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Original research article

microRNA expression profile in Smooth Muscle Cells isolated from thoracic aortic aneurysm samples

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ABSTRACT

Purpose: Thoracic aortic aneurysm (TAA) is a cardiovascular disease characterized by increased aortic diameter, treated with surgery and endovascular therapy in order to avoid aortic dissection or rupture. The mechanism of TAA formation has not been thoroughly studied and many factors have been proposed to drive its progression; however strong focus is attributed to modification of smooth muscle cells (SMCs). Latest research indicates, that microRNAs (miRNAs) may play a significant role in TAA development – these are multifunctional molecules consisting of 19–24 nucleotides involved in regulation of the gene expression level related to many biological processes, *i.e.* cardiovascular disease pathophysiology, immunity or inflammation.

Materials and methods: Primary SMCs were isolated from aortic scraps of TAA patients and age- and sex-matched healthy controls. Purity of isolated SMCs was determined by flow cytometry using specific markers: α-SMA, CALP, MHC and VIM. Real-time polymerase chain reaction (RT-PCR) was conducted for miRNA analysis.

Results: We established an isolation protocol and investigated the miRNA expression level in SMCs isolated from aneurysmal and non-aneurysmal aortic samples. We identified that let-7 g (0.71-fold, $p = 0.01$), miR-130a (0.40-fold, $p = 0.04$), and miR-221 (0.49-fold, $p = 0.05$) significantly differed between TAA patients and healthy controls.

Conclusions: Further studies are required to improve our understanding of the pathophysiology underlying TAA, which may aid the development of novel, targeted therapies. The pivotal role of miRNAs in the cardiovascular system provides a new perspective on the pathophysiology of thoracic aortic aneurysms.

1. Introduction

Data accumulated by the World Health Organization show that about 17.5 million people die annually due to cardiovascular diseases [1]. Thoracic aortic aneurysm (TAA) is a cardiovascular disease characterized by enlargement of the aortic diameter, by at least 50% of its normal size. The thoracic aorta diameter is influenced by age, gender, body size or blood pressure and may expand about 0.9 mm in men and 0.7 mm in women per decade of life [2]. As the aneurysm grows, the risk of acute aortic rupture increases, leading to internal hemorrhaging and life-threatening condition [3]. Aortic aneurysm tends to be an asymptomatic disease in about 90–95% of cases [4] and up to now has been treated surgically in order to avoid complications related to aortic dilatation or rupture [3,5–8]. The aortic wall consists of three fundamental layers: tunica intima, or the internal layer built of endothelial cells (ECs), connective tissue and supportive cells; the middle layer known as tunica media that consists of smooth muscle with connective tissue elements arranged circularly; and the outer layer, tunica adventitia or externa, mainly composed of connective tissue with smooth muscle cells (SMCs) arranged longitudinally [9]. Various cellular processes, including differentiation and proliferation, contribute to the development of vascular disease [10–12].

The pathomechanism of TAA formation is influenced by many factors and modifications in the phenotype of the main blood vessel wall components –EC and SMC, alongside inflammation and extracellular matrix degradation play a pivotal role in this process [8]. SMCs undergo phenotypic changes to a synthetic form, showing more endocytic, migratory and proliferative characteristics, furthermore they are responsible for synthesis and secretion of extracellular matrix [10,13–16].

MicroRNAs (miRNAs) are non-coding RNAs consisting of 19–24

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Table 1
Patient clinical characteristics.

	Patient group (n = 6)	Control group (n = 4)	*p* Value
Age (Years)	45.00 ± 2.37	39.00 ± 9.90	0.29
Female / Male	3/3	1/3	0.49
Aortic diameter (mm)	50.33 ± 5.32	31.25 ± 2.99	0.01*
Obesity, n (%)	4 (66.67)	0 (0)	0.07
eGFR (ml/min/ 1,73 m^2)	85.75 ± 4.44	100.25 ± 59.37	0.01*
Creatinine (μmol/L)	73.33 ± 14.50	116.69 ± 67.20	0.20
Glucose (mmol/L)	6.42 ± 2.40	9.95 ± 4.12	0.09
Diabetes, n (%)	2 (33.33)	1 (25)	0.81
Hypertension, n (%)	5 (83.33)	0 (0)	0.02*
Ever smoker, n (%)	2 (33.33)	0 (0)	0.29

