MyomiRs in cultured muscle cells from patients with idiopathic inflammatory myopathy are modulated by disease but not by 6-month exercise training

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Abstract

Objective

Idiopathic inflammatory myopathies/IIM are associated with changes in muscle-specific microRNA/miR. Exercise improves muscle function and metabolism in parallel with changes in miR expression. We investigated the effects of disease and exercise on miRs in differentiated muscle cells/myotubes from IIM patients and controls.

Methods

Samples of m. vastus lateralis were obtained by needle biopsy from IIM patients before/after 6-month training and from matched sedentary healthy controls. Muscle cell cultures were established and exposed to saturated fatty acid during differentiation. MiR-133a,-133b,-206,-1 and their target genes (qPCR), fat oxidation (FOx), lipids (chromatography) and mitochondrial oxidative phosphorylation (OxPHOS) complexes (immunoblotting) were measured. Interrelations between in vitro miRs and metabolism of myotubes as well as clinical parameters and disease activity/MITAX were explored.

Results

Levels of miRs were higher in myotubes derived from IIM patients compared to healthy controls (up to 3.5-fold, $p<0.05$). Neither 6-month training (IIM patients) nor in vitro palmitate treatment modulated myomiRs in myotubes. However, miR-133a,-133b, and miR-1 correlated negatively with FOx ($p<0.01$), triacylglycerols ($p<0.05$) and OxPHOS complex-V ($p<0.05$) and positively with OxPHOS complex-I ($p<0.05$) in myotubes. MiR-133a and miR-133b in myotubes were related to disease activity and fasting glycaemia in vivo (both $p<0.05$).

Conclusion

Upregulation of microRNAs involved in myogenesis and regeneration in muscle cells derived from IIM patients indicates activation of compensatory epigenetic mechanisms, potentially aimed to counteract disease progression. Relationships of microRNAs with in vitro metabolic profile of muscle cells as well as with clinical parameters support the role of muscle-specific microRNAs in modulating muscle metabolism and clinical state of patients.

Key words

myositis, exercise, muscle cells, microRNAs, lipid metabolism

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Introduction

Idiopathic inflammatory myopathies (IIM) are chronic autoimmune diseases, which include dermatomyositis, polymyositis, immune mediated necrotising myopathy, and sporadic inclusion body myositis. IIM typically present with proximal muscle weakness, fatigue, and extramuscular manifestations (1, 2). Skeletal muscle infiltration with inflammatory cells, which indicates chronic inflammation, is commonly observed in IIM and is being considered the major cause of muscle damage (3). However, owing to the fact that disease symptoms and muscle damage often persist even in the absence of inflammation, other non-immune pathomechanisms have been implicated in the IIM, including the stress of endoplasmic reticulum, oxidative stress as well as mitochondrial and metabolic dysfunction (4, 5).

Physical exercise was shown to be an effective and well-tolerated therapeutic tool in the management of IIM, improving muscle function and clinical state even in patients with active disease (6). Endurance exercise increases aerobic fitness, promotes angiogenesis and mitochondrial biogenesis in skeletal muscles while simultaneously suppressing the chronic inflammatory state in IIM patients (7, 8). Immunosuppressive treatment combined with physical exercise is recommended for patients with IIM to reduce disease activity and improve muscle performance (9, 10).

Epigenetic mechanisms regulate patterns of gene expression in a tissue-specific manner, representing means of gene-environment interactions, capable of inducing changes that could be transferred to next generations of cells (11). Epigenetic mechanisms include DNA methylation, histone modification, and microRNAs. Besides their capacity to modulate the complexity of exercise adaptations, microRNAs can be used as biomarkers of adaptive response to exercise (12). MicroRNAs (miRNAs) are 19–23 nucleotides long RNAs with the capacity to regulate gene expression at the post-transcriptional level by inhibiting translation or degrading transcripts by binding to target mRNAs (13). The levels of specific microRNAs in both circulation (14-16) and in skeletal muscles (17, 18) were shown to be specifically regulated in patients with different IIM subtypes, thus reflecting the specific epigenetic signature which could distinctly modulate patient's clinical state and disease progression.

Tissue-specific miRNA are characterised by >20-fold higher levels in the tissue of interest as compared to global expression levels across other tissues. Muscle-specific miRNAs (miRNA-1, miRNA-133, miRNA-206, miRNA-208, miRNA-499), also called myomiRs, are involved in the regulation of myogenesis and muscle regeneration capacity, related to muscle hypertrophy or dystrophy (19) as well as to the adaptive response of muscle to exercise (20). MiR-1, miR-133a, miR-133b, and miR-206 together account for nearly 25% of all miRNA expression in skeletal muscle (21) and are profoundly regulated by exercise, ageing, and disease (12, 21, 22). It has been shown recently that skeletal muscle microRNA profile can be altered by 12-week endurance training in patients with polymyositis or dermatomyositis (23). Differential expression of myomiRs following exercise (24) or in skeletal muscle of patients with IIM (17, 18) likely contributes to the adaptive process induced by exercise and/or muscle inflammation/atrophy.

Human primary skeletal muscle cells represent a model for studying physiological phenotypes intrinsic to skeletal muscle, which are independent of the whole-body neuro-humoral environment and retained *in vitro* by epigenetic mechanisms (25). Cultured differentiated muscle cells (myotubes) display morphological, metabolic, and biochemical similarities to adult skeletal muscle (26), and can be used to explore mechanisms of muscle insulin resistance, lipid and glucose metabolism, secretory activity or microRNA role in epigenetic regulation (25, 27-30). Previously, we demonstrated that differentiated muscle cells derived from muscle of patients with IIM displayed a reduced capacity to handle the increased load of saturated fatty acids *in vitro* (31). Importantly, relatively short, 8-to-12-week exercise interventions are associated with metabolic adaptations in fatty acid and glucose metabolism detectable also in hu-

man primary myotubes *in vitro* (32, 33). We showed that the 6-month intensive supervised exercise nearly normalised altered lipid metabolism and oxidative capacity in cultured muscle cells derived from IIM patients (31).

The aim of this study was to identify the impact of IIM and 6-month supervised training as well as *in vitro* saturated fatty acid (palmitate) treatment on expression levels of selected myomiRs in muscle cells, derived from skeletal muscle of IIM patients. Therefore, we compared cells obtained from IIM patients before and after 6-month training, and cells from muscle of age/gender/BMI-matched sedentary healthy individuals. We hypothesised that IIM, patients' clinical characteristics, and/or exercise training-related and palmitate-induced adaptive response would be linked to parallel changes in the expression of selected myomiRs in cultured human primary myotubes.

