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Research paper

miRNAs may change rapidly with thoughts: The Relaxation Response after myocardial infarction

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ABSTRACT

Introduction: Mental stress is potentially a major cardiovascular risk factor. Meditation and listening to music may be able to compensate by eliciting the Relaxation Response (RR) with a beneficial prognostic impact after myocardial infarction (MI), reducing the progression of the arteriosclerotic process and improving coronary blood flow. We aimed to study a possible epigenetic mechanism of the RR speculating that circulating microRNAs levels could change during relaxation.

Methods: We enrolled 150 consecutive patients after MI. 50 were trained to meditate, 50 given music appreciation and 50 served as controls. In addition, in order to rule out that the disease state could interfere with the possible movement of microRNAs, we enrolled 50 healthy volunteers (25 were trained to meditate and 25 had music appreciation). After training, and after 60 days of RR practice, we studied the individual variation, before and after the relaxation session, of some important cardiovascular circulating microRNAs: the microRNA-1, −16, −24, −33, −92, −144, −146, −155.

Results: As the RR appeared to be triggered in the same way irrespective of whether this was by music or meditation data was combined. After the RR, a reduction in microRNA-16, −33, −92, −144, −146, −155 ($p < 0.01$) and an increase in the levels of microRNA-1 and −24 ($p < 0.01$) from baseline was observed both at the first observation and after 2 months.

Conclusions: The RR modulates some microRNAs levels suggesting that psychic activity may be an important epigenetic and pathophysiological factor in the arteriosclerotic process and in ischemic heart disease. In particular, the analyzed microRNAs levels seems to vary in relation to the state of stress or relaxation of the subjects.

1. Introduction

Mental stress damages the brain [1] and the immune system [2,3] by affecting hormones and metabolism [4] and underlies many cardiovascular diseases [5–7], particularly myocardial infarction and its risk factors [8]. Many of the molecular mechanisms associated with such evidence are related to epigenetic markers that affect cellular activity in response to environmental or psychic stimuli [9]. These processes (DNA methylation, histone coding, chromatin conformation modification, non-coding RNAs) change cellular functioning in the absence of gene mutations or “defective” genes and can be stable, reversible, and inheritable [10]. Circulating microRNAs are key epigenetic elements that can convey significant physical or psychic stress signals at cellular level, modulating the adaptive response of each cell, tissue, organ, and of the whole individual to the environment [5]. Moreover, circulating microRNAs (miRNA) are at the center of research for their possible diagnostic and therapeutic ability in many cardiovascular diseases [11,12].

As we recently described [13], meditating and listening to classical or meditative music, are two techniques that are able to turn off the brain areas that carry stress signals [14–16] (the so called Default Mode Network, (DMN)) which evoke the Relaxation Response (RR) through specific neuronal circuits (called Attention Network, (AN)). The RR is aroused when an individual focuses on a word, a sound or a song, a phrase, a repetitive prayer, or a movement, disregarding everyday thoughts [17]. These two steps break the mind wandering and train of thoughts of everyday life. The practice of anti-stress methods, such as meditation or music appreciation, correlates with a significant decrease of adverse cardiac events in patients with myocardial ischemia, stroke, atherosclerosis, hypertension and heart failure [18] and are

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recommended by the American Heart Association (AHA) [13] as "an adjunct to guideline-directed cardiovascular risk reduction by those interested in this lifestyle modification even if the benefits of such intervention remain to be better established"[19].

Therefore we wanted to study a possible mechanism, not yet described in literature, to the best of our knowledge, with the purpose to evaluate whether the stress axis activity downregulation related to the practice of meditation or music appreciation translates into a modification of some cardiovascular miRNAs, important in regulating cardiomyocyte function, vascular endothelial function and atherosclerotic process and lipid metabolism.

In fact, as described by Leung et al. [20], "*responding to stresses* −physical or psychological stressors trigger the same responses on the biological level [21,22]- *cells either choose to restore or reprogram their gene expression patterns. This decision is partly mediated by miRNA functions, in particular by modulating the amount of miRNAs, the amount of mRNA targets, or the activity/mode of action of miRNA-protein complexes. In turn, these changes determine the specificity, timing, and concentration of gene products expressed upon stresses. Dysregulation of these processes contributes to chronic diseases, including cancers*" and cardiovascular disease [11,12] and may be transmitted transgenerationally [23].

Is the movement and action of miRNAs a passive/automatic response to the stressor or can it be guided directly by mental activity?

This study aims to verify if the mental activity (which can only go either in the direction of stress or relaxation) is able to determine significant epigenetic variations related to some microRNAs chosen to explain the observations made in a previous work [13]. In other words, our aim is to see if the psychological activity (we have used meditation and music appreciation as two fairly reproducible examples of self- or stimulated relaxation-oriented psychological activity) could directly have an impact on microRNA levels. Our previous work [13] demonstrated that psychological activity associated with meditation and music appreciation results in a significant down regulation of neuroendocrine and inflammatory molecules and genes. In addition, Nuclear factor Kappa B (NfKB), and toll like receptor 4 (TLR4)) appears to improve the lipid profile of the atherosclerotic process and stimulation of a regenerative cardiovascular potential (increasing in circulating endothelial stem cells). The behavior of microRNA-1, −16, − 24, −33, −92, −144, −146 and −155, were selected on the basis of their mechanisms of action (see "discussion") as they may correspond and help to explain the results of our previous study.

2. Methods

2.1. Recruitment

In this paper we expanded the population studied previously [13], as follows. According to Professor Benson's work at Harvard [29], the RR is evoked in an equal way through a process of attention to a "internal" psychological (meditation) or a "external" stimulus (like music appreciation). So, on the one hand we wanted to confirm this aspect again (as happened in our previous study [13]) and thus we enrolled 150 patients post MI, creating three groups of 50 patients (meditation, music and "sick controls no intervention"-see below) based on the expected effect size (see statistical justification in the dedicated paragraph below). Once we had this confirmation, we also enrolled 50 more healthy subjects and taught them the RR (25 through meditation and 25 through music appreciation). This was to see if the state of the disease could alter or not the excursion of the analyzed markers during RR. Even in healthy subjects trained in RR we wanted to verify any potential difference between the methods used to elicit the RR itself.

For the comparison between groups, at least 50 subjects per group were needed (again, please see statistical justification in the dedicated paragraph below).

