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Rapid changes of miRNAs-20, -30, −410, −515, −134, and −183 and telomerase with psychological activity: A one year study on the relaxation response and epistemological considerations

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ABSTRACT

Background and aim: Mental stress represents a pivotal factor in cardiovascular diseases. The mechanism by which stress produces its deleterious effects is still under study, but one of the most explored pathways is inflammation-aging and cell senescence. In this scenario, circulating microRNAs appear to be regulatory elements of the telomerase activity and alternative splicing within the nuclear factor kappa-light-chain-enhancer (NF-κB) network. Anti-stress techniques appeared to be able to slow down the inflammatory and aging processes. As we recently verified, the practice of the relaxation response (RR) counteracted psychological stress and determined favorable changes of the NF-κB, p53, and toll-like receptor-4 (TLR-4) gene expression and in neurotransmitters, hormones, cytokines, and inflammatory circulating microRNAs. We aimed to verify a possible change in the serum levels of six other micro-RNAs of cardiovascular interest, involved in cell senescence and in the NF-κB network (miRNAs −20, −30, −410, −515, −134, and −183), and tested the activity of telomerase in peripheral blood mononuclear cells (PBMCs).

Experimental procedure: We measured the aforementioned molecules in the serum of patients with ischemic heart disease (and healthy controls) immediately before and after a relaxation response session, three times (after the baseline), in one year of follow-up.

Results: According to our data, the miRNA-20 and -30 levels and PBMCs-telomerase activity increased during the RR while the −410 and −515 levels decreased. During the RR sessions, both miRNA-134 and -183 decreased.

Conclusion: The mediators considered in this exploratory work appeared to vary rapidly with the psychological activity (in particular when focused on relaxation techniques) showing that psychological activity should be part of the future research on epigenetics. Epistemological perspectives are also discussed.

1. Introduction

Aging is a normal process for every living species. Chronic pathologies, such as cardiovascular, neurological disorders and metabolic diseases, immune system dysfunction, cancer, and skin diseases may be the result of deregulated aging.[1] Research reported that multiple aberrant cellular functions (including oxidative damage, mitochondrial and telomere dysfunction, and imbalanced activation of p53 and nuclear factor kappa-light-chain-enhancer (NF-κB) pathways) disturb the cell metabolism, derail autophagy and other housekeeping actions, inhibit cell division, induce inflammation and promote aging,[2] cause stem cell exhaustion, and induce either senescence,[3] apoptosis, or cancer.[4] The cellular senescence molecular processes are closely linked to the aging of

Abbreviations: RR, Relaxation Response.

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the entire organism.[5,6]

In this contest, mental stress plays a major role in health deterioration[7,8] and in the induction of cellular senescence.[9] Chronic stress leads to the accumulation of senescent cells and promotes aging[10] through endocrine[11] and immune[2,7,8] mechanisms.[12]

Research has widely confirmed that psychological stress determines important changes in the neural activity and gene expression of multiple brain areas.[13] In particular, the stress reaction involves amygdala hyperactivity associated with the emotions of fear, anxiety or anger with the triggering of a low-grade chronic inflammatory process that determines important negative cardiovascular consequences.[7,8,14]

On the other hand, different "mind–body" techniques (involving the focusing of the attention of the subject to particular tasks—breathing, music appreciation, body postures, words repetition, visualizations, etc.) counteract the adverse effects of stress[12] due to specific changes in brain activity[15] (resulting, over time, in a change in brain structure and downregulation of the amygdala[15,16]) and play a favorable role in the prevention of many chronic degenerative pathologies.[17]

A recent statement from the American Heart Association, based on the available scientific literature, even cautiously advised implementing meditation in clinical practice *"as an adjunct to guideline-directed cardiovascular risk reduction by those interested in this lifestyle modification with the understanding that the benefits of such intervention remain to be better established"*.[18]

Trying to answer this question by explaining some molecular mechanisms, we recently verified how the daily practice of the relaxation response (RR) determines a favorable change in the expression of certain inflammatory genes in leukocyte precursors (NF-κB, p53, and toll-like receptor-4 (TLR-4)) through the action of neurotransmitters, hormones, cytokines, and high-mobility group box 1 protein (HMGB-1).[19] We also documented a reduction of DNA methylation, an emerging estimator of biological aging, and of leukocyte telomere length with the RR practice.[20]

Recent molecular biology discoveries have revealed that microRNAs (miRNAs) are involved in aging and cellular senescence.[1] miRNAs are small, non-coding RNA molecules approximately 22 nucleotides in length that act as post-transcriptional regulators of gene expression.[21] They are almost ubiquitous and are also present in the blood circulation.[22] Individual miRNAs have been shown to regulate the expression of multiple genes. Conversely, the expression of individual genes can be regulated by multiple miRNAs.[23]

We previously described how the practice of RR modifies the levels of certain miRNAs that are directly involved in the NF-κB transcription factor pathway,[24] a pivotal mediator of inflammatory responses,[25] involved in different degenerative pathologies,[26] inflammation and aging,[27] and cell senescence.[28]

With this exploratory research, we wanted to further investigate these aspects, searching in the literature and in the "miRNABase"[29] and "miRandola"[30] databases for other miRNAs of cardiovascular interest (ischemic heart disease[31,32]), potentially involved in the NF-κB–p53–TLR4 axis and that could be linked to the processes of cellular senescence.[33] The miRNAs −20,[22,29] −30,[34] −410,[35] −515,[34] −134,[23] −183[36,37] met all these characteristics.[38–54]

Given these premises, we wanted to verify a possible change in the serum levels of these miRNAs with the RR. To validate our observations we also tested the activity of telomerase in leukocyte precursors (peripheral blood mononuclear cells-PBMCs) for the previously described variation with meditation,[55] due to the key role in cellular senescence[56] given its interplay with NF-κB.[57]

We emphasize that, in the same blood samples used in this work, we previously assessed the malondialdehyde and galectin-3 levels as aging[58,59] and cellular senescence markers,[60–63] demonstrating a decrease in the course of RR.[64] Numerous studies in the literature and our previous studies[12,19] have shown that RR causes a decrease in the levels of inflammatory cytokines and stress hormones.

