Original Research

miR-34a and miR-9 are overexpressed and *SIRT* genes are downregulated in peripheral blood mononuclear cells of aging humans

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Impact statement

High expression of sirtuins, particularly SIRT1, lowers the risk of age-related diseases and probably slows down the rate of aging; therefore, their sustained expression should be one of the features of longevity. However, in this work we show that in peripheral blood mononuclear cells (PBMC) of long-lived individuals, expression of majority of the *SIRT* genes is significantly lower than in cells of young study subjects. In long-lived individuals, downregulation of *SIRT1* coexists with upregulation of *SIRT1* mRNA-interacting miR-34a and miR-9, indicating the role of epigenetic drift in age-dependent deregulation of *SIRT1* expression. Such constellation of *SIRT1*, miR-34a, and miR-9 expression in PBMC of successfully aging long-lived individuals indicates that, at least in these individuals, it is not a risk factor for morbidity and mortality. It might however affect the function of the immune system and, therefore, aging individuals can profit from interventions increasing the level of SIRT1.

Abstract

Increased expression of sirtuins lowers the risk of age-related diseases, while their role in the regulation of longevity is not firmly established. Since aging is associated with immunosenescence, we tested whether sirtuin expression was modified in peripheral blood mononuclear cells (PBMC) in an age-related manner and whether this might result from altered expression of the selected miRNAs. The expression of seven *SIRT* genes and of *SIRT1* mRNA-interacting miR-9, miR-34a, miR-132, and miR-199a-5p was evaluated by real-time PCR in PBMC originating from young (Y, $n = 57$, mean age 27 ± 4.3 years), elderly (E, $n = 52$, 65 ± 3.4 years), and long-lived (L, $n = 56$, 94 ± 3.5 years) individuals. Older age was associated with a decreased expression of the majority of the *SIRT* genes. Most severely affected were median expressions of *SIRT1* ($P = 0.000001$ for the whole studied group, Y *vs.* E: $P < 0.000001$, Y *vs.* L: $P < 0.000001$), and of *SIRT3* ($P = 0.000001$, Y *vs.* E: $P = 0.000004$, Y *vs.* L: $P = 0.000028$). Older age was also associated with the increased median expression of miR-34a ($P = 0.000001$, Y *vs.* E: $P = 0.001$, Y *vs.* L: $P = 0.000004$) and of miR-9 ($P = 0.05$, Y *vs.* L: $P = 0.054$). In functional studies, miR-9 interacted with the 3′UTR of *SIRT1* mRNA. The *SIRT1* mRNA level negatively correlated with the expression of miR-34a ($r = -0.234$, $P = 0.003$). In conclusion, age-related decrease of *SIRT1* expression in PBMC might in part result from overexpression of miR-34a and miR-9. In addition, the sustained expression of the *SIRT* genes in PBMC is not a prerequisite to longevity in humans but might be one of the reasons for the immune system dysfunction in the elderly.

Keywords: SIRT, peripheral blood mononuclear cells, epigenetic drift, aging, longevity

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Introduction

Aging and its clinical course depend on multiple factors.[1–3] A hundred or so genes have so far been implicated in the regulation of human aging, including *SIRT* genes (*SIRT1–SIRT7*) encoding NAD$^+$-dependent deacetylases that control metabolism, production of the reactive oxygen species (ROS), response to oxidative stress and to DNA damage, and inflammatory responses.[4–6] Enhanced function and/or overexpression of sirtuin orthologs have been reported to extend the lifespan of *Caenorhabditis elegans*, yeast, and *Drosophila melanogaster*; this view, however, is not unanimous.[7–10] The lifespan of male mice overexpressing SIRT6 is increased, but the lifespan of SIRT1 transgenic mice is not increased.[11,12] In humans, only select SIRT1 and SIRT3 polymorphisms are associated with longevity.[13,14] Thus, it is still not firmly established whether or not sirtuins