Finally, as the RR appeared to be triggered in the same way irrespective of whether this was by music or meditation (see below) data was combined and three groups were created (namely "INTERVENTION", group of post MI patients subjected to RR −nr. 100 subjects-, "CONTROL", group of "control patients-no intervention" −nr. 50 subjects- and "INTERVENTION HEALTHY CONTROLS", 50 healthy controls subjected to RR).

More in detail, between October 2015 to February 2017, 150 consecutive patients (116 males, mean age 52.7 ± 11.1) who had consulted in our Cardiologic Clinic for ST elevated (STEMI) or non ST elevated myocardial infarction (NSTEMI) and suffering from carotid atherosclerosis were enrolled in the study, after providing informed consent. All patients were free of cognitive deficit and had no other comorbidities. Patients over 65 years of age were excluded. In addition, in order to rule out that the disease could interfere with the possible movement of microRNAs, we also enrolled 50 healthy control subjects matched for age and gender of the MI patients. These healthy control participants were enrolled from amongst the acquaintances of the research group, and agreed to a medical examination in order to verify their "good state of health".

Of the 150 consecutive patients after MI, 50 were trained to meditation, 50 to music appreciation and 50 served as controls (no intervention, please see below). Of the 50 healthy subjects, 25 were trained to meditate and 25 had music appreciation.

After training, and after 60 days of RR practice, we studied the individual variation, before and after the relaxation session, of some important cardiovascular circulating microRNAs: the microRNA-1, −16, −24, −33, −92, −144, −146, −155.

2.2. Training and blood sampling time

These patients were consecutively trained as follows: 50 to Pneumomeditazione® and 50 to music appreciation. A brief description of each relaxation method is available below. Another 50 patients constituted the control group and were not subjected to any intervention. They were asked to relax in a way that felt more appropriate for a period of time corresponding to the relaxation session. Most people sat in the chair with eyes closed. None of the participants had practiced meditation or listened to music aimed at relaxation before the study. Each subject has expressed his free and autonomous choice to participate in the study giving a written informed consent.

The initial four days of training took place in our hospital before discharge and the rest of the relaxation sessions were carried out independently by the subjects at home for 20 min, 2 times a day. After four days of training, we studied partecipants during the two daily relaxation sessions. At 8:00 a.m. we performed blood samples before and immediately after the end of the session. We repeated the same scheme after 60 days of daily practice at home.

Thus, as the primary endpoint, measured was whether there had been any changes in the microRNAs (estimated effect size of at least 0.49 as described below). In blood samples we assessed the following molecules: microRNA-1, −16, −24, −33, −92, −144, −146 and −155.

The environmental conditions at the time of data collection were the same for all the subjects. In particular, the training, the relaxation sessions and blood withdrawals have taken place in the classroom of our clinic situated alongside our echocardiography laboratory. Patients had three days to decide whether or not to participate in the study and their enrollment occurred in the seventh day after infarction. The four days of training were held in our classroom before discharge. All patients received optimal medical therapy in accordance with European Society of Cardiology (ESC) and AHA guidelines for the treatment of ischemic heart disease and followed the same cardiac rehabilitation program (physical training and nutrition education).

The acute variation of the parameters studied can be attributed to the practice of relaxation according to the methods used because the precise timing of blood sampling (before and immediately after the end of the session) prevents any other influences. As previously described

[13], the home daily practice is served to promote and enhance the neuro-hormonal effect of relaxation in order to be able, after 8 weeks, to confirm or increase the sensitivity to that observed at baseline. All groups were subjected to the same environmental conditions: in particular, also the control patients were taken in our classroom for 20 min and were not subjected to any intervention. As already stated, we simply asked them to relax and most of them has sat down with eyes closed.

Investigators collecting data were blind to the intervention that had been delivered to each individual. Only one investigator (CDL) was aware of the allocation and did not take part in data collection or the blood samples collection and analysis.

2.3. The interventions − instructions for meditation and for music listening

The Relaxation Response (RR) is a relaxation technique described by Benson [24] that falls into the category of meditative techniques and that uses different conditioning stimuli. Indeed, this procedure provides that the subject, comfortably seated, repeats a sound, a word or a phrase or concentrates to a music [25]. Thus, to this category of meditative techniques belongs prayer, repetition of mantras or the "focusing of attention" practices [26,27]. According to Benson [24], the attention that the subject lends to the repeating word leads him to a level of concentration that reduces and regulates the rhythm of breathing, producing relaxation [25,28].

In other words, the meditative techniques that involve the repetition of a word, a phrase or a sound, or listening to a music or a sound frequency, disregarding everyday thoughts, evoke the general RR [29], with slight effects (and not always described) superimposed upon the general RR by the particular relaxation technique or conditioning stimulation choosed [30].

As previously reported [13], the Pneumomeditazione® method involves the mental repetition of a sound/word drawn from the Aramaic tradition. The meditation techniques that we used in this study were taught by two physicians of our research group, experts in meditative techniques and RR.

As other forms of Relaxing Response[24], the meditation technique is taught in four days of training, has a duration of 20 min and is performed twice a day, comfortably seated with eyes closed. Besides teaching the technique, over the four training days, subjects are taken on a "life-coaching" journey. In fact, they are taught to identify the types of thoughts that can trigger and fuel a stress reaction facing life events, in order to modify them and improve their psychological, emotional and behavioral reactions in the life of every day and increase their personal well-being.

Even the instruction format for music listening takes place in four days of training with an introductory and preparatory session to outline the benefits and mechanics of the neuro-physiological response that is connected to the relaxation induced by listening to classical music and ambient or meditative sounds (we employed sequences of songs lasting 20 min taken from W.A. Mozart K448, K425, K445, from "Avun" by P. Spoladore and natural sounds). We did not perform "music therapy", but sessions of "focusing the attention on the sounds heard". Then, the subject is trained to take 20 min twice a day for listening, sitting comfortably in a quiet place, with his eyes closed. Besides teaching the listening technique, over the four training days, patients are taken on a "life-coaching" journey. In fact, they are taught to identify the types of thoughts that can trigger and fuel a stress reaction facing the events experienced, in order to modify them and improve their psychological, emotional and behavioral reactions in the life of every day and increase their personal well-being.

In conclusion, these two paths leverage on the teaching of a technique able to induce an "acute" Relaxation Response and knowledge to use for stress management in everyday life.