Materials and methods

Ethics

Study protocol was approved by the Ethics Committee of the Institute of Rheumatology on June 16th, 2015 (approval no. 5688/2015) and it conforms to the standards set by the latest version of the WMA Declaration of Helsinki, as adopted by the 64th WMA General Assembly (Fortaleza, Brazil, 2013). Clinical trial was retrospectively registered on May22nd, 2020 (registration No. ISRCTN35925199). All the volunteers signed the written informed consent prior entering the study.

Clinical examination and training protocol

The study protocol was approved by the ethics committee of the Institute of Rheumatology, Prague. All volunteers provided written informed consent prior to entering the study. The comprehensive clinical examination was performed in both IIM patients (before and after the 6-month intervention) and sedentary age, gender, BMI-matched controls (n=7/7). Clinical examination included BMI and body composition (Lunar iDXA, GE Healthcare, USA; BIA 2000-M, Datainput, Germany), 2-hour oral glucose tolerance test (oGTT), the lipid serum profile (cholesterol; triacylglycerols/TAG), profile of myositis-specific autoantibodies (Supplementary Table S1), a composite score of disease activity (myositis intention to treat activity index, MITAX), muscle functional capacity assessment (manual muscle testing of 8 muscles, MMT8; functional index-2, FI-2).

Patients with IIM underwent the 6-month, intensive, exercise/physiotherapy programme, consisting of supervised individually tailored training performed twice a week (1 hour a week focused on Activities of Daily Living/ADL, 1 hour a week of strength training), while patients were encouraged to perform home-based exercise routine also for the remaining 5 days of the week (0.5 hour/day ADL-training), as described in detail by Spiritovic *et al*. (36), clinical trial registration number: ISRCTN35925199.

Skeletal muscle biopsy

Samples of *m. vastus lateralis* were taken from IIM patients by Bergström needle biopsy after an overnight fast, under local anaesthesia, both before and after the 6-month exercise programme, as described in detail elsewhere (34). Biopsy has also been taken from sedentary age-, gender, BMI- and 2h-glycaemia (oGTT)-matched healthy controls who were not subjected to the exercise programme. Muscle samples were immediately cleaned from excessive blood, fat, and connective tissue and frozen in liquid nitrogen. A piece of muscle tissue (~80mg) was used to establish human primary skeletal muscle cell cultures.

Human primary skeletal muscle cell culture

A sample of freshly obtained muscle was minced, preplated on an uncoated petri dish, and incubated for 60 min (37°C, 5%CO_2) in Dulbecco's modified Eagle's medium (DMEM, Lonza) with 15% heat-inactivated FBS (Gibco) to remove adherent cells (fibroblasts). The minced tissue was subsequently transferred to T-25 collagen-coated flask and incubated in DMEM containing FBS (9%), L-glutamine, human epidermal growth factor, dexamethasone, BSA, fetuin, gentamycin, and fungizone (34, 24). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2. Differentiation was initiated at 80–90% confluence by switching to α-MEM containing Penicillin-Streptomycin, heat-inactivated FBS (2%), and fetuin (34). Media were changed every 48 hours. After 5 days of differentiation, when 70–80% of myoblasts formed multinuclear myotubes, cells were harvested and used for microRNA/RNA/DNA, proteins isolation, and fatty acid oxidation radiometric assays.

Saturated fatty acid treatment of human primary muscle cells

Forty-eight hours after the induction of differentiation, cells were treated with either palmitic fatty acid (100 μM), coupled to a fatty acid-free BSA (in 5:1 molar ratio), or with BSA alone (control cells). Palmitate treatment was performed to assess the adaptive response of muscle cells to 3 days of saturated fatty acid overload (metabolic challenge).

RNA and DNA isolation and real-time PCR

Total RNA/DNA was isolated from myotubes, using Qiazol (Qiagen, USA), as previously described in (28, 34). RNA was treated by DNase I (New England BioLabs, France). Quantity and purity of RNA and DNA were determined with NanoDrop 2000c (Thermo Fisher Scientific, USA). RNA was reverse transcribed to cDNA (miScript II RT-kit, Qiagen, USA) and used for qPCR on Quant Studio 5 (Applied Biosystems, USA) with the aid of Fast SYBR™ Green Master Mix (Applied Biosystems, USA) and sets of specific primers designed with PrimerExpress. *Ribosomal protein L13a*, *18S-rRNA,* and *β-2-microglobulin* were used as an internal reference for *Myh1, Myh7, Myogenin, BDNF, PGC1α, CPT-1, Frataxin*. Relative mitochondrial DNA (mtDNA) content was determined by quantifying the expression of mitochondrial genome-encoded gene *ND1* (NADH-dehydrogenase-subunit-1) relative to genomic DNA content of lipoprotein lipase (*LPL*) and *18S*-rRNA (Suppl. Table S2).

Relative gene expression was calculated (dCT-method).

MicroRNA isolation and quantification

Total RNA was extracted from myotubes after 5 days of differentiation using Qiazol (Qiagen). MiRNA was reversely transcribed using the miScript II RT kit (Qiagen) and purified by DNase I (New England BioLabs, France). Levels of muscle-specific miRNAs were assessed with real-time PCR using the miScript primer assays specifically detecting miR-1, miR-133a, miR-133b, miR-206 (Suppl. Table S2). SNORD44 was used as the internal reference gene to calculate ΔCT expression values.

Fatty acid oxidation (FOx) assay

The radiometric FOx assay was described in greater detail in (25, 31). Briefly, differentiated cells were pre-incubated in glucose- and serum-free media, followed by a 3-hour incubation with [1-^{14}C]-labelled palmitate (0.5 mCi/ml; ARC, USA), with/without a fatty acid challenge provided by 100μM „cold"/non-labelled palmitate (Sigma, USA), coupled to a fatty acid-free BSA. Following incubation, the medium was transferred into the 48-well custom-made CO^2-trapping plate. CO^2 released from media by perchloric acid was collected in 1M NaOH. Acidified media were spun twice/4°C, and radioactivity of supernatant containing intermediate FOx metabolites (ASP/Acid-Soluble-Products) was measured (Ecolite/Germany; TriCarb2910TR/Perkin-Elmer/USA). Cells were washed twice (ice-cold-PBS), harvested (0.25ml 0.05%SDS), and used to measure protein (BCA, Thermo-Fisher-Scientific, USA) and to detect neutral lipids content by thin layer chromatography.