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extend the length of life. In contrast, it is generally accepted that a higher activity of sirtuins is associated with protection against the majority of age-related diseases such as type 2 diabetes, cardiovascular disease, and Alzheimer's disease.[15–18] Notably, a calorie restriction diet (CR), the only intervention known so far to extend healthspan and lifespan, leads to SIRT1 and SIRT3 overexpression.[19,20] It has been also shown that SIRT1 transgenic mice present features similar to those of animals fed a CR: they are lean, resistant to cancer, as well as to the diet-induced metabolic syndrome.[12,21] In SIRT3 transgenic mice, antioxidant mechanisms are enhanced.[22] Conversely, CR leads to SIRT1 and SIRT3 overexpression.[19,23] In primates, implementation of CR decreased morbidity and mortality,[24,25] but this was not replicated by another study.[26] Such inconsistency could result from a different composition and source of diet and a slight calorie restriction in the control group of the latter study. In humans, the Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE) trials showed that mild CR reduces weight and improves cardiometabolic risk factors, while self-imposed severe CR with optimal nutrition (CRON) lasting for up to 15 years resulted metabolic, hormonal, and molecular effects previously detected in long-lived CR animals and reduced metabolic and cardiovascular risk factors. However, effects of CR on survival in humans are still not known (reviewed in Most et al.[27]).

Gene expression is controlled by numerous mechanisms, including epigenetic modifications such as DNA methylation, covalent histone modifications, and the action of miRNAs. Notably, one of the characteristics of aging is epigenetic drift, a subtle but progressive change of the epigenome.[28–30]

With this in mind, we decided to determine whether successful aging is associated with changes in the expression of sirtuin genes in peripheral blood mononuclear cells (PBMC). We show that even in individuals whose aging is not complicated by age-related diseases, older age is associated with a decreased expression of the majority of the *SIRT* genes in these cells. We also wished to elucidate whether an altered expression of certain miRNAs might be responsible for the decreased expression of *SIRT1* and we found that age-related overexpression of miR-34a and miR-9 might be involved in its downregulation.

Materials and methods

Study subjects

Polish Caucasians were divided into young (Y, $n = 57$, age range 19–42 years, mean age 27 ± 4.3 years), elderly (E, $n = 52$, 60–73 years, 65 ± 3.4 years), and long-lived (L, $n = 56$, 90–102 years, 94 ± 3.5 years). Study subjects were non-obese (body mass index [BMI] $< 30\,\text{kg/m}^2$) and healthy/relatively healthy: moderate hypertension was allowed for older age groups, and a mild degree physical (the Activities of Daily Living score ≥ 3) and cognitive disability (the Mini Mental State Evaluation score ≥ 20) were also allowed for the long-lived group.[31,32] The study protocol was approved by the Bioethics Committee of the Medical University of Warsaw. All participants gave a written informed consent for participation in the study. The anonymity of patients has been preserved at all stages of this investigation.

Isolation of RNA from PBMC

PBMC and RNA were isolated as previously described.[33]

Reverse transcription, real-time quantification of gene and miRNA expression

Reverse transcription, analysis of *SIRT1–SIRT7* mRNA expression, and miRNA expression were performed as previously described, with one modification regarding different annealing temperatures (65°C for *SIRT1, 2, 3, 6*; 67°C for *SIRT4*, and 60°C for *SIRT5* and *7*).[34] The list of primers can be found in Table 1.

Functional analysis of miRNA

Candidate miRNAs were searched for with the TargetScanHuman, the miRanda-mirSVR, and the Pictar programs and verified using the rules indicated by Bartel.[36–39] We selected miR-9, miR-34a, miR-132, and miR-199a-5p for analysis regarding SIRT1.

