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Circulating miR-146a in healthy aging and type 2 diabetes: Age- and gender-specific trajectories

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ABSTRACT

To evaluate the combined effect of age and glycemic state on circulating levels of the inflamma-miR-146a levels, 188 healthy subjects (CTR) aged 20–104 years and 144 type-2 diabetic patients (T2DM), aged 40-80 years, were analyzed. In CTR subjects, miR-146a levels showed a significant age-related decline. When a gender-stratified analysis was ran, the miR-146a age-related trajectory was confirmed only in men and a negative correlation with PAI-1, uric acid, and creatinine was also observed. In women, miR-146a circulating levels showed negative correlations with azotemia, uric acid, waist/hip ratio and ferritin. A significant miR-146a decline with aging was also observed in T2DM patients. Significant positive correlations were found between miR-146a in diabetic patients and total cholesterol, LDL-C, ApoA1, ApoB, and platelets, and negative correlations with serum iron and ferritin. Notably, miR-146a was significantly overexpressed in T2DM patients treated with metformin. MiR-146a levels were significantly lower in diabetic patients than in age-matched CTR and negatively correlated to both fasting glucose and HbA1c in males. Finally, age-related trajectories for circulating miR-146a levels showed an inverted U-shaped relationship; however, in T2DM patients the trajectory was significantly shifted towards lower levels. Our findings support the hypothesis that miR-146a could be a functional biomarker of healthy/unhealthy aging.

1. Introduction

Aging is one of the main risk factors for the most common age-related diseases (ARDs), including cardiovascular diseases (CVDs), type 2 diabetes mellitus (T2DM), neurodegenerative diseases and cancer (Kennedy et al., 2014). ARDs, in turn, accelerate the aging process suggesting that aging and ARDs share a common set of basic biological mechanisms. (Franceschi et al., 2018; Lopez-Otin et al., 2013; Shakeri et al., 2018; Wang and Bennett, 2012). Several studies have reported that the pro-inflammatory NF-κB pathway, which is involved in orchestrating the defense of the host against a variety of endogenous and exogenous stimuli, plays a causative role in aging process (Salminen et al., 2008). Chronic inflammation induced by the sustained activation of NF-κB can have detrimental effects in humans, promoting cellular senescence characterized by the acquisition of a senescence-associated secretory phenotype (SASP) and the aging-related degenerative processes (Chien et al., 2011). SASP is characterized by the secretion of a plethora of cytokines, growth factors and metalloproteinases, which can induce senescence or even tumorigenesis in neighboring cells (Coppe et al., 2010; Tasdemir and Lowe, 2013). The increased burden of senescent cells during aging contribute to spread pro-inflammatory molecules at systemic level, thus contributing to fuel inflammaging and promoting ARDs development and progression (Fulop et al., 2018). Both *in vitro* and *in vivo* studies extensively reported that a diabetic microenvironment promotes premature cellular senescence, through self-propagating, SASP-mediated mechanisms (Chang et al., 2015;

Abbreviations: ARD, age-related disease; CTR, control subject; CVD, cardiovascular disease; EV, extracellular vesicle; IRAK1, Interleukin-1 receptor-associated kinase 1; MACE, major adverse cardiovascular event; miRNA/miR, microRNA; PBMC, peripheral blood mononucleated cell; SASP, senescence-associated secretory phenotype; TRAF6, TNF receptor-associated factor 6; T2DM, type-2 diabetes mellitus

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Table 1
Biochemical and anthropometric characteristics of 188 healthy control subjects (CTR) divided into four groups according to age.

Variables	≤55 yrs N = 32	56-65 yrs N = 61	66-75 yrs N = 50	> 75 yrs N = 45	p-value
Age (years)	39.1 (8.4)	62.0 (2.3)	69.9 (2.8)	84.5 (7.1)	< 0.001
Gender (Males)	17	27	23	25	0.638
BMI (Kg/m^2)	25.0 (4.7)	27.9 (3.5)*	27.0 (3.6)	25.3 (3.3)#	0.004
Weight (Kg)	74.4 (15.5)	75.2 (12.4)	72.3 (10.6)	64.6 (9.5)#	0.016
Waist-hip ratio	0.84 (0.08)	0.89 (0.08)*	0.90 (0.06)*	0.91 (0.06)*	0.004
Total cholesterol (mg/dL)	202.9 (33.8)	225.1 (41.8)	220.1 (36.4)	206.3 (42.4)	0.050
HDL-C (mg/dL)	56.5 (13.9)	56.1 (13.3)	62.6 (16.2)	50.9 (17.4)°	0.016
Tryglicerides (mg/dL)	84.4 (53.7)	132.3 (109.8)*	102.1 (52.5)	107.1 (43.3)	0.047
ApoA1 (mg/dL)	172.0 (32.1)	177.7 (30.0)	182.2 (39.0)	165.0 (37.0)	0.283
ApoB (mg/dL)	102.3 (36.2)	108.5 (27.6)	106.5 (34.4)	98.2 (24.0)	0.610
Glucose (mg/dL)	88.7 (7.2)	96.7 (9.4)*	95.6 (9.3)*	89.2 (8.0)#,°	< 0.001
HbA1C (%)	5.5 (0.4)	5.8 (0.4)*	5.8 (0.4)*,	5.6 (0.4)	0.015
Insulin (UI/mL)	5.9 (4.5)	5.9 (3.3)	5.9 (8.0)	5.7 (3.0)	0.999
HOMA index	1.29 (0.96)	1.39 (0.85)	1.44 (2.30)	1.30 (0.70)	0.971
WBC (n/mm^3)	6.4 (1.7)	6.3 (1.6)	6.0 (1.6)	6.0 (1.5)	0.692
Platelets (n/mm^3)	246.2 (52.7)	227.6 (65.5)	223.7 (62.6)	225.6 (80.6)	0.511
Hs-CRP (mg/L)	2.3 (3.9)	2.8 (3.2)	3.0 (2.6)	3.5 (3.0)	0.595
PAI-1 (ng/mL)	17.8 (8.5)	22.5 (12.3)	17.9 (8.4)	26.1 (14.9)°	0.014
Fibrinogen (mg/dL)	252.5 (67.7)	300.8 (68.8)	303.4 (73.6)	378.2 (138.0)*	0.002
Iron (μg/dL)	85.4 (32.7)	81.3 (28.5)	78.0 (20.6)	83.5 (31.9)	0.700
Ferritin (ng/mL)	74.8 (79.4)	113.2 (74.8)	117.1 (92.3)	96.2 (70.4)	0.130
Creatinine (mg/dL)	0.84 (0.19)	0.82 (0.17)	0.88 (0.22)	0.97 (0.43)	0.113
Azotemia (mg/dL)	34.0 (8.7)	38.1 (8.1)	40.8 (9.5)*	41.7 (10.6)*	0.007
Uric acid (mg/dL)	4.4 (1.5)	4.9 (1.1)	5.0 (1.3)	5.3 (1.6)	0.143
Telomere lenght (T/S, a.u.)	0.55 (0.21)	0.45 (0.14)	0.49 (0.19)	0.46 (0.16)	0.073
MiR-146a (rel. expression a.u.)	5.76 (4.75)	5.87 (4.34)	4.97 (4.29)	2.33 (2.92)*,#,°	< 0.001

Variables are expressed as mean (standard deviation). P value from ANOVA for continuous variables and from chi-squared tests of association for categorical variables.