Thin layer chromatography (TLC)

Lipids were extracted from the cell lysates after FOx assays. Dried lipid fractions were dissolved in chloroform:methanol (2:1; v:v) and applied to TLC-Silica-gel60 plates (Merck, Germany). Neutral lipids were separated by the ascending two-step thin layer chromatography/TLC (Folch *et al.)*. Individual spots representing phospholipids-PL, diacylglycerols-DAG, and triacylglycerols-TAG were identified by lipid standards run on the same plate and scraped off into scintillation vials to measure radioactivity. The procedure is described in greater detail by Nemec *et al*. (31). Data were normalised to the protein content of the corresponding well. The impact of IIM and the 6-month training on fatty acid oxidation, lipid accumulation, and mitochondrial OxPHOS proteins was reported elsewhere (31), and in this work, these results were used solely for the exploratory correlation analyses.

Immunoblotting

Cell protein lysates (40 μg) containing complete protease inhibitor cocktail (Roche, Germany) were mixed with a loading dye (240 mM Tris-HCl pH 6.8, 40% glycerol, 8% SDS, 5% β-mercaptoethanol, and 0.4% bromophenol blue), incubated (95°C for 5 min), separated on the 10%-SDS-PAGE and transferred to PVDF membrane (Millipore, USA). After blocking (Odyssey blocking buffer, LI-COR, USA), membranes were incubated overnight with OxPHOS human antibody cocktail (Abcam/UK, 1:250). Appropriate IRDye 680RD or 800CW antibodies (LI-COR, USA, 1:10000) were used to visualise protein content (LI-COR, USA). Protein ladder 10–180kDa (Thermo-Fisher-Scientific, USA) was used. Relative content of individual OxPHOS protein complexes was calculated as the percentage of the sum of protein signals for all five complexes (%OxPHOS).

Statistics

The *in vitro* data on the microRNA expression and metabolic phenotypes were combined with the clinical data to construct the database. Normality of the data distribution was tested, and paired and unpaired t-tests were used as appropriate (GraphPad Prism version 6.01, USA). Pearson's bivariate correlations were used to determine relationships of *in vitro* myomiRs levels with the clinical parameters and cellular *in vitro* metabolic phenotypes (JMP 4.0.2, SAS, USA). The data are presented as mean±SEM, with $p<0.05$ indicating statistical significance.

Results

Characteristics of the study population

The characteristics of the study population, including patients with IIM before and after the 6-month training programme and sedentary healthy controls matched for age, gender, BMI, and glucose metabolism (2h-glycaemia, oGTT) have already been presented (31), together with characteristics of individual patients (disease subtype, duration, dose of prednisone and muscle functional test/MMT8). The 6-month intensive supervised exercise programme improved muscle functional capacity and other clinical parameters of patients with IIM, as well as lipid metabolism in myotubes *in vitro*. Importantly, training intervention did not increase the systemic inflammatory response (31, 36). These observations point to both the efficacy and safety of physical exercise in patients with IIM.

Effects of IIM and 6-month training on myomiRs in human myotubes in vitro

Expression levels of selected myomiRs; miR-133a, miR-133b, miR-1, and miR-206, were higher in myotubes from IIM patients as compared to cells from sedentary healthy controls at both baseline conditions and after 3-day palmitate challenge (Fig. 1A-D). There was no significant effect of the 6-month training to IIM patients on myomiRs expression levels in patients' cultured myotubes (Fig. 1A-D). Palmitate exposure (3-day/100 μM) did not affect expression levels of miR-1, miR-133a, and miR-133b in differentiated muscle cells from neither IIM patients nor the healthy controls (Fig. 1A-C). However, there was a significant decline of miR-206 expression related to the 3-day palmitate challenge in myotubes derived from healthy controls (Fig. 1D). Myotubes derived from the healthy control individuals displayed higher levels of *BDNF* (target gene of miR-1 & miR-206) mRNA compared to patients with idiopathic inflammatory myopathy after 6-month training (healthy controls: 10.53±5.63 A.U. *vs*. IIM after training: 3.46±1.19 A.U., $p=0.02$). *In vitro* exposure to saturated palmitic fatty

