Mechanisms of Ageing and Development 133 (2012) 675–685

Contents lists available at SciVerse ScienceDirect

Mechanisms of Ageing and Development

journal homepage: www.elsevier.com/locate/mechagedev

Age-related differences in the expression of circulating microRNAs: miR-21 as a new circulating marker of inflammaging

Fabiola Olivieri [a,b,1,*], Liana Spazzafumo [c,1], Gabriele Santini [a], Raffaella Lazzarini [a], Maria Cristina Albertini [g], Maria Rita Rippo [a], Roberta Galeazzi [f], Angela Marie Abbatecola [e], Fiorella Marcheselli [b], Daniela Monti [j], Rita Ostan [h,i], Elisa Cevenini [h,i], Roberto Antonicelli [d], Claudio Franceschi [h,i], Antonio Domenico Procopio [a,b]

[a] Department of Clinical and Molecular Sciences, Università Politecnica delle Marche, Ancona, Italy
[b] Center of Clinical Pathology and Innovative Therapy, INRCA-IRCCS, Ancona, Italy
[c] Biostatistical Center, INRCA-IRCCS, Ancona, Italy
[d] Cardiology Unit, INRCA-IRCCS, Ancona, Italy
[e] Scientific Direction, INRCA-IRCCS, Ancona, Italy
[f] Clinical Laboratory & Molecular Diagnostics, INRCA-IRCCS, Ancona, Italy
[g] Dipartimento di Scienze Biomolecolari, Sezione di Biochimica e Biologia molecolare, Università degli Studi di Urbino "Carlo Bo", Urbino, Italy
[h] Department of Experimental Pathology, "Alma Mater Studiorum" University of Bologna, Bologna, Italy
[i] Centro Interdipartimentale Galvani "CIG", "Alma Mater Studiorum", University of Bologna, Bologna, Italy
[j] Department of Experimental Pathology and Oncology, University of Florence, Florence, Italy

ARTICLE INFO

Article history:
Received 22 April 2012
Received in revised form 13 September 2012
Accepted 24 September 2012
Available online 2 October 2012

Keywords:
Circulating microRNA
Centenarians
Centenarians offspring
Circulating miR-21

ABSTRACT

Circulating microRNAs (miRs) have been investigated as diagnostic/prognostic biomarkers in human diseases. However, little is known about their expression throughout the aging process.

Eleven healthy individuals aged 20, 80 and 100 years underwent miR plasma profiling. The validation cohort consisted of 111 healthy adults (CTR) aged 20–105 years and included 30 centenarians. In addition, 34 patients with cardiovascular disease (CVD) and 15 healthy centenarian offspring (CO) were enrolled.

An exploratory factorial analysis grouped the miRs into three main factors: factor 1 primarily higher in 20-year-old subjects, but these differences did not reach statistical significance, factor 2 primarily higher in octogenarians and factor 3 primarily higher in centenarians. MiR-21, the most highly expressed miR of factors 2 and 3, was further validated, confirming the differences in the age groups. MiR-21 expression was higher in the CVD patients and lower in the CO compared to the age-matched CTR. MiR-21 was correlated with C-reactive protein and fibrinogen levels. TGF-β signaling was the predicted common pathway targeted by miRs of factors 2 and 3. TGF-βR2 mRNA, a validated miR-21 target, showed the highest expression in the leukocytes from a subset of the octogenarians.

Our findings suggest that miR-21 may be a new biomarker of inflammation.

1. Introduction

Human aging is a highly complex process characterized by the remodeling of many molecular pathways involved in cellular and tissue homeostasis (Spazzafumo et al., 2011; Cevenini et al., 2010; Bonafè et al., 2003). An important goal in this field of research is to identify innovative cellular and tissue level biomarkers of aging that may also be useful for diagnosing age-related diseases. MicroRNAs (miRs) are small non-coding RNAs involved in the epigenetic regulation of coding gene expression. They provide an additional level of control for important cellular processes such as growth, differentiation, the stress response and remodeling (Garzon et al., 2009; Zampetaki et al., 2012). They can safeguard the robustness of biological systems, modulating in turn the chance to achieve longevity or develop age-related diseases. Several recent reports have suggested that circulating miRs have relevant diagnostic and prognostic implications for age-related diseases, but their clinical relevance is still controversial (Cortez and Calin, 2009; D'Alessandra et al., 2010; Zampetaki et al., 2012; Olivieri et al., 2012a,b). At present, only a few studies have identified significant differences in the circulating miR levels of healthy adults and centenarians

* Corresponding author at: Department of Clinical and Molecular Sciences, Università Politecnica delle Marche, Via Tronto 10/A, 60020 Ancona, Italy. Tel.: +39 071 2206242; fax: +39 071 2206240.
E-mail address: f.olivieri@univpm.it (F. Olivieri).
[1] These authors contributed equally to the manuscript.

0047-6374/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.mad.2012.09.004

(Elsharawy et al., 2012). Data obtained from models such as *Caenorhabditis elegans* and mouse have shown that specific miRs exhibit age-dependent expression patterns (Noren Hooten et al., 2010; Ibanez-Ventoso et al., 2006; Li et al., 2011a; Maes et al., 2008). It is important to recall that exceptional survival requires the dynamic maintenance of physiological variables at optimal levels and that the levels of many aging biomarkers have been shown to change throughout one's life (Yashin et al., 2009; Franceschi et al., 2007; Spazzafumo et al., 2011). Interestingly, recent data have demonstrated that the expression of miRs in human peripheral blood mononuclear cells changes with aging, suggesting that miRs and their predicted targets may be potential diagnostic indicators of aging and/or age-related diseases (Noren Hooten et al., 2010; Li et al., 2011b). Furthermore, gender specific signatures in the circulating miR profiles of young male and female individuals were recently reported (Duttagupta et al., 2011).

Studies have tested if lysed cells contribute to blood circulating miRs or if an active exchange of miRs occurs across cell membranes (microvesicles or exosomes) (Valadi et al., 2007; Turchinovich et al., 2011; Ramachandran and Palanisamy, 2011). An interesting aspect related to the "active secretory hypothesis" is the possibility that circulating miRs may influence the intercellular signaling of health status (Cocucci et al., 2009; Iguchi et al., 2010; Zampetaki et al., 2012; Zhu and Fan, 2011). It was recently demonstrated that exosomes are readily taken up by macrophages, supporting the notion that exosomal RNA can be shuttled between cells (Losche et al., 2004; Lasser et al., 2011). Subpopulations of plasma microvesicles and their miRs have also been reported to regulate the immune response and hematopoiesis (Hunter et al., 2008). These findings underline the urgent need to investigate the biological role of circulating miRs.

We aimed at identifying the plasma expression levels of miRs in differently aged healthy persons (20 to more than 100 years of age) to determine if healthy aging is characterized by differences in circulating miR expression levels. Moreover, because cellular miRs use well-connected networks to control many important biological functions, one may hypothesize that groups of miRs could modulate common target pathways. Therefore, we aimed at identifying the common pathways targeted by circulating miRs that are differentially expressed at different ages.

Finally, the circulating miRs that were differentially expressed in healthy subjects of different ages were also analyzed in older CVD patients and centenarian offspring. Geriatric CVD patients are one of the most studied models of unsuccessful aging, while centenarian offspring are a model of successful aging due to their significantly reduced risk of CVD development and mortality.

2. Materials and methods

2.1. Study participants

The study participants were recruited from the Italian National Research Center on Aging (I.N.R.C.A.) at the Institute of Ancona, Italy, which is involved in the Cardiovascular Diseases Prevention Program, and from the Universities of Bologna and Florence (Italy), which are involved in the "Biomarkers of Longevity" research project. All of the subjects provided their informed consent to participate in the study, which was approved by the Ethics Committee of the I.N.R.C.A. and the Ethical Committee of the Sant'Orsola-Malpighi Hospital, University of Bologna.

Eleven subjects who were aged 20 ($n = 4$), 80 ($n = 4$) and 100 ($n = 3$) years and selected for their health status underwent plasma miR profiling. The validation cohort consisted of 111 healthy control subjects (CTR) with an age range of 20–105 years of age. The CTR group also included 30 centenarians. The validation cohort consisted of approximately 50% men and 50% women; however, among the centenarians, more women than men were enrolled (75% vs. 25% respectively).

Each participant's health status was assessed using questionnaires, laboratory assays and a physical examination. Individuals were considered healthy if they did not have any major acute and/or chronic age-related diseases, such as acute myocardial infarction, congestive heart failure (CHF), Alzheimer's disease (AD), type 2 diabetes mellitus (T2DM) or cancer, at the time of blood collection. Moreover, the subjects with a Cumulative Illness Rating Scale (CIRS) > 2, which is indicative of a co-morbidity, were excluded (Mistry et al., 2004).

In addition, 15 healthy centenarian offspring (CO) and 34 patients with cardiovascular disease (CVD), including acute congestive heart failure (CHF) and acute non-ST myocardial infarction (NSTEMI), were enrolled. The NSTEMI and CHF patients were hospitalized in the Coronary Care Unit (CCU) of the IRCCS-INRCA Hospital, Ancona, Italy.

NSTEMI was diagnosed according to the European Society of Cardiology (ESC) guidelines (Alexander et al., 2007). The criteria for NSTEMI diagnosis have previously been reported in detail (Olivieri et al., 2012a,b). Echocardiography was performed on all of the enrolled NSTEMI patients to confirm the presence of myocardial infarction. The regional wall motion index (WMI) was also assessed. The CHF patients were selected from patients presenting with a CHF exacerbation that required hospitalization. Only those patients with systolic CHF from a post-ischemic etiology were enrolled, while those with an isolated diastolic dysfunction were excluded. The diagnosis of all the acute CHF patients was confirmed by trans-thoracic echocardiography (TTE) and an increase in NT-proBNP. The left ventricular ejection fractions (LVEF) (%) in the NSTEMI and acute CHF patients were 42 ± 7 and 40 ± 5, respectively.