Values are presented as means ± SD, *p value ≤0.05.

nucleotides, which modulate gene expression, to control numerous signaling pathways and cellular processes [17]. In the cardiovascular system, miRNAs control functions of various cells such as cardiomyocytes, ECs, SMCs and fibroblasts; they are also involved in processes such as angiogenesis, cellular communication, inflammation and immune response [18–22]. Therefore, the aim of our study was to establish a protocol for isolation and culture of SMCs from human aortic samples and investigate the miRNAs potentially contributing to TAA pathology.

2. Materials and methods

2.1. Patient characteristics

Primary aortic vascular SMCs were isolated from aortic samples of patients diagnosed with TAA undergoing vascular replacement surgery (n = 6) in the Department of Cardiac Surgery and Transplantology. Control cell lines (n = 4) were isolated from aortic scraps of age (TAA patients = 45.00 ± 2.37 years; control = 39.00 ± 9.90 years, p = 0.29) and sex-matched heart graft specimens (Table 1).

2.2. Primary human aortic SMC isolation and culture

The excised aortas were immediately transferred to a sterile laminar-flow cabinet and rinsed in HBSS with Ca^{2+} and Mg^{2+} (*Corning, NY,* USA) supplemented with Antibiotic-Antimycotic (HBSS-AA, *Sigma Aldrich, St. Louis, MO,* USA). After the ECs separation by gently scraping the intima layer, the rinsed tissue was placed in a culture dish and the adventitia was removed using tweezers. The remaining layer – tunica media, was then transferred to another culture dish and cut into small, 2–4 mm pieces. The obtained fragments were placed into 50 ml Falcon, suspended in 4.5 ml of HBSS-AA, then 250 μl of 2 mg/ml collagenase type I (*Gibco, Thermo Fisher Scientific, Waltham, MA,* USA) and 250 μl of elastase type I (*Sigma Aldrich, St. Louis, MO,* USA) was added, followed by incubation on a thermo-shaker (300 rpm) at 37 °C for 30 min. After incubation, 5 ml of HBSS-AA was added and the sample was centrifuged for 5 min at 300*g* (4 °C). The supernatant was gently discarded and the remaining material resuspended in 5 ml of HBSS-AA and centrifuged. Subsequently, the sample was resuspended in 2 ml HBSS without Ca^{2+} and Mg^{2+} (*Corning, NY,* USA) and filtrated in order to remove the remaining tissue fragments using Falcon® 100 μm Cell Strainer (*Corning, NY,* USA) and centrifuged for 5 min (300*g*, 4 °C). Finally, the supernatant was discarded and freshly isolated cells were cultured in 1 ml of Smooth Muscle Basal Medium with Smooth Muscle Growth Medium-2 and 5% fetal bovine serum (FBS, *Lonza, Basel,* Switzerland) in a 12-well plate and incubated at 37 °C in 5% CO_2. The culture medium was changed every 2–3 days. Microscopic observations were completed using inverted microscope Olympus CKX41 with DP74 camera (*Olympus, Shinjuku, Tokyo,* Japan).

2.3. Flow cytometry

Expression of α-smooth muscle actin (α-SMA), calponin (CALP), myosin heavy chain (MHC) and vimentin (VIM) were analyzed in SMC culture using MoFlo Astrios EQ Cell Sorter (Beckman Coulter, *Brea*, *CA*, USA). Briefly, the cells were harvested at confluence, and then fixation and permeabilization were done with IC Fixation Buffer (*eBioscience*, *San Diego, CA,* USA) and 1X Permeabilization Buffer (*eBioscience, San Diego, CA,* USA), respectively. Samples were incubated with primary antibodies against human α-SMA (R&D Systems, #IC1420 A), CALP (Abcam, #ab700), MHC (Miltenyi Biotec, #130-106 − 253) and VIM (R &D Systems, #IC2105 G) for 40 min, washed with Permeabilization Buffer (1X) and centrifuged, followed by incubation with secondary antibodies Anti-Mouse IgG1 (eBioscience, #11-4015-82). Finally, the cells were suspended with Flow Cytometry Staining Buffer (1% FBS and 0.09% sodium azide in phosphate-buffered saline) and analyzed. The obtained results were examined with Kaluza analysis software (*Beckman Coulter, Brea*, *CA*, USA).