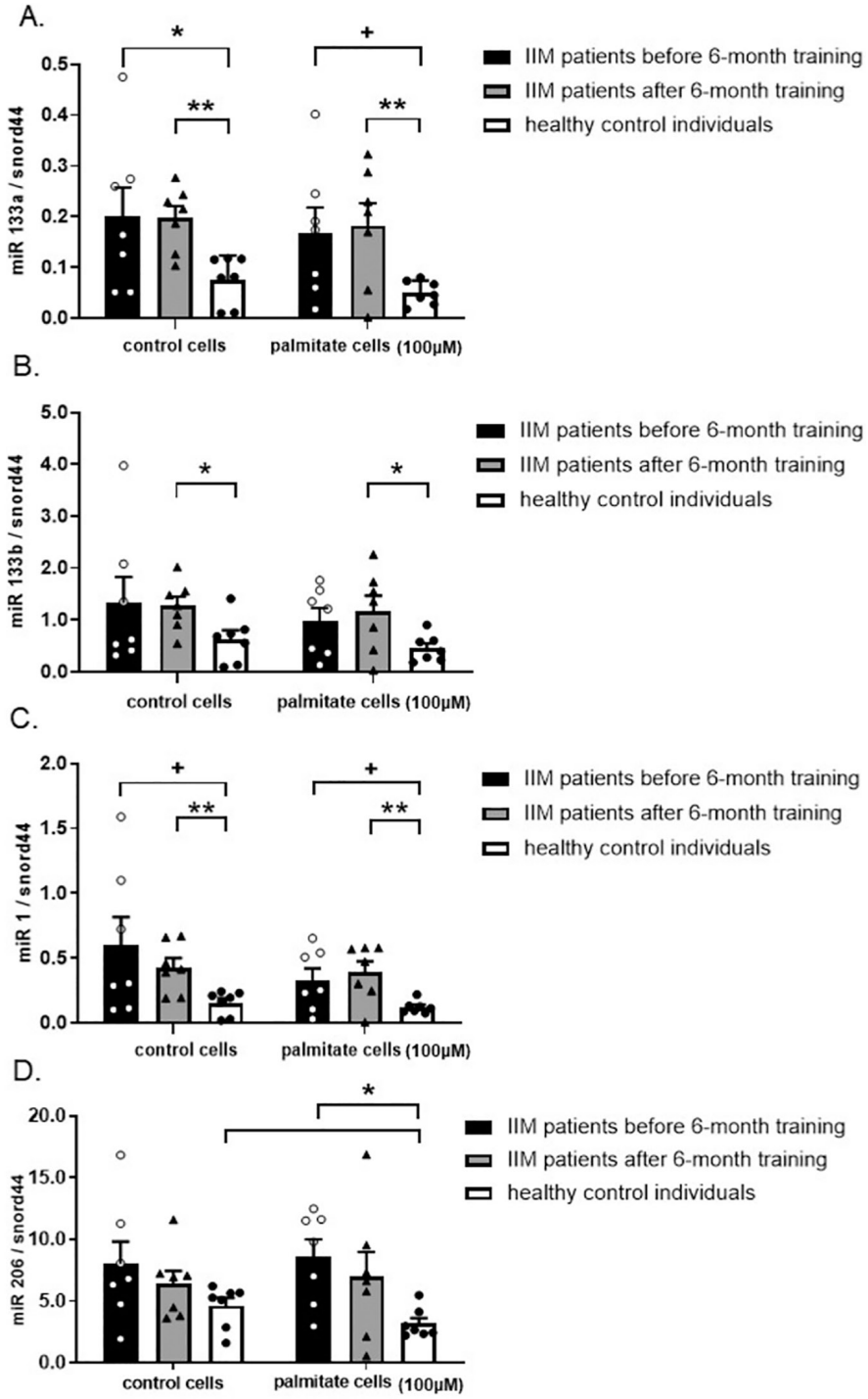

Fig. 1. Idiopathic inflammatory myopathy/IIM but not 6-month training programme modulates myomiRs in skeletal muscle cells. Muscle cell cultures were established from samples of *vastus lateralis* muscle of patients with IIM both before and after 6-month intense supervised exercise training intervention and from sedentary healthy age-, gender-, and BMI-matched individuals (controls). Expression levels of miR-133a (**A**), miR-133b (**B**), miR-1 (**C**), miR-206 (**D**). Differentiation was induced when cells reached ~80–90% confluence. After 48h of differentiation, the 3-day treatment with saturated palmitic fatty acid (100 µM, coupled to BSA), or BSA alone (control to palmitate), was initiated. Myotubes were harvested after 5 days of differentiation. MyomiRs were quantified by qPCR. Data are presented as means±SEM, n=7 per group, *p<0.05, **p<0.01, ^{+}p<0.1.

acid increased *BDNF* mRNA in myotubes from trained patients with IIM (3.46±1.19 A.U. *vs*. 9.07±5.35 A.U., p=0.025), but not in sedentary IIM patients or in healthy controls (p>0.1 for both). *Frataxin* (predicted target of miR-206) mRNA levels, measured in a smaller cohort because of reduced RNA availability, did not differ across the groups (data not shown).

In vitro metabolic phenotypes are linked to myomiR levels in human myotubes

The impact of disease (IIM), as well as changes related to 6-month training intervention and *in vitro* palmitate treatment of myotubes on oxidative metabolism, including fatty acid oxidation, lipid synthesis, OxPHOS complexes protein content, mitochondrial DNA content, and mRNA expression of genes involved in oxidative metabolism (PGC1-α, CPT-1, Myh-7) and muscle differentiation (myogenin) has already been described (31). Herein, relationships between *in vitro* muscle cell metabolic phenotypes and selected myomiRs were explored. In palmitate-treated cells, miR-133a, miR-133b, and miR-1 expression levels correlated negatively with incomplete fatty acid oxidation (accumulation of ASPs) in IIM patients as well as in the entire population (Fig. 2A and 3A) but not in healthy individuals (Fig. 3B). The similar correlation pattern has been also found for CO_2, the end-product of mitochondrial fatty acid oxidation. These relationships were less pronounced under the baseline cell culture conditions (Fig. 3). Accumulation of neutral lipids (TAGs, DAGs, PL) within muscle cells was negatively associated with levels of miR-133a, miR-133b and miR-1 in the entire population as well as in IIM patients, but not in healthy controls (Fig. 3). MiR-133a, miR-133b, and miR-1 were positively related to the relative content of mitochondrial OxPHOS complex-I in palmitate-treated cells from the entire cohort and from IIM patients before but not after the training intervention (Fig. 2B and 3). In contrast, reciprocal pattern was found for miR-133a, miR-133b & miR-1, and OxPHOS complex-V relationships (Fig. 2C and 3). It is important to note that the reciprocal relationship between ATP-synthase and other OxPHOS complexes found upon metabolic challenge with palmitate in muscle cells of IIM patients before training was ameliorat-