2.2. RNA extraction

Peripheral venous blood samples from all of the subjects were collected in EDTA-coated tubes (Venoject, Terumo Europe NV). After two subsequent spins, total RNA was extracted from 100 μl of plasma using an RNA purification kit (Norgen Biotek Corporation, Thorold, ON, Canada) that isolates enriched miR species. The RNA was stored at −80 °C until use.

2.3. MicroRNA profiling

The mature miR analysis was performed using an Applied Biosystems 7900 HT real-time PCR instrument and human MicroRNA Array pool A (Applied Biosystems, Foster City, CA), which contains 365 different human miRNA assays, in addition to selected small nucleolar RNAs (snoRNAs). The RNA extracted from 100 μl of plasma was converted to cDNA by priming with a mixture of looped primers (MegaPlex kit, Applied Biosystems, Foster City, CA) using the manufacturer's instructions. Pre-amplification was performed using 3 μl of input RNA with the PreAmp kit (Applied Biosystems, Foster City, CA). Nine microliters of pre-amplified cDNA was used for mature miR profiling with a real-time PCR instrument equipped with a 348-well reaction plate (7900 HT, Applied Biosystems, Foster City, CA) and human MiR Array pool A (Applied Biosystems, Foster City, CA).

2.4. RTq-PCR validation

A modified real-time quantification of miR expression was performed with the TaqMan miRNA reverse transcription kit and a miRNA assay (Applied Biosystems, Foster City, CA). Briefly, total RNA was reverse transcribed (RT) with a TaqMan MicroRNA RT kit. The 5 μl RT reactions contained 1 μl of each miR specific stem-loop primer, 1.67 μl of input RNA, 0.4 μl of 10 mM dNTPs, 0.3 μl of reverse transcriptase, 0.5 μl of 10× buffer, 0.6 μl of RNAse inhibitor diluted 1:10 and 0.5 μl of H_2O. The mixture was incubated at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min. The quantitative real-time PCR was subsequently performed. The 5 μl PCR reaction included 0.25 μl of 20x Taqman MicroRNA Assay, which contained the PCR primers and probes (5′-FAM), 2.75 μl of 2x TaqMan Universal Master mix no UNG (Applied Biosystems) and 2.25 μl of RT product. The reaction was first incubated at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The data were analyzed with Real Time PCR OpticonMonitor version 2 (MJ Research, Bio-Rad, Hercules, CA), with the automatic Ct setting for adjusting the baseline and threshold for Ct determination.

To date, very few validation reports have measured normalized miRNA levels in the serum (Peltier and Latham, 2008; Chen et al., 2008). Some reports have demonstrated that the endogenous non-miRNA controls used in miRNA expression studies, such as RNU6B, RNU44 and RNU48, are degraded in the serum. Therefore, to obtain accurate and reproducible results, the miR expression in the different age groups was quantified with various normalization methods. The relative expression of each miR was determined using miR-17-5p as the reference (D'Alessandra et al., 2010; Olivieri et al., 2012a,b). The absolute miR concentration (expressed as number of molecules/ml) was determined by running a dilution series containing a known input quantity of synthetic miRs simultaneously with the experimental samples.

The synthetic *C. elegans* miR, cel-miR-39, was spiked into the human plasma before RNA extraction. Only those samples with a cel-miR-39 recovery higher than 95% were used in the subsequent analysis. Each reaction was performed in duplicate.

2.5. RT-qPCR validation of mRNA expression levels in leukocytes

The mRNA levels of TGF-βR2 were analyzed by qPCR. The following oligonucleotides (5′–3′) were used for the TGF-βR2 mRNA assay:

TGF-βR2 forward:
CTG GGC TCC TGA TTG CTC

TGF-βR2 reverse:
TGA AAC GCT GTG CTG ACC

The data were normalized to β-actin expression levels and reported as the mean value ± S.D.

2.6. Laboratory assays

The plasma concentrations of cardiac troponin T (cTnT) and Nt-proBNP were determined with ElectroChemiLuminescence ImmunoAssays (ECLIA) using the Modular Analytics E170/Cobas immunoanalyzer (Roche Diagnostics, Indianapolis, Indiana, USA) according to the manufacturer's instructions. CTnT values ≤ 0.03 ng/ml were considered normal. The total cholesterol, HDL, triglycerides and fasting glucose were measured by an enzymatic colorimetric test (Roche-Hitachi). The level of high sensitive C-reactive protein (hsCRP) was determined by the particle-enhanced immunoturbidimetric assay (CRP High Sensitive, Roche-Hitachi). It is worth noting that, unless otherwise specified, the same blood sample was used for the miRNA, cTnT, NT-proBNP and CRP determinations.

2.7. Statistical analysis

The statistical analysis approach applied to our data was reported in detail as "supplementary statistical analysis".

The expression levels of the all miRs detected in each sample were reported as the relative expression with regards to the overall miR expression on each array using the median normalization analysis (ΔCt = Ct miR X-median value of all Cts of detectable miRs). The relative expression of each miR was reported as $2^{-\Delta CT}$.

The variables that did not exhibit a normal distribution, such as CRP and the relative and absolute expression of miR-21, were log transformed prior to the statistical analyses.

2.7.1. Exploratory factor analysis

An exploratory factor analysis (EFA) was used to reduce the number of variables (expression values for circulating miRs), revealing common patterns in the complex data sets. EFA is able to identify underlying structures, which are named factors, based on the correlation of miR expression levels. A principal component analysis (PCA) was used to extract the initial set of components. The PCA transformed a set of observed variables into a linear combination that accounted for both the maximum proportion of the total variance and a new set of uncorrelated components. The eigenvalues with a value >1.0 were retained in the analysis. A varimax orthogonal rotation was used in this analysis to obtain the factors. To interpret the results from the factor analysis, the factor loading pattern was examined to determine which original variables represented primary constituents of each factor. The patterns were depicted in a Venn diagram that included miRs with a factor loading greater than or equal to 0.8. This cut-off value is indicative of a very high correlation between the latent factor and the variables.

In addition, standardized factor scores for each subject were extracted from the latent factors to compare the mean values in the three different age groups with a non-parametric Kruskal–Wallis test. The miR subsets identified from a factorial loading greater than or equal to 0.8 were chosen to identify the common targeted pathways.

2.7.2. MicroRNA pathway prediction

A fast and efficient identification of the common pathways targeted by multiple circulating miRs was obtained with the DIANA microT v3.0 target prediction program (http://diana.cslab.ece.ntua.gr/pathways), which has the highest ratio of correctly predicted targets of several prediction tools (Maragkakis et al., 2009). Multiple criteria were used to identify the pathways with the highest probability of being targeted by a specific subset of circulating miRs. DIANA-microT v3.0 calculates the miRNA-targeted gene score −ln(*p* value) that reflects the weighted sum of the scores of all the conserved and non-conserved miRNA recognition elements on the 3′UTR of the target mRNA. This score correlates well with the fold changes in the suppression of protein expression and it is also indicated for clusters of miRs (Maragkakis et al., 2009; Satoh and Tabunoki, 2011). A −ln(*p* value) higher than 18 was used as a cut-off value to establish a high probability of being targeted by a specific subset of miRs (Satoh and Tabunoki, 2011). Moreover, the targeting of more than 30% of the genes belonging to the same pathway was considered a measure of the goodness of the prediction. Finally, the number of miRs with more than 80% of the targeted genes belonging to the same factors was considered a measure of the likelihood that the entire pathway could be modulated.

2.7.3. Correlation and regression analysis

The correlations between the parameters were calculated using the Spearman's rho correlation coefficient. To understand the mathematical relationship between miR-21 (*y*) and age (*x*), the data were plotted, and the best fit function (linear or polynomial) was chosen based on the highest R^2 value of the estimated regression curves.

The data analysis was performed with the SPSS/Win program version 18 (SPSS Inc., Chicago, IL). Statistical significance was defined as a two-tailed *p* value <0.05.

3. Results

3.1. The profiling of circulating miRs

To test for the differential expression of circulating miRs across different ages, we isolated miRs from the plasma of 4 subjects aged 20 years, 4 subjects aged 80 years and 3 subjects aged 100 years. All of the enrolled subjects were selected for their health status. We initially profiled 365 miRs to identify the miRs that exhibited differences in expression. We then validated the expression of the most relevant miRs in a larger cohort. The characteristics of the profiling and validation cohorts are reported by age group in Table 1 of the supplementary data (panels a and b). Due to the small number of subjects profiled, the men and women were not analyzed separately.

Of the 365 miRs profiled, only 127 showed detectable levels and were included in the subsequent analyses. MiR-223 showed the highest expression of circulating miRs, confirming previous results (Hunter et al., 2008).

3.2. Exploratory factor analysis (EFA) of the profiled circulating miRs

EFA is used to reduce the set of variables based on a correlation matrix, identifying uncorrelated variables called factors. Supplementary Table 2 reports the 10 factors identified by the EFA that

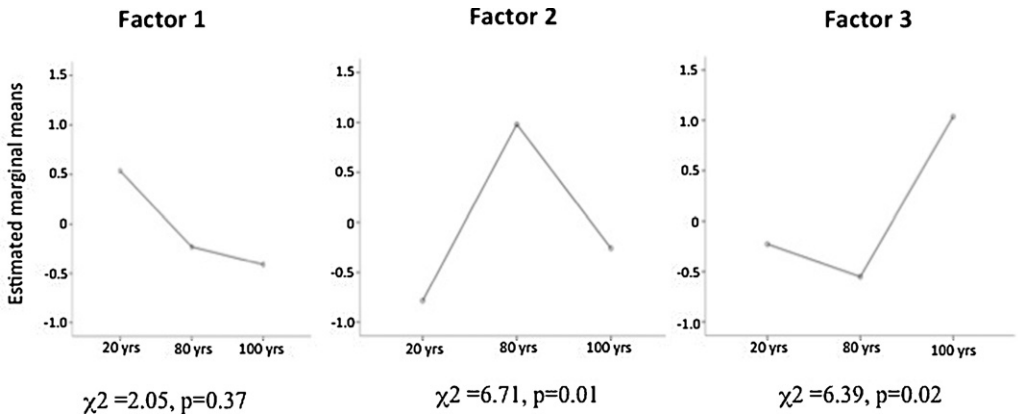

Fig. 1. The estimated marginal mean plots for factors 1, 2 and 3 across the age groups. Line plots showing the estimated marginal means for factors 1, 2 and 3 across the three age groups (20-, 80- and 100-year-old subjects). Factor 1 was lower in octogenarians and centenarians than in the 20-year-old subjects, but these differences did not reach statistical significance (Kruskal–Wallis $\chi^2 = 2.05$, $p = 0.36$). Factor 2 showed higher expression in the octogenarians than in the 20-year-old subjects (Kruskal–Wallis $\chi^2 = 6.71$, $p = 0.01$). Factor 3 was significantly higher in the centenarians than the 20-year-old subjects and octogenarians (Kruskal–Wallis $\chi^2 = 6.39$, $p = 0.02$).