2.4. Sample size and statistical analysis

In this study, the sample size was calculated based on the following scenario: type-I error rate of 0.05, power 0.95, pre-post changes in the circulating miRNAs levels, after intervention, from baseline, represented by an effect size of 0.49 (on the basis of the studies in the literature recently reviewed (5–7) and of a previous exploratory study performed in our clinic). Using a Wilcoxon signed-rank test (matched pairs), 49 sick subjects are required to detect this difference. Then, we analyzed the difference in the change of the parameters between patients' intervention groups and patients' control group and between patients' intervention group and the groups of healthy control subjects. In exploratory terms, a sample size of 50 persons allows to detect an effect size of 0.43 or higher with a type-I error of 0.05 and a power of 0.90, using a non-parametric test. Data are expressed as median and interquartile range (variables don't have a normal distribution as assessed by the Shapiro-Wilk test). Categorical variables, used for the description of the population studied (Table 1), are expressed as percentages and compared using the χ^2 test or Fisher's exact test. The comparison between the pre-post intervention changes was performed by means of Wilcoxon test. Moreover, we compared the extent of the percentage changes of each parameter occurring during each relaxation session and after 8 weeks by means of the Mann-Whitney test. An initial comparison between groups was performed by means of Kruskal-Wallis test for independent samples or by Friedman test for paired data. Statistical significance was assumed if the null hypothesis could be rejected at $p = .05$. The statistical analysis was performed using software SPSS version 22.0 (Chicago, SPSS, Inc., Chicago, IL).

2.5. miRNA and total RNA extraction

For the miRNA assay and RNA extraction we have faithfully followed the procedure described in the Exiqon kit [56]. Total RNA was isolated from plasma by miRCURYTM RNA Isolation kit-Biofluids (Exiqon, Denmark), following the manufacturer's instructions. RNA was treated with rDNase (Exiqon) before reverse transcription (RT). For miRNAs expression, 10 ng of RNA was reversely transcribed using miRCURY LNATM Universal RT microRNA PCR reverse transcription kit (Exiqon) according to the given protocol. miRNAs were detected using ExiLENT SYBR® Green master mix (Exiqon) and miRCURY LNATM Universal RT microRNA PCR LNATM PCR primers set (Exiqon) in a Bio-Rad CFX96 Real Time PCR detection system. A negative control containing all reagents but no cDNA template was included in all runs. The specific primers were (Exiqon): hsa-miR-1-3p, has-miR-16-5p, hsa-miR-24-3p, hsa-miR-92a-3p, hsa-miR-144-3p, hsa-miR-146a-5p, hsa-miR-155-5p, hsa-miR-33a-5p (Table 1). We used hsa-miR-103a-3p as stably expressed miRNA and reference gene based on the advice given by the primer manufacturer.

Validation of specificity of Real-Time PCR assay was performed by melt-curve analysis. For each target miRNA, a calibration curve was generated with threshold cycle (Cq) values from serial dilutions of cDNA (from 106 to 10 copies/reaction) to determine reaction

Table 1
The primer sequence of each microRNA.

miRNA	sequence
hsa-miR-1-3p	5′UGGAAUGUAAAGAAGUAUGUAU
hsa-miR-144-3p	5′UACAGUAUAGAUGAUGUACU
hsa-miR-155-5p	5′UUAAUGCUAAUCGUGAUAGGGGU
hsa-miR-33a-5p	5′GUGCAUUGUAGUUGCAUUGCA
hsa-miR-16-5p	5′UAGCAGCACGUAAAUAUUGGCG
hsa-miR-24-3p	5′UGGCUCAGUUCAGCAGGAACAG
hsa-miR-146a-5p	5′UGAGAACUGAAUUCCAUGGGUU
hsa-miR-92a-3p	5′UAUUGCACUUGUCCCGGCCUGU
hsa-miR-103a-3p	5′AGCAGCAUUGUACAGGGCUAUGA

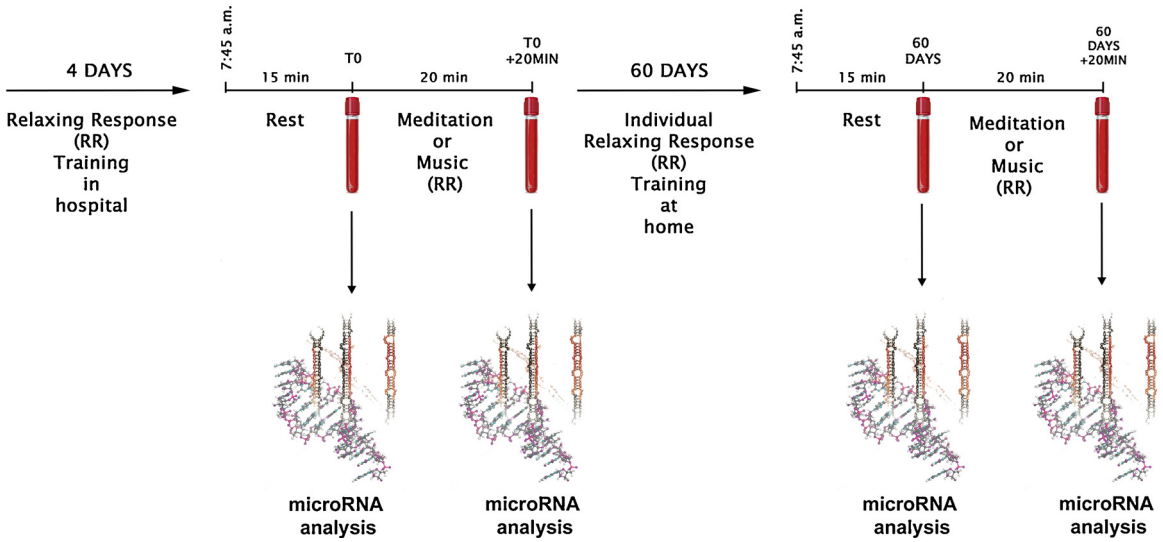

Fig. 1. The study design. Explanation in the text.

efficiencies, linearity, detection and quantification limits. Data analyses were performed with the Bio-Rad CFX Manager. The comparative cycle threshold method (ΔΔCq), which compares the difference between groups in cycle threshold values, was used to obtain the relative fold change of miRNA expression.