* $p < 0.05$ vs. [≤55 yrs].

\# $p < 0.05$ vs. [56–65 yrs].

° $p < 0.05$ vs. [66–75 yrs] from Bonferroni multiple comparison test.

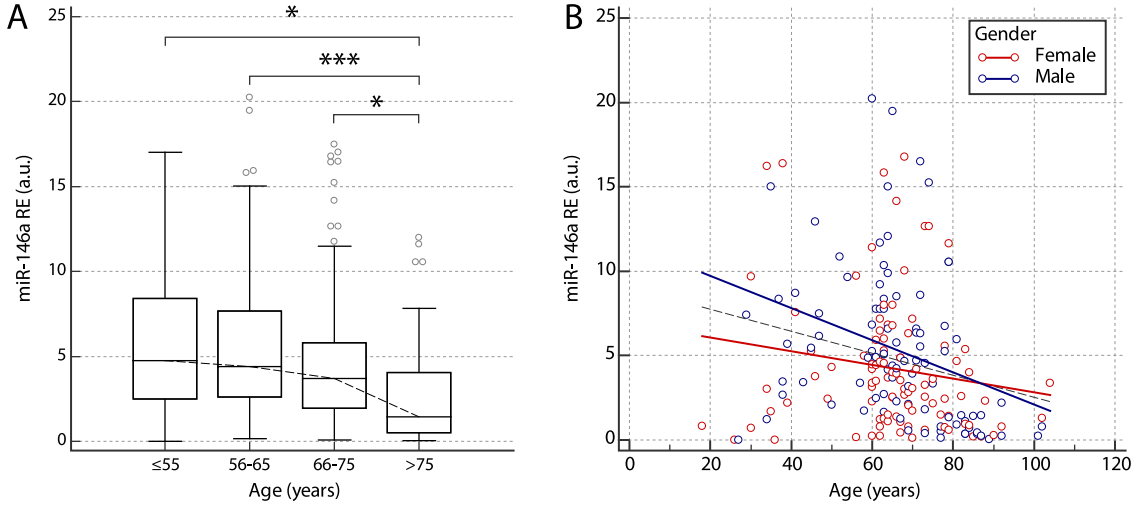

Fig. 1. Relative miR-146a expression in plasma from 188 healthy subjects (CTR). (A) Box-plot showing relative miR-146a expression (in a.u.) in plasma of CTR subjects, divided into four subgroups (≤55 years, n = 32; 56–65 years, n = 61; 66–75 years, n = 50; > 75 years, n = 45). * $p < 0.05$, *** $p < 0.001$ from *t*-test with Bonferroni correction for multiple comparisons. (B) Scatter plot showing relative miR-146a expression levels (in arbitrary units, a.u.) stratified by age and gender in CTR subjects. Data are expressed as mean of $2^{-\Delta\Delta Ct}$ normalized with cel-miR-39. Regression lines are displayed for males (solid blue, n = 92, R= -0.32, p = 0.002), females (solid red, n = 96, R= -0.16, p = 0.129) and all CTR subjects (dotted black, n = 188, R= -0.23, p = 0.001) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Kitada et al., 2014; Prattichizzo et al., 2018a). We previously reported specific age-related trends of circulating biomarkers associated to cellular senescence, such as Beta-galactosidase activity and leukocyte telomere length, in healthy subjects and in T2DM patients (Spazzafumo et al., 2017; Testa et al., 2011).

Also microRNAs (miRNAs), which are short noncoding single-stranded RNA that can modulate gene expression primarily at the post-transcriptional level, have been extensively investigated in relation to the aging process and ARDs, including T2DM (Huntzinger and Izaurralde, 2011). Given their ability to affect a broad range of targets, miRNAs can potentially be involved in every cellular process (Bayraktar et al., 2017; Tkach and Thery, 2016). Moreover, given that they are secreted by extracellular vesicles (EVs) and lipoproteins or transported by specific RNA binding proteins, miRNAs can participate in cell-to-cell communication (Turchinovich et al., 2011; Zhang et al., 2015). The evidence that miRNAs are present not only at the intracellular level but also in a very stable circulating form in many biological fluids has opened the field to their potential application as diagnostic/prognostic