2. Materials and methods

2.1. Study design

The main objective of our work was to verify if, in the same person (suffering from a stress-related and age-related pathology, such as myocardial infarction), the levels of certain pathophysiological mediators linked to aging, inflammation, and cellular senescence changed according to psychological activity. As explained in the introduction, in this proof-of-concept study, we focused our attention on miRNA-20, -30, −410, −515, −134, and −183 and telomerase.

In an attempt to make the subjects' "psychological activity" objective and reproducible, we decided to study the RR (a description of this phenomenon is beyond the scope of this paper, please refer to[65] and to our previous papers[12,19,20,24,64]). In this context, we know the mental operations performed by the subjects, unlike studies on stress where the stressful stimulus is known (called the "stressor"), a physical response is measured, without knowing the thoughts made by the subject under study. It is not the stimulus-event that produce the stress reaction but the thoughts that the subject produces in those situations that trigger the stress reaction in the body. It also appears ethically beneficial to study the possible benefits of relaxation, especially in the context of a stress-related illness.

We collected serum samples of 120 subjects following an approved protocol (Comitato Etico per la Sperimentazione Clinica-Azienda Sanitaria di Padova; protocol number 3487/AO/15–13/7/2015 updated number 4895/AT/20–23/7/2020).[19] Briefly, we enrolled 90 consecutive patients after myocardial infarction and 30 healthy controls. A total of 30 patients were taught to meditate, 30 were taught to appreciate music, and 30 did not carry out any intervention and served as controls.

As a secondary end-point, to rule out that the disease state could interfere with the relaxation effect, we enrolled 30 healthy volunteers (15 were trained to meditate and 15 had music appreciation). Therefore, we attempted to understand any differences related to the pathological process, comparing patients and healthy subjects undergoing the same RR.

The practices of meditation and music appreciation can produce the so-called relaxation response (RR) in the same way.[65] The details of the RR techniques that we used and the description of their pathophysiological mechanism is described extensively in our previous works.[19,24] Briefly, the techniques we used involve the active participation of subjects who learned to focus their attention on the mental repetition of a sound-phoneme for 20 min (meditation) or to pay attention to their breathing while listening to music. These techniques were taught over 4 days, in meetings of about 90 min, as part of a 4-day Rational–Emotional–Education intervention.[64]

Each participant, except for the controls, continued to perform the RR at home two times a day for 20 min for the duration of the study, filling in a daily diary in which to write their experience.

At the baseline (before any intervention), after the initial four-days-training and after 6 and 12 months of RR practice, we collected a blood sample immediately before and after the relaxation session (according to the scheme reported in Fig. 1) to describe any variation of the markers in this study.

Each subject arrived at our center at 6 p.m.; a cannula was

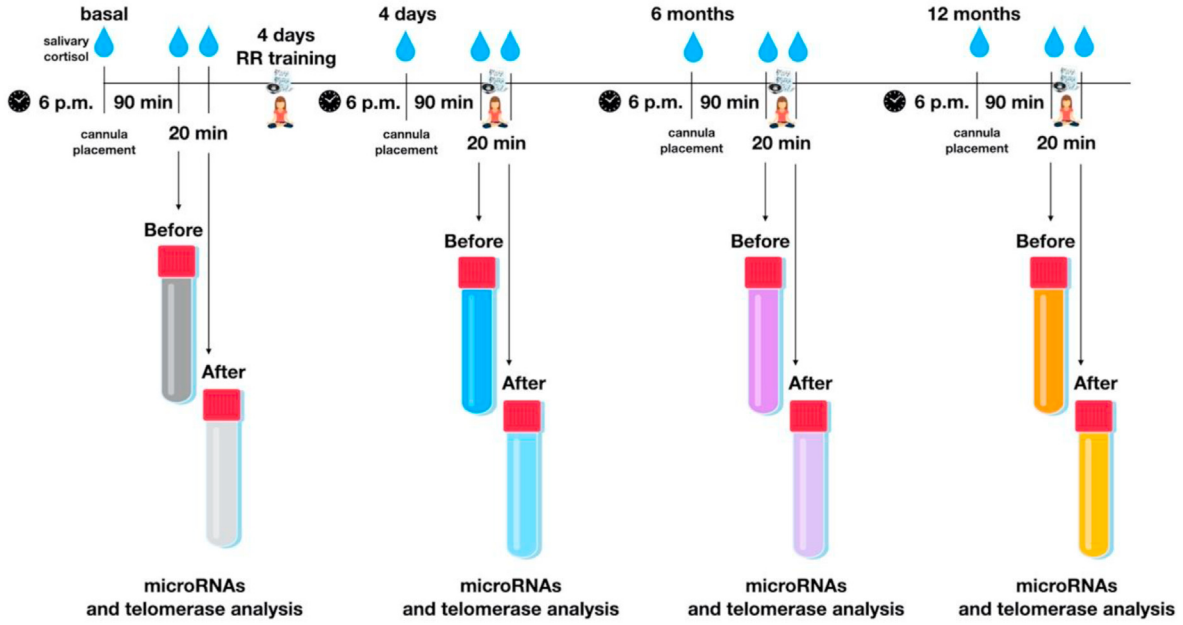

Fig. 1. The study design. Explanation in the text. RR: Relaxation Response. RR 20 min: after 4 days of training, each subject relaxed through meditation or music appreciation for 20 min. A blood sample was taken immediately before and immediately after. The acute variation of the studied parameters can be attributed to the practice of relaxation according to the used methods because the precise timing of blood sampling (before and immediately after the end of the session) prevents any other influences. All groups were subjected to the same environmental conditions. For more details please see our previous works.[19,24]