2.6. Research ethics

Written informed consent was obtained from each patient included in the study, the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and the study protocol has been priorly approved by the Institution's ethics committee on research on humans (Comitato Etico per la Sperimentazione Clinica-Azienda Sanitaria di Padova; protocol number 3487/AO/15).

3. Results

This is a prospective, blinded non-randomized study. The design of our study follows what has already been published in literature [13,15] and is shown in Fig. 1. Each patient admitted to our hospital with a MI was consecutively enrolled blindly, the first group of 50 patients were trained to meditation, the second group of 50 patients to music appreciation and the third group of 50 patients served as controls (no intervention, please see above). Finally, of the 50 healthy controls, 25 were allocated to either training in meditation and 25 to music appreciation. The same musical compositions as described previously [13] were used and Pneumomeditazione® as the only meditative procedure [13].

The groups were homogeneous (Table 2) and the characteristics of the studied population did not change significantly compared to the data already presented previously (Table 2) [13].

To obtain the number of 150 subjects, 190 were initially invited and of the 150, 100 were allocated to either music or meditation and 50 served as “sick” controls. This gave a response rate for participation in the study of 78,9%.

Confirming Benson's research [29] and as in our previous pilot study on 30 patients [13], there were no significant differences between relaxation techniques in terms of variance of each parameter before or after the sessions, both in the diseased population and in the healthy one (Table 3).

Therefore, we merged into a single “intervention” group all patients treated with meditation and music and into a single “intervention healthy controls” group all healthy subjects.

We want to emphasize that in our work we observed the RR using two conditioning thechniques, meditation and music, which have to be considered as two ways leading to the same relaxation effect [29]. Therefore, even from a strictly methodological point of view, we used a unique technique −precisely the RR-, from which also the need to unite in a single “intervention group” the treated subjects. We checked our observations twice: at the fourth day of RR training and after 60 days of regular individual practice at home.

Indeed, all subjects enrolled in the study have continued the practice at home, twice a day, as they taught. During the 8 weeks of autonomous home practice, each subject reported to have pleasantly performed more than 95% of the meditation or music listening sessions.

The study design, with the execution of blood draw immediately before and after the relaxation session, implies that the results observed could only be related to RR. Moreover, all conditions that could have affected the variation of the circulating miRNAs did not change and were comparable in the different groups both at baseline and after 60 days (blood sampling at the same time of the day both at baseline and after 60 days, same time of follow-up and same environmental conditions at the time of sampling).

The study results in Table 4 highlight a highly significant important difference in miRNAs changes during the relaxation session (expressed as Δ%) between intervention group and control group, that indicate variations opposite to the considered parameters.

The RR results also significantly reduced microRNA-16, −33, −92, −144, −146, −155 ($p < 0.01$) both during the first observation and after 2 months (Fig. 2).

The RR also significantly increased the levels of microRNA-1 and −24 ($p < 0.01$) both at the baseline observation and after 2 months of practsing the intervention(Fig. 2).

4. Discussion

Every human being can be more or less stressed or relaxed and never at the same time. What we think does not remain confined to the brain but could be constantly written in the body through neuroendocrine-immune molecules and miRNAs.

In particular, this study seems to demonstrate that the practice of meditation and specific music frequencies affect the levels of some important cardiovascular miRNAs, describing indirectly a way through which psychological activity could positively modulate the expression of our genome with a potentially beneficial clinical impact after a myocardial infarction [13].

We chose to measure eight specific miRNAs (microRNA-1, −16, −24, −33, −92, −144, −146, −155) that are related to stress axis

Table 2
Characteristics of the population at the time of enrollment and pharmacological regimen after two months. Patients were taking similar dosages of drugs (beta-blockers, ACE-inhibitors and statins) in all groups ($p > 0.05$ Mann-Whitney). Moreover, both at discharge and after 60 days, all subjects enrolled were taking antiplatelet drugs as per ESC/AHA guidelines; After 60 days, medical therapy, blindly adapted by our colleagues of cardiac rehabilitation, did not differ significantly from the baseline ($p > 0.05$ Wilcoxon), with a tendence towards reduction of the statin dosage for all the patients. We want to specify that some single patients in the intervention group significantly reduced the dose of beta-blockers and statins due to hypotension or excessive reduction of total cholesterol, even if, on the whole, we did not appreciate significant differences in drug dosage between groups. The black circle indicates $p < 0.05$ with the χ^2 test for categorical variables.

	CONTROL				INTERVENTION				INTERVENTION HEALTHY CONTROLS			
GENDER MALE (%)		76 (38)				78 (78)				75 (37)		
AGE (years)	51.64 ± 10.93				53.77 ± 11.01				50.73 ± 12.1			
FAMILIARITY FOR CAD (%)	54				57				12●			
SMOKE (%)	34			EX = 22	38			EX = 15	0●			0●
BMI (kg/m^2)	25.44 ± 2.82				24.56 ± 3.53				22.67 ± 2.73			
HYPERTENSION (%)	18				20				0●			
DIABETES (%)	0				0				0●			
DYSLIPIDEMIA (%)	42				40				0●			
ALLERGY (%)	28				32				30			
NSTEMI (%)	58				62				–			
NUMBER OF CORONARY DISEASED (n = %)	1 = 30	2 = 50		3 = 20	1 = 28	2 = 48		3 = 24	–	–	–	
TROPONIN (ug/L)	45.86 ± 25.12				48.17 ± 31.79				–			
MEDITERRANEAN DIET (%)	100				100				100			
HOURS EXERCISE/WEEK	4.5 ± 1.5				4 ± 1.5				4 ± 1			
mg of drug at discharge	50 (50–100)	2.5 (1.76–5)	2,5 (2.5–2.5)	60 (40–80)	50 (50–100)	2,3 (1.25–5)	2,5 (2.5–2.5)	60 (40–80)	–	–		
mg of drug after 2 months	100 (100–100)	2,5 (2.5–3.75)	2,5 (2.5–5)	40 (40–50)	50 (25–100)	1,25 (0.61–2.5)	2,5 (1.25–2.5)	30 (20–40)	–	–		
	METOPROLOL	**BISOPROLOL**	**RAMIPRIL**	**ATORVASTATIN**	**METOPROLOL**	**BISOPROLOL**	**RAMIPRIL**	**ATORVASTATIN**	–	–		

activity and are involved in NFkB signal transmission, cholesterol metabolism and myocardial and vascular regeneration based on what clinically and biochemically highlighted in our previous pilot study [13]. Further studies will require the dosage of a miRNA panel to see what other molecules could move in relation to specific thoughts and mental processes and the activity of the stress axis. As one example (obviously the schematization of all the possible signal transmission paths in which these microRNAs are involved is outside the scope of the paper), in Fig. 3 is depicted a part of NFkB signal transduction pathway involving microRNA-92, −146 and −155.