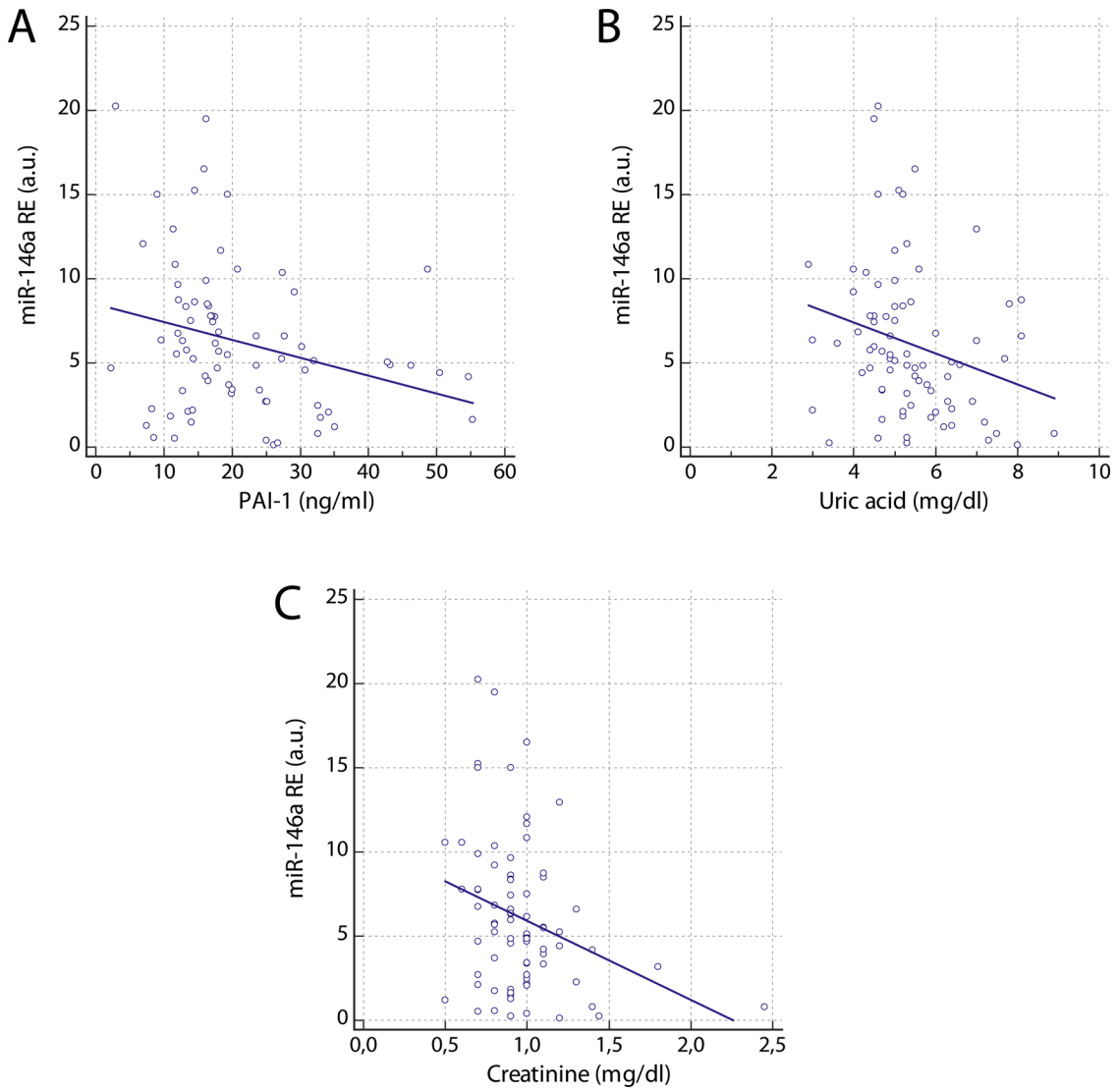

Fig. 2. Correlations between circulating miR-146a levels and selected biochemical variables in male CTRs. Scatter plots showing the relationship between miR-146a plasma levels and (A) PAI-1, (B) uric acid, (C) creatinine in 92 male CTR subjects. Data are expressed as mean of $2^{-\Delta\Delta Ct}$ normalized to cel-miR-39. Regression lines are displayed in solid blue (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

biomarkers for a number of diseases. A large body of evidence suggest that several miRNAs are modulated during human aging and many of them can modulate target genes involved in age-related pathways (Huan et al., 2018; Noren Hooten et al., 2013; Olivieri et al., 2012). MiR-146a is one of the first miRNAs reported to restrain NF-κB activation and down-regulate IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) (Taganov et al., 2006). Specifically, miR-146a appears to reduce NF-κB DNA binding activity, thereby restraining the transcription of SASP-related genes (Bhaumik et al., 2009). The increase in the expression of miR-146a in the inflammatory process and in cellular senescence might be the result of a negative-feedback loop able to avoid excessive production of proinflammatory cytokines (Olivieri et al., 2013a). MiR-146a has been named *inflamma-miR* because of its ability to fine tune NF-κB signaling (Olivieri et al., 2013b).

The modulation of miR-146a in aging and ARDs has been investigated in both cellular and animal models, as well as in patients affected by cardiovascular diseases (Olivieri et al., 2013a), neurodegenerative disorders (Alexandrov et al., 2014) and autoimmune diseases (Zhou et al., 2015). It has also been suggested that miR-146a exerts a tumor-suppressive effect, preventing malignant transformation (Boldin et al., 2011; Zhao et al., 2011). Although many studies have investigated the association between circulating miR-146a expression levels and T2DM, their role in T2DM is still controversial. Although the expression levels of miR-146a were lower in peripheral blood mononuclear cells (PBMC) of T2DM patients compared to nondiabetic control subjects (Corral-Fernandez et al., 2013), other studies showed contradictory results in plasma or serum (Baldeon et al., 2014; Rong et al., 2013). The complex effect of hyperglycemia on different blood cell types, as well as the impact of different treatments and the effect of complications on the natural history of the disease are likely the main reasons for the contradictory findings. Moreover, to the best of our knowledge, there are no studies that have evaluated the impact that aging might have on circulating miR-146a levels in healthy subjects. Therefore, to disentangle the complex relationship between age, hyperglycemia, and miR-146a circulating levels, in the present study we investigated the levels of miR-146a plasma levels in healthy subjects and in T2DM patients at different ages and in both genders.