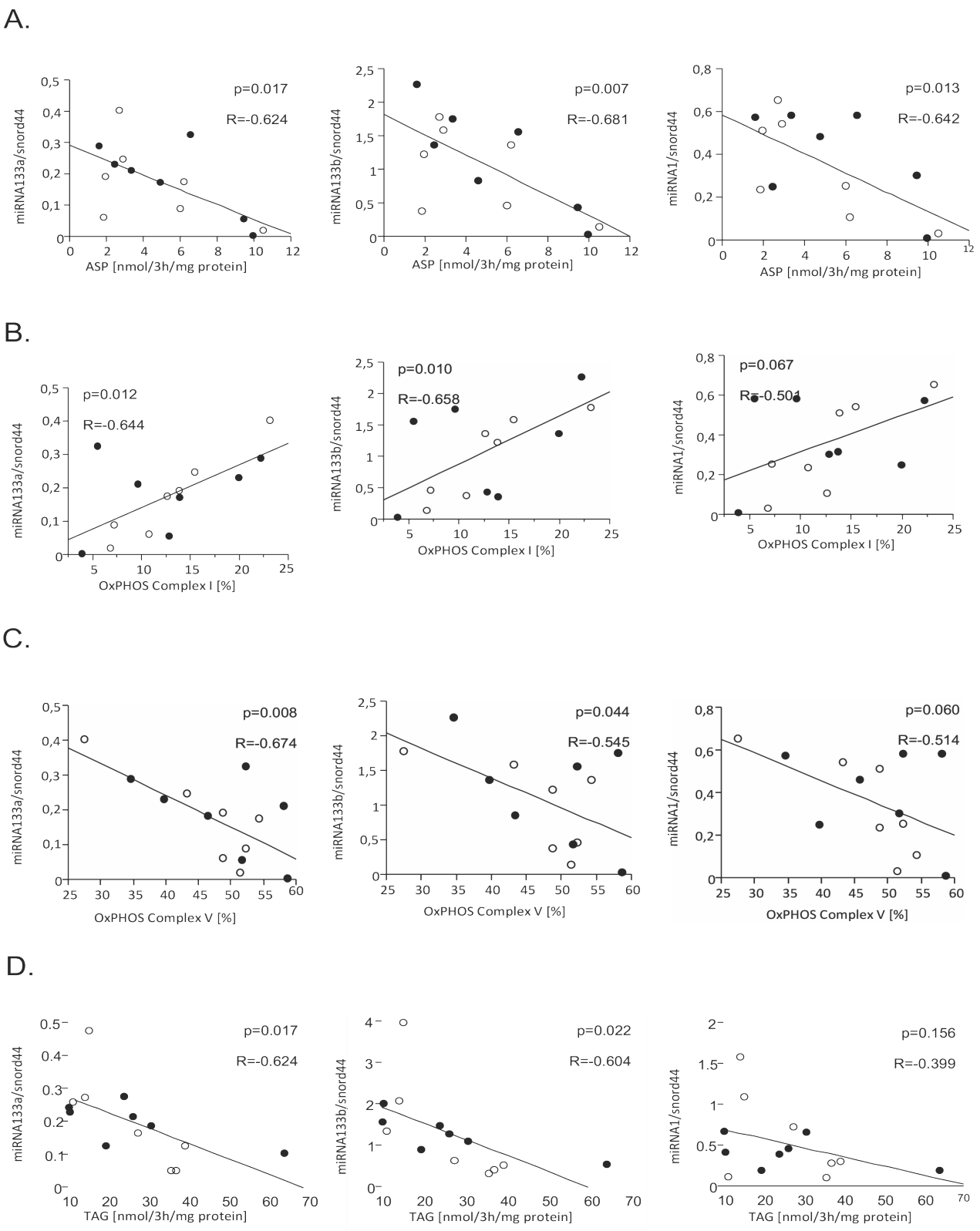

Fig. 2. Training-independent relationships of myomiRs and metabolic parameters in differentiated muscle cells derived from patients with idiopathic IIM with fatty acid oxidation (Acid Soluble Products/ASP, incomplete fatty acid oxidation) (**A**); mitochondrial complexes of oxidative phosphorylation (OxPHOS): complex-I (**B**); and complex-V (**C**); triacylglycerols (TAG) (**D**). Fatty acid oxidation was measured by radioactive assay with [1-14C]-palmitate, protein levels of individual OxPHOS complexes were assessed by immunoblotting and expressed as percentage from the total signal (the sum of all 5 proteins' signal); diacylglycerols were assessed by thin layer chromatography. ASPs and DAGs were normalised to protein levels of individual wells. Muscle-specific microRNAs (myomiRs) were quantified by qPCR and normalised to a housekeeper gene snord44. Data were obtained using palmitate-treated cells (A-C) and cell cultured at baseline conditions (D). Open circles: patients with IIM before training intervention (n=7); Solid circles: patients with IIM after 6-month training intervention (n=7).

A. The entire population

Entire cohort	miRNA-133a		miRNA-133b		miRNA-1		miRNA-206	
n=21	Control	Palmitate	Control	Palmitate	Control	Palmitate	Control	Palmitate
Parameters of fatty acid oxidation								
ASPs		**		**		**		*
CO_2		†		†	*	*		
$\%CO_2$								†
OXPHOS								
Complex I.	*	*	*	*		*		
Complex II.		*		*		*	†	
Complex III.								
Complex IV.						*	†	
Complex V.		**		*		*		*
Lipids								
DAG				*		*		
TAG	*		*					
PL				†		†		
Myogenesis, differentiation & oxidative markers								
Myh1								
Myh7		*		*	†	†		
Myogenin	†			**				
mtDNA	**	*	*		*	†		
CPT1						†		
PGC1α								
MyomiR´s target genes								
Frataxin	*	*		*				†
BDNF		*		*		†		*

B. Healthy controls

Healthy controls	miRNA-133a		miRNA-133b		miRNA-1		miRNA-206	
n=7	Control	Palmitate	Control	Palmitate	Control	Palmitate	Control	Palmitate
Parameters of fatty acid oxidation								
ASPs								
CO_2								
$\%CO_2$	†		*					
OXPHOS								
Complex I.		†			†			
Complex II.								
Complex III.	†				*			
Complex IV.								
Complex V.					†			
Lipids								
DAG						†		
TAG								
PL						†		
Myogenesis, differentiation & oxidative markers								
Myh1								
Myh7	†				*			
Myogenin								
mtDNA								
CPT1								
PGC1α	*	†	†		†			
MyomiR´s target genes								
Frataxin								
BDNF								

C. Patients with IIM before 6-month training intervention

IIM patients before intervention	miRNA-133a		miRNA-133b		miRNA-1		miRNA-206	
n=7	Control	Palmitate	Control	Palmitate	Control	Palmitate	Control	Palmitate
Parameters of fatty acid oxidation								
ASPs						*		
CO_2								
$\%CO_2$					†			
OXPHOS								
Complex I.		***		**		*		
Complex II.			†	†			†	
Complex III.								
Complex IV.		**		†		*		†
Complex V.						*		
Lipids								
DAG								
TAG	*		†					
PL				†				
Myogenesis, differentiation & oxidative markers								
Myh1								
Myh7								†
Myogenin		†		**				
mtDNA	*		†					
CPT1								
PGC1α	*		†					
MyomiR´s target genes								
Frataxin				†				*
BDNF		†		*				

D. Patients with IIM after 6-month training intervention

IIM patients after intervention	miRNA-133a		miRNA-133b		miRNA-1		miRNA-206	
n=7	Control	Palmitate	Control	Palmitate	Control	Palmitate	Control	Palmitate
Parameters of fatty acid oxidation								
ASPs	*	†		*	**		†	
CO_2	†	*		*	†		†	
$\%CO_2$								
OXPHOS								
Complex I.							†	
Complex II.								
Complex III.								
Complex IV.	†		†				**	
Complex V.							*	
Lipids								
DAG								
TAG			*	†		†		
PL	†		†					
Myogenesis, differentiation & oxidative markers								
Myh1								
Myh7	†						†	
Myogenin		*		**		†		†
mtDNA								
CPT1						†		
PGC1α								
MyomiR´s target genes								
Frataxin							*	
BDNF		***		*		*		*

