Circulating miRNAs and miRNA shuttles as biomarkers: Perspective trajectories of healthy and unhealthy aging

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Abstract

Human aging is a lifelong process characterized by a continuous trade-off between pro-and anti-inflammatory responses, where the best-adapted and/or remodeled genetic/epigenetic profile may develop a longevity phenotype. Centenarians and their offspring represent such a phenotype and their comparison to patients with age-related diseases (ARDs) is expected to maximize the chance to unravel the genetic makeup that better associates with healthy aging trajectories. Seemingly, such comparison is expected to allow the discovery of new biomarkers of longevity together with risk factor for the most common ARDs. MicroRNAs (miRNAs) and their shuttles (extracellular vesicles in particular) are currently conceived as those endowed with the strongest ability to provide information about the trajectories of healthy and unhealthy aging. We review the available data on miRNAs in aging and underpin the evidence suggesting that circulating miRNAs (and cognate shuttles), especially those involved in the regulation of inflammation (inflamma-miRs) may constitute biomarkers capable of reliably depicting healthy and unhealthy aging trajectories.

Keywords

Centenarians; Offspring of centenarians; Circulating microRNAs; Aging trajectories; miR-21-5p

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1. Introduction

Human aging is a lifelong process that likely begins *in utero* (Menon, 2016) and is characterized by a dynamic phenotype that changes over time. Continuous interactions among genetic and epigenetic makeup and environmental (*e.g.* microbiota, lifestyle and diet) and stochastic factors influence the possibility to reach the limit of the human lifespan. The main feature of the aging process is a chronic, progressive proinflammatory status that has been named "inflammaging" (Franceschi et al., 2000, 2007; Cevenini et al., 2013; Salvioli et al., 2013), and it is currently considered as a major risk factor for the most common age-related diseases (ARDs) (Xia et al., 2016). Aging involves the entire organism; however, not all cell types, tissues, organs, and systems age at the same rate (Cevenini et al., 2008). Indeed, it is conceivable that different cells types are characterized by different rates of aging, and that senescent cells may contribute to spread senescence to younger cells (Olivieri et al., 2015a). The build-up of senescent cells during aging sustains and propagates inflammaging. A variety of different conditions can contribute to the systemic spread of inflammaging: i) the release of a number of soluble factors from senescent cells that have acquired a senescence-associated secretory phenotype (SASP); ii) the release of apoptotic/necrotic cell-derived molecules (Danger Associated Molecular Patterns, DAMPs) induced by age-damage-related modifications (Franceschi and Campisi, 2014); and iii) the release of proinflammatory factors from cells infected by pathogens *e.g.* viruses, (Long et al., 2016). Very recent data also suggest an effect due to inflammatory molecules, such as IL-6, on tissue remodeling and cell reprogramming emerging from cell senescence process in *in vivo* models (Mosteiro et al., 2016).

Inflammation related molecules seem to be shuttled into blood or lymph by different nano/microvesicles and to be captured by immune system cells, thus contributing to immunosenescence (Su et al., 2013). On the other hand, preservation of healthy state during aging may be ensured by efficient enzymatic and cell repair systems, that counteract the changes induced by harmful, chronic interactions with external and internal exposomes, i.e. all the types of exposures which an individual encounter during the lifetime (Wild, 2005; Nakamura et al., 2014). It is thus conceivable that the trade-off between pro-and anti-inflammatory molecules stimulates organism adaptation, and that the best adapted and/or remodeled genetic/epigenetic profile leads to development of the longevity phenotype. In this regard, centenarians and, especially, supercentenarians, who have avoided or postponed most ARDs, provide the best example of adaptation/remodeling and represent the paradigm of successful aging (Franceschi et al., 1995).

We review the evidence suggesting that circulating molecules such as microRNAs (miRNAs, miRs), particularly those involved in the modulation of inflammation (inflamma-miRs), and their shuttles may also act as modulators of inflammaging. Studies of their expression and targets have the potential to supply critical information on the aging process and its propagation through cells, tissues, and organs (Franceschi et al., 2016). However, circulating miRNAs (c-miRs) and their shuttles may serve not only as markers/mediators of healthy and pathological conditions, but also as tools, that can help monitor the aging trajectory. Ideally, early detection of deviation from a healthy trajectory would enable adopting therapeutic interventions to counteract or delay the onset of ARDs such as type 2 diabetes (T2DM) and

cardiovascular disease (CVD). Since c-miRs reflect an individual's biological adaptation to environmental exposures, their determination allows evaluating the biological changes induced by lifestyle interventions such as exercise and dietary changes (Flowers et al., 2015).