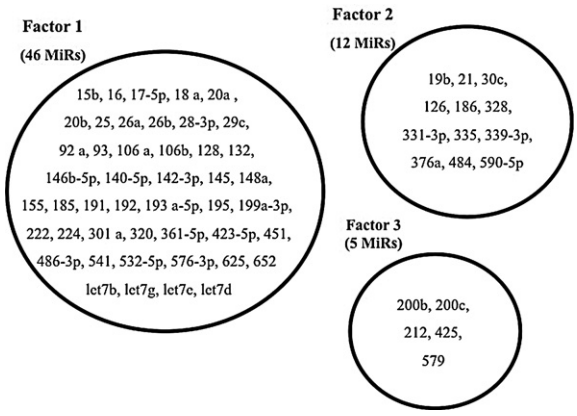

Fig. 2. The miRs included in factors 1, 2 and 3 based on a factorial loading ≥0.8. A Venn diagram showing the three main factors identified by the EFA. The miRs included in each factor showed a factorial loading greater than or equal to 0.8, which is indicative of a very high correlation between the latent factor and the variables.

applied to all 127 of the detectable circulating miRs. Applying the inclusion criterion of a percentage of explained variance higher than 10% of the total variance, three main factors were selected. Factor 1 explained approximately 43% of the total variance, factor 2 explained approximately 18% of the total variance, and factor 3 explained approximately 10% of the total variance. Thus, the EFA identified three main factors that jointly accounted for 71% of the variance in the data. Next, the marginal mean values of standardized factor scores were compared in the three age groups, as shown in Fig. 1. Factor 1 showed age-related differences in miR expression, but these differences did not reach statistical significance ($\chi^2 = 2.05$, $p = 0.36$). However, some of the miRs belonging to this factor, such as miR-18a, -142-3p, -192, -423-5p, -576-3p, -652, -let7e, and -let7d, showed significant lower expression in the oldest when analyzed separately (Kruskal–Wallis χ^2 for trend, $p < 0.05$) (data not shown).

Factor 2 showed significant higher levels in the 80-year-old group of subjects and slightly higher levels in the centenarians compared to the 20-year-old subjects (Kruskal–Wallis $\chi^2 = 6.71$, $p = 0.01$).

Factor 3, which explained 10% of the total variance, was higher in centenarians vs. both the 20-year-old subjects and the octogenarians (Kruskal–Wallis $\chi^2 = 6.39$, $p = 0.02$).

The miRs with a factorial loading greater than or equal to 0.8 are highlighted in bold (Table 1 supplementary data).

3.3. Target and pathway analysis of the miRs belonging to the main factors identified by EFA

Because each miR can regulate many target genes and a group of miRs may modulate specific pathways, we explored the

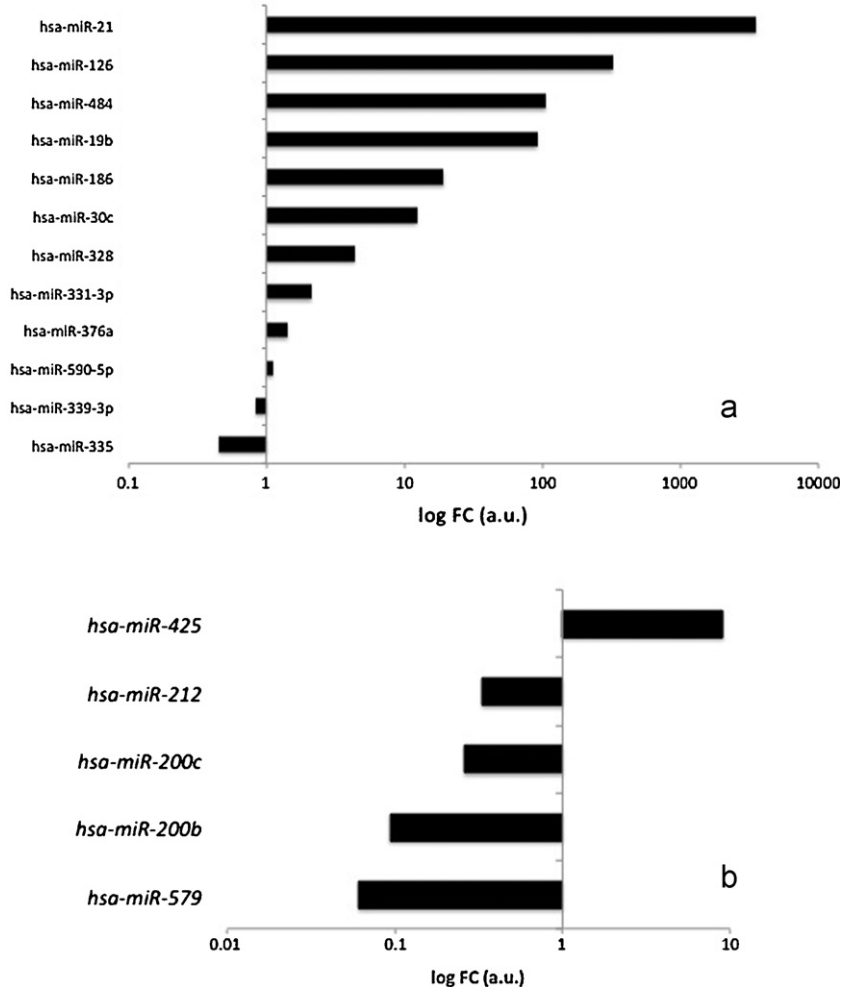

Fig. 3. The relative expression of the miRs belonging to factors 2 and 3 (factorial loading ≥ 0.8). (a) Relative expression levels of the miRs belonging to factor 2. (b) Relative expression levels of the miRs belonging to factor 3. The relative expression levels of the miRs belonging to factors 2 and 3 are expressed as the logarithmic transformation of fold changes (FCs) in arbitrary units (a.u.). The FCs were calculated with the equation $2^{-\Delta CT}$, in which ΔCt = Ct miR X − the median value of all Cts for the detectable miRs.

Table 1
KEGG (Kyoto encyclopedia of genes and genomes) common pathways targeted by miRs belonging to factor 2 and 3.

KEGG pathways (has-ID)	No. (%) of gene-target	No. (%) of miRs	−ln(*p* value)
Factor 2			
Axon guidance (4360)	41 (32)	11 (92)	22
TGF-beta signaling pathway (4350)	31 (37)	10 (83)	18
Factor 3			
TGF-beta signaling pathway (4350)	26 (31)	5 (100)	28
Renal cell carcinoma (5220)	21 (30)	4 (80)	25

KEGG pathways and −ln(*p* value) were identified by DIANA microT v3.0.

pathways that are potentially regulated by the miRs found in factors 1, 2 and 3 using the DIANA microT v3.0 database. To discriminate the miRs in these factors, only those miRs with a factorial loading ≥0.8 (indicative of a very high correlation between the latent factor and the variables) were considered. According to this criterion, 46, 12 and 5 miRs were included in factors 1, 2 and 3, respectively. The patterns of the miRs belonging to the three main factors are reported in a Venn diagram (Fig. 2), and their relative expression is depicted in Fig. 3 (panels a and b). The miRs belonging to factor 3 showed lower relative expression than the miRs belonging to factor 2.

To screen for the pathways that are potentially targeted by the miRs identified by the DIANA-microT v3.0 prediction analysis, we defined the following inclusion criteria: (i) a −ln(*p* higher than 18, (ii) targeting over 30% of the genes belonging to the same pathway and (iii) the number of miRs belonging to the factors higher than 80%. Based on these inclusion criteria, 2 KEGGS pathways were found to be targeted by the miRs belonging to factor 2, and 2 pathways were found to be targeted by the miRs belonging to factor 3 (Table 1). The pathways targeted by the miRs belonging to factor 2 included the axon guidance and transforming growth factor-beta (TGF-β) signaling pathways, the pathways targeted by the miRs belonging to factor 3 included the transforming growth factor-beta (TGF-β) signaling and renal cell carcinoma pathways, and the pathways targeted by the miRs belonging to factor 1 included the MAPK signaling, focal adhesion, WNT signaling and axon guidance pathways (data not shown).

3.4. Validation of circulating miR-21 expression

The level of circulating miR-21, the most highly expressed miR belonging to factors 2 and 3, was measured in the validation cohort (CTR). The CTR subjects were categorized into three groups according to years of age: 20–65 ($n = 33$), 66–96 ($n = 40$) and >96 ($n = 38$).

