**Article**

**Rapid Senectome and Alternative Splicing miRNAs Changes With the Relaxation Response: A One Year Follow-Up Study**

Carlo Dal Lin 1*, Mariela Marinova 2, Laura Brugnolo 2, Giorgio Rubino 1, Mario Plebani 2, Sabino Iliceto 1; Francesco Tona 1

1 Department of Cardiac, Thoracic and Vascular Sciences, Padua University School of Medicine, Via Giustiniani 2, 35100 Padua, Italy.
2 Department of Laboratory Medicine, Padua University School of Medicine, Via Giustiniani 2, 35100 Padua, Italy

* Correspondence: Email: carlodallin@libero.it. Carlo Dal Lin, Department of Cardiac, Thoracic and Vascular Sciences, Padua University School of Medicine, Via Giustiniani 2, 35100 Padua, Italy. Fax: +39-049 8211802 Phone: +39-049 8218642.

**Abstract:** 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 pathway is cell senescence. In this scenario, circulating microRNAs appear to be mobile regulatory elements of the telomerase activity and alternative splicing within the “senectome” network. Anti-stress techniques seem to be able to slow-down aging process. As we have recently verified how the practice of Relaxation Response (RR), counteracting psychological stress, determines favorable changes of some inflammatory genes expression, of some neurotransmitters, hormones, cytokines and inflammatory circulating microRNAs, we aimed to verify a possible change even in serum levels of 4 senectome micro-RNAs (SE-miRNAs -20, -30, -410, -515), testing the activity of telomerase in peripheral blood mononuclear cells-PBMCs. We analyzed also alternative splicing microRNAs 134 and 183. According to our data, miRNA-20 and -30 levels and PBMCs-telomerase activity increase during the RR while -410 and -515 levels decrease. Moreover, during the RR sessions both miRNA-134 and -183 decrease. The mediators considered in this work seem to vary rapidly according to a (stress)-relaxation condition showing that psychic activity should be part of the study of aging factors.

**Keywords:** cardiovascular disease; inflammation; aging; senectome, telomerase; alternative splicing; Relaxation Response; microRNA.

1. Introduction

The close connection between chronic stress and deterioration of health has been amply demonstrated with the relative increase in the risk of cardiovascular diseases and immune system dysfunction[1,2]. It has already been widely confirmed that psychological stress determines important changes in neural activity and in gene expression of multiple brain areas[3]. In particular, the stress reaction involves amygdala hyperactivity associated with emotions of fear, anxiety or anger [4,5][6] with the triggering of a low-grade chronic inflammatory process that determines important negative cardiovascular consequences[7][8][9].

The exact mechanism by which stress produces such effects is still under study but one of the most explored molecular pathways is linked to cell senescence[10]. The aging process involves the
coordination of various cellular mechanisms, from the balance of telomeres to DNA damage, the increase of inflammatory signals and oxidative stress, up to metabolic and cytoskeletal modifications[11].

Psychic activity seems able to influence these processes. Indeed, Epel et al. have described[10] how psychological stress is closely linked to greater oxidative stress, lower telomerase activity and lower telomere length in leukocyte precursors.

On the other hand, different techniques designed to counteract the adverse effects of stress[12] thanks to specific changes in brain activity[13] (resulting, over time, in a change in brain structure[14–16] and to “silence” the amygdala[13,17]) play a favorable role in preventive terms in many chronic-degenerative pathologies[18].

Even a recent statement from the American Heart Association cautiously advises to implement 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”[19].

Trying to answer this question by explaining some molecular mechanisms, we have recently verified how the daily practice of Relaxation Response determines a favorable change in the expression of some inflammatory genes in leukocyte precursors through the action of neurotransmitters, hormones, cytokines[20] and circulating microRNAs[21].

Again, these mechanisms triggered by psychological activity seem to influence cellular aging processes. Several researches have described the practice of meditation as correlating with an improvement in telomerase activity in leukocyte precursors[22,23,24].

Since the simple identification of correlations does not imply a causal link between phenomena, to study the complexity of the interaction between psychological orientation (stress or relaxation) and aging, different researchers are trying to identify all the possible molecular protagonists in play, tracing their reciprocal influences with the creation of a network called "senectome”[11].

Circulating microRNAs appear to be mobile regulatory elements of this senectome-network and, currently, have been grouped into 4 families based on shared action targets: the microRNA family -154, -17, -515 and -30[11].

Moreover, among the mechanisms of senescence alternative splicing is indicated[25,26]. It’s known that under stress, cholinergic transcription is modified[27] through post-transcriptional mechanisms[28] involving the production of proteins with different and even opposite functions starting from a common primary transcript, via alternative splicing[29,30]. This process would appear to be regulated by microRNAs 134 and 183 through the SC35 splicing factor[31].

It is interesting to note that the same microRNAs are hyper-expressed during an acute coronary syndrome[32,33] and in some oncologic pathologies[34].