2.4. RNA isolation and qRT-PCR assay

For miRNA analysis, cells were harvested at 90% confluence and total RNA with miRNA was isolated using miRNeasy mini kit (*Qiagen, Valencia, CA,* USA) according to the manufacturer's protocol, followed by RNA quantification with NanoPhotometer (*Implen, Munich,* Germany). 10 ng of total RNA was then reversely transcribed to cDNA using TaqMan Advanced miRNA cDNA synthesis kit (*Applied Biosystems, Foster City, CA,* USA). The relative miRNA expression levels were measured using quantitative Reverse Transcriptase-PCR (qRT-PCR) on Viia7 (*Applied Biosystem*, *Foster City, CA,* USA) with miRNA classified into 6 groups, previously described as associated with heart disease, including hypertrophy (miR-21, miR-199a), coronary artery disease and acute coronary syndrome (let-7 g, miR-17, miR-21, miR-26a, miR-148, miR-155, miR-181a, miR-301, miR-769), heart failure (let-7e, miR-21, miR-22, miR-93, miR-103a, miR-130b, miR-193, miR-301, miR-423), hypertension (let-7e, miR-22, miR-130a), inflammation (miR-21, miR-22, miR-148, miR-155, miR-221/222) and other biological processes such as cell growth, proliferation or apoptosis (let-7 g, miR-26a, miR-28b, miR-148, miR-335, miR-769), as described in Table 2 [23]. According to the literature, the candidate housekeeping genes were: miR-16, miR-23a, and miR-423 [24–27], and based on the Bestkeeper stability score [28], the miR-23a was used as a reference. All primers were purchased from Thermo Fisher Scientific (*Thermo Fisher Scientific, Waltham, MA,* USA). The obtained results were calculated by the ΔΔCt method.

2.5. Ethical issues

The study protocol conformed to the 1964 Helsinki declaration and was approved by the Institutional Ethics Committee at the Institute of Cardiology (Warsaw, Poland) (IK-NP-0021-10/1542/16 and IK-NP-0021-7/1614/17). Written informed consent was obtained from each participating patient.

2.6. Statistical analysis

All results are presented as means ± SD or SEM where *n* describes the number of patients and controls included into the analysis. Data were analyzed using Student's *t* test or Mann-Whitney test when appropriate and performed using MedCalc software (Version 18). Values of $p \leq 0.05$ were considered statistically significant.

3. Results

Clinical characteristics of patients included into this study are shown in Table 1. Patients diagnosed with CAD, genetic connective

Table 2
Role of miRNAs involved in the regulation of the vascular SMC proliferation, migration, phenotype, apoptosis and autophagy.

Function	miRs	Targets	References
Promote proliferation	miR-21	PTEN, Bcl-2	[63]
	miR-17	MFN2	[64]
	miR-26a	SMAD1/4	[65]
	miR-29a	YY1	[66]
	miR-130a	GAX	[52]
	miR-155	eNOS	[67]
	miR-221/222	p27(Kip1), p57(Kip2)	[51]
	miR-199a	Wnt2	[68]
Inhibit proliferation	let-7 g	LOX-1	[61]
	miR-22	HMGB1	[48]
	miR-148b	HSP90	[66]
	miR-155	AT1R	[69]
	miR-181a		[70]
Promote migration	miR-29a	YY1	[66]
	miR-155	eNOS	[67]
Inhibit migration	let-7g	LOX-1	[61]
	miR-22	HMGB1	[48]
	miR-148b	HSP90	[66]
	miR-181a		[70]
Promote contractile phenotype	let-7g	PDGFB, MEKK1	[60]
	miR-21	SP-1	[71]
	miR-145	ACE, KLF4/5, MRTF-B	[72,73]
	miR-199a	Myocardin, Wnt2	[68]
Promote synthetic phenotype	miR-21	PDCD4	[74]
	miR-26a	SMAD1/4	[65]
	miR-181a	SRF	[75]
	miR-221/222	p27, p57	[51]
Inhibit apoptosis	let-7g	LOX-1	[76]
	miR-17	STAT3, MFN2	[64,77]
	miR-21		[63]
	miR-26	SMAD1/4	[65]
	miR-155	eNOS	[67]
Induce autophagy	miR-17	STAT3	[77]