Currently, there is no consensus on how to normalize a microarray or RT-qPCR, and the choice of normalization method could represent an important bias (Meyer et al., 2010). We measured miR-21 expression using both absolute and relative quantification. For the relative expression measure, miR-17-5p was used as the reference because it is highly expressed and does not show gender or age-related changes. In our analysis, miR-17-5p was included in factor 1, which was characterized by a non-significant age-related trend. To confirm the appropriateness of miR-17-5p as a reference, the validation cohort was used to provide an absolute quantification of miR-17-5p, confirming that its expression was not correlated with age (Fig. 4). However, to avoid any potential bias due to the choice of reference, the absolute quantification of miR-21 was also performed. An age-related non-monotonic trend in miR-21 expression was confirmed in both the relative and absolute quantifications (Fig. 5, panels a and b). The

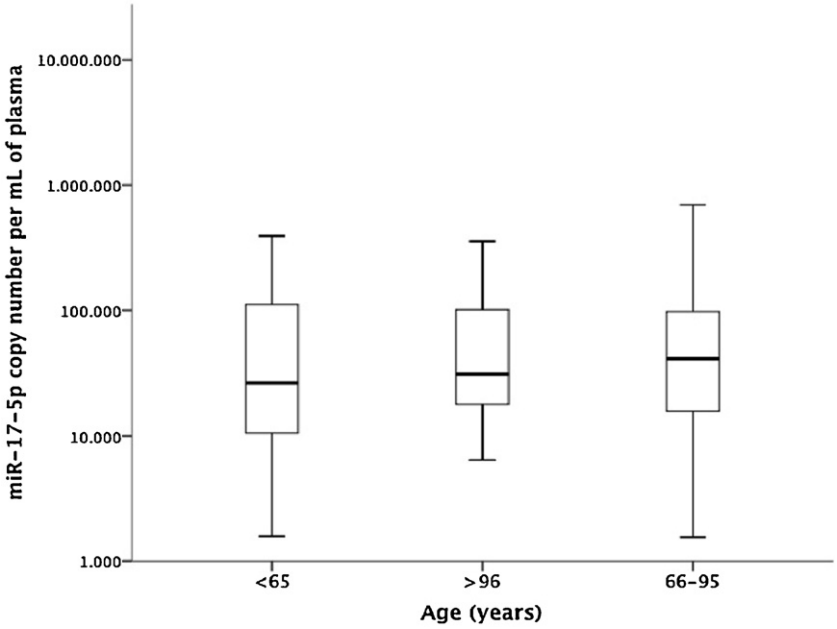

Fig. 4. The absolute expression of circulating miR-17-5p in the CTR subjects (stratified by age group). The absolute miR-17-5p concentration (expressed as the "number of molecules per ml of plasma") was determined by diluting known input quantities of synthetic miR-17-5p.

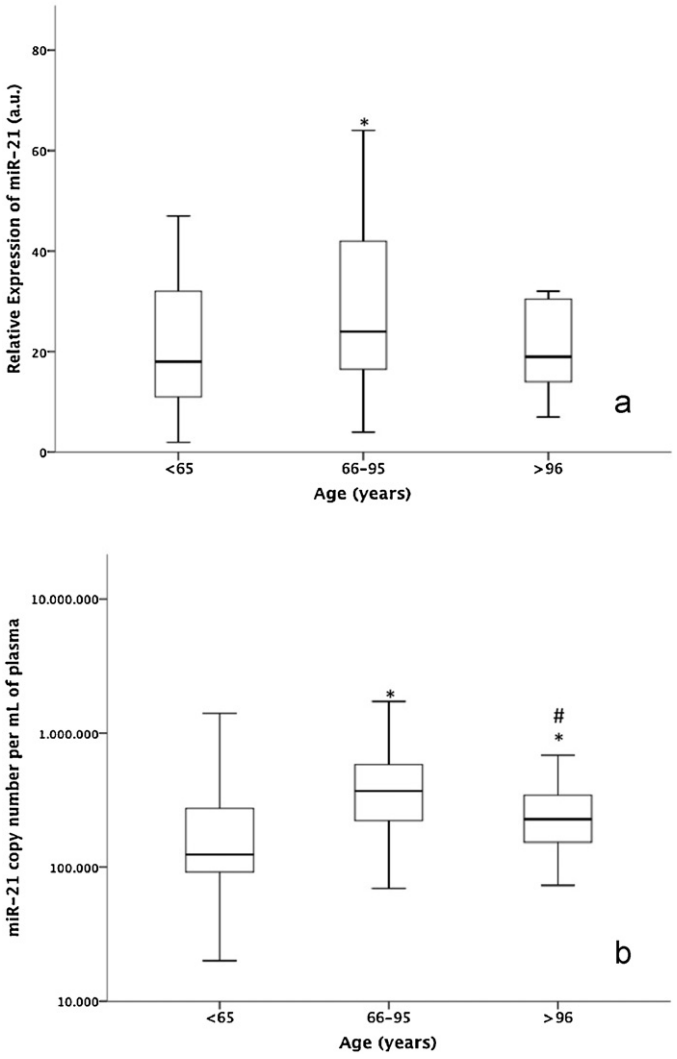

Fig. 5. Circulating miR-21 expression in the CTR subjects (stratified by age group). (a) The relative expression of circulating miR-21. $^{*}p < 0.05$ vs. <65 years. The relative expression of miR-21 was calculated using the following equation: $2^{-\Delta CT}$, in which ΔCt = Ct miR-21 − Ct miR-17-5p. (b) The absolute expression of circulating miR-21. $^{*}p < 0.05$ vs. <65 years, $^{\#}p < 0.05$ vs. <66–95 years. The absolute concentration of miR-21 (expressed as the "number of molecules per ml of plasma") was determined by diluting known input quantities of synthetic miR-21.

66–96 years age group showed significantly higher absolute and relative miR-21 levels than the younger group (20–65 years) (miR-21 relative expression, 33.7 ± 29.8 vs. 22.6 ± 13.7, $p < 0.05$; miR-21 absolute expression, 317,012 ± 226,804 vs. 189,206 ± 200,759, $p < 0.05$).

The relative expression of miR-21 in centenarians was not significantly different from the relative expression of miR-21 in the 20–65 years group (relative expression, 30.18 ± 26.51 vs. 22.6 ± 13.7, $p > 0.05$) (Fig. 5, panels a and b), whereas the absolute expression of miR-21 in centenarians was significantly higher than that of the younger 20–65 years group (miR-21 absolute expression, 289,548 ± 178,549 vs. 189,205 ± 200,759, $p < 0.05$) and lower than the absolute miR-21 expression in the 66–95 years group (absolute expression, 289,548 ± 178,549 vs. 317,012 ± 226,804, $p < 0.05$).

No significant differences were observed between the men and women in each age group (data not shown).

Moreover, the regression model analysis (linear or polynomial) showed that a quadratic curve fit the data better than a linear curve (relative miR-21 expression: linear regression $R^2 = 0.03$ vs. quadratic regression $R^2 = 0.14$. Absolute miR-21 expression: linear regression $R^2 = 0.03$ vs. quadratic regression $R^2 = 0.07$) (Fig. 7, panels a and b).

A significant correlation between the miR-21 (relative and absolute expression) and CRP levels was observed in the validation cohort (relative miR-21 expression: Spearman's rho = 0.33, $p < 0.01$; miR-21 absolute expression: Spearman's rho = 0.47, $p < 0.01$).

A significant correlation between the miR-21 (relative and absolute expression) and fibrinogen levels was also observed (miR-21 relative expression: Spearman's rho = 0.25, $p < 0.01$; miR-21 absolute expression: Spearman's rho = 0.36, $p < 0.01$).

3.5. Circulating miR-21 expression in healthy centenarian offspring and geriatric patients with cardiovascular disease

We recently reported that patients with acute NSTEMI and CHF had significantly higher circulating miR-21 levels than age-matched healthy CTR subjects (Olivieri et al., 2012b). Therefore, we compared the miR-21 levels in an additional sample of 34 patients with CVD (Fig. 6). The characteristics of the CVD patients are compared to the characteristics of the age-matched CTR subjects in Supplementary Table 1c. The CVD patients exhibited significantly higher miR-21 expression than the CTR subjects (CVD vs. CTR, 165.4 ± 161.1 vs. 41.6 ± 5.00, $p < 0.01$) (Fig. 6). A significant correlation was observed for the miR-21 and CRP levels in CVD patients and the age-matched CTR group (relative miR-21 expression: Spearman's rho = 0.27, $p < 0.01$; miR-21 absolute expression: Spearman's rho = 0.38, $p < 0.01$).

The relative miR-21 expression of the CVD patients, age-matched CTR subjects and CO was also compared. As expected, the CO showed significantly lower miR-21 expression than the CVD patients (relative miR-21 expression: CO vs. CVD, 23.8 ± 5.8 vs. 165.4 ± 161.1). Surprisingly, the CO also showed lower miR-21 expression than the aged-matched CTR subjects (relative miR-21 expression: CO vs. CTR, 23.8 ± 5.8 vs. 41.6 ± 5.00, $p > 0.05$). Thus, the CO showed the lowest miR-21 expression levels and the lowest variability of the three groups (Fig. 6).

3.6. MiR-21 targets the TGF-β signaling pathway

The TGF-β signaling pathway emerged as the most reliable pathway targeted by the miRs belonging to factors 2 and 3. This pathway is detailed in Fig. 8. The genes targeted by the miR subsets belonging to factors 2 and 3 are reported. The TGF-β signal is transduced by a pair of transmembrane serine-threonine kinase receptors, TGF-β-R1 and TGF-β-R2 (Bacman et al., 2007). As shown in Fig. 8, miR-21 targets both TGF-β-R1 and TGF-β-R2.

We analyzed the expression of miR-21 and TGF-βR2 mRNA in the leukocytes of 5 individuals aged 20–30 years, 5 individuals aged 75–85 years and 5 centenarians. The miR-21 expression was significantly higher in the octogenarians compared to the 20-year-old subjects, but not in the centenarians (Fig. 9). Accordingly, the TGF-βR2 mRNA levels was lower in the octogenarians than in the centenarians compared to the younger subjects (Fig. 9).