placed, which was used for subsequent blood sampling. Salivary cortisol was measured. After about 90 min, the subjects performed the RR-technique and the salivary cortisol and blood samples were taken immediately before and after the session. This was repeated at 6 and 12 months. At baseline, the same timings were respected without performing the RR. The timing was calculated to check and avoid venipuncture stress bias.[66] The salivary cortisol data also served to monitor this eventuality. All groups were subject to the same environmental conditions. The control patients did not perform any technique. We simply asked them to relax, and most of them sat down with eyes closed, telling us that, while waiting, their minds often wandered to their daily, social, relational, or health problems.

Clear variation of the physical characteristics of the serum samples (Fig. 2), was observed.

According to Benson's research[65] and our previous study,[19,24] there were no significant differences found between the relaxation techniques. Therefore, we merged into a single "intervention" group (called "RELAXATION RESPONSE") all patients treated with meditation and music and into a single "intervention healthy controls" group (called "RELAXATION RESPONSE HEALTHY CONTROLS") for all healthy subjects. Finally, the patients that did not carry out any intervention constituted the "CONTROLS" group. Our work aimed to study the RR using two conditioning techniques, meditation and music, which have to be considered as two methods leading to the same relaxation effect.[65] Therefore, even from a strictly methodological point of view, we used a unique effect—precisely the RR—from which arose the need to unite the treated subjects in a single "intervention group".

Indeed, all subjects enrolled in the study continued the practice at home, twice a day, as they were taught, and, during the follow-up period, each subject reported having pleasantly performed more than 80% of the meditation or music listening sessions.

2.2. Marker analysis

MicroRNAs were analyzed following the same procedure previously described.[24] Briefly, the total RNA was isolated from the serum using a miRCURYTM RNA Isolation kit-Biofluids (Exiqon, Denmark), following the manufacturer's instructions. The RNA was treated with rDNase (Exiqon) before reverse transcription (RT). For the miRNA expression, 10 ng of RNA was reverse transcribed using a 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 the 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-20–3p, has-miR-30–5p, hsa-miR-410–5p, hsa-miR-515–5p, hsa-miR-134–5p, and hsa-miR-183–5p. We used hsa-miR-103a-3p as stably expressed miRNA and a reference gene based on the advice given by the primer manufacturer. Validation of the specificity of the real-time PCR assay was performed by a 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 the reaction 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 the miRNA expression.

PBMCs were obtained from fresh whole blood as previously described in detail,[19] and the telomerase activity was assayed with the commercially available kit, TRAPeze® (Chemicon, USA). The reaction was carried out according to the TRAPeze kit manual strictly following the procedure described in the literature by Jacobs et al.[55] The salivary cortisol was determined using a chemiluminescence immunoassay (IBL International, Hamburg, Germany), with a lower limit of detection at 0.14 nmol/L and intra-

Fig. 2. Variation of the physical characteristics of the serum of the same patient after 20 min of meditation (representative image). Left test tube: the blood sample (after 4 min of centrifugation at 5000 rpm, room temperature) before meditation is opalescent. Right test tube: the blood sample immediately after meditation was clearer. Considering all the samples before the RR, the mean turbidity (assessed with the Turbidimeter Hach 2100Q) was 324 ± 33.2 NTU, and after the RR was 266 ± 23.7 NTU ($p < 0.05$, *t*-test dependent samples). In healthy subjects, the mean turbidity was 223 ± 44.5 NTU, and after the RR was 206 ± 31.9 NTU ($p < 0.05$, *t*-test dependent samples). No significant variations were assessed in the controls whose mean turbidity remained almost unchanged during the 20 min of observation (318 ± 38.3 NTU before and 323 ± 33.7 NTU after 20 min ($p > 0.05$, *t*-test dependent samples). All the subjects were fasting for more than 5 h before the RR. This modification may be related to some physical processes as well as biochemical quantitative variations of serum components (lipids[19]) as detailed in.[64,67] The data on turbidity could reveal the emergence of a spatial order between the molecular plasmatic constituents after the RR (for more details about this biophysical issue please see our previous works[64,67–69]).

and interassay coefficient of variation below 4%.

2.3. Statistical analysis

The data are expressed as the mean and standard deviation or median and interquartile range (if variables do not have a normal distribution as assessed by the Shapiro–Wilk test). The comparison between the pre-post intervention changes was performed using the *t*-test for dependent samples or the Wilcoxon test (primary end-point). The comparison between the RELAXATION RESPONSE group and CONTROLS (same disease state, comparison between the RR-oriented mind activity and default "mind-wandering" activity) and RELAXATION RESPONSE group and RELAXATION RESPONSE HEALTHY CONTROLS (same RR-oriented mind activity, comparison between disease state and health) was performed using the *t*-test for independent samples or the Mann–Whitney test. An initial comparison between groups was performed through the Kruskal–Wallis test for independent samples or by the Friedman test for paired data. Bivariate correlation was performed using the Spearman test. Statistical significance was assumed if the null hypothesis could be rejected at $p = 0.05$. The statistical analysis was performed using the software SPSS version 22.0 (Chicago, SPSS, Inc., Chicago, IL).