Given these premises, as we have recently described how the expression of some circulating microRNAs linked to inflammation varies in relation to psychological relaxation[21], we designed this study to verify a possible change even in serum levels of 4 senectome micro-RNAs (SE-miRNAs), representative of the 4 families described above (respectively miR-20 for -154 family, miR-30, miR-410 for -17 family, and miR-515) [11].

To validate our observations we also tested the activity of telomerase in leukocyte precursors (Peripheral blood mononuclear cells-PBMCs). We wondered if the practice of RR could also
determine a coherent change in the circulating levels of the alternative splicing microRNAs 134 and 183.

2. Results

In figures 3 to 9 are reported the variations of the microRNAs analyzed and telomerase activity in PBMCs.

We want to highlight the opposite variations in miRNAs changes during the 20 minutes sessions between RELAXATION RESPONSE group and CONTROL group (p<0.01, Mann-Whitney test of the delta (PRE-POST) comparisons at every timepoint). On the other hand, similar behavior seems to be present between patients and healthy volunteers performing the RR (p>0.05 Mann-Whitney test of the delta (PRE-POST) comparisons at every timepoint).

2.1 SE-miRNAs (miRNA-20, miRNA-30, miRNA-410, miRNA-515)

The RR results in a significative increasing 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 opposite significant behavior in CONTROLS (p<0.01 Wilcoxon test at every time point).

2.2 Telomerase activity

Telomerase activity seems 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 happens in CONTROLS (p>0.05 Wilcoxon test at every time point).

2.3 Alternative splicing miRNAs (miRNA-134, miRNA-183)

During the RR sessions both miRNA-134 and -183 seem to significantly decrease both in patients and healthy volunteers (p<0.01 Wilcoxon test at every timepoint) with opposite behavior in CONTROLS (p<0.01 Wilcoxon test at every time point).
Figure 3 miRNA-20. 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 20 minutes of waiting in case of CONTROLS). It is possible to notice in every session the significant increasing of miRNA-20 with RR with opposite behavior in CONTROLS (p<0.01 Wilcoxon test at every timepoint).

Figure 4 miRNA-30. 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 20 minutes of waiting in case of CONTROLS). It is possible to notice in every session the significant increasing of miRNA-30 with RR with opposite behavior in CONTROLS (p<0.01 Wilcoxon test at every timepoint).

**Figure 5 miRNA-410.** 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 20 minutes of waiting in case of CONTROLS). It is possible to notice in every session the significant decreasing of miRNA-410 with RR with opposite behavior in CONTROLS (p<0.01 Wilcoxon test at every timepoint).
Figure 6 miRNA-515. 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 20 minutes of waiting in case of CONTROLS). It is possible to notice in every session the significant decreasing of miRNA-515 with RR with opposite behavior in CONTROLS (p<0.01 Wilcoxon test at every timepoint).

Figure 7 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 20 minutes of waiting in case of CONTROLS). It is possible to notice in every session the significant increasing of telomerase activity with RR (p<0.01 Wilcoxon test at every timepoint).
Wilcoxon test at every timepoint). No significant variation seems to happen in CONTROLS ($p>0.05$).

**Figure 8 miRNA-134.** 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 20 minutes of waiting in case of CONTROLS). It is possible to notice in every session the significant decreasing of miRNA-134 with RR with opposite behavior in CONTROLS ($p<0.01$ Wilcoxon test at every timepoint).
Figure 9 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 (boxes with dashed lines represent the values immediately after the RR session or 20 minutes of waiting in case of CONTROLS). It is possible to notice in every session the significant decreasing of miRNA-183 with RR whit opposite behavior in CONTROLS (p<0.01 Wilcoxon test at every timepoint).

3. Discussion

The “stress response” and the RR represent 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 but recently it has been seen that a process of cellular senescence can be favored by a condition of psychological stress or slowed down by relaxation. The orientation of mental processes, either towards stress or towards relaxation, can impact cellular aging through at least 3 main recognized pathways: the immune system[35,36], the oxidative balance[37,38],[39] and the activity of telomerase[40].

Telomerase is a cellular enzyme that adds the necessary telomeric DNA (T\textsubscript{2}AG\textsubscript{3} repeats) to the 3’-end of the telomeres, protecting their degeneration[41].

The activity of this enzyme, together with the length of the telomeres, represent consolidated markers of cellular aging[42]. However, compared to telomere length, telomerase function seems to correlate more faithfully with stress-related psychological mechanisms[42]. Stress leads to a decrease in its activity[10] while relaxation favors its functioning[43], as our results seem to confirm.

Recently, a substantial role for circulating microRNAs in regulating senescence has emerged[11], although we are only at the beginning of the discoveries[44] about their precise action of co-regulation of multiple target genes in different metabolic pathways[45]. They 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[46], finely regulating various biological processes[47].