We focused our analysis on the short-term variations of the molecules considered because their balance in the long-term is still difficult to study since no normal values or absolute quantitative thresholds have been identified for miRNAs. Thus, in our study, many external and internal influences, such as social stress, nutrition, physical activity, etc. (and therefore not necessarily the RR) may have affect miRNAs balance and their baseline values at two months.

The in-depth description of the molecular effects and pathways of each of the miRNAs considered is out of the scope of the work but it is possible to find all the information consulting a dedicated database [31]

MiRNA-1 plays a pivotal role in cardiogenesis [32], cardiac hypertrophy, heart failure [33] and arrhythmias [34], performing numerous functions in the heart, including regulation of cardiac growth and differentiation, electrical conduction, and cell-cycle control through modulation of transcription factors like Irx4, Hrt2, Hand1 and Gata6 [35]. Adequate values of miRNA-1 are able to counteract the vasoconstrictor effects of endothelin-1[36] and reverse pressure-induced cardiac hypertrophy preventing maladaptive cardiac remodeling [37]. Finally, Jayawardena et al. [38] demonstrated that miRNA-1 is sufficient to induce reprogramming of fibroblast into cardiomyocytes for a possible regeneration of the heart after a damage [39].

MiRNA-16 increases, along with miRNA-144 and endothelin-1 (ET-1), in response to chronic mental stress [40], and represents a signature able to differentiate Takotsubo cardiomyopathy from acute myocardial infarction [41].

MiRNA-24 is a pro-survival molecule that inhibits the apoptosis of cardiomyocytes [42].

MiRNA 33 regulate high density lipoprotein (HDL) biogenesis, cellular cholesterol efflux, and HDL cholesterol (HDL-C) uptake in the liver, thereby controlling all of the steps of reverse cholesterol transport [43,44]. Interestingly, in vivo, while the short term miRNA-33 antagonization seems to enhance the regression and reduces the progression of atherosclerosis [45], its long term therapeutic silencing increases circulating triglyceride (TG) levels, lipid accumulation in the liver and the expression of genes involved in fatty acid synthesis [46].

Thus, the self-regulating system of the body (the stress axis) seems to be based on adjustment signals that require to be modulated and not necessarily extinguished following a reductionistic and mechanistic view of human biology.

MiRNA-92a has a pro-inflammatory and pro-atherogenic function in the endothelium [47] enhancing nuclear factor of κ light polypeptide gene enhancer (NF-κB) signaling at two levels: by repressing KLF2/KLF4, antagonists of NF-κB-dependent transcription, and by promoting the activation of p65 [48].

MiRNA-146a and miRNA-155 [49] are NF-κB-dependent microRNAs, that impinge on various stages of the NF-κB signaling pathway, playing critical roles in attenuating activation of this pathway[48] acting on TRAF-6 and κB-RAS1.

From the mechanisms just described, we can deduce that RR, modifying some key miRNA values, may determine beneficial cardiovascular effects reducing inflammation, improving endothelial function and by triggering a regenerative cardiac potential.

Particularly, we want to emphasize the decrease in microRNA 33 with relaxation that could be involved in the favorable variation of lipid profile described in our foregoing work [13] and probably also in the rapid change of the plasma physical characteristics during the 20 min of

Table 3
The table shows the mean variations (expressed as percentage of 2dct) of each miRNA after the meditation and music appreciation sessions, both in the intervention group and in healthy volunteers. The data show no significant differences between relaxation techniques in terms of variance of each parameter before or after the sessions, both in the diseased population and in the healthy one. T0 = first blood sample at the fourth day of training; T0 + 60 days = second observation after 60 days of regular RR practice.

	INTERVENTION A			INTERVENTION - HEALTHY CONTROLS C		
	% VARIATION WITH MEDITATION	% VARIATION WITH MUSIC	p Mann-Whitney	% VARIATION WITH MEDITATION	% VARIATION WITH MUSIC	p Mann-Whitney
miRNA 24						
T0	+60%	+65%	$p > 0.05$	+63%	+58%	$p > 0.05$
T0 + 60 days	+23%	+19%		+105%	+110%	
miRNA 155						
T0	−42%	−36%	$p > 0.05$	−52%	−46%	$p > 0.05$
T0 + 60 days	−50%	−57%		−61%	−66%	
miRNA 146						
T0	−56%	−52%	$p > 0.05$	−22%	−15%	$p > 0.05$
T0 + 60 days	−9%	−13%		−165%	−178%	
miRNA 144						
T0	−42%	−37%	$p > 0.05$	−70%	−64%	$p > 0.05$
T0 + 60 days	−16%	−21%		−186%	−198%	
miRNA 1						
T0	+167%	+163%	$p > 0.05$	+205%	+196%	$p > 0.05$
T0 + 60 days	+144%	+133%		+178%	+184%	
miRNA 92						
T0	−45%	−55%	$p > 0.05$	−35%	−44%	$p > 0.05$
T0 + 60 days	−63%	−56%		−97%	−94%	
miRNA 16						
T0	−51%	−47%	$p > 0.05$	−62%	−58%	$p > 0.05$
T0 + 60 days	−16%	−19%		−37%	−42%	
miRNA 33						
T0	−49%	−46%	$p > 0.05$	−40%	−34%	$p > 0.05$
T0 + 60 days	−23%	−28%		−33%	−30%	

meditation or music appreciation (Fig. 4).

These results confirm and expand our previous pilot study(13) where meditation and listening to particular music frequencies down-regulated inflammatory genes expression (p53,NF-κB, Toll-like receptor 4) in circulating peripheral blood mononuclear cells, by means of a highly significant variation (and below the current values of “normality”) of neurotransmitters, hormones and cytokines resulting in a clinically favorable impact (improvement in endothelial function and the initial regression of carotid atherosclerosis) in patients with coronary artery disease.