R	-1	-0.5	-0.25	0	0.25	0.5	1

Fig. 3. The heat maps depicting the distinct patterns of relationships between muscle-specific microRNAs and *in vitro* characteristics of lipid metabolism, mitochondrial and differentiation markers as well as expression of myomiRs' target genes in myotubes representing (**A**) the entire study population (n=21; healthy controls, patients with IIM before and after the intervention, n=7 per group); (**B**) healthy controls n=7; (**C**) patients with IIM before and (**D**) IIM patients after the 6-month training intervention. Lipid metabolism was measured by radiometric assay and neutral lipid content by thin layer chromatography. Individual OxPHOS complexes were assessed by immunoblotting and are presented as the percentage from total OxPHOS signal. Gene expression levels of Myh1, Myh7, myogenin, mtDNA, CPT1, PGC1a, frataxin and BDNF were measured by qPCR, and normalised to internal reference genes ribosomal protein L13a, 18S-rRNA, and β-2-microglobulin.
ASP: acid soluble products; BDNF: brain derived neurotrophic factor; CPT: carnitine palmitoyl transferase; DAG: diacylglycerol; IIM: idiopathic inflammatory myopathy; Myh: myosin heavy chain; OxPHOS: complexes of mitochondrial oxidative phosphorylation; PGC1-α: peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; PL: phospholipids; TAG: triacylglycerol.

ed after training intervention and tended to be reversed in cells from healthy control individuals (Fig. 3B-C-D). When evaluating the entire cohort, levels of miR-133a, miR-133b and miR-1 correlated positively with markers of oxidative muscle fibres *Myh7* in cells exposed to palmitate (Fig. 3 A). This indicates that myomiRs could modulate the metabolic capacity of muscle cells in accordance with the prevailing metabolic substrate availability.

To summarise, there was a distinct pattern of relationships between selected myomiRs and *in vitro* markers of muscle lipid metabolism, mitochondrial function, muscle cell differentiation and regeneration in myotubes derived from patients with IIM compared to the healthy controls (Fig. 3B-C-D). The negative trends or associations between myomiRs and parameters of fatty acid oxidation (CO_2 and ASPs), found in cells from IIM patients but not in healthy controls, were enhanced by the 6-month training intervention in IIM patients (Fig. 3B-C-D). Similarly, there was a distinct pattern of the relationships between myomiRs 133a and 133b and relative OxPHOS complexes