2. Healthy and unhealthy aging trajectories

The debate on whether aging is or is not programmed (Kowald and Kirkwood, 2016) appears to have been overtaken by the "quasi-programmed" theory (Blagosklonny, 2013), which is based on the main feature of aging, *i.e.* the close relationship between organism development and the deregulation of molecular pathways. Accordingly, over-activation of signal transduction pathways such as MTOR and exacerbation of normal cellular functions leading to alteration of homeostasis, malfunction, disease, and organ damage are likely the driving forces of aging processes.

A way to characterize aging-related biomarkers would be the use of longitudinal studies from birth to the extreme limit of human lifespan, i.e. centenarians and supercentenarians (Fig. 1). Since this happens an almost unfeasible approach, the best alternative one is to examine healthy individuals of different ages, in a cross-sectional design. Given that, age-related functional decline is associated with an increased vulnerability to a variety of degenerative and chronic diseases, thus aging may be depicted as dichotomized into two divergent, healthy and unhealthy, trajectories (Fig. 1). Healthy elderly people (60–80 year olds) are not easily recruited because this age group is highly heterogeneous for the likelihood to achieve extreme old age. Moreover, pre-frail and ostensibly healthy elderly subjects may not exhibit ARD traits at the time of observation, but may develop ARDs soon after recruitment, and, finally, only a small proportion of such population will become centenarian. The health status of elderly people can be viewed as a track that eventually veers to a poorer health trajectory. Based on these premises, any sample of healthy subjects aged more than 60 years will necessarily include individuals who will eventually develop ARDs, and whose biomarkers will deviate away from a healthy trajectory. The deviation will be represented by the number of apparently "healthy" subjects, who do go on to develop disease (depicted in Fig. 1 as red and green people, respectively).

In this context, comparing elderly unhealthy people to extreme phenotypes (healthy centenarians and their offsprings) is expected to maximize the chance to find out any difference in the genetic/epigenetic make up. According to this tenet, this approach has been successfully undertaken to identify T2DM genetic risk variants (Garagnani et al., 2013). In fact, centenarians are characterized by well-preserved glucose metabolism and their offspring are endued with a better functional status, a lower risk of developing ARDs, and a reduced use of medications than the offspring of non-long-lived parents (Gueresi et al., 2013). Notably, the risk of mortality from CVD of centenarians' offspring is 30% lower than in aged-matched people from the general population (Terry et al., 2004; Westendorp et al., 2009).

The view that the unhealthy aging trajectory can be traced by investigating patients with the most common ARDs is supported by the consideration that aging is the single largest risk

factor for ARD development, and that studies of animal models have identified conserved pathways that modulate both the aging rate and ARD onset (Johnson et al., 2015).

In western countries, the most common ARDs are CVD, T2DM, cancer, and neurodegenerative diseases. The prevalence of diabetes is increasing at all ages, mostly due to rising overweight and obesity rates. T2DM patients experience early aging (Spazzafumo et al., 2013) and have distinctive genetic characteristics (Garagnani et al., 2013). Notably, 50% of T2DM patients die from CVD (primarily acute myocardial infarction and stroke), and their overall mortality risk is at least twice that of their peers without diabetes (Viña et al., 2013). On the other hand, the main predictors of mortality after acute myocardial infarction are diabetes and age (Wagner et al., 2014).

A sound approach to the study of aging is to establish at which point the aging trajectory begins to diverge and to identify the non-modifiable and modifiable factors that may defer it. If the genetic background is a non-modifiable risk factor for ARD development, epigenetic factors such as DNA/histone methylation and circulating non-coding RNA are emerging as functional biomarkers that can help monitor an individual's aging trajectory (Bacalini et al., 2016; Wagner et al., 2016). Such epigenetic markers are the main mediators between genetic make-up and environmental factors (*e.g.* dietary restriction, exercise) influencing genomic stability and expression.