DNA corresponding to the full-length 3′UTR of *SIRT1* mRNA was amplified from 50 ng of PBMC-originating cDNA with Dream Taq polymerase (Thermo Scientific, Vilnius, Lithuania) using the forward 5′ACTA**GAGCTC**TAGTGTAATAATTGTGCAGG3′ (added *Sac*I restriction site shown in bold; the STOP codon is underlined) and reverse 5′CTAA**CTCGAG**AACAGAAAAAAGTCAAATGAC3′ (additional *Xho*I restriction site shown in bold) primers. The PCR reaction was: 3 min at 94°C, 40 cycles of: 30 s at 94°C, 30 s at 62°C, and 2 min 30 s at 72°C, and final extension for 10 min at 72°C. The PCR product was cloned into the pmirGLO reporter vector (Promega, Madison, WI) and sequenced (pmirGLO_SIRT1).

Table 1 Primers used for analysis of the expression of *SIRT1-SIRT7* in human PBMC

	Primers	Exons
SIRT1	F: 5′ACAGGTTGCGGGAATCCAAAGG3′ R: 5′CCTAGGACATCGAGGAACTACCTG3′	E5 E7
SIRT2[a]	F: 5′CCTCGCCTGCTCATCAACA3′ R: 5′TCCTCCGAGGCCCATAATC3′	E13 E14
SIRT3	F: 5′GCTGACGTGATGGCAGACA3′ R: 5′AACCACATGCAGCAAGAACCT3′	E5 E5
SIRT4	F: 5′ACAGGGTCCTGTGCTTGGATTG3′ R: 5′TTCAGGACTTGGAAACGCTCTTGC3′	E2/3 E3
SIRT5	F: 5′AAGGCTGGCACCAAGAAC3′ R: 5′GCCACAACTCCACAAGAG3′	E5 E6
SIRT6	F: 5′TGGTCTCCAGCTTAAACAG3′ R: 5′AAGGCAGTGCAAGCCTCT3′	E8 E8
SIRT7[a]	F: 5′CGTCCGGAACGCCAAATAC3′ R: 5′GACGCTGCCGTGCTGATT3′	E3 E3/4
ACTB	F: 5′TTCTACAATGAGCTGCGTGTG3′ R: 5′CAGCCTGGATAGCAACGTACA3′	E3 E4

F: forward; PBMC: peripheral blood mononuclear cells; R: reverse.

[a] Primers as in Ashraf et al.[35]

Human embryonic kidney (HEK) 293 cells were cultured and transfected as previously described.[34] Forty nanograms of the reporter plasmid and 5 pmol or 10 pmol (30 nmol/L or 60 nmol/L final concentration, respectively) of pre-miRNA (pre-miR-9 or pre-miR miRNA Precursor Negative Control #1; Ambion, Life Technologies, Carlsbad, CA) were used. Cells were then cultured, lysed, and the luminescence was assessed as previously described.[34] Each experiment was repeated nine times.

Immunoblot

HEK 293 cells were cultured and transfected with pre-miR-9 or with a negative control miRNA precursor (each at 30 nmol/L or 60 nmol/L final concentration) in a six-well dish. Cells were harvested 24 h after transfection. Total protein was isolated with radioimmunoprecipitation assay (RIPA) buffer and an aliquot of 20 µg was resolved using 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was incubated with rabbit anti-SIRT1 primary antibody (1:250, sc-15404, Santa Cruz Biotechnology, TX) and with goat anti-rabbit secondary antibody (1:10,000, 401393-2ML, Merck Milipore, Germany). The control reaction was performed with rabbit anti-GAPDH primary antibody (1:500, sc-25778, Santa Cruz Biotechnology). Proteins were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, IL) and the GeneGnome XRQ bio imaging system (Syngene, UK). Densitometric measurements of three independent immunoblots were performed with the Image Studio Lite software (LI-COR Biosciences, NE).