tissue disorders, acute aortic dissections and ruptured aneurysms were excluded from this study. We identified statistically significant variables in aortic diameter ($p = 0.01$), estimated glomerular filtration rate (eGFR) values ($p = 0.01$) and hypertension occurrence ($p = 0.02$) among the groups.

3.1. Human aortic SMCs culture

Current protocols for isolation of human arterial SMCs are relatively complicated and time consuming, resulting in low percentage of viable cells. We established a novel, simple and time-effective protocol for isolation and culture of SMCs derived from human aortic samples obtained from patients undergoing ascending aorta aneurysm excision (Fig. 1A). After removing the intima layer (Fig. 1B), isolation based on double collagenase-elastase digestion lasted for 60 min. The first SMCs were visible after 24–48 h, and grew up to 20–30% confluency within the next few days (Fig. 1C). The culture media was replaced every two days, and first passage done within 9–15 days of culture with TrypLE™ Express Enzyme (12604013, *Gibco, Thermo Fisher Scientific, Waltham, MA,* USA). The cells were further subcultured to a confluency of 80% (approximately 7 days) and used for further studies or frozen in SMC freezing medium containing culture media supplemented with 10% dimethyl sulfoxide (DMSO) and 10% FBS. The obtained SMCs displayed characteristic spindle-shaped morphology and were morphologically similar to control cell lines obtained from the aortic samples of heart graft donors (Fig. 1D).

3.2. Flow cytometry analysis

Purity of TAA isolated SMCs was determined using MoFlo Astrios EQ Cell Sorter (Beckman Coulter, *Brea, CA*, USA). The population of non-treated cells was used as a negative control. The 88.95%–99.10% primary isolated SMCs demonstrated expression of SMC-specific markers: α-SMA, CALP, MHC and VIM, respectively (Fig. 2A–D).

3.3. miRNAs expression profile in human aortic SMCs

We compared miRNA expression levels between SMCs isolated from TAA tissue samples (n = 6) and age- and sex-matched controls (n = 4). Twenty two miRNAs associated with heart disease and vascular SMC functioning were tested and 3 were found to be differentially expressed ($p \leq 0.05$) in patient *versus* control samples. Let-7 g (MI0000433), miR-130a (MI0000448) and miR-221 (MI0000298) were significantly down-regulated (0.71 $p = 0.01$, 0.40 $p = 0.04$ and 0.49-fold $p = 0.05$ differential expression, respectively) suggesting a potential role of these miRNAs in aortic aneurysm pathology (Fig. 3). Based on the qRT-PCR data analysis, the expression profile of other 19 miRNAs mentioned above as connected with heart or cardiovascular disease did not change significantly.