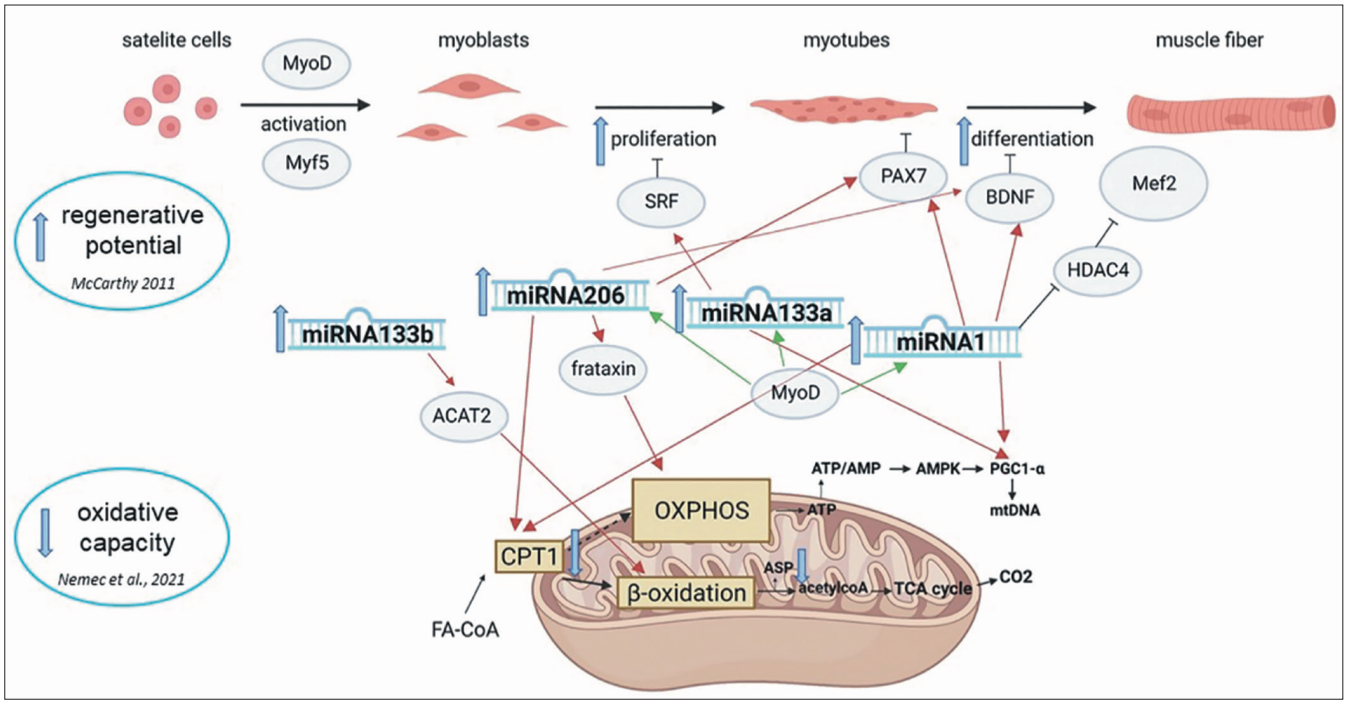

Fig. 4. Schematic representation of the putative role of specific myomiRs in the regulation of muscle cell regeneration and oxidative capacity in patients with idiopathic inflammatory myopathy (IIM). MyomiRs reduce the expression of myogenic regulatory factors that have an inhibitory effect on proliferation and differentiation of muscle cells (62). Increased myomirs could thus represent a compensatory mechanism, aimed at enhancing muscle regenerative potential in patients with IIM. Negative regulation of factors important for mitochondrial oxidative capacity by increased levels of myomiRs could contribute to the reduced fat oxidative capacity (63, 24) observed in muscle cells from patients with IIM (31). MyomiRs' target genes: ACAT2, CPT1, Frataxin & PGC1-α (predicted target genes according to miRNA target prediction database miRDB.com); BDNF (64, 65), HDAC4 (66, 67, 68), PAX7, SRF (69).
ACAT2: Acetyl-CoA Acetyltransferase 2; AMPK: AMP-activated protein kinase; ASP: acid soluble products; BDNF: brain derived neurotrophic factor; CPT1: carnitin palmitoyl transferase-1; FA-CoA: Fattyacyl-Coenzyme A; HDAC4: Histon deacetylase-4; OXPHOS: complexes of oxidative phosphorylation; mtDNA: mitochondrial DNA; MyoD, Myf5, Mef2: transcription factors involved in myogenesis. PAX7: paired box 7; PGC1-α: Peroxisome proliferator-activated receptor-γ coactivatro-1-alpha; SRF: serum response factor; TCA: tricarboxylic acid cycle.
The red arrows indicate inhibition/downregulation and the green arrows activation/upregulation. Blue arrows indicate parameters regulated in muscle cells from patients with IIM, and their putative significance for the cellular phenotype. Created with BioRender.com.

protein content between patients and controls, which was more prominent in palmitate-treated cells, and ameliorated by the 6-month training. The differences in patterns of associations with the markers of myogenesis and oxidative capacity were more pronounced in palmitate treated cells, without a substantial impact of the training intervention (Fig. 3B-C-D). Negative associations of miRs with *BDNF*, pronounced in palmitate-treated cells, were enhanced by the training intervention. The relationships with lipid synthesis were mostly negative, without significant difference in patterns across the groups. Moreover, miR-133a and miR-133b correlated/tended to correlate with the gene expression of *myogenin*, supporting the relationship of myomiRs to the differentiation of muscle cells derived from IIM patients but not healthy controls.

In palmitate-treated myotubes, miR-133a, miR-133b and miR-206 correlated negatively with the expression of *BDNF,* a target of miR-1 and miR-206 and myokine modulating muscle differentiation and fatty acid oxidation (Fig. 3) in IIM patients but not in healthy controls. MiR-133a, miR-133b, miR-1, miR-206 were negatively associated with mitochondrial protein *frataxin*, a target of miR-206 and an activator of OxPHOS, which could modulate mitochondrial membrane potential and cellular ATP content in IIM patients (Fig. 3). MiR-133a, miR-133b and miR-1 were positively associated with mitochondrial DNA content in the entire population (Fig. 3A). MiR 133a and miR133b were related to the expression of *PGC1α*, the transcription cofactor regulating mitochondrial biogenesis in IIM patients before intervention and in healthy controls. Expression of carnitine palmitoyl transferase-1, protein of the inner mitochondrial membrane representing the rate limiting step for fatty acid transport/oxidation in mitochondria was not correlated with myomiRs (Fig. 3). The potential role of myomiRs in the regulation of muscle regeneration and oxidative capacity in patients with IIM is depicted in Figure 4.

Interrelations between in vitro myomiRs and clinical characteristics of the cell donors

Next, we analysed associations between myomiRs in cultured myotubes and clinical characteristics of the cell donors. Levels of miR-133a and miR-133b in muscle cells cultured under baseline conditions were positively associated with fasting glycaemia (n=21, R=0.521, p=0.016; R=0.435, p=0.049). In palmitate-treated myotubes derived from muscle of patients with IIM, miR-133a and miR-133b tended to positively correlate with serum levels of LDL-cholesterol (n=14, R=0.531, p=0.051; R=0.504, p=0.066) and lipoprotein(a),

a risk factor for atherosclerosis (n=14, R=0.539, p=0.047; R=0.533, p=0.050). Similar correlations of miR-133a and miR-133b with total serum cholesterol levels were observed in baseline, untreated myotubes (n=14, R=0.563, p=0.036; R=0.629, p=0.016). Myositis Intention to Treat Activity Index (MITAX), indicating both muscular and extramuscular disease activity in patients with IIM, tended to positively correlate with miR-133b and miR-206 levels in myotubes cultured under baseline conditions (n=14, R=0.517, p=0.059; R=0.630, p=0.016). However, functional indices of muscle strength (MMT8) and endurance (FI-2), disease duration, the dose of glucocorticoids and circulating levels of proinflammatory cytokine TNF-α were not associated with levels of myomiRs in cultured myotubes from patients with IIM (p>0.1).

Discussion

MicroRNAs have been implicated in many disease states, including sarcopenia, muscular dystrophy and IIM (15, 17, 37, 38). Being involved in modulating the whole spectrum of cellular phenotypes, including skeletal muscle differentiation, maintenance, metabolism and regeneration, it can be assumed that alterations in microRNAs could contribute to the pathogenesis of muscle dysfunction (39) and/or to the muscle regeneration/compensatory response to damage (17). The major finding of our study is the substantial upregulation of four muscle-specific miRNAs (myomiRs), miR-133a, miR-133b, miR-1 and miR-206, in the primary cultures of differentiated skeletal muscle cells (myotubes) derived from muscle of IIM patients, when compared to the age-, gender-, BMI- and glycaemia-matched healthy sedentary individuals. Higher levels of myomiRs were present both in control (baseline, palmitate-untreated) and palmitate-treated muscle cells that were challenged by the 3-day exposure to saturated fatty acid during differentiation. A few studies pointed at differences in the expression levels of skeletal muscle microRNAs in patients with IIM compared to healthy individuals. In the microarray study, Eisenberg *et al*. described distinct regulation of 185 microRNAs in skeletal muscle of patients with 10 major muscular disorders, including upregulation of 37 miRNAs in polymyositis and 35 miRNAs in dermatomyositis (17).

Others demonstrated the inhibition of myogenic miRNAs, including miR-1, miR-133 and miR-206, by elevated levels of inflammatory cytokines in muscle samples from IIM patients as well as *in vitro*, in both human skeletal muscle cells and murine C2C12 myoblasts (40). The great majority (36 *vs*. 8) of the patients in this study were yet untreated, which could be responsible for the discrepancy between their results and our *in vitro* observations. Based on the role of myomiRs in the regulation of myogenesis and the presence of changes in patients' muscle cells *in vitro*, it is plausible to speculate that the expression of myogenic microRNAs can be elevated in skeletal muscle of patients treated for IIM as a potent compensatory epigenetic mechanism that combats muscle degeneration (22, 41-42).