2. Materials and methods

2.1. Study population

One hundred eighty-eight (188) healthy subjects (CTRs) and 144

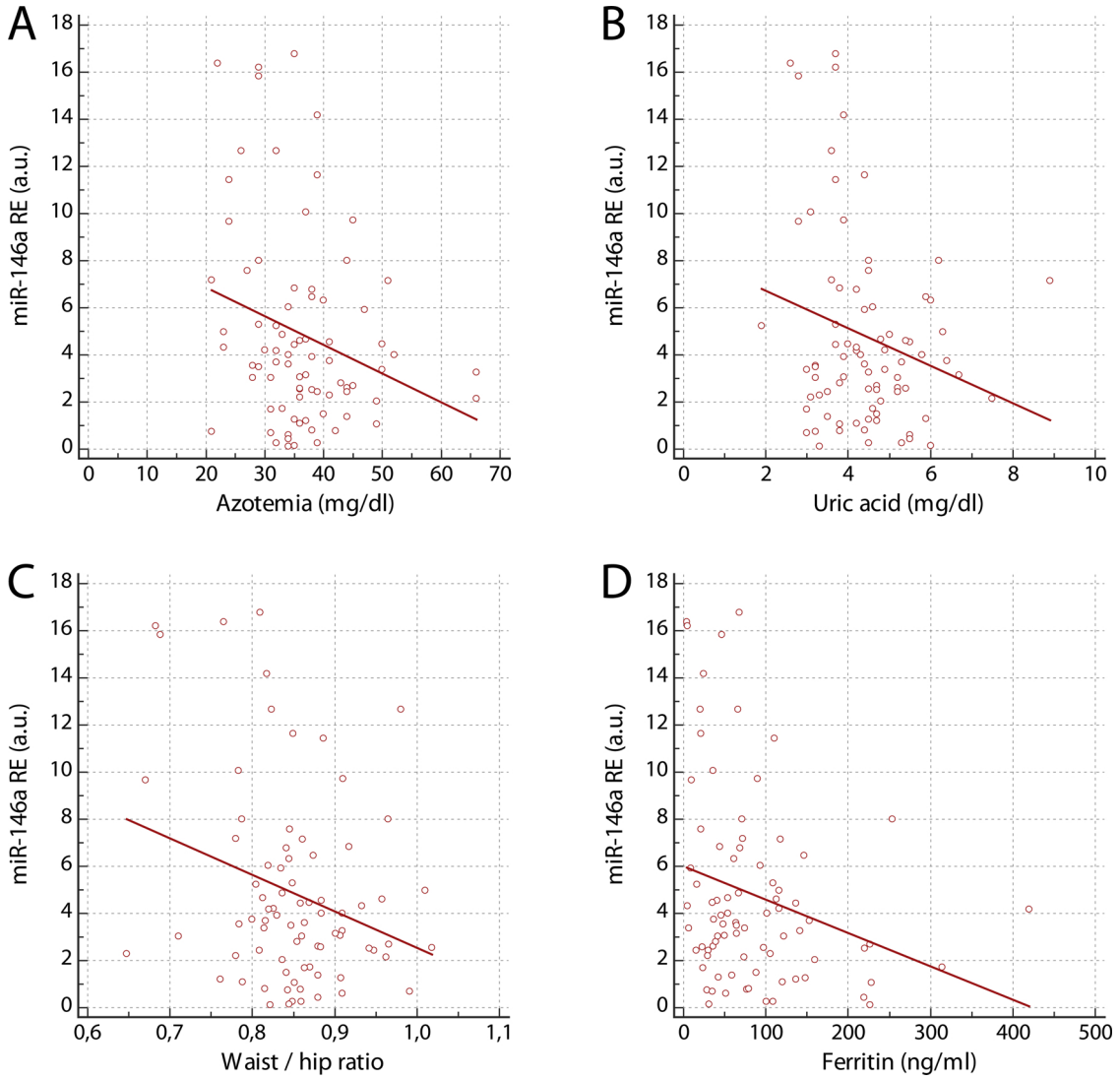

Fig. 3. Correlations between circulating miR-146a levels and selected biochemical variables in female CTRs. Scatter plots showing the relationship between miR-146a plasma levels and (A) azotemia, (B) uric acid, (C) waist/hip ratio, (D) ferritin in 92 male CTR subjects. Data are expressed as mean of $2^{-\Delta\Delta Ct}$ normalized to cel-miR-39. Regression lines are displayed in solid red (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

T2DM patients were recruited from the Italian National Research Center on Aging (INRCA), Ancona. The Institutional Review Board of INRCA approved the study protocol and all enrolled subjects provided a written informed consent. The inclusion criteria and the clinical information collected from each subject were as described in Testa et al. (Testa et al., 2011).

The presence/absence of diabetic complications was established as follows:

- retinopathy was defined as dilated pupils detected on fundoscopy and/or fluorescence angiography;
- incipient nephropathy was defined as a urinary albumin excretion rate > 30 mg / 24 h and normal creatinine clearance;
- chronic renal failure was defined as an estimated glomerular filtration rate < 60 mL/min per 1.73 m^2;
- neuropathy was established by electromyography;
- ischemic heart disease was diagnosed by clinical history and/or ischaemic electrocardiographic alterations; these patients had ST- or non-ST-elevation myocardial infarction, which was defined as a major adverse cardiac event (MACE). Mean time from the MACE was 9 ± 8 years;
- peripheral vascular disease, including arteriosclerosis obliterans and cerebrovascular disease, was diagnosed based on history, physical examination, and Doppler imaging.

Among the 144 T2DM patients, no complication was documented in 73 patients, whereas at least one complication was found in 71 subjects, specifically neuropathy (n = 22), MACE (n = 39), nephropathy (n = 15), chronic renal failure (n = 6), retinopathy (n = 49), lower limb arteriopathy (n = 6). Each patient could have more than one complication. At the time of enrollment, 61 patients were treated with metformin, 84 with sulfonylurea, 3 with glinides, 29 with insulin. Some patients were treated with more than one medication.

2.2. Laboratory assays

Total white blood cells, monocyte and platelet counts were performed by standard automated procedures. Serum concentrations of HbA1c, fasting insulin, fibrinogen, apolipoprotein A–I and B, total and HDL cholesterol, triglycerides, creatinine, fasting glucose and highly sensitive C-reactive protein were measured by standard procedures in all subjects.

Table 2
Biochemical and anthropometric characteristics of 144 patients with type 2 diabetes mellitus (T2DM) divided into four groups according to age.

Variables	≤55 yrs N = 13	56-65 yrs N = 38	66-75 yrs N = 77	> 75 yrs N = 16	p-value
Age (years)	49.8 (3.5)	61.6 (2.6)*	70.1 (2.8)*,#	77.4 (1.5)*,#,°	< 0.001
Gender (Males)	9	15	34	8	0.303
Disease duration (years)	22.7 (5.3)	26.1 (9.5)	33.1 (12.3)*,#	42.5 (14.9)*,#,°	< 0.001
BMI (Kg/m^2)	27.6 (3.0)	28.1 (4.2)	28.4 (3.8)	26.4 (3.4)	0.278
Weight (Kg)	78.1 (12.8)	75.1 (13.2)	75.5 (11.3)	68.8 (10.1)	0.147
Waist-hip ratio	0.91 (0.06)	0.93 (0.09)	0.92 (0.07)	0.93 (0.06)	0.768
Total cholesterol (mg/dL)	193.3 (45.2)	220.6 (38.1)	212.9 (35.4)	205.9 (33.2)	0.122
HDL-C (mg/dL)	47.8 (10.0)	56.0 (19.8)	54.9 (15.6)	57.7 (19.0)	0.405
Tryglicerides (mg/dL)	118.2 (75.2)	154.7 (135.5)	131.1 (83.5)	143.0 (141.6)	0.626
ApoA1 (mg/dL)	154.8 (28.7)	168.2 (37.3)	171.4 (36.5)	179.4 (45.2)	0.340
ApoB (mg/dL)	102.4 (34.9)	105.5 (27.2)	109.2 (26.5)	99.6 (27.3)	0.563
Glucose (mg/dL)	139.7 (25.4)	172.1 (53.8)	165.3 (44.7)	175.0 (49.9)	0.149
HbA1C (%)	7.4 (0.9)	7.8 (1.3)	7.4 (1.1)	7.5 (1.4)	0.519
Insulin (UI/mL)	5.8 (3.6)	7.0 (4.3)	6.2 (3.7)	8.0 (9.8)	0.497
HOMA index	1.94 (1.10)	3.07 (2.51)	2.65 (2.14)	3.65 (4.64)	0.275
WBC (n/mm^3)	6.9 (1.6)	7.0 (1.9)	6.6 (1.4)	6.4 (1.2)	0.372
Platelets (n/mm^3)	201.3 (45.8)	216.9 (43.6)	219.3 (65.4)	210.3 (52.1)	0.737
Hs-CRP (mg/L)	2.6 (2.4)	4.5 (4.4)	4.0 (4.7)	3.5 (2.5)	0.552
PAI-1 (ng/mL)	17.1 (7.8)	21.3 (7.4)	19.7 (9.6)	21.3 (10.5)	0.465
Fibrinogen (mg/dL)	271.3 (60.8)	294.4 (75.6)	297.1 (83.4)	305.0 (54.1)	0.679
Iron (μg/dL)	81.2 (27.0)	80.1 (31.2)	80.5 (21.4)	90.3 (39.7)	0.596
Ferritin (ng/mL)	128.9 (93.3)	140.7 (190.2)	111.8 (94.5)	106.6 (105.7)	0.671
Creatinine (mg/dL)	0.81 (0.18)	0.84 (0.19)	0.94 (0.40)	1.10 (0.51)	0.061
Azotemia (mg/dL)	39.1 (9.1)	36.7 (8.7)	43.2 (15.2)	48.1 (17.2)#	0.021
Uric acid (mg/dL)	4.6 (0.7)	4.6 (1.1)	4.7 (1.2)	5.2 (1.7)	0.293
Telomere lenght (T/S, a.u.)	0.48 (0.16)	0.45 (0.19)	0.49 (0.23)	0.35 (0.14)	0.092
MiR-146a (rel. expression a.u.)	5.94 (4.29)	4.76 (3.59)	4.54 (3.86)	3.58 (3.14)	0.409

Variables are expressed as mean (standard deviation). P value from ANOVA for continuous variables and from chi-squared tests of association for categorical variables.