Complex trajectories involving a number of parameters, such as U–shaped trajectories, have been described where the combined effect of age, genetic profile, and diet/nutrients have been seen to have a role in determining the overall health status of elderly subjects (Jack et al., 2012). Nevertheless, limited data are available on the age-related trajectories of circulating miRs in healthy subjects including the oldest old, as summarized in Table 1a and as described in the next paragraph.

3. Circulating miRs as biomarkers of healthy and unhealthy aging trajectories

Even though extracellular nucleic acids were first described in the human bloodstream more than 50 years ago, they have only recently been recognized as significant agents in intercellular signaling rather than as mere 'spill-over' (Dhahbi, 2014; Vickers and Remaley, 2012). Circulating nucleic acids, in particular small RNAs such as miRs (17–25 nt long), provide additional inter-tissue and inter-organ communication besides the classic mechanisms (hormones, cytokines, growth factors). Recent evidence has confirmed that some features of aging reflect the profile of circulating miRs (Weilner et al., 2013; Olivieri et al., 2012, 2013; Serna et al., 2012; Ghai and Wang, 2016). By applying Principal Component Analysis to centenarians' results (representing "extraordinary aging"), we have found that their miRNome is similar to that of young individuals and different from that of septuagenarians ("ordinary aging") (Serna et al., 2012). This finding demonstrates that centenarians are indeed on a "healthy trajectory" that has led to their long and remarkably disease free life.

Tables 1a and b analyse the most relevant researches on human blood (whole blood, plasma, serum, peripheral mononuclear cells, and immortalized B cell lines) and the effect of healthy aging on miR expression levels. The increase of miR levels (up-regulation) with age appears to be the most relevant result, particularly evident in centenarians. Significantly, the biological samples of centenarians are characterized by a distinctive miR profile. The tables also show the normalization procedure adopted in each work and being different in all cases, any comparative conclusion about the quantity of expression is very difficult. Additional variables have been taken into account, such as biological sample, method and group age making more complex definitive conclusions. Nevertheless, our investigations showed that the levels of c-miRs-21-5p and 126-3p increase during aging (Olivieri et al., 2012; Olivieri et al., 2014a), findings recently confirmed by other researchers (Ameling et al., 2015), as shown in the tables.

Moreover, miR-126-3p, which is highly expressed in endothelial and hematopoietic progenitor cells, is reduced in diabetic patients suffering from the complications of diabetes (Olivieri et al., 2014a), highlighting the role of miR levels in distinguishing healthy from unhealthy status.

Furthermore, the c-miR-146a is an important regulator in the interplay among DNA damage response (DDR), cell senescence and inflammaging (Olivieri et al., 2015a). We have designated c-miRs-21-5p, −126a and −146a as inflamma-miRs (Olivieri et al., 2013) for their role in inflammaging, but many other c-miRs with a possible pro-inflammatory role have recently been identified (Noren Hooten et al., 2013; Zhang et al., 2015; Ameling et al., 2015), as reported in Table 1(a and b).

The comparison of miR expression in serum from young (mean age 30 years) and older (mean age 64 years) individuals has demonstrated miR-151a-5p, miR-181a-5p and miR-1248 down-regulation in the latter subjects (Noren Hooten et al., 2013). Intriguingly, miR-1248 has been reported to regulate the expression of mRNAs involved in inflammatory pathways, and miR-181a has been found to correlate negatively with the pro-inflammatory cytokines IL-6 and TNFα, and positively with the anti-inflammatory cytokines TGFβ and IL-10. These data further document that the interaction among inflammatory and anti-inflammatory molecules can help understand lifelong remodeling and the attainment of longevity (Franceschi et al., 2007).

Very recently, a cross-sectional design in 50, 55 and 60 years old individuals with documented life span (from 58 to 92 years) were analyzed. They were divided into two groups. i.e. long and short –lived, and a lifespan signature of c-miRs was proposed (miR-211-5p, 374a-5p, 340-3p, 376c-3p, 5095, 1225-3p) (Smith-Vikos et al., 2016). This work highlights the possibility to trace healthy and unhealthy trajectories starting at a critical age when the evolution driving force is decreased and age-related diseases start to develop, and concomitantly gene expression regulation is less finely tuned. Importantly, these dichotomized lifespan trajectories and miRs expression have also been identified in animal models, such as C. *Elegans* (Pincus et al., 2011), thus suggesting similar aging mechanisms in phylogenetically distant organisms.