Our work seems to suggest that neuro-endocrine-immune mediators and miRNAs vary rapidly depending on the orientation of our psychological activity, thus increasing the degree of complexity in the study of epigenetic phenomena. The results presented reveal that the study of miRNAs and other epigenetic factors involved in many cardiovascular pathologies should take into account the action that the psyche constantly exercises on the epigenome network in order to avoid arbitrary associations. In fact, a condition of cellular oxidative stress, potentially triggered by mental stress [5–7], may lead to a wrong folding of proteins [50] with relative function deficits. Numerous genetic linkage studies that do not take into account the effects of mental stress on oxidative stress may have determined for different pathologies different gene-diseased protein associations that are completely arbitrary because the problem may not necessarily reside in coding information but in the environment where the transition to the quaternary-functional protein structure occurs. This study appears to confirm this consideration and helps to explain in more detail the regression of the atherosclerotic process related to the practice of stress modulating methods, as evidenced by Ornish [51–53] and Castillo Richmond [54] in their works.

As previously stated, for microRNAs, thresholds or “normal” values have not yet been established, and we can only describe their trends and variations that, in our work, seem to vary according to the degree of activation of the stress axis. In addition, taking into account the possible psyche's impact on biology, it is necessary to evaluate the biochemical parameters not only quantitatively but also qualitatively. Indeed, the same molecule can mediate different functions depending on the degree of oxidative stress, which varies with the degree of mental stress [13]. Could it also be the case for microRNAs? Since deregulated miRNA expression is an early event in many chronic diseases such as cardiovascular pathologies and tumorigenesis [57], our description of their movement with psychological activity could be of paramount importance for prevention.

In conclusion, our work reveals that thoughts may constantly affect the expression of our genome and epigenome. Indeed, the practice of meditation or music appreciation, being able to downregulate stress axis activity (from brain cortical level to neuroendocrine and immunitary molecules [5–7,17]) seems to shape the levels of some important cardiovascular miRNAs. We described a possible epigenetic mechanism involving miRNAs, underlying the favorable cardiovascular epidemiological evidence related to the practice of the anti-stress methods considered. These two interventions suggest that they can be used as techniques which are able to induce an “acute” Relaxation Response and this can be used for stress management in everyday life.

Study limitations

In statistics we need to pay attention that correlations do not imply causation; our study was not randomized, but the practice of relaxation techniques requires a patient's voluntary decision/intention which may hinder a true randomization process. We tried to overcome this aspect enrolling patients consecutively assigning them to meditation or music appreciation training after obtaining their consent to the study and studying subjects immediately before and after the RR section in the same conditions. Moreover, each patient was studied twice and the second observation has reinforced the findings of the first. Finally, our findings seem to be coherent with the movement of the messengers described in our previous study [13] and with the rapid change of the plasma physical characteristics shown in Fig. 4. Although statistics may have epistemological limitations, it is unlikely that these results are the

Table 4
micro-RNA levels expressed as 2-dtc and the comparison of their variations (Δ) during the relaxation sessions (Mann-Whitney test). We want to highlight the significantly important difference in miRNAs changes during the relaxation session (expressed as Δ%) between intervention groups and control group, that indicate variations opposite to the considered parameters. This trend could be suggestive of the fact that mental activity proceeds either in the direction of relaxation either of tension and that, normally, seems to be set in a tensioned way (as shown by the controls).

	CONTROL A				INTERVENTION B				INTERVENTION - HEALTHY CONTROLS C	
	T0	T0 + 20MIN	60 days	60 days + 20MIN	T0	T0 + 20MIN	60 days	60 days + 20MIN	T0	T0 + 20MIN
miRNA 24	1 (1–1)	0,47 (0,16–0,81)	1,53 (1,07–2,62)	0,84 (0,44–1,25)	1 (1–1)	1,63 (1,54–1,83)	0,65 (0,53–0,95)	0,86 (0,59–1,1)	1 (1–1)	1,6 (1,53–1,69)
miRNA 155	1 (1–1)	4,16 (2,85–5,32)	2,37 (2,01–2,8)	3,29 (3,01–3,73)	1 (1–1)	0,62 (0,34–0,89)	0,88 (0,69–1,01)	0,34 (0,28–0,45)	1 (1–1)	0,52 (0,51–0,62)
miRNA 146	1 (1–1)	1,57 (1,46–1,93)	0,61 (0,54–0,82)	1,32 (1,26–1,58)	1 (1–1)	0,46 (0,31–0,63)	0,77 (0,6–1,48)	0,67 (0,54–0,8)	1 (1–1)	0,84 (0,8–0,88)
miRNA 144	1 (1–1)	2,16 (1,56–3,3)	0,9 (0,83–1,06)	1,8 (1,34–2,55)	1 (1–1)	0,62 (0,43–0,74)	1,36 (0,92–2,93)	1,19 (0,76–1,7)	1 (1–1)	0,36 (0,34–0,4)
miRNA 1	1 (1–1)	0,73 (0,65–0,82)	0,8 (0,77–0,82)	0,51 (0,42–0,62)	1 (1–1)	2,65 (1,97–3,01)	1,29 (1–1,55)	2,68 (1,98–3,74)	1 (1–1)	3 (2,78–3,89)
miRNA 92	1 (1–1)	1,53 (1,43–1,82)	0,71 (0,57–0,97)	1,09 (0,85–1,59)	1 (1–1)	0,49 (0,33–0,63)	1,48 (1,27–1,81)	0,88 (0,65–1,12)	1 (1–1)	0,6 (0,39–0,83)
miRNA 16	1 (1–1)	2,97 (2,81–3,49)	0,95 (0,86–1,16)	2,2 (1,82–2,68)	1 (1–1)	0,52 (0,36–0,74)	1,36 (0,92–2,93)	1,19 (0,76–1,7)	1 (1–1)	0,41 (0,4–0,43)
miRNA 33	1 (1–1)	1,82 (1,69–2,23)	1,57 (1,50–1,86)	2,34 (2,23–2,80)	1 (1–1)	0,52 (0,35–0,71)	0,68 (0,53–1,31)	0,43 (0,35–0,51)	1 (1–1)	0,64 (0,61–0,67)