4. Discussion

This is one of the first studies to test for differences in the expression of circulating miRs over a wide range of ages. Many data support the hypothesis that diverse age-related biological changes occur in advanced age (Sebastiani et al., 2012; Perls et al., 2002; Bonafe and Olivieri, 2009; Franceschi et al., 2005). We applied an innovative statistical approach, the exploratory factorial analysis (EFA), to analyze our circulating miR profiling data. The EFA

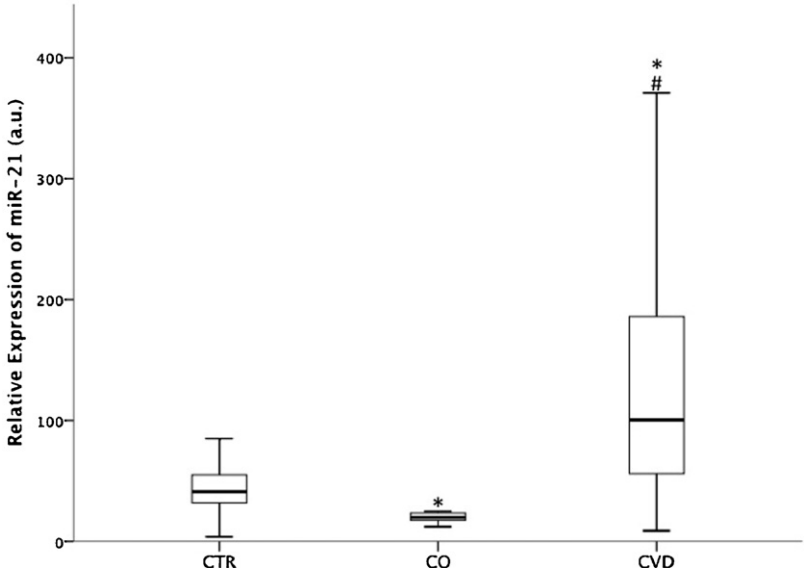

Fig. 6. The relative expression of circulating miR-21 in the offspring of centenarians (CO) and patients affected by cardiovascular disease (CVD) compared to age-matched CTR subjects. $^{*}p < 0.05$ vs. <CTR, $^{\#}p < 0.05$ vs. <CO. The relative levels of miR-21 were calculated using the equation $2^{-\Delta CT}$, in which ΔCt = Ct miR-21 − Ct miR-17-5p. CTR = age-matched healthy subjects; CO = offspring of centenarians; CVD = patients affected by cardiovascular disease.

categorized the miRs into three main underlying factors that explained approximately 70% of the total variance in all of the detectable circulating miRs.

This is the first report to show that the miRs belonging to factors 2 and 3 are more highly expressed in centenarians than in 20-year-old subjects. Interestingly, the miRs belonging to factor 2 showed an higher expression in octogenarians and a lower expression in centenarians relative to 20-year-old subjects. Non-monotonic related trends have previously been reported for variables related to longevity and inflammation (De Benedictis et al., 1998a,b; Yashin et al., 1999; Bonafè et al., 2002). Some studies have suggested that complex trajectories can be expected when "frail" and "robust" longevity-associated parameters are both present and the mortality rates for the carriers of different longevity-associated parameters overlap (Toupance et al., 1998; Yashin et al., 1999; Bonafe et al., 1999). Studies have also suggested that the aging phenotype is mainly due to an imbalance between inflammatory and anti-inflammatory networks; thus, an inflammatory imbalance may be considered one of the major driving forces of frailty and common age-related diseases (Franceschi et al., 2007). We hypothesized that specific non-monotonic age-related trends in circulating miR expression could be involved in the modulation of inflammation and the development of age-related diseases.

Notably, miRs that have previously been linked to inflammation and CVD, such as mir-146a (Olivieri et al., 2012a; Bhaumik et al., 2009) and miR-133a (Olivieri et al., 2012b; D'Alessandra et al., 2010), were found in factor 2. Despite the fact that miR-21 is known as an 'onco-miR' (due to its aberrant expression in numerous cancers), it has been recently reported to play a pivotal role as a modulator of many inflammatory pathways (Zhou et al., 2011; Kumarswamy et al., 2011). Moreover, miRs that have recently been reported to be involved in the modulation of cellular senescence, the oxidative-stress response and inflammation, such as miR-200c and miR-200b, were included in factor 3, which showed a significant age-related increase in centenarians as compared to in 20-year-old subjects (Magenta et al., 2011; Reddy et al., 2012). Our findings may have different implications in basic and clinical research. The clinical relevance of age-related trends in the expression of specific circulating miR subsets is derived from the use of circulating miRs as reliable biomarkers of human disease. Age-tailored miR expression cut-off values must be defined to optimize the diagnostic accuracy of circulating miRs in the clinic.

This study has also highlighted mechanisms that are potentially involved in the aging process. While the function of intracellular miRs can be elucidated through mRNA target prediction, this strategy has not yet been applied to circulating miRs. Circulating miRs may not simply represent 'spill-over' from the cellular miR content, but may also contribute to intercellular signaling (Iguchi et al., 2010; Kosaka et al., 2010; Zampetaki et al., 2012). Thus, identifying the pathways that are targeted by age-related miRs will improve our knowledge of the specific biological networks that are differentially modulated throughout the aging process. Our prediction analysis indicated that the TGF-β signaling pathway is targeted by the circulating miRs in factors 2 and 3. The levels of the miRs belonging to factor 3 were significantly higher in centenarians, whereas the miRs in factor 2 were primarily increased in octogenarians. Interestingly, the miRs belonging to factor 2 had higher plasma levels than the miRs belonging to factor 3. Thus, the combined effect of the miRs belonging to factors 2 and 3 could be increased expression in octogenarians and lower expression in centenarians. TGF-β is one of the most potent endogenous modulators of inflammation, with a well-documented anti-inflammatory effect (Breit et al., 2011; Hebel et al., 2011). Moreover, the inactivation of different components of the TGF-β signaling pathway caused CVD-related vascular dysfunction in both mice and humans (Pardali and Ten Dijke, 2012; Aihara et al., 2010). The TGF-β signaling pathway has also been reported as a pathway that is common to both cellular senescence/aging and age-related diseases such as CVD (Tacutu et al., 2011). We observed the increased expression of circulating miRs that negatively modulate TGF-β signaling pathway in older persons who exhibited an increased pro-inflammatory state and risk of CVD development (Franceschi et al., 2007; Song et al., 2012). On the contrary, centenarians are characterized by high levels of anti-inflammatory cytokines and a low incidence of CVD (Salvioli et al., 2006, 2009). Thus, circulating age-related miRs may contribute to an individual's pro-inflammatory status, which impacts aging successfully. Indeed, TGF-β signaling appears to be one of the most relevant pathways influencing aging in diverse eukaryotic species

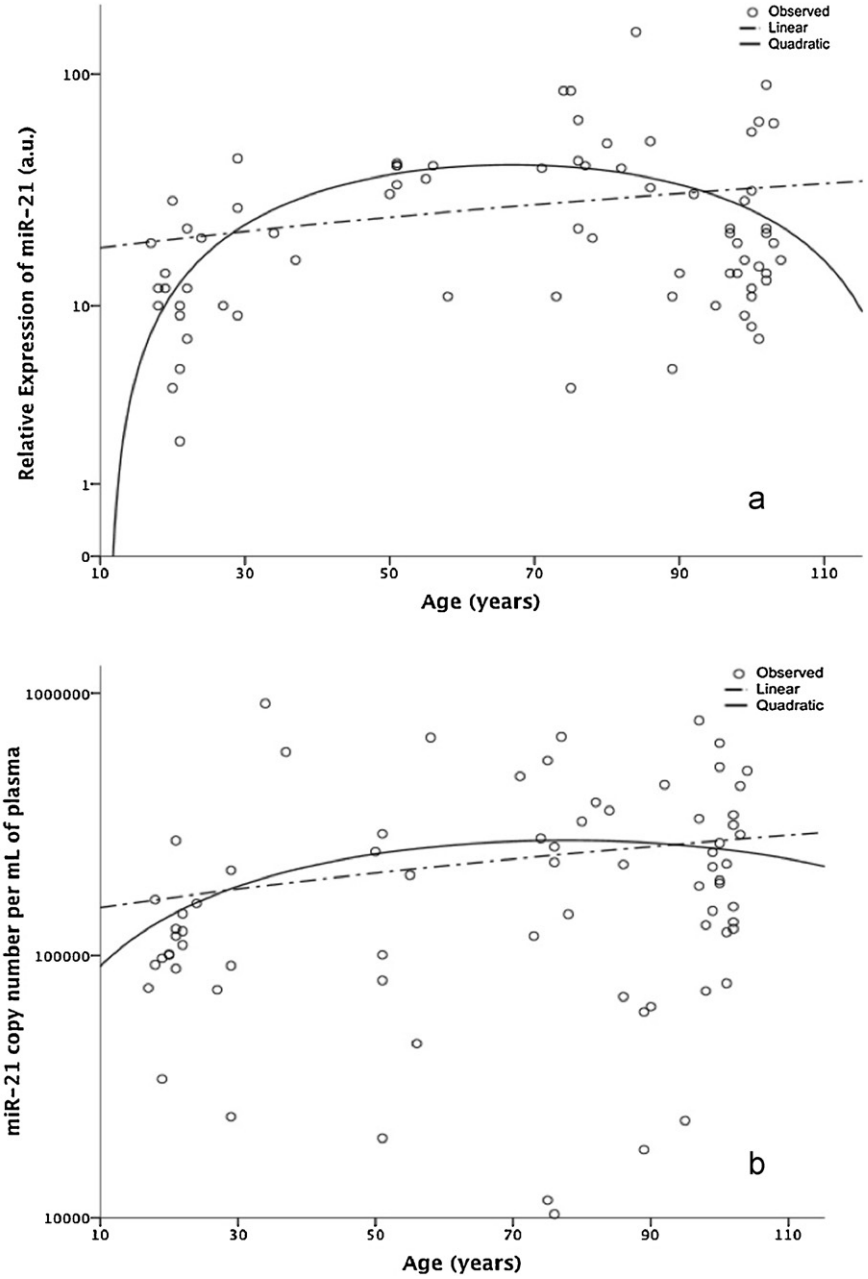

Fig. 7. The age-related expression of miR-21 in the CTR group. (a) The relative age-related expression of miR-21 in the CTR group. The relative miR-21 expression values were plotted against age. The relative expression levels of miR-21 were calculated using the equation $2^{-\Delta CT}$, in which ΔCt = Ct miR-21 − Ct miR-17-5p. (b) The absolute age-related expression of miR-21 in the CTR group. The absolute miR-21 expression values were plotted against age. The absolute miR-21 concentration (expressed as the "number of molecules per ml of plasma") was determined by running a dilution series containing known input quantities of synthetic miR-21 simultaneously with the experimental samples.

(Cheng et al., 2005; Salvioli et al., 2009; Bonafe and Olivieri, 2009; Vellai and Takacs-Vellai, 2010; Carrieri et al., 2004).