Statistical analysis

Statistical calculations were performed using Statistica v. 10. To assess normality of the distribution, the Shapiro-Wilk test was used. For *SIRT1* expression, statistical analysis was performed with the analysis of variance (ANOVA) and *post hoc* Fisher's tests. Since distribution of expressions of the remaining *SIRTs* and of miRNAs was not normal, statistical analyses were performed with the Kruskal–Wallis test. Correlation between the *SIRT1* mRNA and miRNA expressions was calculated by the Spearman's rank correlation coefficient. The effect of miRNA interaction with the *SIRT1* mRNA on the reporter protein activity was analyzed by the two-sided Student's *t*-test. For all tests the level of significance was established at 0.05.

Results

Expression of the SIRT1-SIRT7 mRNA in PBMC of young, elderly, and long-lived individuals

We have previously established that age did not affect the mean Cp values for the *ACTB* control gene in PBMC.[36] We also determined that the expression of *SIRTs* was similar in PBMC of women and men and, therefore, further analyses were performed for all study subjects together.

The median expressions of the *SIRT* genes, presented in arbitrary units (Figure 1) showed that the expressions of *SIRT1* and *SIRT3* were most severely affected by age ($P = 0.000001$ and $P = 0.000001$, respectively) and were reduced approximately two-fold in the E and L groups compared to young controls. The median expression of *SIRT4* was significantly lower in the L group than in the

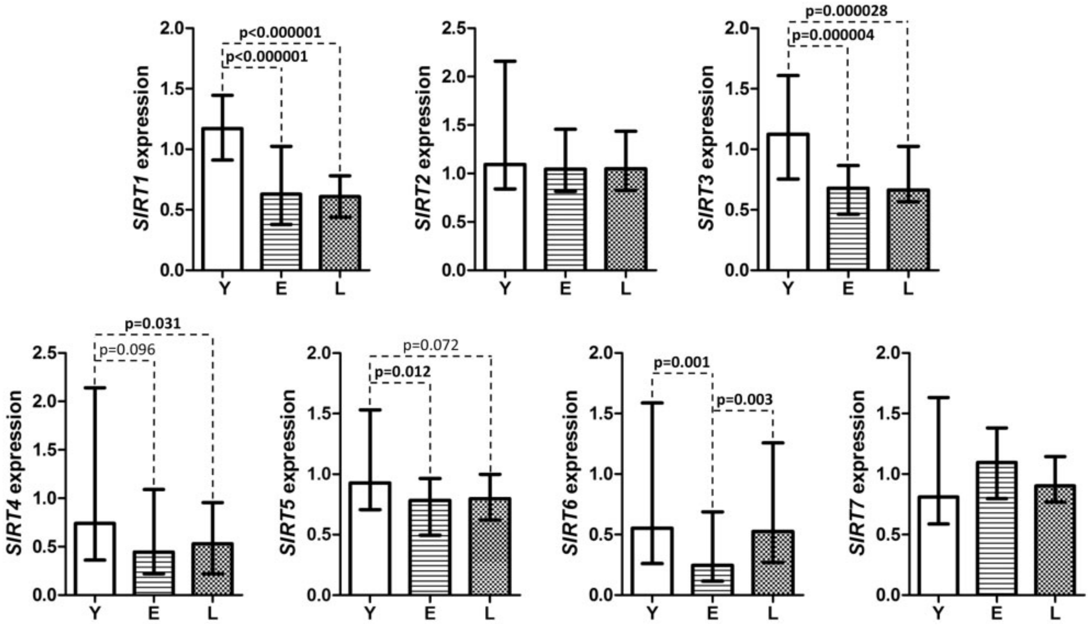

Figure 1 The median expressions of the *SIRT1-7* genes in PBMC of individuals of various ages. Results are presented in arbitrary units (specific for each gene) as median (25th, 75th percentile) values. For *SIRT1* expression, statistical analysis was performed with the ANOVA and *post hoc* Fisher's tests, while for other genes the Kruskal–Wallis test was used. The level of significance was established at 0.05.
Y: young; E: elderly; L: long-lived study participants; PBMC: peripheral blood mononuclear cells

Y group, while the median expression of *SIRT5* was significantly lower in the E than in the Y group. The expression of *SIRT6* was significantly lower in the E group than in the Y and L groups. Finally, age did not affect the expressions of *SIRT2* and *SIRT7*.