	INTERVENTION - HEALTHY CONTROLS C		p value A vs B			p value B vs C		
	60 days	60 days + 20MIN	p Δ 20MIN T0	p Δ 20MIN T1	p Δ 60 days	p Δ 20MIN T0	p Δ 20MIN T1	p Δ 60 days
miRNA 24	1 (0,59–2,07)	2,07 (1,6–2,44)	< **0,001**	< **0,001**	**0,02**	0,298	0,142	0,49
miRNA 155	1,63 (1,37–3,22)	0,99 (0,62–1,02)	< **0,001**	< **0,001**	**0,049**	0,68	0,091	**0,026**
miRNA 146	2,74 (0,67–3,15)	1 (0,34–2,26)	0,41	< **0,001**	0,41	0,237	**0,032**	0,237
miRNA 144	4,66 (3,92–5,85)	2,69 (1,88–4,79)	< **0,001**	**0,027**	0,307	**0,014**	0,185	**0,021**
miRNA 1	1,5 (1,01–2,01)	3,3 (2,61–3,46)	< **0,001**	< **0,001**	**0,027**	0,21	0,945	0,407
miRNA 92	3,17 (2,93–4,02)	2,21 (2,19–2,8)	< **0,001**	< **0,001**	**0,027**	0,49	0,21	**0,007**
miRNA 16	0,79 (0,72–0,89)	0,4 (0,27–0,41)	< **0,001**	**0,014**	0,307	0,407	1	**0,026**
miRNA 33	0,83 (0,54–1,12)	0,52 (0,32–0,73)	< **0.001**	< **0.001**	< **0.001**	0,584	0,382	0,631

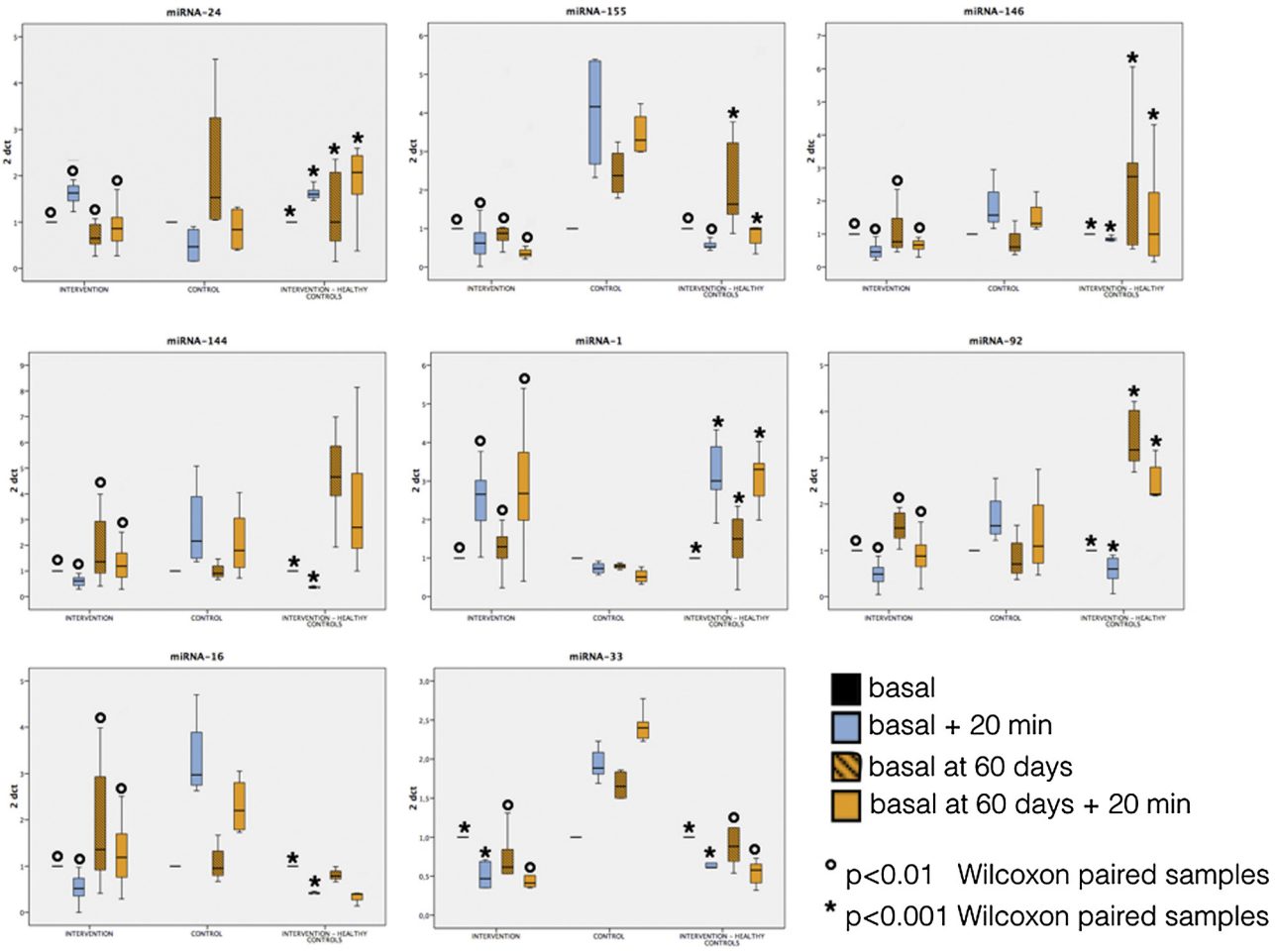

Fig. 2. micro-RNA variations during the relaxation session, expressed as 2-dtc. On the axis of the abscissas are indicated the three groups of analysis that we have created: Intervention (Meditation or Music appreciation), Control, and Intervention − Healthy controls (details in the text). For each group are drawn the box diagrams related to the medians and interquartile ranges of each microRNA dosage. In particular, the blue boxes represent the data relating to the first observation, before (blue boxes with diagonal lines) and after (blue boxes) the 20 min of relaxation. In orange the data of the second blood sample performed after 60 days, always before (orange boxes with diagonal lines) and after (orange boxes) the relaxation session of 20 min. The black circles or the asterisks indicate if the difference between the medians of the two adjacent boxes of the same color (relative to the same observation), is significant according to the Wilcoxon test (p value indicated in the figure). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

result of a random and non-causal association with the RR.

Author contributions

CDL: contributed to conception and design of the study; contributed to acquisition, analysis, or interpretation; drafted the manuscript; gave final approval; agrees to be accountable for all aspects of work ensuring integrity and accuracy.