3. Results

3.1. Salivary cortisol

Table 1 shows the data relating to salivary cortisol. After 90 min, at each observation, the levels were comparable. A significant decrease was observed after RR. In the controls, the values tended to increase over the 20 min wait.

Figs. 3–5 report the variations of the telomerase activity in the PBMCs and of the microRNAs analyzed.

3.2. Telomerase activity in PBMCs

The telomerase activity (Fig. 3) appeared to increase during the RR sessions ($p < 0.01$ Wilcoxon test at every time point) both in patients and in healthy volunteers. No significant variation was found in the CONTROLS ($p > 0.05$ Wilcoxon test at every time point). The basal values (before and after 20 min) of telomerase activity were comparable in all groups and were merged into a single starting basal point.

3.3. miRNA-20, miRNA-30, miRNA-410, miRNA-515, miRNA-134, and miRNA-183

In Fig. 4, within both the RELAXATION RESPONSE group and healthy controls, we want to highlight the opposite variations of the miRNAs after the 20 min RR sessions (coloured boxes) from the baseline (grey boxes) when no intervention was performed ($p < 0.001$, Wilcoxon test of the percentage change ((POST-PRE)%) comparisons at every timepoint vs. the basal). We can see the opposite behavior of the same markers between the RELAXATION RESPONSE group and CONTROL group ($p < 0.01$, Mann–Whitney test of the percentage change ((POST-PRE)%) comparisons at every timepoint). On the other hand, similar behavior appeared to be present between patients and healthy volunteers performing the RR ($p > 0.05$ Mann–Whitney test of the percentage change ((PRE-POST)%) comparisons at every timepoint).

In particular, the RR resulted in a significant increase of miRNA-20 and -30 ($p < 0.01$ Wilcoxon test at every time point), and in a significant reduction of microRNA-410, -515 ($p < 0.01$ Wilcoxon test at every time point) both in patients and healthy volunteers, with the opposite significant behavior in the CONTROLS ($p < 0.01$ Wilcoxon test at every time point). After the RR sessions, both miRNA-134 and -183 were found to significantly decrease both in patients and healthy volunteers ($p < 0.01$ Wilcoxon test at every timepoint) with the opposite behavior found in the CONTROLS ($p < 0.01$ Wilcoxon test at every time point).

Fig. 5 presents the basal values of miRNA-20, miRNA-30, miRNA-410, miRNA-515, miRNA-134, and miRNA-183 over time. In the RELAXATION RESPONSE group, miRNA-20 significantly increased at 4 days and 12 months ($p < 0.01$ Wilcoxon test); miRNA-30 significantly increased at every time point ($p < 0.001$ Wilcoxon test); miRNA-410 and 183 significantly decreased at 6 and 12 months ($p < 0.01$ Wilcoxon test); miRNA-134 significantly increased at 6 and 12 months ($p < 0.001$ Wilcoxon test); and miRNA-515 significantly decreased at 12 months ($p < 0.05$ Wilcoxon test). The same

Table 1
Salivary cortisol in nmol/L. Mean ± standard deviation. • = the significant ($p < 0.05$) comparisons for dependent samples (*t*-test), at baseline, after 4 days and 6 and 12 months. # = the significant ($p < 0.05$) comparisons for independent samples (*t*-test), at baseline, after 4 days and 6 and 12 months.

Groups	Basal	+90min	+110min	4 days	+90min	+110min	6 months	+90min	+110min	12 months	+90min	+110min
Relaxation Response	9.4 ± 2.1	9.3 ± 1.8	8.9 ± 3.1	9.2 ± 2.4	9.3 ± 2.3•	7.1 ± 2.8•#	9 ± 2.7	9.2 ± 2.4•	7.5 ± 2.3•#	9.5 ± 2.5	9.3 ± 2.2•	7.2 ± 3.1•#
Controls	9.5 ± 2.7	9.2 ± 2.1	9.1 ± 2.8	9.3 ± 2.3	9.1 ± 2.4	9.6 ± 2#	9.2 ± 2.2	9.4 ± 2.6	9.5 ± 2.4#	9.4 ± 2.3	9.2 ± 2.5	9.5 ± 2.7#
RR Healthy Controls	9 ± 2.5	9.2 ± 2.2	9.1 ± 2.3	8.9 ± 2.5	9 ± 2.3•	6.9 ± 2.9•	9.1 ± 2.6	9.2 ± 2.1•	6.9 ± 2.4•	8.9 ± 2.2	9.1 ± 2.6•	6.8 ± 2.4•