Patients with CVDs are unlikely to become centenarians, and their circulating miR patterns are believed to considerably differ from those of healthy subjects. Recently, a c-miR signature has been described in patients with acute heart failure (Ovchinnikova et al., 2016) and T2DM (Guay and Regazzi, 2013). However, among the c-miRs identified to date, miR-21-5p is the one that seems to characterize best the longevity phenotype, since it is under expressed in successful aging (Olivieri et al., 2012). In fact, we analyzed c-miR-21-5p levels in samples from healthy subjects with an age-range from 20 to more than 100 years old (Olivieri et al., 2012). This analysis allowed us to estimate the "physiological" modification of c-miR-21-5p associated with the "aging process". Surprisingly, we observed a non-monotonic age-related trajectory characterized by an age-related increase until 80 years old, and by a slight decrease in subjects older than 80 years. Non monotonic changes similar to those observed for c-miR-21 were reported in different physiological parameters, such as blood glucose, diastolic blood pressure, serum cholesterol and body mass index (BMI) among others (Arbeev et al., 2011). Centenarians showed c-miR-21-5p levels lower than those of healthy subjects of 80 years old, suggesting that low levels of c-miR-21-5p are beneficial for longevity. This hypothesis was reinforced by results obtained in samples of patients affected by different ARDs, such as cancers and cardiovascular diseases (CVD). C-miR-21-5p levels were significantly increased in patients affected by acute myocardial infarction compared with age-matched healthy subjects (Zhang et al., 2016; Wang et al., 2014; Olivieri et al., 2014b). Significant increased levels of c-miR-21 have also been reported in patients affected by different types of cancers (Gao et al., 2016).

On the contrary, reduced levels of miR-21 in plasma and circulating cells were observed in patients affected by metabolic syndrome, T2DM and obesity (He et al., 2016; Zampetaki et al., 2010; Olivieri et al., 2015b; Mazloom et al., 2016). The reduced expression of miR-21 in circulating cells of obese subjects appears to be associated with an increased secretion of pro-inflammatory cytokines, and likely, it suggests a similar role in centenarians, who are also characterized by anti-inflammatory balancing processes (Franceschi et al., 2007).

Overall, the concordance of the results obtained by a number of independent groups, confirms that c-miR-21-5p levels measured in patients affected by the most common ARDs are different from those observed in healthy subjects of the same age. Therefore c-miR-21-5p could be a promising candidate as biomarker of deviation from the healthy trajectory (as illustrated in Fig. 2), especially at old age, when the effects of adaptation and remodeling strongly interact with the selective effects due to mortality (Arbeev et al., 2016).

Since in the majority of the studies related to c-miR-21-5p levels were measured with relative expression method, that allows to obtain values expressed in arbitrary units, is quite difficult to identify an age-tailored threshold value to discriminate a "physiological aging" from a "pathological aging". Further studies on largest samples of healthy subjects and with standardized methods for miR-21-5p detection, will allow to identify the age-tailored reference range of normal c-miR-21 levels.

Additionally, mounting evidence has been confirming that c-miR levels have the potential to monitor the efficacy of therapeutic interventions. Dynamic changes in their trends have suggested that some miRs are able to discriminate between the types of response to

antitumor therapy (Ponomaryova et al., 2016; Chen et al., 2016) and postmenopausal estrogen-based hormone replacement therapy (Kangas et al., 2014).

It has recently been demonstrated that the levels of miR-140-5p and miR-650, which are markers for a wide range of human conditions, depend significantly on physical exercise training mode (Hecksteden et al., 2016). Therefore, if exercise is an important confounding factor for miR-based disease diagnosis, c-miRs modulated by training could prove useful to monitor rehabilitation compliance.

Altogether, a greater understanding of c-miR changes occurring during aging and of the conditions that affect them is needed before such novel markers can be applied in clinical practice.