EG: design of the study, acquisition of data; critically revised the manuscript; gave final approval; agrees to be accountable for all aspects of work ensuring integrity and accuracy.

AB: acquisition of data; drafted part of the manuscript; gave final approval; agrees to be accountable for all aspects of work ensuring integrity and accuracy.

GR: acquisition of data; critically revised the manuscript; gave final approval; agrees to be accountable for all aspects of work ensuring integrity and accuracy.

MM: design of the study, acquisition of data; critically revised the manuscript; gave final approval; agrees to be accountable for all aspects of work ensuring integrity and accuracy.

LB: acquisition of data; critically revised the manuscript; gave final approval; agrees to be accountable for all aspects of work ensuring integrity and accuracy.

MP: acquisition of data; critically revised the manuscript; gave final approval; agrees to be accountable for all aspects of work ensuring integrity and accuracy.

SI: acquisition of data; critically revised the manuscript; gave final approval; agrees to be accountable for all aspects of work ensuring integrity and accuracy.

FT: data analysis and interpretetation; critically revised the manuscript; gave final approval; agrees to be accountable for all aspects of work ensuring integrity and accuracy.

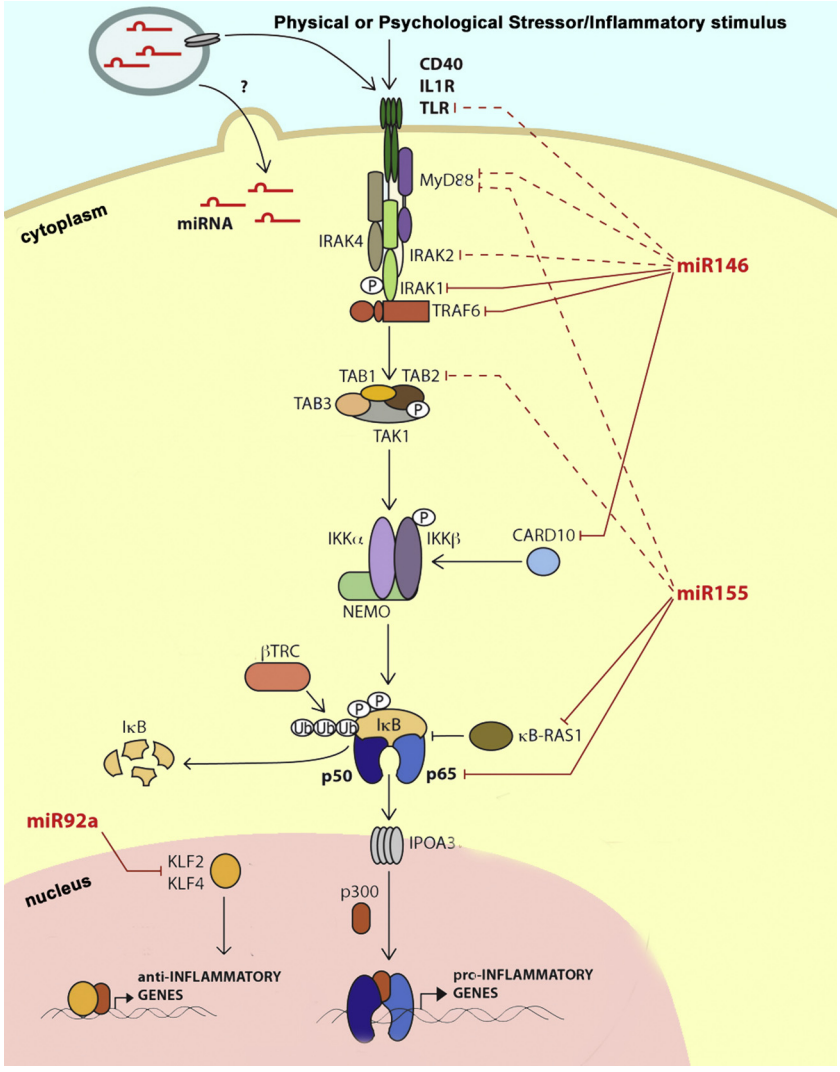

Fig. 3. The picture shows the signal transduction pathway leading to the activation of the NF-κB gene which, in turn, leads to the transcription of over 400 other genes related to inflammation [55]. The figure, therefore, represents one of the possible mechanisms by which a physical stimulus or a stress thought could be transmitted at the cellular level, triggering an inflammatory response. Obviously, contrary stimuli (as meditation or music appreciation in our study) produce reverse responses. For the details on the action of the molecules mentioned in the figure, please consult the work of Cheng et al. [48]. Biefly, as they describe [48]: "*NF-κB-dependent microRNAs, such as miR-146 and miR-155 impinge on various stages of the NF-κB signaling pathway, and play critical roles in attenuating activation of this pathway.The microRNA targets that have been verified in vascular endothelial cells are shown as solid lines, and targets validated in other cell types are indicated with dashed lines. The overexpression of miR-92a, which targets KLF2 and KLF4, leads to increased inflammation. MicroRNAs can be found at high levels within microvesicles (MVs) in the circulation*". Modified from [48].

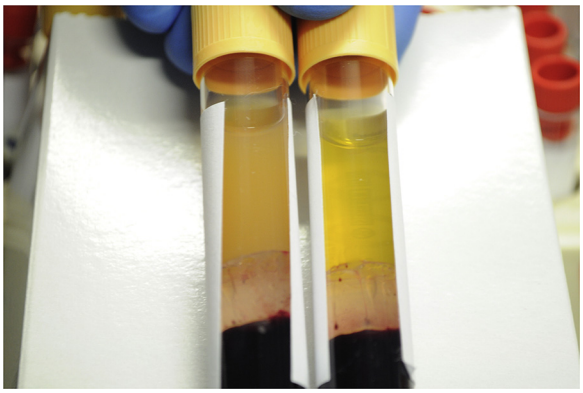

Fig. 4. Variation of the physical characteristics of the plasma of the same patient during 20 min of meditation. On the left: the blood sample (after 4 min of centrifugation at 5000 rpm) before meditation is opalescent. On the right, the blood sample immediately after meditation is clearer. The patient was fasting for more than 5 h before meditating.

Conflict of interest and disclosures

none for all the authors. No sex-based or race/ethnicity-based differences were present. No conflict of interest to declare for all the authors.

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