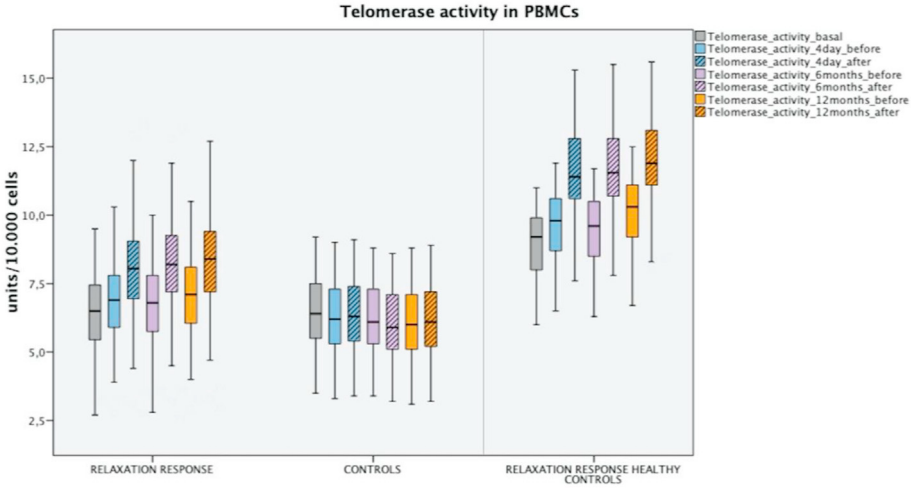

Fig. 3. Telomerase activity in PBMCs. In light blue, the results after the initial 4 days of RR training; in light violet, after 6 months of regular practice of RR at home; and in orange, after 12 months (boxes with dashed lines represent the values immediately after the RR session or after 20 min of waiting in the case of the CONTROLS). The vertical line separates the healthy controls, the secondary end-point of the study.

behavior was found in the healthy controls. An opposite trend was present in the CONTROLS with significant results for miRNA-410, miRNA-183, and miRNA-134 at every time-point ($p < 0.01$ Wilcoxon test); miRNA-30 at 4 days and 12 months ($p < 0.05$ Wilcoxon test); miRNA 515 at 6 months ($p < 0.001$ Wilcoxon test).

4. Discussion

The "stress response" and the "RR" represent two terms describing two sides of the same coin: the continuous process of adaptation to the environment that the brain must organize. The mechanism that connects the mind to cellular functions is still unknown; however, recently, a process of cellular senescence was seen to be favored by a condition of psychological stress or slowed down by relaxation. The orientation of mental processes, either toward stress or toward relaxation, can impact cellular ageing through at least three main recognized pathways: the immune system,[70] the oxidative balance,[71] and the activity of telomerase.[72] Although telomere length is implicated in cellular ageing, the evidence suggesting telomere length is a biomarker of ageing in humans is equivocal,[73] if not invalid.[57]

As stated, we previously assessed malondialdehyde (oxidative-stress marker) and galectin-3 (immune system-inflammatory marker) levels as aging[58,59] and cellular senescence markers,[60–63] demonstrating a decrease in the course of RR.[64] Numerous studies in the literature and our previous studies[12,19] have shown that RR causes a decrease in the levels of inflammatory cytokines, stress hormones, inflammatory genes expression,[19] and epigenetic markers of ageing.[20]

In this work, we continued to investigate the effects of RR measuring other molecular markers that may be involved in cellular senescence. RR was induced by the practice of meditation and music listening in subjects with ischemic heart disease and healthy subjects, with non-specific resting as a control intervention in a similar group of patients. The variations of the studied markers were coherent with our previous findings.

Telomerase is a cellular enzyme that adds the necessary telomeric DNA (T_2AG_3 repeats) to the 3 ′-end of the telomeres, protecting their degeneration.[74] The activity of this enzyme represents a marker of cellular aging[75] and is implicated in aging-associated diseases.[76] However, compared to telomere length, telomerase function seems to correlate more faithfully with stress-related psychological mechanisms.[75] Stress leads to a decrease in its activity[77] while relaxation favors its functioning,[78] as our results appear to confirm.

Telomerase participates in a variety of biological pathways independently of its telomere-lengthening function.[57] Telomerase is now well recognized to act as a transcription (co-)factor to regulate gene expression (such as Wnt/β-catenin and the NF-κB pathways).[57] The telomerase behavior in this work appears to be consistent with the trend of NF-κB in our previous work (decreased with RR).[19] As NF-κB is a key regulator of inflammatory and developmental processes, telomerase is likely to regulate inflammation and development through its interplay with NF-κB.[57]

The main pathway in response to telomere erosion is the p53-dependent tumor suppressive mechanism that downregulates the expression of several factors involved not only in cell cycle control but also in DNA repair and telomere maintenance.[57] Again, the telomerase behavior in this work appears to be consistent with the trend of p-53 in our previous work (decreased with RR).[19]

Our data on telomerase activity, which was consistently affected by RR across the whole study timeframe, but not by non-specific resting, suggest that the biological effects of RR may be maintained over time and not necessarily blunted by the preexisting cardiovascular condition, thus, raising an obvious therapeutic interest.