4. Perspective contribution of circulating miR-extracellular vesicles to aging trajectories

Albeit conventional cell–cell communication modes depend mostly on extracellular ligand concentration and cell-surface receptor availability, the magnitude of circulating miR-mediated signaling responses relies on capture and uptake by target cells and their antigens/receptors. MiRs are functional after the uptake by target cells. These phenomena suggest that epigenetic modifications may be induced by a highly regulated compartment of circulating miRs (García-Olmo et al., 2010). Serum miRs are a pool of RNA molecules that are likely to be associated with different circulating carriers, such as exosomes and ectosomes (Cocucci and Meldolesi, 2015). Exosomes are small (30–120 nm in diameter), membrane vesicles that originate through the endocytic pathway, *via* the multivesicular bodies of the endosomal compartment (Simpson et al., 2008, 2009; Mathivanan et al., 2010). At variance, ectosomes are microvesicles that directly bud from the cellular plasma membrane and have an irregular shape and variable size (100–1000 μm). The study of exosomes and ectosomes, collectively called extracellular vesicles (EVs), has disclosed not only a different proteomic cargo, but also different biological characteristics (Choi et al., 2015). Besides their role in c-miR transport, they also carry a variety of bioactive molecules (*e.g.* cytokines, proteins, DNA) (Kim et al., 2013; Kahlert et al., 2014), thus providing an attractive research field for diagnostic and therapeutic applications (Thakur et al., 2014).

EVs act both on adjacent acceptor cells and on cells lying at considerably greater distance *via* the circulatory and lymphatic system. Such communication system provides control of cell development, cell fate, and tissue homeostasis, likely protecting the functional state of biological systems and affecting the chance of achieving longevity or developing ARDs. EVs may also mediate the extracellular spread of misfolded/pathogenic or garbage proteins in case of lysosome malfunction (Eitan et al., 2016) as well as viral or bacterial proteins and nucleic acids. EV-shuttled molecular communication undergoes major age-related modifications due to factors such as the increase of senescent cells within tissue and organs (Weilner et al., 2013). C-miRs can also be carried by protein complexes (containing Argonaute, or AGO1-2, proteins), nucleophosmin and lipoproteins (*e.g.* HDL) (Turchinovich et al., 2011; Turchinovich and Burwinkel, 2012; Wagner et al., 2013; Weilner et al., 2013). Even though some miR-protein associations may occur in a non-specific way

(adsorption, entrapment), and some may reflect passive release from dead or damaged cells rather than active secretion, these data lead to hypothesize the existence of a multilevel system of communication and regulation.

In vitro studies have found that miRs released by senescent cells *via* EVs into the extracellular environment induce senescence in surrounding cells. In particular, cells expressing high miR-433 levels released it into growth media within EVs and induced cell senescence and chemoresistance in ovarian cancer cells (Weiner-Gorzel et al., 2015). Thus, miR-433 modulates the tumor microenvironment by inhibiting growth and inducing senescence in nearby cells. On the other hand, miRs released within EVs are also able to suppress senescence in recipient cells. For instance, miR-214, which controls endothelial cell function and angiogenesis, plays a dominant role in vesicle-mediated signaling between endothelial cells (Van Balkom et al., 2013); indeed, endothelial cell derived vesicles stimulated migration and angiogenesis in recipient cells, preventing senescence, whereas vesicles from miR-214-depleted endothelial cells failed to stimulate this process. According to a recent study (Weilner et al., 2016), EVs isolated from young donors enhance osteoblastogenesis *in vitro* compared with those from elderly subjects; in particular, reduced galectin-3 levels in the latter vesicles correlated with an altered osteoinduction potential.

In another study, miR microarray analysis of salivary exosomes from 15 young healthy individuals and 13 old subjects has identified miR-24-3p as a novel candidate biomarker of aging (Machida et al., 2015).

Since circulating EVs conceivably arise from all tissues and organs, they may provide a circulating representation of successful aging that may be difficult to obtain when the single tissue is examined. It can be expected that the assessment of their “normal levels” and the identification of the subpopulation(s) or EVs in the liquid biopsy will be relevant to provide reliable estimates of the miRs trajectories in healthy and unhealthy aging.