4. Discussion

Aortic aneurysm rapture is a life threatening disease. This condition is a consequence of chronic aortic diseases, which frequently remain asymptomatic. Currently, the first line of treatment is surgical repair. Despite advancements in surgical techniques, the survival rate of patients with a ruptured aneurysm has not improved and in 2004 was reported by the International Registry of Aortic Dissection at 25% [29]. Data regarding epidemiology of thoracic aorta disease are not so optimistic at present and an increase in the mortality rate from acute aortic syndromes has been observed [30]. According to Global Burden Disease 2010 project data, global mortality caused by aortic aneurysms and dissections increased from 2.49 per 100,000–2.78 per 100,000 inhabitants between 1990 and 2010 [31]. There are various additional risk factors that may impact the pathologic enlargement of aorta including aortic atherosclerosis, aortic valve stenosis, chest injuries, hypertension or blood vessels inflammation. Patients with genetic mutations demand special care and separate criteria for intervention. Syndromes such as Marfan or Loeys-Dietz, TGFBR1 or TGFBR2 mutations are reflected in the European Society of Cardiology (ESC)/ European Association for Cardio-Thoracic Surgery (EACTS) and American College of Cardiology Foundation (ACCF)/ American Heart Association (AHA) guidelines and should be treated more invasively. Following current ESC/EACTS guidelines, the therapeutic approach depends on localization of the lesion. Aneurysms of the ascending aorta and the aortic arch are an indication for surgical treatment because operation risk is acceptable and common medical treatments have dismal prognosis. Thus, there is an urge to broaden the current knowledge on the mechanisms underlying aneurysm development given the poor possibility of non-invasive treatment of this condition [32,33]. Therefore, in the present study we investigated and established the protocol for rapid and effective SMCs isolation from TAA and healthy aortic tissue. Our screening data revealed differentially expressed miRNAs in human TAA tissue *versus* healthy donor tissue, suggesting a potential role of these molecules in TAA pathogenesis.

Available protocols of SMC isolation refer mainly to various vascular arteries of common mice and rat species using both explant or enzyme digestion method mostly with Dulbecco's Modified Eagle Medium (DMEM) or Smooth Muscle Basal Medium (*Lonza, Basel,* Switzerland) [34–37]. Currently, there is still only a limited number of papers presenting thoroughly the isolation protocol and characterization of the obtained human aortic SMCs, so we aimed to investigate and describe this technique for further research purposes. Our *in vitro*

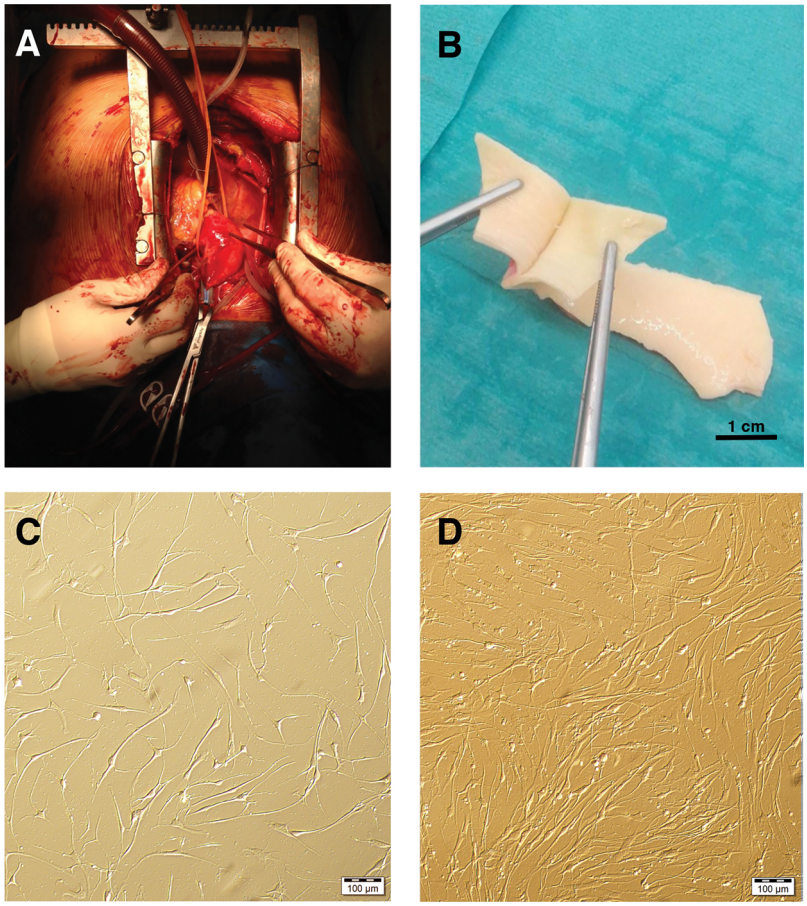

Fig. 1. Primary human aortic SMCs preparation, isolation and culture.
A. TAA – surgical repair view.
B. Excised TAA tissue prepared for removing intima for SMCs isolation.
C. Culture of isolated cells at 20–30% confluency.
D. Culture of isolated cells at 80% confluency.