* $p < 0.05$ vs. [≤55 yrs].

# $p < 0.05$ vs. [56–65 yrs].

° $p < 0.05$ vs. [66–75 yrs] from Bonferroni multiple comparison test.

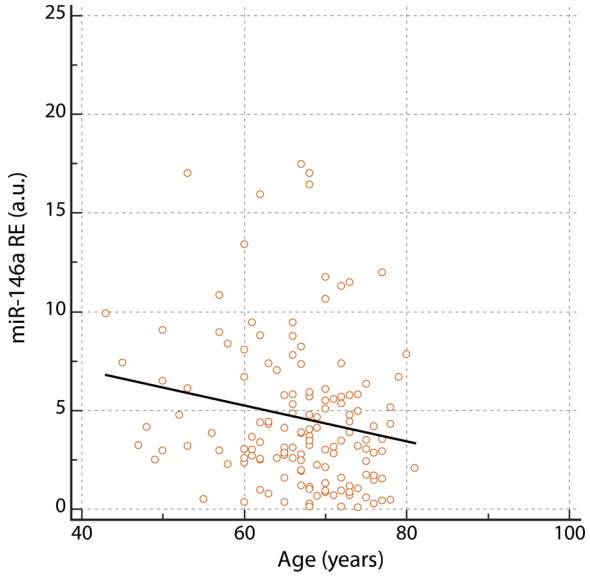

Fig. 4. Relative miR-146a expression in plasma from 144 T2DM patients. Scatter plot showing relative miR-146a expression (in arbitrary units, a.u.) by the age of T2DM patients. Data are expressed as mean of $2^{-\Delta\Delta Ct}$ normalized to cel-miR-39. Regression line is displayed for all patients (n = 144, R = -0.19, p = 0.026).

2.3. RNA isolation and quantification by RT q-PCR

Total RNA was extracted from plasma and miR-146a was analyzed as described in Olivieri et al. (Olivieri et al., 2014). The plasma levels of circulating miR-146a are reported as relative expression normalized to the mean of spiked-in miRNA cel-miR-39. The relative expression of each miR was reported as $2^{-\Delta\Delta Ct}$.

2.4. Measurement of telomere length

Telomere length was measured by quantitative real-time PCR as abundance of telomeric template (T) vs. a single gene copy (S) on high molecular weight DNA isolated from white blood cells, as previously described (Testa et al., 2011).

2.5. Statistical analysis

Continuous data were tested for normality by Shapiro Wilk's test and are reported as mean ± standard deviation (SD). Categorical variables are reported as relative frequencies and compared using Chi-squared tests. Partial correlation was used to test for correlations between miR-146a and other biochemical variables, after adjustment for age in CTR subjects, and for age and gender in T2DM patients. Analysis of covariance (ANCOVA) followed by post-hoc tests for multiple comparisons was used to compare the mean differences in biochemical, clinical, and anthropometric variables after adjustment for age and sex. Data analysis was performed using IBM SPSS Statistics for MacOS, version 25.0 (IBM Corp, Armonk, NY, USA). Statistical significance was defined as two-tailed p-value < 0.05.

3. Results

3.1. MiR-146a plasma levels in healthy individuals

The clinical, anthropometric, and biochemical variables of 188 healthy subjects are reported in Table 1. Subjects were grouped into four subgroups according to age: ≤55 years (n = 32), 56–65 years (n = 61), 66–75 years (n = 50) and > 75 years (n = 45). Notably, the latter group included four centenarians, two of whom are women and