To validate the miR profiling and EFA results, we measured the miR-21 levels in the validation cohort (CTR) because it was the most highly expressed miR in factor 2. To avoid any possible bias due to the choice of a reference plasma miR, we analyzed both relative and absolute miR-21 expression. Both methods confirmed an age-related increase in the plasma miR-21 levels in the 65–95 years age group compared to the younger age group. This is the first report to show age-related differences in the expression of a circulating miR subset in older persons (65–95 years) and centenarians. To reinforce our hypothesis that some of the miRs belonging to factors 2 and 3 have inflammatory/anti-inflammatory properties, we tested for a correlation between miR-21 and inflammatory markers such as CRP and fibrinogen. Interestingly, significant correlations between CRP, fibrinogen and miR-21 were found in the validation cohort. Because of the pro-inflammatory status of miR-21, the plasma levels of this miR were also measured in patients affected by CVD. We recently published data showing the increased expression of circulating miR-21 in elderly patients with acute NSTEMI and CHF (Olivieri et al., 2012a,b). The present study confirms the higher miR-21 expression in CVD patients compared to the age-matched CTR subjects.

Considering the fact that the offspring of centenarians have a 62% and 85% reduced risk for all-cause and CVD mortality, respectively, we enrolled a sample of centenarian offspring (CO) in

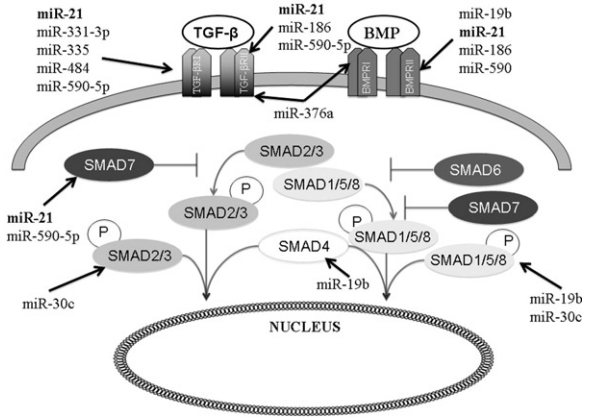

Fig. 8. The genes in the TGF-beta signaling pathway that are also targets of the miRs belonging to factors 2 and 3. The genes in TGF-beta signaling pathway that are targeted by the miR subsets belonging to factors 2 and 3 are depicted.

this study (Terry et al., 2004; Westendorp et al., 2009). Interestingly, we found a lower expression of miR-21 in the CO compared to CVD patients, as well as to the age-matched CTR subjects.

Overall, the findings of this study support the idea that circulating miR-21 may be an inflammatory biomarker linking the aging process to age-related diseases such as CVD. MiR-21 was recently reported to target multiple genes associated with the immunological antimicrobial response, providing an effective mechanism to escape from the antimicrobial pathway (Liu et al., 2012). Previous findings showing correlations between miR-21 expression and IL-6 and IL-8 levels suggest a potential role of miR-21 in inflammatory gene expression (Schetter et al., 2009). The pro-inflammatory markers IL-6, TNF-α and CRP have been shown to increase with age and predict disease and disability in older adults (Singh and Newman, 2011; Franceschi et al. 2007; Olivieri et al., 2006, 2008).

Interestingly, recent data indicated that miR-21 secreted by cancer cells in exosomes can bind to TLR8 in macrophages at the tumor interface inducing the secretion of proinflammatory cytokines, such as TNF-α and IL-6 (Fabbri et al., 2012). These data suggest a direct link between miR-21 expression levels and inflammatory response.

Moreover, miR-21 has been shown to directly modulate TGF-β signaling by targeting the TGF-βR2 in different cellular models (Kim et al., 2009; Wang et al., 2012; Yu et al., 2012; Schaefer et al., 2011; Sheedy et al., 2010). We found that the levels of TGF-βR2 mRNA were lower in the leukocytes of octogenarians than in the leukocytes of younger subjects. Interestingly, the centenarians showed miR-21 and TGF-βR2 mRNA levels similar to that of 20-year-old subjects. Overall, these preliminary results are indicative of a reduced "TGF-β resistance" in centenarians, even if these data need to be confirmed in a larger sample.

In conclusion, our results depict a very complex scenario in which circulating miRs, including miR-21, may play a role in "inflammaging", thus affecting the risk of major age-related diseases.

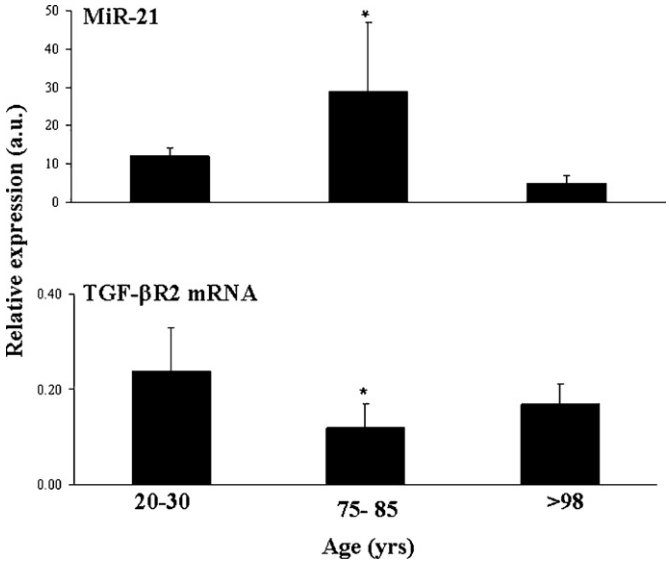

Fig. 9. The expression of miR-21 and TGF-βR2 mRNA in the leukocytes of young subjects, old subjects and centenarians. The levels of miR-21 and TGF-βR2 mRNA in the leukocytes of 5 individuals aged 20–30 years, 5 individuals aged 75–85 years and 5 centenarians. The data were normalized against β-actin levels and are reported as the mean value ± S.D.

5. Limitations

We chose healthy persons for our validation cohort to lower the probability of finding increased expression of disease-related miRs in this group. The potential impact of miRs controlling for unrecognized comorbidities in the CTR cannot be excluded. Indeed, we excluded those with any major acute and/or chronic age-related diseases, such as acute myocardial infarction, CHF, AD, T2DM or a CIRS > 2. Unfortunately, MR imaging was not available.

Research funding

The present study was supported by the following grants from the Italian Health Ministry: Università Politecnica delle Marche RFPS-2007-6-654027 and INRCA – Programma Strategico RFPS-2007-6-654027. This work was also supported by grants from the "Università Politecnica delle Marche" to ADP and FO.

Author contributions

All of the authors contributed significantly to the submitted work: (1) conception and design of the study: FO, LS, MCA, RA, CF and ADP; (2) data collection, analysis and interpretation: FO, LS, RL, RO, GS, RG, EC, FM, DM and ADP; and (3) drafting and revising the manuscript for intellectual content: MRR, DM, AMA, RA, CF and ADP.

Conflict of interest

Nothing to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mad.2012.09.004.

References

Aihara, K., Ikeda, Y., Yagi, S., Akaike, M., Matsumoto, T., 2010. Transforming growth factor-beta1 as a common target molecule for development of cardiovascular diseases, renal insufficiency and metabolic syndrome. Cardiology Research and Practice 2011 175381.

Alexander, K.P., Newby, L.K., Cannon, C.P., Armstrong, P.W., Gibler, W.B., Rich, M.W., Van de Werf, F., White, H.D., Weaver, W.D., Naylor, M.D., Gore, J.M., Krumholz, H.M., Ohman, E.M., American Heart Association Council on Clinical C, Society of Geriatric C, 2007. Acute coronary care in the elderly. Part I. Non-ST-segment-elevation acute coronary syndromes: a scientific statement for healthcare