Interaction of miR-9 with the 3′UTR of SIRT1 mRNA

Of all sirtuins, SIRT1 is the one most clearly associated with the phenotype of aging; therefore, we selected this sirtuin for further analysis. We assessed the association of its expression with age-related changes of miR-9, miR-34a, miR-132, and miR-199a-5p levels. The interaction between human *SIRT1* mRNA and miR-34a, miR-132 and miR-199a-5 p was demonstrated earlier; therefore, we tested the interaction with only miR-9.[40–42] We found that transfection of pre-miR-9 increased the expression of miR-9 in HEK 293 cells while transfection of a negative control miRNA precursor did not, as Cp values for miR-9-overexpressing cells were lower by approximately 10 than those for not transfected cells and cells overexpressing negative control miRNA (Figure 2(a)). Overexpression of miR-9 was associated with the decrease of endogenous SIRT1 protein (by 18% at 30 nmol/L final pre-miR-9 concentration, $P = 0.05$ and by 33% at 60 nmol/L final concentration, $P = 0.03$, 70% transfection efficiency, Figure 2(b)). The mean relative luminescence induced by firefly luciferase expressed from the pmirGLO_SIRT1 in the presence of negative control miRNA was normalized to 100%. Co-transfection of pmirGLO_SIRT1 with pre-miR-9 decreased luminescence by 42.6% ($P = 0.000001$) (Figure 2(c)), suggesting that this miRNA interacted with its binding site present in the 3′UTR of *SIRT1* mRNA and decreased the translation of the reporter protein.