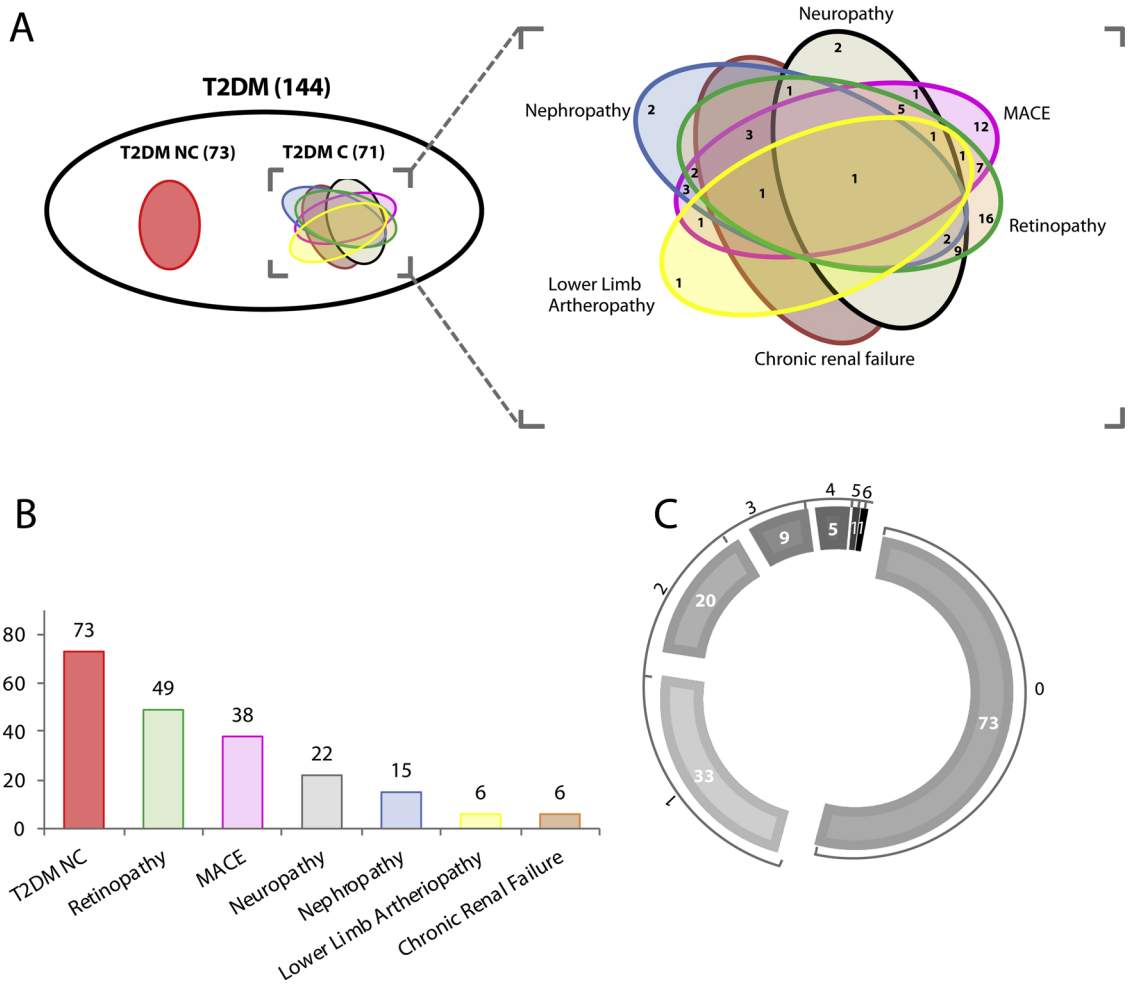

Fig. 5. Overview of diabetic complications in T2DM patients. (A) Venn diagram showing the overlaps between different complications (T2DMC). (B) Bar graph displaying the frequency of each complications in the T2DM cohort. (C) Pie chart showing number of T2DM patients without complications and with one or more complications.

Table 3
Circulating miR-146a levels in T2DM patients in relationship with different diabetic complications and antidiabetic treatments.

Complication	N	Mean (SE) Absent	Mean (SE) Present	p-value
At least one complication	71	4.542 (0.452)	4.685 (0.445)	0.822
Neuropathy	22	4.610 (0.338)	4.642 (0.808)	0.971
Nephropathy	15	4.601 (0.330)	4.732 (1.011)	0.903
Chronic renal failure	6	4.701 (0.317)	2.632 (1.677)	0.231
Retinopathy	49	4.355 (0.387)	5.119 (0.547)	0.266
Lower limb arteriopathy	6	4.636 (0.318)	4.121 (1.561)	0.748
MACE	39	4.608 (0.377)	4.630 (0.591)	0.976

Medication	N	Mean (SE) Untreated	Mean (SE) Treated	p-value
Metformin	61	4.128 (0.332)	5.277 (0.395)	0.027
Sulphonylureas	84	4.023 (0.478)	5.037 (0.404)	0.109
Glinides	3	4.634 (0.313)	3.708 (2.158)	0.672
Insulin	29	4.400 (0.346)	5.464 (0.698)	0.177

MACE, major adverse cardiovascular events. Variables are expressed as estimated marginal mean (standard error). P value from post-hoc tests with Bonferroni corrections after analysis of covariance (ANCOVA).

two men, with an age range of 101–104 years.

Plasma miR-146a levels showed a significant decline with age (miR-146a levels expressed as arbitrary units [a.u.]: 5.76 ± 4.75, 0.45 ± 0.14, 0.49 ± 0.19 and 0.46 ± 0.16, respectively; one-way ANOVA, $F(3, 184) = 7.445$, $p < 0.001$). Bonferroni post hoc analysis revealed significant differences between the > 75 yrs group and ≤ 55 yrs ($p = 0.02$), 56–65 yrs ($p < 0.001$) and 66–75 yrs ($p = 0.012$) groups (Fig. 1A). To investigate potential gender-specific differences in the age-related trajectory, the analysis was conducted separately for women (n = 96) and men (n = 92). The significant decrease of circulating miR-146a levels with aging was confirmed in men (one-way ANOVA, $F(3, 88) = 7.916$, $p < 0.001$) but not in women (one-way ANOVA, $F(3, 92) = 1.584$, $p = 0.199$) (Fig. 1B).

After controlling for age, miR-146 levels were correlated with

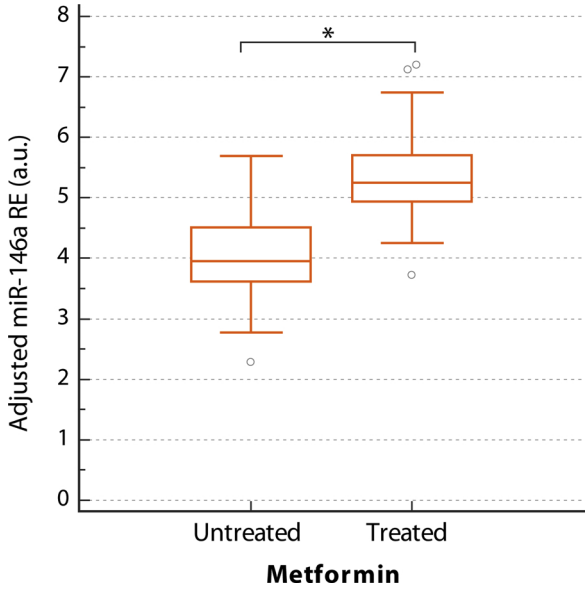

Fig. 6. Effect of metformin treatment on circulating miR-146a levels in 144 T2DM patients. Box-plot showing relative miR-146a expression (in a.u.) in plasma of T2DM patients treated (n = 61) or not (n = 83) with metformin. Data are expressed as mean of $2^{-\Delta\Delta Ct}$ normalized to cel-miR-39 and adjusted for age, gender, and azotemia. * $p < 0.05$ following analysis of co-variance (ANCOVA) with Bonferroni correction.

Table 4
Comparison of biochemical and anthropometric characteristics between healthy control subjects (CTR) and patients with type 2 diabetes mellitus (T2DM).

Variables	CTR N = 188	T2DM N = 144	p-value
Age (years)	65.6 (15.5)	66.8 (7.7)	0.382
Gender (Males)	92	66	0.576
BMI (Kg/m^2)	26.8 (3.9)	28.0 (3.8)	0.006
Weight (Kg)	73.0 (12.5)	74.9 (12.0)	0.175
Waist-hip ratio	0.89 (0.07)	0.92 (0.07)	< 0.0001
Total cholesterol (mg/dL)	217.3 (39.6)	212.4 (37.2)	0.269
HDL-C (mg/dL)	57.5 (15.3)	54.8 (16.8)	0.152
Tryglicerides (mg/dL)	111.3 (80.4)	137.5 (105.7)	0.015
ApoA1 (mg/dL)	176.8 (34.4)	169.9(37.2)	0.099
ApoB (mg/dL)	105.7 (31.1)	106.5 (27.5)	0.795
Glucose (mg/dL)	94.0 (9.4)	165.8 (47.0)	< 0.0001
HbA1C (%)	5.7 (0.4)	7.5 (1.2)	< 0.0001
Insulin (UI/mL)	5.9 (5.4)	6.6 (4.9)	0.224
HOMA index	1.38 (1.47)	2.81 (2.57)	< 0.0001
WBC (n/mm^3)	6.2 (1.6)	6.7 (1.6)	0.004
Platelets (n/mm^3)	229.3 (64.7)	216.0 (57.1)	0.061
Hs-CRP (mg/L)	2.8 (3.1)	3.9 (4.2)	0.012
PAI-1 (ng/mL)	20.6 (11.2)	20.1 (9.0)	0.652
Fibrinogen (mg/dL)	296.7 (78.4)	294.8 (76.8)	0.837
Iron (μg/dL)	81.2 (27.3)	81.5 (27.0)	0.915
Ferritin (ng/mL)	105.7 (81.7)	120.4 (14.7)	0.227
Creatinine (mg/dL)	0.86 (0.24)	0.92 (0.36)	0.113
Azotemia (mg/dL)	38.6 (9.2)	41.7 (13.9)	0.026
Uric acid (mg/dL)	4.9 (1.3)	4.7 (1.2)	0.165