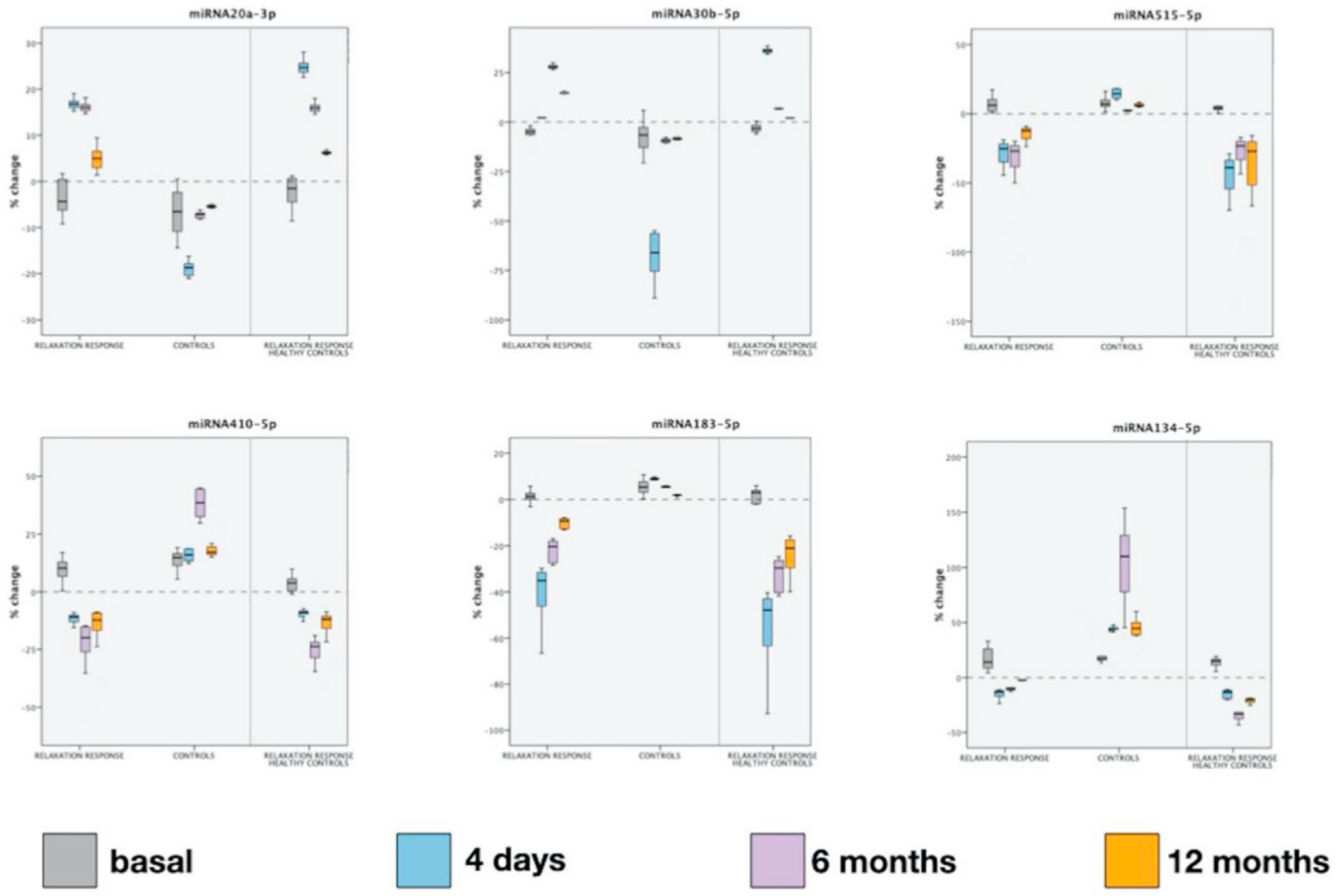

Fig. 4. Percent change at baseline and during the 20 min RR or waiting of miRNA-20, miRNA-30, miRNA-410, miRNA-515, miRNA-134, and miRNA-183. In light blue, the results after the initial 4 days of RR training; in light violet, after 6 months of regular practice of RR at home; and in orange, after 12 months. The vertical lines separate the healthy controls, the secondary end-point of the study.

Recently, a substantial role for circulating microRNAs in regulating cellular senescence has emerged,[33] although we are only at the beginning of the discoveries[79] regarding their precise action of co-regulation of multiple target genes in different metabolic pathways.[80] There are 20–28 nucleotide non-coding RNAs encoded in the genome able to repress or degrade target mRNAs of proteins responsible for different signaling pathways,[81] finely regulating various biological processes.[30]

Currently, four families of miRNAs (−154, −17, −515, and −30) have been recognized to regulate the senescence process by modifying the expression of genes related to growth, cell differentiation, migration, angiogenesis, apoptosis, DNA repair, calcium metabolism, oxidative stress, and telomere homeostasis.[33]

In our work, we considered one representative miRNA for each of these families (respectively miR-20 for −154 family, miR-410 for −17 family, miR-30, and miR-515) due to their specific cardiovascular interest in the coronary disease setting[31,32] and their emerging role in the NF-κB–p53–TLR4 axis. Our data appeared to describe a rapid change in their expression according to the psychological orientation of an individual (in our case as a function of relaxation). This variation, reverberating in specific signaling pathways (still under study[30]), including that of telomerase, could likely alter the cellular behavior favoring its homeostasis or its functional degeneration and senescence.

Considering all of the independent research conducted globally thus far, we can infer that alternative splicing may be involved among the mechanisms promoting cellular senescence.[82,83] Under stress, cholinergic transcription is modified[84] through post-transcriptional mechanisms[85] involving the production of proteins with different and even opposite functions starting from a common primary transcript, via alternative splicing.[86] This process would appear to be regulated by microRNAs 134 and 183 through the SC35 splicing factor.[87] As has been demonstrated in animal models, miRNAs 134 and 183 vary in acute stress and can alter cholinergic neurotransmission via alternative splicing.[87] Our data seem to further expand this evidence by describing a counter-regulation of the same microRNAs with relaxation in humans. We speculate that this variation could, in turn, act on the SC35 factor favoring in the brain the alternative splicing of the acetylcholinesterase transcript associated to the synapse instead of the soluble form linked to stress.[87] This could be very interesting if we consider that it has already been shown that, during relaxation, the levels of cholinergic neurotransmitters vary.[88]

The action of microRNAs 134 and 183 does not stop only at the brain but involves different organs in different pathologies (http://mirandola.iit.cnr.it/visualizations.php). Therefore, the specific action of the mediators considered in this work and the fact that they appear to vary according to mental activity could also mean that the structural alteration of the proteins underlying many chronic-degenerative pathologies could be linked not to alleged “errors in the genome” but to post-transcriptional epigenetic mechanisms partly linked to individual psychological activity.[89]