5. Challenges and perspectives

C-miRs have great potential as biomarkers in aging and ARD management. Moreover, miRs showing dysregulation in pathological conditions are emerging as novel tools that could be harnessed in therapeutic interventions against a broad array of cellular targets. Numerous preclinical and clinical trials are testing their effect on a variety of targets including cancer, CVD, and virus infection and proliferation. The challenges that need to be addressed before miRs can be used as biomarkers, and ultimately as therapeutic targets, must clearly be recognized. First and foremost, c-miR preparation and measurement should be standardized. Even though most studies have failed to consider the influence of factors like serum and plasma preparation, generalizing findings from patients of different age and sex and from different groups or labs (Sato et al., 2009) requires standardization of sample preparation. This is a precondition to reduce lab-to-lab variations and maximize the prospects for clinical use. Accurate determination of c-miR levels in a given cell type or body fluid is crucial to establish miRs as diagnostic biomarkers. Furthermore, standardization of data processing and analytical methods, including normalization, is critical to minimize technical variation and maximize the reliability of experimental outcomes (Meyer et al., 2010; Witwer, 2015).

6. Conclusions

Research of aging biomarkers and attempts to harness them to carry out personalized therapeutic interventions and monitor their efficacy are at the core of several European projects (Capri et al., 2015). At present, no biomarker alone can monitor healthy or unhealthy aging, even though recent data emphasize the value of molecular and DNA/RNA-based biomarkers. The study of c-miRs and their shuttles as biomarkers and functional modulators of aging-related pathways is generating interesting data. Among the panels of candidate miRs being proposed to assess the healthy aging trajectory, miR-21-5p appears to be one of the most powerful, given its different levels in subjects of different age with and without ARDs. Even though longitudinal cohort studies would clarify the significance of c-miR changes and better establish their predictive value, cross-sectional studies and maybe the combination of both experimental designs could be a suitable approach for miR-biomarker identification, as well as prognostic and diagnostic assessment, thus favoring translational medicine applications.

Altogether, current data suggest that different c-miRs have the ability to identify subjects experiencing healthy or unhealthy aging conditions, such as patients with CVD, T2DM, cancer, and neurodegenerative diseases. C-miRs may display non-monotonic, U–shaped trends suggesting that prediction of deviation from the healthy trajectory may be very complex. Future studies should elucidate how miRs can be better applied, not only as biomarkers of aging rate and disease, but also as predictive, diagnostic, and eventually therapeutic approaches for gene expression regulation. The ability to predict ARD onset using c-miRs, also in combination with other molecules, would be a great scientific achievement. Another critical objective is to harness the exosome transport system to the modulation of tissue-specific molecules and deliver therapeutic interventions, a goal that requires reliable identification of EV origin and a comprehensive understanding of organ cross-talk. Achievement of these objectives would involve a huge number of patients with a broad spectrum of diseases.

Acknowledgments

Funds were received from FARB2-2014-University of Bologna (project RFBO120790) to MC, from FP7 European Commission–grant agreement no. 602757 “HUMAN”-, and from Università Politecnica delle Marche, RSA 2015–2016 to FO.

We are grateful to Dr. Marzio Marcellini for his assistance with the preparation of Fig. 1. The authors are grateful to Word Designs for the language revision (www.silviamodena.com).

Abbreviations

ARDs	age-related diseases
CVD	cardiovascular disease
EVs	extracellular vesicles
miRs	microRNAs
SASP	senescence-associated secretory phenotype