Currently, there seem to be 4 families of SE-miRNAs (-154, -17, -515 and -30) that regulate the senescence process by modifying the expression of genes related to growth, cell differentiation and
migration, angiogenesis, apoptosis, DNA repair, calcium metabolism, oxidative stress and telomere homeostasis[11].

In our work we considered one representative SE-miRNAs for each of these families (respectively miR-20 for -154 family, miR-410 for -17 family, miR-30 and miR-515) and our data seem 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[47]), including that of telomerase, could likely alter the cellular behavior favoring its homeostasis or its functional degeneration and senescence.

Among the mechanisms of senescence alternative splicing is indicated[25,26]. As it has been demonstrated, in the animal model, that miRNAs 134 and 183 vary in acute stress and can alter cholinergic neurotransmission via alternative splicing[31], 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 acetylcholesterasease transcript associated to the synapse instead of the soluble form linked to stress[31]. This can be very interesting if we consider that it has already been shown that during relaxation the levels of cholinergic neurotransmitters vary[48].

Moreover, 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 seem to vary according to a (stress)-relaxation condition, 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[49].

The results described in this paper could be very important from a preventive and therapeutic point of view. In fact, chronic/degenerative diseases represent one of the main challenges of modern medicine and one of the most significant costs for world health systems[50]. Many of these conditions, characterized by early cellular senescence, recognize a condition of chronic stress at their origin[8]. In perspective, the evidence of a possible reversibility or at least of a slowing of this condition could be really considerable, even if the actual clinical implications of our observations remain to be extensively verified further[51].

Finally, our data seem to highlight the absolute need to pay close attention to the possible post-transcriptional regulation of the genome linked to the psychic activity of a subject involved in any future scientific study in order not to 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.

4. Materials and Methods

4.1 Study Design

We collected a 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)[20]. Briefly, we enrolled 90 consecutive patients after myocardial infarction and 30 healthy controls. 30 patients were taught to meditate, 30 to appreciate music and 30 did not carry out any intervention and served as controls. In order 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). The practices of meditation and music appreciation are able to produce the so called
Relaxation Response (RR) in the same way[52]. The details of the RR techniques that we used and the description of their pathophysiological mechanism is described in our previous works [20][21].

After the initial four-days-training, 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 figure 1) in order to describe any variation of the markers object of this study.

**Figure 1 The study design.** Explanation in the text. RR: Relaxation Response. RR 20 min: after 4 days of training, each subject relaxes through meditation or music appreciation for 20 minutes. A blood sample is 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: in particular, also the control patients were taken in our classroom for 20 minutes and were not subjected to any intervention. We simply asked them to relax and most of them sat down with eyes closed. For more details please see our previous works[20,21].

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

According to Benson’s researches[52] and to our previous study [20,21], there are no significant differences between 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”) all healthy subjects. Finally, the patients that did not carry out any intervention constituted the “CONTROLS” group. We emphasize that our work aims to study the RR using two conditioning techniques, meditation and music, which have to be considered as two ways leading to the same relaxation effect[52]. 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.

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

4.2 Markers analysis

MicroRNAs were analyzed following the same procedure previously described[21]. Briefly, 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-20-3p, has-miR-30-5p, hsa-miR-410-5p, hsa-miR-515-5p, hsa-miR-134-5p, hsa-miR-183-5p. 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 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.

PBMCs were obtained from fresh whole blood as described previously in detail[20], and telomerase activity was assayed with the commercially available kit, TRAPEze® (Chemicon, USA). The reaction was carried out according to the TRAPEze kit manual following strictly the procedure described in literature by Jacobs et al[22].

4.3 Statistical analysis

Data are expressed as median and interquartile range (variables don’t have a normal distribution as assessed by the Shapiro-Wilk test). The comparison between the pre-post intervention changes was performed by means of Wilcoxon test. The comparison between RELAXATION RESPONSE group and CONTROLS and RELAXATION RESPONSE group and RELAXATION RESPONSE HEALTHY CONTROLS was performed 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. Bivariate correlation was performed by Spearman test. Statistical significance was assumed if the null hypothesis could be rejected at p=0.05. The statistical analysis was performed using software SPSS version 22.0 (Chicago, SPSS, Inc., Chicago, IL).

We focused our analysis on the single follow-up sessions and we did not compare the basal values of the markers over time because other factors (other than RR) could have influenced the one year trend of the markers (alimentation, physical activity, smoking, different levels of stress between people etc.).

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

Funding: this research received no external funding, the entire study was funded by the Department of Cardiac, Thoracic and Vascular Sciences, Padua University School of Medicine.

Acknowledgments: we thank the Pneumomeditazione© teachers and the Entolé staff for the recording of audio files used for meditation and for their support; the meditation music used in this study was composed by Paolo Spoladore.

Conflicts of Interest: the authors declare no conflict of interest.

References