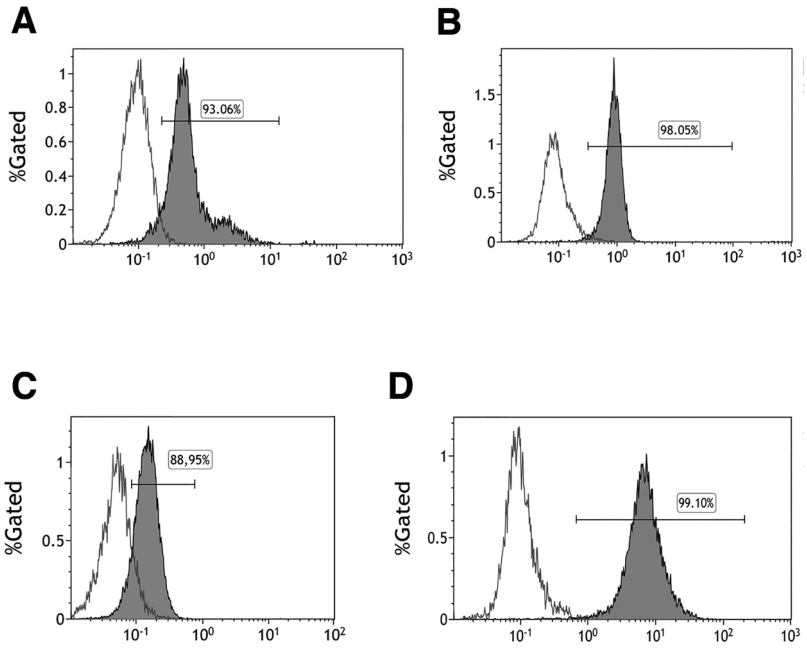

Fig. 2. Flow cytometry analysis of isolated SMCs. Expression of specific markers: (A) α-SMA; (B) CALP; (C) MHC; (D) VIM.

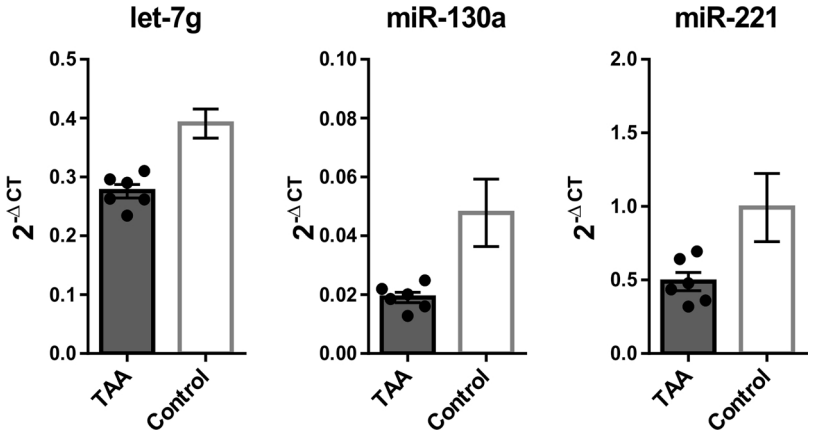

Fig. 3. miRNAs expression profile in TAA patients *vs.* control samples.
Values are presented as means ± SEM, *p value ≤0.05, ** p value ≤0.01.

studies allow for establishing a straightforward and time-efficient method to obtain human aortic SMC culture from both TAA and healthy donor samples. The isolation of SMCs should begin within 24 h after excision and based on our present study, the early start of the isolation procedure results in the obtaining of high quality cell lines, as noticed also by Patel et al. [38]. Lu et al. [39] also showed that isolation and culture of SMCs from older patients is more difficult and time-consuming, providing a smaller number of recovered viable cells. The purity of isolated cells tested with α-SMA was significantly higher in comparison to Lu et al. [39] and similar to Moss et al. [40], reaching over 93%. Purity was additionally confirmed with other SMC markers: CALP, MHC and VIM and marked positive in 88.95%–99.10% of cells. Similar results from mouse and rat aorta were described by Kwartler et al. [33] and Xu et al. [37]. It should be noticed that microscopic observations of SMC growth and proliferation is consistently a valuable technique for confirmation of cells characteristics and phenotype [41].

