).

work also revealed *SMAD2* as the most important node, being targeted by five different miRNAs (Figure 3).

Discussion

The present study reports new evidence of a broad dysregulation of several sncRNA biotypes in the skeletal muscle of patients suffering from GI cancer. Among sncRNAs, miRNAs (myomiRs in particular) were proposed to play important roles in skeletal muscle homeostasis, including the regulation of muscle size, metabolism and regeneration.[S14] Alterations of miRNA expression in the muscle of cancer patients with cachexia were previously reported.[14,16] Yet, to the best of our knowledge, this is the first study investigating miRNAs levels in both skeletal muscle and circulation in a single cohort of GI cancer patients stratified according to the level of muscularity and compared with control subjects.

The results here reported highlighted that some of the miRNAs down-regulated in the muscle of cancer patients participate to pathways relevant to the regulation of ubiquitin ligase activity, thus likely impinging on muscle proteostasis. The most repressed miRNA was miR-223-3p, and this was in agreement with a report showing its down-regulation in mice affected by myotonic dystrophy type 1.[21] Previous studies showed that miR-223-3p expression was comparable in cachectic versus non-cachectic cancer patients, but it was up-regulated in the muscle of mice bearing the Lewis lung carcinoma[22] or the colon 26 carcinoma (unpublished data). From the pathway enrichment analysis, miR-223-3p resulted as a player in the regulation of E3-ligase activity, being able to down-regulate *FOXO1* expression.[S15] Importantly, miR-223-3p also represses *FOXO3*,[S16] a transcription factor that induces the expression of muscle-specific E3-ligases.[23] *FOXO3* is a validated target of other three miRNAs (miR-96-5p, miR-182-5p and miR-221-3p),[S17-S19] down-regulated in the muscle of GI cancer patients. As FOXO3 was not included in the 'Ubiquitin protein ligase binding' (GO:0031625) GO biological process, this gene was not reported in the network analysis shown in Figure 3. Finally, miR-223-3p also reduced the expression of IL-6,[S20] a cytokine that activates the STAT3 pathway promoting the expression of atrogenes,[24] and its direct binding to *Stat3* was validated in mice.[S21] In addition to

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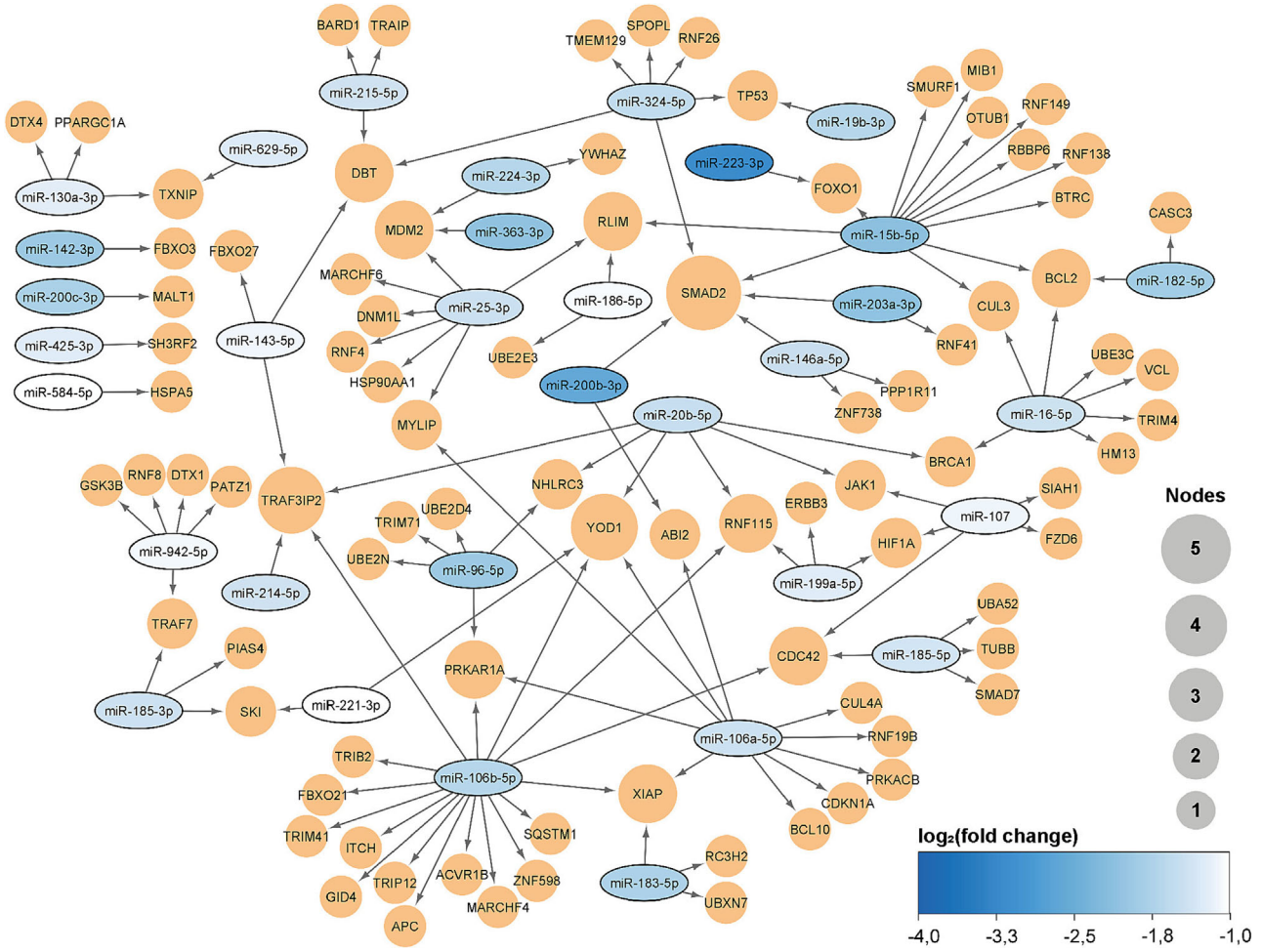