T2DM type 2 diabetes

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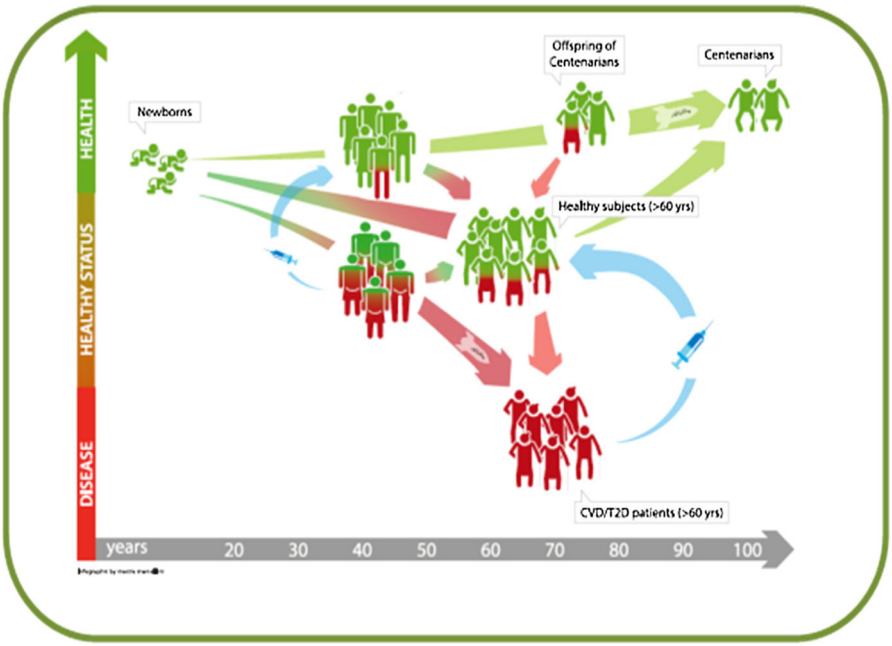

Fig. 1.
Trend of a hypothetical biomarker of aging: the deviation from a healthy (green) to a non-healthy (red) aging trajectory could be reversed by early disease prediction and personalized treatment (blue arrows). At present, the deviation toward T2DM and CVD cannot be reversed in late disease stages, but it is conceivable that in the future these patients could be recovered to a healthy trajectory. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

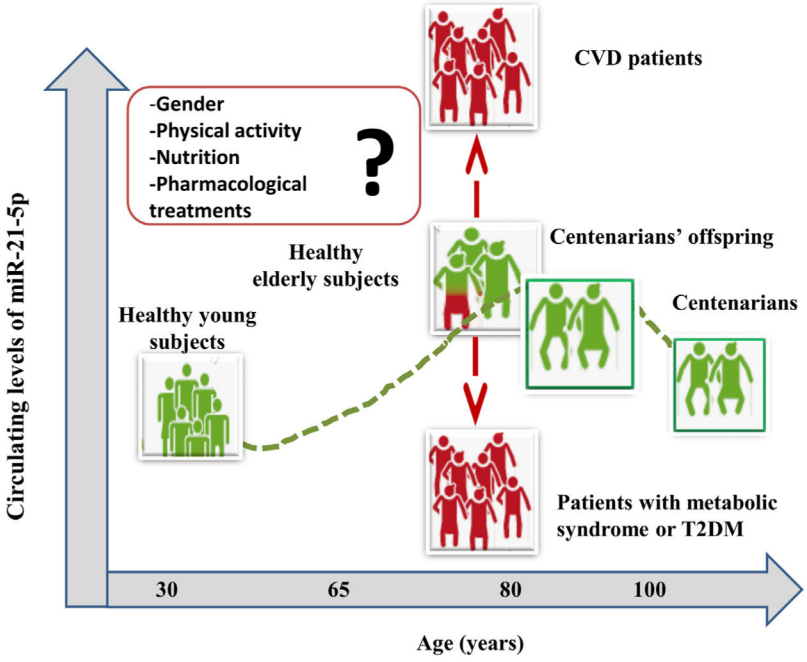

Fig. 2.
Trend of circulating miR-21-5p levels: the deviation from a healthy (green) to a non-healthy (red) condition can be monitored by circulating miR-21-5p levels. The figure presents circulating miR-21-5p levels in healthy subjects of different age and in groups of patients suffering from the most common age-related diseases (metabolic syndrome, T2DM, CVD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

a. Circulating miRs in centenarians. b. Circulating miRs in human aging.