Variables are expressed as mean (standard deviation). P value from *t*-test for continuous variables and from chi-squared tests of association for categorical variables.

parameters reported in Table 1. Significant negative correlations were observed between miR-146a and PAI-1 (p = 0.015), uric acid (p = 0.032) and creatinine (p = 0.030) plasma levels in men, whereas weaker but significant negative correlations between miR-146a and azotemia (p = 0.049), uric acid (p = 0.043), waist/hip ratio (p = 0.032) and ferritin (p = 0.034) were observed in women (Supplementary Table 1). Figs. 2 and 3 show scatter plots for each significant pairwise correlation in men and women, respectively.

3.2. MiR-146a plasma levels in diabetic patients

Plasma levels of miR-146a were investigated in T2DM patients, after dividing them into four subgroups according to age (≤55 yrs, n = 13; 56–65 yrs, n = 38; 66–75 yrs, n = 77; > 75 yrs, n = 16). The clinical, anthropometric and biochemical variables of the four groups are reported in Table 2. A significant age-related decline in miR-146a was observed (F = 5.08, R = -0.19, p = 0.026; Fig. 4) even if the differences among the four age-groups did not reach statistical significance. No gender specific differences were found in the cohort of diabetic patients (data not shown).

To highlight correlations between T2DM associated variables and miR-146a plasma levels, additional analyses were performed. After adjusting for age and gender, significant positive correlations were observed between miR-146a circulating levels and total cholesterol (p = 0.047), LDL cholesterol (p = 0.049), ApoA1 (p < 0.001), ApoB (p = 0.040) and platelet count (p < 0.001). On the other hand, significant negative correlations were between observed between miR-146a and serum iron (p = 0.012) and ferritin (p = 0.026) (Supplementary Table 2).

When T2DM complications, specifically neuropathy, nephropathy, chronic renal failure, retinopathy, lower limb arteriopathy and major adverse cardiovascular events (MACE), were also included in the analysis, no significant difference between patients with or without T2DM complications was observed (31 patients with one complication, 40 patients with two or more complications) (Fig. 5 and Table 3). MiR-146a levels were analyzed also in relation to different treatments (metformin, sulphonylureas, glinides, insulin). The circulating levels of miR-146a were significantly higher in patients treated with metformin (ANCOVA, F[1, 140] = 4.995, p = 0.027; Fig. 6), whereas no difference was observed between mono- and combination therapy regimens (data not shown).

To determine whether miR-146a circulating levels were associated with T2DM, the groups of T2DM patients and CTR subjects were compared. Clinical and biochemical characteristics of the two groups are reported in Table 4. Comparisons were made after adjustment for biochemical variables that were significantly different between T2DM and CTR, with the exception of those strictly related to diabetes diagnosis (fasting glucose, HbA1C, HOMA-index). No significant differences were found between healthy CTR subjects and T2DM patients (F[1, 290] = 1.936, p = 0.165).However when the analysis was performed on males and females separately, significant lower miR-146a levels were observed in male patients compared to CTR (F[1, 133] = 17.884, p < 0.0001) (Fig. 7A). This finding was further substantiated by the fact that negative correlations were observed between circulating miR-146a and both fasting glucose and HbA1c in male subjects (miR-146a/glucose, $r_{partial}$ = -0.21, p = 0.013; miR-146a/HbA1c, $r_{partial}$ = -0.21, p = 0.013). Moreover, the gender-stratified regression analysis (linear or polynomial) showed that a quadratic curve fits the data better than a linear curve (relative miR-146a expression: CTR linear regression r^2 = 0.11 vs. quadratic regression r^2 = 0.19; T2DM linear regression r^2 = 0.08 vs. quadratic regression r^2 = 0.11). Circulating miR-146a levels exhibited an inverted U-shaped trajectory with age in both CTR and T2DM groups, but the age-related trend in T2DM patients was significantly shifted towards lower levels compared to the CTR (Fig. 7B).

4. Discussion

Over the past decade, there has been an increasing interest in the study of specific circulating miRNA signatures that can predict healthy aging and the onset of ARDs and their complications. Previous studies from our group demonstrated a non-monotonic age-related trend for miR-21 and miR-126-3p, which were both upregulated in octogenarians

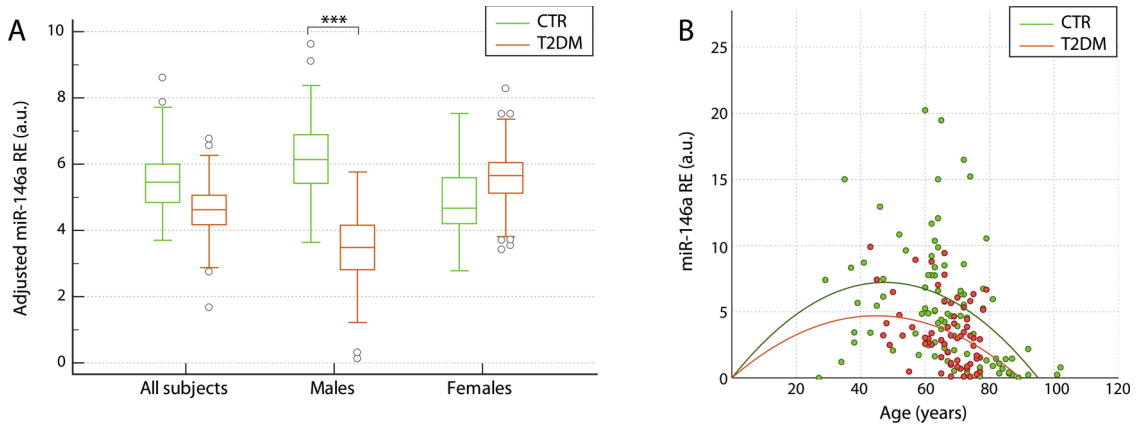

Fig. 7. Age- and gender-related expression of miR-146a in plasma from CTR subjects and T2DM patients. (A) Box-plots showing multiple comparisons of relative miR-146a expression (in a.u.) in plasma of CTR subjects (green) vs. T2DM patients (orange), overall and by gender. Data are expressed as mean of $2^{-\Delta\Delta Ct}$ normalized with cel-miR-39 and adjusted for age, BMI and biochemical variables significantly different between groups. *** $p < 0.001$ following analysis of co-variance (ANCOVA) with Bonferroni correction. (B) Scatter plot showing relative miR-146a expression (in arbitrary units, a.u.) according to age in male CTR subjects and male T2DM patients. Data are expressed as mean of $2^{-\Delta\Delta Ct}$ normalized with cel-miR-39. Regression curves are displayed for male CTR subjects (solid green, n = 92, $r^2 = 0.19$) and male T2DM patients (solid orange, n = 66, $r^2 = 0.11$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