Figure 3 Network of validated mRNA-miRNA interactions annotated within GO pathways regarding ubiquitin protein ligase/transferase activity. Schematic representation showing the potential interaction between down-regulated miRNAs in the skeletal muscle of GI cancer patients and their validated targets with 'ubiquitin protein ligase binding' (GO:0031625) and 'ubiquitin-protein transferase activity' (GO:0004842) functions (performed with Cytoscape). Colour of miRNAs indicates their log2(fold change) in GI cancer patients vs control samples. Size of the circumference indicates the number of different miRNAs targeting a single gene (nodes).

miR-223-3p, miR-15b-5p was also markedly down-regulated in the muscle of GI cancer patients of our cohort. This miRNA accounts for up to 12 validated targets related to 'ubiquitin protein ligase/transferase activity' functions, including *FOXO1* and *SMAD2*, this latter being targeted by five different miRNAs (Figure 3).

The analysis of circulating miRNAs showed that miR-21-5p and miR-133a-3p levels were higher in cancer patients than in controls and performed as good biomarkers for cancer diagnosis. As in the muscle, miR-15b-5p was reduced also in plasma confirming previous observations in colorectal cancer patients[25] and associated with poor prognosis in patients with liver cancer.[26] The pattern of miR-15b-5p expression here observed suggests that its plasma levels could be a good indicator of its expression in the skeletal muscle, although the relevance of circulating miR-15b-5p to cancer-induced muscle wasting is unknown. The increased circulating levels of miR-21-5p in GI cancer patients are consistent with previous studies showing that this miRNA is up-regulated in tumour cells as well as in the circulation of tumour hosts and is involved in the regulation of cellular processes such as proliferation, death and invasiveness.[S22-S24] In addition, miR-21-5p was proposed to play a role in mediating muscle wasting in cancer cachexia.[20] Consistently, high levels of miR-21-5p were inversely correlated with *psoas* muscle mass in patients with colorectal cancer.[27] However, these observations did not perfectly fit with the data here reported, considering that the increase of circulating miR-21-5p was significant in patients with moderate-high muscularity versus controls but not in the low muscularity group versus controls, a discrepancy that could be determined by the heterogeneity of the GI cancer patients.

As for myomiRs, the results here reported conform to previous studies showing that they are frequently modulated in

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pathological states associated with muscle wasting.[28] As an example, myomiRs such as miR-133a-3p, miR-133b-3p and miR-206-3p are up-regulated in the circulation of patients affected by Duchenne muscle dystrophy, while miR-133 is down-regulated at the muscle level in the same patients.[13] Similarly, some myomiRs are up-regulated in experimental models of muscle damage.[29] By contrast, circulating myomiRs are very frequently reduced in cancer patients. In this regard, an interesting hypothesis is that the progressive loss of muscle mass might account for such a decrease.[13] In the present study, circulating miR-486-5p was decreased in moderate-high muscularity patients but unchanged in the low muscularity group compared to controls, performing as a good predictor of muscularity among cancer patients. Previous data revealed a reduction of circulating levels of miR-486-5p in sarcopenic older adults, positively correlating with the skeletal muscle index but not with muscle function.[30] Similarly, miR-486-5p levels were reduced in both skeletal muscle and plasma of tumour-bearing animals.[31] Circulating levels of miR-133a-3p were increased in GI cancer patients versus controls consistently with previous data showing that high miR-133a-3p expression levels were associated with reduced whole body protein synthesis in overweight volunteers exposed to energy restriction.[32] The observation that miR-133a-3p up-regulation occurred in patients with moderate-high muscularity but not in those with low muscularity could reflect a different regulation of muscle proteostasis between the two groups.