Growing evidence supports the efficiency of regular moderate-to-intensive resistance or aerobic exercise in reducing inflammation, muscle weakness, and disability in IIM patients (6, 43). Until recently, patients with myositis were advised to avoid exercise because of fears of exacerbation of inflammation and muscle damage. In the last few years, numerous intervention studies have shown that regular exercise exerts anti-inflammatory effects and represents a viable therapeutic intervention for patients with multiple chronic conditions (24, 44-45) that can act in synergy with standard immunosuppressive pharmacotherapy (9, 23). It was shown that endurance training upregulated molecular pathways of aerobic metabolism in skeletal muscle of IIM patients (8). Both acute and regular exercise alter several miRNAs in skeletal muscle (46) and immobilisation and space flight reduced levels of several myomiRs' (22). Boehler *et al*. demonstrated that the 12-week endurance training altered levels of 39 miRNAs, implicated in the regulation of immune response, aerobic metabolism and muscle atrophy, in skeletal muscle of patients (23). In our study, the 6-month intensive exercise training programme significantly improved muscle functional capacity and clinical state of the patients (36).

However, we did not observe any effect of the training intervention on the levels of selected muscle-specific microRNAs in primary myotubes derived from *vastus lateralis* muscle of IIM patients. Small sample size and heterogeneity of the study population (47), as well as the fact that exercise-induced changes can differ between different types of IIM, could have prevented us from detecting the regulation of selected myomiRs (4, 48). On the other hand, other microRNAs (17, 23) or other epigenetic mechanisms such as methylation or histone modification could be involved in the exercise-induced modulation of the muscle phenotype and its maintenance *in vitro*, in myotubes derived from IIM patients (33, 49, 50).

MiRNAs are involved in muscle regeneration and mitochondrial biogenesis (51). We found positive associations of miR-1, 133a and 133b with protein levels of mitochondrial OxPHOS (relative content of complex-I, II, IV), and negative correlations with relative content of complex-V, parameters of fat oxidation and lipid accumulation in primary myotubes, indicating the interrelations between myomiRs and muscle oxidative/lipid metabolism. The relationships were present or more pronounced in cells that were exposed to long chain saturated fatty acid during the last three days of differentiation, indirectly indicating that myotubes from IIM patients with increased levels of miR-1, miR-133a and miR-133b could have a lower ability of ATP synthesis via oxidative phosphorylation and possibly higher levels of oxidative stress (48, 52) in response to palmitate challenge. Reduced capacity to handle the increased load of saturated fatty acid *in vitro* can represent an attribute of impaired substrate switching (25), which could contribute to the disease progression. In our previous work, we have identified altered dynamics of lipid metabolism and reduced capacity to handle the increased saturated fatty acid load in primary cultures of muscle cells derived from II patients (31). Non-immune mechanisms, including metabolic and mitochondrial

dysfunction, increased oxidative stress, and impaired ATP production, have been implicated in the pathogenesis of IIM, contributing to muscle weakness and degeneration even after the suppression of inflammation (53).

Increased levels of saturated fatty acids were observed in the circulation of patients with IIM, which correlated positively with disease (54). In our work, we did not observe the regulation of selected myomiRs by saturated palmitic fatty acid in myotubes *in vitro*. However, our results support the role of myomiRs in the regulation of glucose and lipid metabolism (55, 56). We observed positive correlations of miR-133a and miR-133b with fasting glycaemia and parameters of lipid metabolism, including total cholesterol, LDL-cholesterol, and lipoprotein(a), markers of increased risk of atherosclerosis. MiRNAs are involved in the regulation of insulin resistance, lipid metabolism and glucose uptake in skeletal muscle cells. Dysregulation of miRNAs can contribute to chronic inflammation and insulin resistance in metabolic tissues (57), including skeletal muscle (55), which could promote the progression of IIM.

It has been previously shown that overexpression of miR-1 and miR-133 strongly enhances myogenesis, as demonstrated by an increase in the expression of early and late myogenic markers, including myogenin and myosin heavy chain (MHC) in C2C12 muscle cells (58). We observed positive correlations of miR-133a, miR-133b and miR-1 with gene expression of myogenin, Myh1 and Myh7, which is in line with the regulatory role of myomiRs in myogenesis, muscle regeneration and muscle cell differentiation. Negative correlations with myokine BDNF and frataxin, target genes of miR-1 and miR-206, indirectly indicate a negative regulatory role of miRs in the expression of selected genes.

Levels of cytokines IL-6, IL-8, IL-10 and TNF-α are associated with global disease activity in IIM (59). TNF-α affects the expression of multiple genes which are involved in pathways responsible for TNF-induced muscle loss, including matrix degradation, NF-ϰB signalling, chemokine networks, apoptosis, and muscle cell proliferation and differentiation (59). Furthermore, specific microRNAs involved in the regulation of the immune system and B-cell function can modulate the production of autoantibodies (60) or other components of the immune response (61) in IIM.

Inflammatory cytokines were shown to downregulate myogenic microRNAs in skeletal muscle of IIM patients *in vivo* and muscle cells *in vitro* (40). In our study, we identified positive correlations between *in vitro* miR-133a, miR-1, and miR-206 and disease activity, suggesting more pronounced activation of potential compensatory mechanisms in muscle cells from patients with more severe disease.

Study limitations

The small number of study participants and heterogeneity of the study population represent the major limitations of our study. The complexity of the disease-related microRNAs regulation could be assessed by measurement of myomiRs in circulation and skeletal muscle samples, in parallel to myomiRs in myotubes *in vitro*. Different IIM subtypes and stages of the disease, as well as disease therapy could distinctly modulate levels of selected myomiRs. The protein content of myomiRs' target genes could better reflect the myomiRs regulatory role. Some of these limitations will be targeted in our future studies.

Conclusions

The substantial consistent increase of specific myomiRs in primary muscle cells derived from patients with idiopathic inflammatory myopathy compared to those of sedentary healthy controls suggest the activation of compensatory epigenetic mechanisms in skeletal muscle of patients treated for IIM with pharmacotherapy or intensive exercise training intervention. Relationships of muscle specific myomiRs levels with *in vitro* and *in vivo* / clinical phenotypes indicate the role myomiRs could play in fine-tuning mitochondrial function, lipid and glucose metabolism at both skeletal muscle and systemic level. These observations provide a novel insight into epigenetic mechanisms that combat muscle degeneration.

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