professionals from the American Heart Association Council on Clinical Cardiology: in collaboration with the Society of Geriatric Cardiology. Circulation 115, 2549–2569.
Bacman, D., Merkel, S., Croner, R., Papadopoulos, T., Brueckl, W., Dimmler, A., 2007. TGF-beta receptor 2 downregulation in tumour-associated stroma worsens prognosis and high-grade tumours show more tumour-associated macrophages and lower TGF-beta1 expression in colon carcinoma: a retrospective study. BMC Cancer 7, 156.
Bhaumik, D., Scott, G.K., Schokrpur, S., Patil, C.K., Orjalo, A.V., Rodier, F., Lithgow, G.J., Campisi, J., 2009. MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8. Aging (Albany, NY) 1, 402–411.
Bonafe, M., Barbieri, M., Marchegiani, F., Olivieri, F., Ragno, E., Giampieri, C., Mugianesi, E., Centurelli, M., Franceschi, C., Paolisso, G., 2003. Polymorphic variants of insulin-like growth factor I (IGF-I) receptor and phosphoinositide 3-kinase genes affect IGF-I plasma levels and human longevity: cues for an evolutionarily conserved mechanism of life span control. Journal of Clinical Endocrinology and Metabolism 88, 3299–3304.
Bonafe, M., Olivieri, F., 2009. Genetic polymorphism in long-lived people: cues for the presence of an insulin/IGF-pathway-dependent network affecting human longevity. Molecular and Cellular Endocrinology 299, 118–123.
Bonafè, M., Barbi, C., Storci, G., Salvioli, S., Capri, M., Olivieri, F., Valensin, S., Monti, D., Gonos, E.S., De Benedictis, G., Franceschi, C., 2002. What studies on human longevity tell us about the risk for cancer in the oldest old: data and hypotheses on the genetics and immunology of centenarians. Experimental Gerontology 37, 1263–1271.
Bonafe, M., Olivieri, F., Mari, D., Baggio, G., Mattace, R., Berardelli, M., Sansoni, P., De Benedictis, G., De Luca, M., Marchegiani, F., Cavallone, L., Cardelli, M., Giovagnetti, S., Ferrucci, L., Amadio, L., Lisa, R., Tucci, M.G., Troiano, L., Pini, G., Gueresi, P., Morellini, M., Sorbi, S., Passeri, G., Barbi, C., Valensin, S., Monti, D., Deiana, L., Pes, G.M., Carru, C., Franceschi, C., 1999. P53 codon 72 polymorphism and longevity: additional data on centenarians from continental Italy and Sardinia. American Journal of Human Genetics 65, 1782–1785.
Breit, S.N., Johnen, H., Cook, A.D., Tsai, V.W., Mohammad, M.G., Kuffner, T., Zhang, H.P., Marquis, C.P., Jiang, L., Lockwood, G., Lee-Ng, M., Husaini, Y., Wu, L., Hamilton, J.A., Brown, D.A., 2011. The TGF-beta superfamily cytokine, MIC-1/GDF15: a pleotrophic cytokine with roles in inflammation, cancer and metabolism. Growth Factors 29, 187–195.
Carrieri, G., Marzi, E., Olivieri, F., Marchegiani, F., Cavallone, L., Cardelli, M., Giovagnetti, S., Stecconi, R., Molendini, C., Trapassi, C., De Benedictis, G., Kletsas, D., Franceschi, C., 2004. The G/C915 polymorphism of transforming growth factor beta1 is associated with human longevity: a study in Italian centenarians. Aging Cell 3, 443–448.
Cevenini, E., Bellavista, E., Tieri, P., Castellani, G., Lescai, F., Francesconi, M., Mishto, M., Santoro, A., Valensin, S., Salvioli, S., Capri, M., Zaikin, A., Monti, D., de Magalhaes, J.P., Franceschi, C., 2010. Systems biology and longevity: an emerging approach to identify innovative anti-aging targets and strategies. Current Pharmaceutical Design 16, 802–813.
Chen, X., Ba, Y., Ma, L., Cai, X., Yin, Y., Wang, K., Guo, J., Zhang, Y., Chen, J., Guo, X., Li, Q., Li, X., Wang, W., Wang, J., Jiang, X., Xiang, Y., Xu, C., Zheng, P., Zhang, J., Li, R., Zhang, H., Shang, X., Gong, T., Ning, G., Zen, K., Zhang, C.Y., 2008. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Research 18, 997–1006.
Cheng, C.L., Gao, T.Q., Wang, Z., Li, D.D., 2005. Role of insulin/insulin-like growth factor 1 signaling pathway in longevity. World Journal of Gastroenterology 11, 1891–1895.
Cocucci, E., Racchetti, G., Meldolesi, J., 2009. Shedding microvesicles: artefacts no more. Trends in Cell Biology 19, 43–51.
Cortez, M.A., Calin, G.A., 2009. MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases. Expert Opinion on Biological Therapy 9, 703–711.
D'Alessandra, Y., Devanna, P., Limana, F., Straino, S., Di Carlo, A., Brambilla, P.G., Rubino, M., Carena, M.C., Spazzafumo, L., De Simone, M., Micheli, B., Biglioli, P., Achilli, F., Martelli, F., Maggiolini, S., Marenzi, G., Pompilio, G., Capogrossi, M.C., 2010. Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. European Heart Journal 31, 2765–2773.
De Benedictis, G., Carotenuto, L., Carrieri, G., De Luca, M., Falcone, E., Rose, G., Cavalcanti, S., Corsonello, F., Feraco, E., Baggio, G., Bertolini, S., Mari, D., Mattace, R., Yashin, A.I., Bonafè, M., Franceschi, C., 1998a. Gene/longevity association studies at four autosomal loci (REN, THO, PARP, SOD2). European Journal of Human Genetics 6, 534–541.
De Benedictis, G., Carotenuto, L., Carrieri, G., De Luca, M., Falcone, E., Rose, G., Yashin, A.I., Bonafè, M., Franceschi, C., 1998b. Age-related changes of the 3′APOB-VNTR genotype pool in ageing cohorts. Annals of Human Genetics 62, 115–122.
Duttagupta, R., Jiang, R., Gollub, J., Getts, R.C., Jones, K.W., 2011. Impact of cellular miRNAs on circulating miRNA biomarker signatures. PLoS One 6, e20769.
Elsharawy, A., Keller, A., Flachsbart, F., Wendschlag, A., Jacobs, G., Kefer, N., Brefort, T., Leidinger, P., Backes, C., Meese, E., Schreiber, S., Rosenstiel, P., Franke, A., Nebel, A., 2012, April. Genome-wide miRNA signatures of human longevity. Aging Cell, http://dx.doi.org/10.1111/j.1474-9726.2012.00824.x.
Fabbri, M., Paone, A., Calore, F., Galli, R., Gaudio, E., Santhanam, R., Lovat, F., Fadda, P., Mao, C., Nuovo, G.J., Zanesi, N., Crawford, M., Ozer, G.H., Wernicke, D., Alder, H., Caligiuri, M.A., Nana-Sinkam, P., Perrotti, D., Croce, C.M., 2012. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. Proceedings of the National Academy of Sciences of the United States of America 31 (109) E2110-6.
Franceschi, C., Capri, M., Monti, D., Giunta, S., Olivieri, F., Sevini, F., Panourgia, M.P., Invidia, L., Celani, L., Scurti, M., Cevenini, E., Castellani, G.C., Salvioli, S., 2007. Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. Mechanisms of Ageing and Development 128, 92–105.
Franceschi, C., Olivieri, F., Marchegiani, F., Cardelli, M., Cavallone, L., Capri, M., Salvioli, S., Valensin, S., De Benedictis, G., Di Iorio, A., Caruso, C., Paolisso, G., Monti, D., 2005. Genes involved in immune response/inflammation, IGF1/insulin pathway and response to oxidative stress play a major role in the genetics of human longevity: the lesson of centenarians. Mechanisms of Ageing and Development 126, 351–361.
Garzon, R., Calin, G.A., Croce, C.M., 2009. MicroRNAs in cancer. Annual Review of Medicine 60, 167–179.
Hebel, K., Rudolph, M., Kosak, B., Chang, H.D., Butzmann, J., Brunner-Weinzierl, M.C., 2011. IL-1beta and TGF-beta act antagonistically in induction and differentially in propagation of human proinflammatory precursor CD4+ T cells. Journal of Immunology 187, 5627–5635.
Hunter, M.P., Ismail, N., Zhang, X., Aguda, B.D., Lee, E.J., Yu, L., Xiao, T., Schafer, J., Lee, M.L., Schmittgen, T.D., Nana-Sinkam, S.P., Jarjoura, D., Marsh, C.B., 2008. Detection of microRNA expression in human peripheral blood microvesicles. PLoS One 3, e3694.
Ibanez-Ventoso, C., Yang, M., Guo, S., Robins, H., Padgett, R.W., Driscoll, M., 2006. Modulated microRNA expression during adult lifespan in *Caenorhabditis elegans*. Aging Cell 5, 235–246.
Iguchi, H., Kosaka, N., Ochiya, T., 2010. Secretory microRNAs as a versatile communication tool. Communicative & Integrative Biology 3, 478–481.
Kim, Y.J., Hwang, S.J., Bae, Y.C., Jung, J.S., 2009. MiR-21 regulates adipogenic differentiation through the modulation of TGF-beta signaling in mesenchymal stem cells derived from human adipose tissue. Stem Cells 27, 3093–3102.
Kosaka, N., Iguchi, H., Yoshioka, Y., Takeshita, F., Matsuki, Y., Ochiya, T., 2010. Secretory mechanisms and intercellular transfer of microRNAs in living cells. Journal of Biological Chemistry 285, 17442–17452.
Kumarswamy, R., Volkmann, I., Thum, T., 2011. Regulation and function of miRNA-21 in health and disease. RNA Biology 8.
Lasser, C., Alikhani, V.S., Ekstrom, K., Eldh, M., Paredes, P.T., Bossios, A., Sjostrand, M., Gabrielsson, S., Lotvall, J., Valadi, H., 2011. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. Journal of Translational Medicine 9, 9.
Li, N., Bates, D.J., An, J., Terry, D.A., Wang, E., 2011a. Up-regulation of key microRNAs, and inverse down-regulation of their predicted oxidative phosphorylation target genes, during aging in mouse brain. Neurobiology of Aging 32, 944–955.
Li, X., Khanna, A., Li, N., Wang, E., 2011b. Circulatory miR34a as an RNAbased, noninvasive biomarker for brain aging. Aging (Albany, NY) 3, 985–1002.
Liu, P.T., Wheelwright, M., Teles, R., Komisopoulou, E., Edfeldt, K., Ferguson, B., Mehta, M.D., Vazirnia, A., Rea, T.H., Sarno, E.N., Graeber, T.G., Modlin, R.L., 2012. MicroRNA-21 targets the vitamin D-dependent antimicrobial pathway in leprosy. Nature Medicine 18, 267–273.
Losche, W., Scholz, T., Temmler, U., Oberle, V., Claus, R.A., 2004. Platelet-derived microvesicles transfer tissue factor to monocytes but not to neutrophils. Platelets 15, 109–115.
Maes, O.C., An, J., Sarojini, H., Wang, E., 2008. Murine microRNAs implicated in liver functions and aging process. Mechanisms of Ageing and Development 129, 534–541.
Magenta, A., Cencioni, C., Fasanaro, P., Zaccagnini, G., Greco, S., Sarra-Ferraris, G., Antonini, A., Martelli, F., Capogrossi, M.C., 2011. miR-200c is upregulated by oxidative stress and induces endothelial cell apoptosis and senescence via ZEB1 inhibition. Cell Death and Differentiation 18, 1628–1639.
Maragkakis, M., Alexiou, P., Papadopoulos, G.L., Reczko, M., Dalamagas, T., Giannopoulos, G., Goumas, G., Koukis, E., Kourtis, K., Simossis, V.A., Sethupathy, P., Vergoulis, T., Koziris, N., Sellis, T., Tsanakas, P., Hatzigeorgiou, A.G., 2009. Accurate microRNA target prediction correlates with protein repression levels. BMC Bioinformatics 10, 295.
Meyer, S.U., Pfaffl, M.W., Ulbrich, S.E., 2010. Normalization strategies for microRNA profiling experiments: a 'normal' way to a hidden layer of complexity? Biotechnology Letters 32, 1777–1788.
Mistry, R., Gokhman, I., Bastani, R., Gould, R., Jimenez, E., Maxwell, A., McDermott, C., Rosansky, J., Van Stone, W., Jarvik, L., Group, U.C., 2004. Measuring medical burden using CIRS in older veterans enrolled in UPBEAT, a psychogeriatric treatment program: a pilot study. Journals of Gerontology. Series A, Biological Sciences and Medical Sciences 59, 1068–1075.
Noren Hooten, N., Abdelmohsen, K., Gorospe, M., Ejiogu, N., Zonderman, A.B., Evans, M.K., 2010. microRNA expression patterns reveal differential expression of target genes with age. PLoS One 5, e10724.
Olivieri, F., Lazzarini, R., Recchioni, R., Marcheselli, F., Rippo, M.R., Di Nuzzo, S., Albertini, M.C., Graciotti, L., Babini, L., Mariotti, S., Spada, G., Abbatecola, A.M., Antonicelli, R., Franceschi, C., Procopio, A.D., 2012a, June. MiR-146a as marker of senescence-associated pro-inflammatory status in cells involved in vascular remodeling. Age (Dordrecht, Netherlands) 13.
Olivieri, F., Antonicelli, R., Lorenzi, M., D'Alessandra, Y., Lazzarini, R., Santini, G., Spazzafumo, L., Lisa, R., La Sala, L., Galeazzi, R., Recchioni, R., Testa, R., Pompilio, G., Capogrossi, M.C., Procopio, A.D., 2012b. Diagnostic potential of circulating miR-499-5p in elderly patients with acute non ST-elevation myocardial infarction. International Journal of Cardiology.
Olivieri, F., Spazzafumo, L., Antonicelli, R., Marchegiani, F., Cardelli, M., Sirolla, C., Galeazzi, R., Giovagnetti, S., Mocchegiani, E., Franceschi, C., 2008. Combination