The data reported in the present study show that miRNA dysregulation in GI cancer patients partially followed a sex-specific pattern. Indeed, when participants were stratified according to sex, differences in specific miRNAs were observed only in males in both muscle and blood. As for circulating miRNAs, miR-206-3p and miR-133a-3p were higher in male but not in female GI cancer patients compared with controls. In this regard, previous data showed that miR-206-3p was more expressed in the blood of post-ischaemic female than male mice,[S25] while no sex difference was shown in experimental amyotrophic lateral sclerosis.[33] By contrast, to the best of our knowledge, this is the first time that a sex-related modulation is reported for miR-133a-3p circulating levels. The reasons underlying such differential regulation are unclear and the possibility that this observation reflects a hormonal regulation cannot be excluded. Along this line, sex was previously shown to impinge on miRNA expression, and a miRNA sex-bias was reported in healthy[34] and pathological states, such as metabolic syndrome, autoimmune diseases and cancer,[35] possibly contributing to the different susceptibility to cancer and cachexia development observed in males and females.[36]

Besides miRNAs, other sncRNAs are emerging as important actors in a broad range of cellular functions. Specific piRNAs are dysregulated in cancer, and some of them were correlated with tumour growth and invasiveness and proposed as biomarkers.[S26] However, the relevance of piRNAs to the homeostasis of fully differentiated tissues, in particular skeletal muscle, is almost completely unknown. A recent study reported that piRNA expression was lost during the programmed degeneration of intersegmental muscles of *Manduca sexta*, suggesting that piRNAs might play a role in the regulation of muscle mass.[18] Given their direct contribution to genome stabilization during germ cell generation, it is assumed that piRNAs contribute to genomic stability also in adult stem cells. Along this line, the inhibition of the transposable element LINE1 improved post-ischaemic functional recovery in the heart by promoting the activity of the Akt-dependent signalling pathway,[37] suggesting that piRNAs might also participate in skeletal muscle regeneration.

The present study also reveals that most of the snoRNAs dysregulated in the muscle of GI cancer patients were overexpressed when compared to controls. SnoRNAs, the most abundant group of intron-encoded ncRNAs, are small RNAs of 60–300 nucleotides in length and are predominantly found in the nucleolus.[S27] In view of their size, snoRNAs are considered borderline small RNAs since some members of this class are larger than the 200 nt limit that differentiate small and long RNAs. At present, snoRNAs are poorly investigated, and only scanty observations are available in the literature suggesting that these sncRNAs could be relevant to muscle homeostasis and, potentially, also to its alterations occurring in cancer cachexia. In this regard, previous data showed that snoRNAs belonging to the SNORD family (C/D box snoRNAs) are highly expressed in the muscle of patients affected by amyotrophic lateral sclerosis. As SNORDs participate in the post-transcriptional modification of rRNA, their increased expression could reflect the enhanced ribosomal turnover rates occurring in amyotrophic lateral sclerosis.[38] SnoRNA up-regulation was also reported in the muscle of patients with myotonic dystrophy type 1, positively correlating with muscle mass and function.[39] Quite recently, mice lactating from mothers fed a low protein diet showed significant deregulation of muscle snoRNAs,[40] adding to the idea that snoRNAs might play a role in the regulation of muscle protein synthesis. These observations seem to highlight that snoRNA up-regulation occurs in pathological states associated with muscle wasting, consistently with the results shown in the present study. However, while suggesting a role for piRNAs/snoRNAs in the regulation of muscle homeostasis, the observations here reported are at present not enough to define a causative link to cancer cachexia, and further studies are warranted in this regard.

While reporting new data in the field of sncRNAs and cachexia, this study has some limitations. These include the higher age of cancer patients versus controls. Although the

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effect of this confounding variable was taken into consideration during the analysis of the sequencing data (see Methods section), it is a plausible main contributor of sample variability. In this regard, the heterogeneity in the patient population might also explain the lack of sncRNA dysregulation in circulating MVs. Beyond that, the results on circulating miRNAs indicate that further sequencing in whole plasma RNA might help to identify other potential plasma miRNAs relevant to the diagnosis, classification and monitoring of muscle dysfunction in cancer patients. Another potential limitation is that the study did not include muscle function assessments which could have been correlated with both modulations of sncRNA profiling and level of muscularity. Finally, due to the very high variability among samples, the attempt to elucidate down-stream targets of miRNAs modulated in GI cancer patients did not provide any appreciable result (data not shown), thus remaining an issue that deserves to be further investigated in the future.

On the whole, the results here reported show the occurrence of a dysregulation of circulating and muscle miRNAs in GI cancer patients, showing that at least miR-15b-5p is consistently regulated in both compartments. Moreover, an altered expression of sncRNA biotypes other than miRNAs (piRNAs, snoRNAs) was present in the skeletal muscle of cancer patients. Interestingly, these data indicate an association of circulating miRNAs with muscularity rather than with body weight loss, as well as a sex-specific dysregulation of muscle and circulating sncRNAs in GI cancer patients to be further elucidated in additional clinical studies.

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The authors of this manuscript certify that they comply with the ethical guidelines for authorship and publishing in the *Journal of Cachexia, Sarcopenia and Muscle*.[S28]

Conflict of interest

All authors declare that they have no conflict of interest.

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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