compared with younger subjects (Olivieri et al., 2014, 2012). Several studies have investigated circulating levels of miR-146a in the context of ARDs, but there is currently no data regarding its modulation during human aging and no conclusive results in T2DM patients. In this study, a significant age-related decline in miR-146a plasma levels was observed in healthy individuals, with a marked reduction in the group of subjects aged 75 years or older. When considering genders separately, this link persisted only in males. This result supports the hypothesis that an impaired balance between anti- and pro- inflammatory players occurs during aging, also in absence of ARDs (Boldin et al., 2011; Jiang et al., 2012; Olivieri et al., 2013a). The decrease in miR-146a circulating levels could partly explain the pro-inflammatory state typically observed in elderly subjects (Monti et al., 2017).

Our study also showed a significant negative correlation between miR-146a levels and creatinine and azotemia in male and female CTRs, respectively. These results are in line with findings from studies in animal models of kidney disease, including diabetic nephropathy, which showed that the suppression of miR-146a expression is associated with a more severe tubulointerstitial damage after ischemia-reperfusion injury (Amrouche et al., 2017). Here, negative correlations were also identified with PAI-1 and uric acid, which have lately received significant attention as potential cardiovascular risk biomarkers in light of their contribution to the pathogenesis of endothelial dysfunction (Sharaf El Din et al., 2017; Shimizu et al., 2016). Likewise, miR-146a levels in healthy women were found inversely correlated with waist-hip ratio, which in turn is associated with an increased risk of T2DM and coronary heart disease (Emdin et al., 2017).

An intriguing finding is a weaker but significant age-related decline in miR-146a plasma levels also in T2DM patients. Our results also revealed positive correlations of miR-146a plasma levels with total cholesterol, LDL-C and ApoA1. Although the role of miR-146a in the modulation of lipid profile is still unknown, miR-146a is strongly associated with specific serum HDL subfractions and exerts an atheroprotective action by inhibiting the uptake of oxidized LDL by macrophages and their subsequent differentiation into foam cells (Yang et al., 2011). Notably, negative correlations were also observed with ferritin and serum iron, which is in line with the fact that the synthesis of ferritin in macrophages is stimulated by several inflammatory cytokines, including IL-1β, IL-6 and TNF-α. These cytokines in turn mediate NF-κB binding to the enhancer element FER2 and the subsequent transcription of ferritin heavy chain (Bertoli et al., 2018). It is also important to point out that elevated serum ferritin is a characteristic feature of inflamm-aging and, according to the EPIC-InterAct study, it is independently associated with an increased risk of T2DM (Cankurtaran et al., 2012; Podmore et al., 2016).

When healthy subjects and T2DM patients were compared, male patients displayed significantly lower miR-146a levels than age-matched controls, while the same difference was not significant in females. The gender-specific difference in miR-146a levels with age and disease could be explained in light of miR-146 modulation by estrogen. Previous work in animal models and in human subjects reported that estrogen treatment impairs the synthesis of miR-146a and other miRs (Dai et al., 2008; Kangas et al., 2014). The sexual dimorphism in miRNAs expression could in part explain the role of estrogen in shaping the immune system responses and the attenuation in the slope of miR-146a reduction observed in elderly women as seen in our study.

In regard to miR-146a circulating levels in T2DM, previous studies observed decreased levels in diabetic patients, with no relationship with age and the clinical variables pertaining to the disease (Baldeon et al., 2014). However, since healthy subjects were younger than T2DM patients, the lack of matching for age could have influenced the results. Conversely, Rong et al. reported increased plasma levels of miR-146a in newly diagnosed T2DM patients (Rong et al., 2013). Interestingly, this latter study included non-elderly patients at the initial stage of disease, with an overall good glycemic control and no complications. Indeed, our data show a strong inverse correlation between miR-146a and both HbA1c and fasting glucose in the male subpopulation, in agreement with other reports describing a decrease in miR-146a levels with worsening glycemic control (Balasubramanyam et al., 2011). It is therefore plausible that the circulating miRNA signature in T2DM is strongly modulated during the natural history of the disease and could be affected by specific interventions or treatments.

Notably, after proper normalization, diabetic patients treated with metformin showed higher circulating levels of miR-146a. Metformin was previously reported to decrease the production of pro-inflammatory cytokines during cell senescence or in response to LPS by preventing NF-κB translocation into nucleus through inhibition of IκB and IKKα/β phosphorylation (Moiseeva et al., 2013). In agreement with this evidence, the upregulation of miR-146a could represent an additional mechanism supporting the emerging anti-inflammatory and anti-aging pleiotropic effects of metformin (Prattichizzo et al., 2018b).

Finally, to investigate the impact of an ARD on the expression of plasma miR-146a, the decreasing trend observed in healthy aging was compared to the one found in T2DM but only in male subjects. A non-

monotonic relationship between age and miR-146a plasma levels was observed both in CTR and in T2DM. Specifically, an inverted U-shaped trajectory was found to fit the miR-146a age-related trend better than a linear one, with a slight increase up to 65 years of age, followed by a marked decrease in elderly subjects. The non-monotonic trends observed for miR-146a are similar to the trends reported for miR-21, reinforcing our hypothesis that the development of ARDs could be monitored by circulating functional biomarkers with non-monotonic age-related trends (Olivieri et al., 2012). Similarly, an inverted U-shaped trend was observed in T2DM, even if it was significantly shifted towards lower levels of miR-146a. The greater discriminant power was observed in the age group of 45–65 years, which is when T2DM is more often diagnosed (American Diabetes Association, 2018). This result further supports the evidence that T2DM patients experience earlier aging, probably through an increased vulnerability towards environmental stressors (Spazzafumo et al., 2013).

5. Conclusions

In conclusion, findings from the present study corroborate the idea that circulating biomarkers of ARDs, including miRNAs, may be useful to early detect deviations from a physiological aging trajectory, allowing to adopt therapeutic or lifestyle interventions to delay the onset of T2DM. In this context, circulating miR-146a could be used as a novel age-related biomarker of healthy/unhealthy aging trajectories. Longitudinal cohort studies and cross-sectional studies are warranted to clarify the significance of circulating microRNA changes and to better establish their prognostic and diagnostic value, thus promoting translational medicine applications.

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