Despite the fact that more than 2500 human miRNAs have been discovered, the molecular mechanisms behind TAA formation and progression are still not completely understood [42–44]. It has been demonstrated that miRNAs are associated with cardiovascular disease, as they alter many cellular functions and affect the expression of multiple genes. To date, miR-21, miR-27b, miR-29b, miR-93, miR-133b, miR-143/miR-145, miR-146a, miR-155, miR-331-3p and miR-486-5p were found to be related to abdominal aortic aneurysm [45,46] while miR-15 family, miR-21, miR-29 and miR-30 family, miR-126, miR-143/145, miR-155, miR-181b, miR-221/222, miR-486-5p to TAA [8,32,47–50]. Two other miRNAs, miR-221 with miR-222, were recognized as being involved in cancer cell proliferation by promoting vascular SMC proliferation and neointimal hyperplasia [51], which is consistent with our results. The miRNAs described above are known to be engaged in SMC regulation by influencing apoptosis, migration, or proliferation and in consequence promoting their remodeling and phenotype switch. In our current study, we found 3 of 22 miRNAs investigated as differentially expressed in patient *vs* control samples. For the first time, we identified miR-130a as differentially expressed in human aortic SMCs, which is consistent with the findings of Wu et al. [52] who indicated the role of miR-130a as a regulator of proliferation in vascular SMCs in a rodent model of pulmonary hypertension. Circulating miR-130a was associated with bicuspid aortic valve disease and aortic dilation by switching on TGF-β1 and VEGF signaling pathways [53]. miR-130a was found to promote the proliferation of ECs and vascular SMCs through ability of modulating the expression of *GAX* and *HOXA5* genes [54]. The miR-221/222 cluster is known for controlling development and differentiation of ECs by inhibiting its proangiogenic activation, migration and proliferation, while switching on the "synthetic" phenotype of SMCs [55]. *In vitro* knockdown of miR-221 and miR-222 resulted in decreased vascular SMC proliferation in the rat model of the abdominal aortic aneurysms disease [56].

In the present study, we also identified let-7 g as differentially expressed in TAA samples, which is compatible with other results reporting *let* family as alternatively expressed in cardiovascular disease including arrhythmia, angiogenesis, atherosclerosis, cardiac fibrosis, heart hypertrophy, hypertension, dilated cardiomyopathy and myocardial infarction. Let-7 g was found downregulated in microRNA profile of thoracic aortic dissection as compared to normal aortic tissue [57]. Another study revealed that let-7 g may be involved in endothelial apoptosis and atherosclerosis [58], and be differentially expressed in human intracranial aneurysms [59]. However, the exact function of let-7 g in TAA formation and progression has not been described yet. Nevertheless, a revised understanding of signaling pathways is crucial regarding let-7 g as a potential molecular target for clinical settings [60–62].

4.1. Limitations of the study

We acknowledge some limitations of the study related to its *in vitro* character. The established SMC isolation protocol required at least 2 passages (2–3 weeks in culture) increasing chances of type II error in SMC miRNA profiling. The lack of *in situ* data is also a limiting factor, as it could strengthen the results by translating *in vitro* to *in vivo* data.

5. Conclusions

We described a simple, yet very effective and time-efficient isolation protocol that may be used as a biotechnological tool to study cellular processes and molecular pathways leading to TAA and other cardiovascular diseases. Further studies are required to support the performance of specific miRNAs in TAA formation. Understanding the molecular background of aneurysm progression may allow development of new and more effective therapeutic strategies, as well as more sensitive and specific diagnostic methods. Ongoing miRNA studies make a foundation to assume that the coming years will provide the use of these molecules in clinical settings.

Conflict of interests

The authors declare no conflict of interests.

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