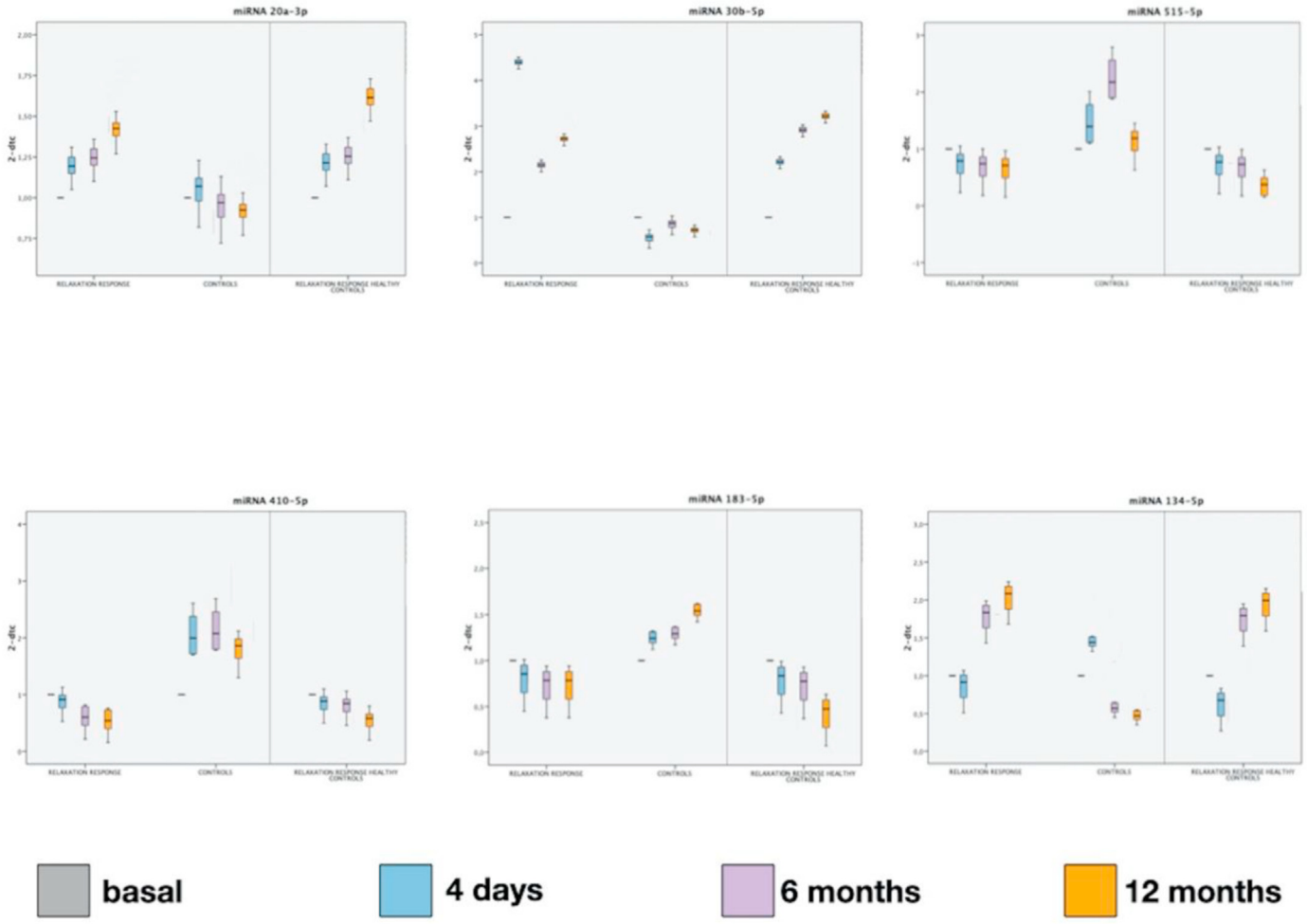

Fig. 5. Basal value (before intervention) trends over time for miRNA-20, miRNA-30, miRNA-410, miRNA-515, miRNA-134, and miRNA-183. In light blue, the results after the initial 4 days of RR training; in light violet, after 6 months of regular practice of RR at home; and in orange, after 12 months. The vertical lines separate the healthy controls, the secondary end-point of the study.

Acute (20 min) changes in miRNAs may not be related to different genetic expression, which would require genomic mechanisms and likely take longer; however, one option is that the measured effects do not relate to expression of the miRNA but rather to their intra-to-extracellular trafficking within microvesicles. We are planning further studies to investigate this hypothesis.

The other option relates to global changes within the plasma, as Fig. 2 would suggest. Based on other data[67] and on the evidence of our in-vitro study,[68] the RR could be due to a biophysical mechanism, that is the creation of a so-called "coherent state" in the organism with the establishment of a long-range correlation (molecular dipole synchronization) between the molecules immersed in an aqueous environment,[90] which could modify their spatial arrangement and their conformation. The greater transparency of the plasma after RR could be due not only to the change in concentration of solutes (that we measured in our works) but also to their different organization in space supported by a different alignment between aqueous molecules,[64,67–69] which allows the light-photons to pass without scattering.

miRNA mediated regulation could serve to manage the impact of noise in gene expression.[91] A number of studies have addressed the relationship between miRNA and noise, and the results suggested that miRNAs could provide precision to protein expression by buffering noise when needed or exploit stochasticity in biological contexts that take advantage of gene expression variability, such as differentiation processes.[91] The coherent, quite uniform variations of the miRNAs in our work appear to be in line with this.