of biomarkers to predict mortality in elderly patients with myocardial infarction. Mechanisms of Ageing and Development 129, 231–237.
Olivieri, F., Antonicelli, R., Cardelli, M., Marchegiani, F., Cavallone, L., Mocchegiani, E., Franceschi, C., 2006. Genetic polymorphisms of inflammatory cytokines and myocardial infarction in the elderly. Mechanisms of Ageing and Development 127, 552–559.
Pardali, E., Ten Dijke, P., 2012. TGFbeta signaling and cardiovascular diseases. International Journal of Biological Sciences 8, 195–213.
Peltier, H.J., Latham, G.J., 2008. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA 14, 844–852.
Perls, T., Kunkel, L.M., Puca, A.A., 2002. The genetics of exceptional human longevity. Journal of the American Geriatrics Society 50, 359–368.
Ramachandran, S., Palanisamy, V., 2011. Horizontal transfer of RNAs: exosomes as mediators of intercellular communication. Wiley Interdisciplinary Reviews. RNA.
Reddy, M.A., Jin, W., Villeneuve, L., Wang, M., Lanting, L., Todorov, I., Kato, M., Natarajan, R., 2012. Pro-inflammatory role of microRNA-200 in vascular smooth muscle cells from diabetic mice. Arteriosclerosis, Thrombosis, and Vascular Biology 32, 721–729.
Salvioli, S., Capri, M., Bucci, L., Lanni, C., Racchi, M., Uberti, D., Memo, M., Mari, D., Govoni, S., Franceschi, C., 2009. Why do centenarians escape or postpone cancer? The role of IGF-1, inflammation and p53. Cancer Immunology, Immunotherapy 58, 1909–1917.
Salvioli, S., Olivieri, F., Marchegiani, F., Cardelli, M., Santoro, A., Bellavista, E., Mishto, M., Invidia, L., Capri, M., Valensin, S., Sevini, F., Cevenini, E., Celani, L., Lescai, F., Gonos, E., Caruso, C., Paolisso, G., De Benedictis, G., Monti, D., Franceschi, C., 2006. Genes, ageing and longevity in humans: problems, advantages and perspectives. Free Radical Research 40, 1303–1323.
Satoh, J., Tabunoki, H., 2011. Comprehensive analysis of human microRNA target networks. BioData Mining 4, 17.
Schaefer, J.S., Montufar-Solis, D., Vigneswaran, N., Klein, J.R., 2011. Selective upregulation of microRNA expression in peripheral blood leukocytes in IL-$10^{-/-}$ mice precedes expression in the colon. Journal of Immunology 187, 5834–5841.
Schetter, A.J., Nguyen, G.H., Bowman, E.D., Mathe, E.A., Yuen, S.T., Hawkes, J.E., Croce, C.M., Leung, S.Y., Harris, C.C., 2009. Association of inflammation-related and microRNA gene expression with cancer-specific mortality of colon adenocarcinoma. Clinical Cancer Research 15, 5878–5887.
Sebastiani, P., Solovieff, N., Dewan, A.T., Walsh, K.M., Puca, A., Hartley, S.W., Melista, E., Andersen, S., Dworkis, D.A., Wilk, J.B., Myers, R.H., Steinberg, M.H., Montano, M., Baldwin, C.T., Hoh, J., Perls, T.T., 2012. Genetic signatures of exceptional longevity in humans. PLoS One 7, e29848.
Sheedy, F.J., Palsson-McDermott, E., Hennessy, E.J., Martin, C., O'Leary, J.J., Ruan, Q., Johnson, D.S., Chen, Y., O'Neill, L.A., 2010. Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. Nature Immunology 11, 141–147.
Singh, T., Newman, A.B., 2011. Inflammatory markers in population studies of aging. Ageing Research Reviews 10, 319–329.
Song, Y., Shen, H., Schenten, D., Shan, P., Lee, P.J., Goldstein, D.R., 2012. Aging enhances the basal production of IL-6 and CCL2 in vascular smooth muscle cells. Arteriosclerosis, Thrombosis, and Vascular Biology 32, 103–109.
Spazzafumo, L., Olivieri, F., Abbatecola, A.M., Castellani, G., Monti, D., Lisa, R., Galeazzi, R., Sirolla, C., Testa, R., Ostan, R., Scurti, M., Caruso, C., Vasto, S., Vescovini, R., Ogliari, G., Mari, D., Lattanzio, F., Franceschi, C., 2011. Remodelling of biological parameters during human ageing: evidence for complex regulation in longevity and in type 2 diabetes. Age (Dordrecht, Netherlands).
Tacutu, R., Budovsky, A., Yanai, H., Fraifeld, V.E., 2011. Molecular links between cellular senescence, longevity and age-related diseases—a systems biology perspective. Aging (Albany, NY) 3, 1178–1191.
Terry, D.F., Wilcox, M.A., McCormick, M.A., Pennington, J.Y., Schoenhofen, E.A., Andersen, S.L., Perls, T.T., 2004. Lower all-cause, cardiovascular, and cancer mortality in centenarians' offspring. Journal of the American Geriatrics Society 52, 2074–2076.
Toupance, B., Godelle, B., Gouyon, P.H., Schachter, F., 1998. A model for antagonistic pleiotropic gene action for mortality and advanced age. American Journal of Human Genetics 62, 1525–1534.
Turchinovich, A., Weiz, L., Langheinz, A., Burwinkel, B., 2011. Characterization of extracellular circulating microRNA. Nucleic Acids Research 39, 7223–7233.
Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J.J., Lotvall, J.O., 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nature Cell Biology 9, 654–659.
Vellai, T., Takacs-Vellai, K., 2010. Regulation of protein turnover by longevity pathways. Advances in Experimental Medicine and Biology 694, 69–80.
Wang, T., Zhang, L., Shi, C., Sun, H., Wang, J., Li, R., Zou, Z., Ran, X., Su, Y., 2012. TGF-beta-induced miR-21 negatively regulates the antiproliferative activity but has no effect on EMT of TGF-beta in HaCaT cells. International Journal of Biochemistry and Cell Biology 44, 366–376.
Westendorp, R.G., van Heemst, D., Rozing, M.P., Frolich, M., Mooijaart, S.P., Blauw, G.J., Beekman, M., Heijmans, B.T., de Craen, A.J., Slagboom, P.E., Leiden Longevity Study G, 2009. Nonagenarian siblings and their offspring display lower risk of mortality and morbidity than sporadic nonagenarians: the Leiden longevity study. Journal of the American Geriatrics Society 57, 1634–1637.
Yashin, A.I., Arbeev, K.G., Akushevich, I., Ukraintseva, S.V., Kulminski, A., Arbeeva, L.S., Culminskaya, I., 2009. Exceptional survivors have lower age trajectories of blood glucose: lessons from longitudinal data. Biogerontology 11, 257–265.
Yashin, A.I., De Benedictis, G., Vaupel, J.W., Tan, Q., Andreev, K.F., Iachine, I.A., Bonafe, M., DeLuca, M., Valensin, S., Carotenuto, L., Franceschi, C., 1999. Genes, demography, and life span: the contribution of demographic data in genetic studies on aging and longevity. American Journal of Human Genetics 65, 1178–1193.
Yu, Y., Kanwar, S.S., Patel, B.B., Oh, P.S., Nautiyal, J., Sarkar, F.H., Majumdar, A.P., 2012. MicroRNA-21 induces stemness by downregulating transforming growth factor beta receptor 2 (TGFbetaR2) in colon cancer cells. Carcinogenesis 33, 68–76.
Zampetaki, A., Willeit, P., Drozdov, I., Kiechl, S., Mayr, M., 2012. Profiling of circulating microRNAs: from single biomarkers to re-wired networks. Cardiovascular Research 93, 555–562.
Zhou, J., Wang, K.C., Wu, W., Subramaniam, S., Shyy, J.Y., Chiu, J.J., Li, J.Y., Chien, S., 2011. MicroRNA-21 targets peroxisome proliferators-activated receptor-alpha in an autoregulatory loop to modulate flow-induced endothelial inflammation. Proceedings of the National Academy of Sciences of the United States of America 108, 10355–10360.
Zhu, H., Fan, G.C., 2011. Extracellular/circulating microRNAs and their potential role in cardiovascular disease. American Journal of Cardiovascular Disease 1, 138–149.