Reference (a)	Biological sample	Profiling methods and donors	Validation methods and donors	miRs	Changes in centenarians	Additional data
ElSharawy et al. (2012)	whole blood	Method: Geniom Biochip miRNA, with quantile normalization Donors: 15 centenarians/nonagenarians (96.4y) and 55 young (45.9y)	Method: RT-qPCR: technical validation and validation on independent samples, normalized to RNU6B Donors: 15 centenarians/ nonagenarians and 14–17–18 young controls	miR-106a	down	
				miR-126	*vs* young	
				miR-20a		
				miR-144*		
				miR-18a		
				miR-320d	up	
				miR-320b	*vs* young	
Gombar et al. (2012)	EBV transformed immortalized B-cells	Method: Illumina sequencing (GA1), normalized to the total reads in each sample Donors: Ashkenazi Jewish female, 3 centenarians and 3 old (63y)	Method: RT-qPCR, normalized to U6 snRNA Donors: same subjects analyzed in the profiling	miR-363*	up	miR-363* down-regulates during aging (three groups: 50–60, 70–80, 80–90 y)
				miR-1974	*vs* old	
				miR-223*		
				miR-148a		
				miR-148a*		
Olivieri et al. (2012)	plasma	Method: MicroRNA Array pool A (Applied Biosystems), data normalized using the median Donors: 3 centenarians, 4 old (80y) and 4 young (20y)	Method: RT-qPCR, normalized to miR-17-5p, and absolute quantification, determined by diluting synthetic miR-21 Donors: 111 subjects (20–105y)	miR-21	down *vs* old (66–95y); up *vs* young (< 65y)	
Serna et al. (2012)	Mononuclear cells	Method: GeneChip Array (Affymetrix), normalized with RMA algorithm Donors: 20 centenarians, 16 old (80y), 14 young (30y)	Method: RT-qPCR, normalized to RNU66 and RPLPO Donors: same subjects analyzed in the profiling	miR-21	up	miR-19b down-regulates in old (80 y)
				miR-130a	*vs* old and young groups	
Reference (b)	Biological samples	Profiling methods and donors	Validation methods and donors	miRs	Changes with age	Additional data
Noren Hooten et al. (2010)	PBMCs	Method: miRnome Array (System Biosciences), normalized to U1 expression Donors: 2 young (30y) and 2 old (64y)	Method: RT-qPCR, normalized to the average of 3 stable miRs (miR-147, miR-574-3p, miR-1469) Donors: 14 young and 14 old	miR-103 miR-107	down	
				miR-128 miR-130a		
				miR-155 miR-24		
				mIR-221 miR-496		
				miR-1538		

Reference (a)	Biological sample	Profiling methods and donors	Validation methods and donors	miRs	Changes in centenarians	Additional data
Noren Hooten et al. (2013)	serum	Method: Illumina small-RNA seq, analyzed with miRDeep software Donors: 11 young (30y) and 11 old (64y)	Method: RT-qPCR, normalized to miR-191 Donors: 20 young and 20 old	miR-151a-3p	down	
				miR-181a-5p		
				miR-1248		
Meder et al. (2014)	blood	Method: Geniom Biochip miRNA, with quantile normalization Donors: 109 subjects (19–105y) Method: Illumina small-RNA seq (HiSeq 2000), analyzed with miRDeep softwar Donors: 58 subjects (44–75y)		miR-1284	up	positive correlation with age
				miR-93-3p		
				miR-1262		
				miR-34a-5p		
				miR-145-5p		
Olivieri et al. (2014a)	plasma		Method: RT-qPCR, normalized to cel-miR-39 Donors: 44 young (20–45y), 57 elderly (46–75y), 35 old (≥75y)	miR-126-3p	up	
Zhang et al. (2015)	serum	Method: Solexa sequencing, total copy number of each sample was normalized to 100,000 Donors: pool serum from four different ages groups (22y, 40y, 59y, 70y)	Method: RT-qPCR, absolute quantification Donors: 31 subjects (22y), 31 subjects (40y), 30 subjects (59y), 31 subjects (70y)	miR-92a	up	Only miR-29b and miR-92a were significantly different for all four aging groups
				miR-222		
				miR-375		
				miR-29b	down	
				miR-106b		
				miR-130b		
				miR-142-5p		
				miR-340		
Ameling et al. (2015)	plasma	Method: Exiqon Serum/Plasma Focus microRNA PCR Panel, normalized to the lower quartile per sample Donors: 372 donors (22–79y)		miR-126-3p	up	Additional variables (gender, BMI)
				miR-21-5p		
				miR-30b-5p		
				miR-30c-5p		
				miR-142-3p		
				let-7a-5p		
				miR-93-5p	down	