The results described in this paper could be very important from a preventive and therapeutic point of view. Chronic/degenerative diseases represent one of the main challenges of modern medicine and one of the most significant costs for world health systems.[92] Many of these conditions, characterized by early cellular senescence, recognize a condition of chronic stress at their origin.[7] In perspective, the evidence of possible reversibility or at least of a slowing of this condition could be considerable, even if the actual clinical implications of our observations remain to be extensively verified.[20]

Finally, our data appear to highlight the absolute need to pay close attention to the possible post-transcriptional regulation of the genome linked to the psychological activity of a subject involved in any future scientific study in order to not create false data associations. It seems that we cannot properly understand the functioning of the body without the mind and of the mind without the body, just as we cannot understand the tides without the moon.

4.1. Study limitations and future perspectives

Clearly, the basal values of the markers over time may be influenced by other factors (other than RR) such as alimentation, physical activity, smoking, different levels of stress between people, etc.

RR in humans has a pleiotropic effect with many intrinsic and extrinsic variables from subject to subject. The fundamental point of our work is the use of reproducible psychological methods capable of inducing RR and the collection of plasma samples immediately before and after. Each subject is confronted with himself before and after the RR. Avoiding venipuncture stress bias, the only variable capable of explaining a possible variation in the markers observed is the change in psychological orientation. Thus, the observed effect on telomerase and the miRNAs levels is a consequence of the psychological activity (in our case leading to a RR). Currently, it has been indicated that the genome should not be viewed as a management center that gives instructions to the body but instead as an adaptive device that responds to psycho-environmental needs by regulating gene expression.[93]

We would like to emphasize that the results of this work are part of a proof of concept study designed only to explore the existence and the possibility of detecting rapid changes in the levels of certain microRNAs that may be implicated in various physiological processes, in this case, cellular ageing.

The intent of this work was not to characterize the role of microRNAs in the relaxation process but, on the contrary, to verify whether the psychological process that leads to relaxation could be accompanied by a measurable quantitative modification of microRNA (based on our previous findings on inflammation, NF-κB genetic expression, and global aging markers) to lead to future studies.

We cannot exclude that other miRNAs are not similarly affected by RR or that the effect is specific of the neurobiological axis under investigation, i.e., inflammation–senescence. Upon evidence of such changes, our results open the door to investigate other miRNAs extensively. We suggest that it is important to allocate economic resources for more complex transcriptomic studies. In the future, this line of research can be expanded by increasing the panel of miRNAs, including negative control miRNAs and analyzing the miRNA expression in blood cells as well as the downstream genomic expression. In collecting Big Data, we have to pay close attention to the "Texas sharpshooter fallacy" and the related "clustering illusion", which is the tendency in human cognition to interpret patterns where none actually exist.

Why is the increase in the expression of a cluster of molecules (or genes) considered as the "cause" of a phenomenon? Could it be instead the sequential and combined effect of single molecular/genes variations in precise moments (where one increases, another decreases, and a third does not change expression?). In our body, there can be many epigenetic processes (e.g., NF-κB genetic expression) that change due to the reciprocal–combined instant variation over time of their constituents (single molecules involved in the network whose levels oscillate), even if their overall absolute mean/median values do not change (mean, mode, and median in this sense may be a limited instrument of knowledge). In this conceptual research frame, we must remember that the variables in our body are not independent (organs, tissues, cells, molecules, and genes).

In every moment, we are studying the results of different interactions of a dynamic, integrated, and dissipative system. Any artificial division of the world neglects or considers only a schematic account of the interaction across the split.[94] The property we measure does not necessarily belong to the part of the system under consideration, and our measurements can hide other reasons and other properties. For example, the microRNAs in our work may not have increased in their single total quantity—i.e., their expression—but instead have a different spatial location/conformation by virtue of a collective motion with other molecules.

It could be interesting to segregate human subjects into different age groups and demonstrate the expression of other miRNAs in combination with RR and stress-induced situations. Do the miRNAs behave in opposite direction in stressed human subjects? "Relaxation Response" is a conceptual label, given by professor Benson at Harvard University at the beginning of his studies on this subject.[65] The psychological activity that leads to RR does not necessarily have to be studied and understood as "opposite" to stress. This activity can present identificative peculiarities of a different state of consciousness (just as sleep is not "opposed" to dreaming or waking).[95] We have to distinguish the concepts of "stress" and "relaxation", which are only concepts/words, from the measurements/data regarding a specific psychologic activity. Extensive research will be required to detect the significance and the precise role of miRNAs on senescence induction or rescue in established cell line models.

In any case, in order to weave new effective studies from the cognitive point of view, we must pay close attention to the scientific concept of "cause", which is still widely debated,[96] to the strong epistemological limits of statistics[96–100] and of the mechanistic–reductionist paradigm.[17] There are no facts without their interpretation. As indicated, it may be interesting to study senescence markers by age group. However, there are subjects who, at the same age, are biologically and phenotypically younger, and so this classification could represent a bias. In addition, with this work, we have shown that some molecules can vary rapidly according to the psychic activity of a subject, beyond age and the subject's biometric or food characteristics.

Author contributions

Conceptualization, CDL; methodology, CDL; formal analysis, CDL and FT; investigation: CDL, LB, MM; resources: FT, SI, and MP; data curation, CDL, LB, and MM; writing—original draft preparation, CDL; writing—review and editing, CDL and FT; supervision, FT and SI; funding acquisition, FT and SI. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

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