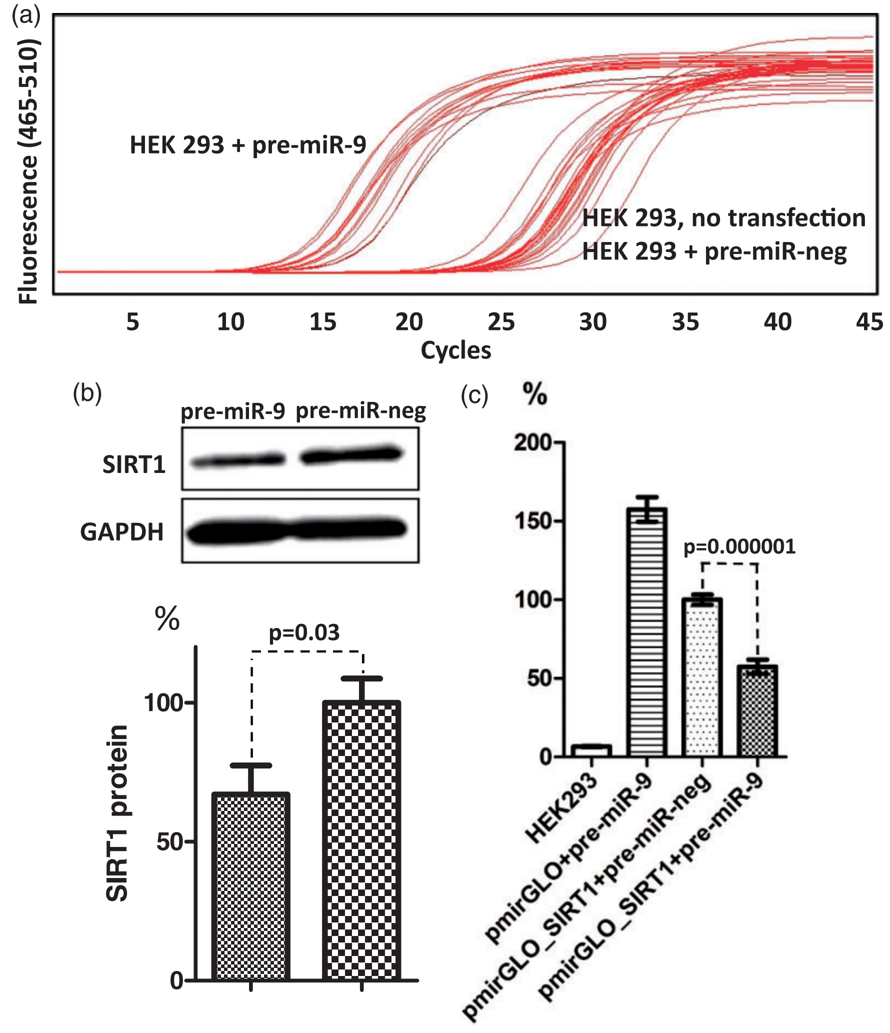

Figure 2 miR-9 interaction with the 3′UTR of *SIRT1* mRNA. (a) Amplification curves for RT-PCR analysis of miR-9 expression in HEK 293 cells transfected with miR-9 precursor or with a negative miRNA precursor (each at 30 nmol/L final concentration). Expression of miR-9 was evaluated 24 h after the initiation of transfection. (b) Representative immunoblot of total protein isolated from HEK 293 cells 24 h after the initiation of transfection with miR-9 precursor or with a negative control miRNA precursor (each at 60 nmol/L final concentration), and the result of quantification of densitometric measurements of three independent immunoblots. (c) HEK 293 cells were co-transfected with the pmirGLO reporter vector with or without the cloned sequence corresponding to the 3′UTR of *SIRT1* mRNA and with miRNA precursors. The mean relative luminescence induced by firefly luciferase in the presence of negative control miRNA was normalized to 100%. Each experiment was repeated nine times. HEK: human embryonic kidney. (A color version of this figure is available in the online journal.)

Age-dependent changes of expression of miRNAs in PBMC

As a first step, we wished to establish whether the mean U6 snRNA Cp values changed with age in PBMC. Since they did not (Y: 23.3 ± 2.4, E: 23 ± 2.4, L: 23.7 ± 2.2), we used U6 snRNA as a control for evaluating the expression of miR-9, miR-34a, miR-132, and miR-199a-5p. The expression of these miRNAs was similar in women and men and further analyses were performed for both genders together.

The median expression of miR-34a increased with age ($P = 0.000001$) and was significantly higher in the E and L groups than in young controls. In addition, the median expression of miR-9 was also affected by age ($P = 0.05$) and was higher in the L than in the Y group, but this difference did not reach the level of significance. The median expressions of miR-132 and miR-199a-5p were not affected by age in PBMC of the examined individuals (Figure 3).

The *SIRT1* mRNA levels negatively correlated with the expression of miR-34a and miR-132 ($r = -0.234$, $P = 0.003$ and $r = -0.156$, $P = 0.049$, respectively), but not with the expression of miR-9 and that of miR-199a-5p in the whole group of study subjects. There was no correlation between SIRT1 and the studied miRs in any of the age groups while analyzed separately.

Discussion

The available data regarding physiological roles of sirtuins suggest that the preservation of their function should be one of the features of longevity. However, contrary to expectation, in this work we found that in PBMC of aging individuals, older chronological age is associated with a significantly lower expression of the majority of the *SIRT* genes at the mRNA level, with the expression of *SIRT1* and *SIRT3* being most severely affected.

SIRT1, mostly located to the nucleus, by deacetylating FOXO transcription factors stimulates the expression of ROS-deactivating enzymes catalase and manganese superoxide dismutase,[44–46] and by deacetylating proliferator-activated receptor coactivator-1α increases mitochondrial biogenesis and decreases ROS production.[43–47] By deacetylating the Lys310 residue of RelA/p65 component of nuclear factor-κB (NF-κB), SIRT1 inhibits the activity of this transcription factor, supresses inflammation,

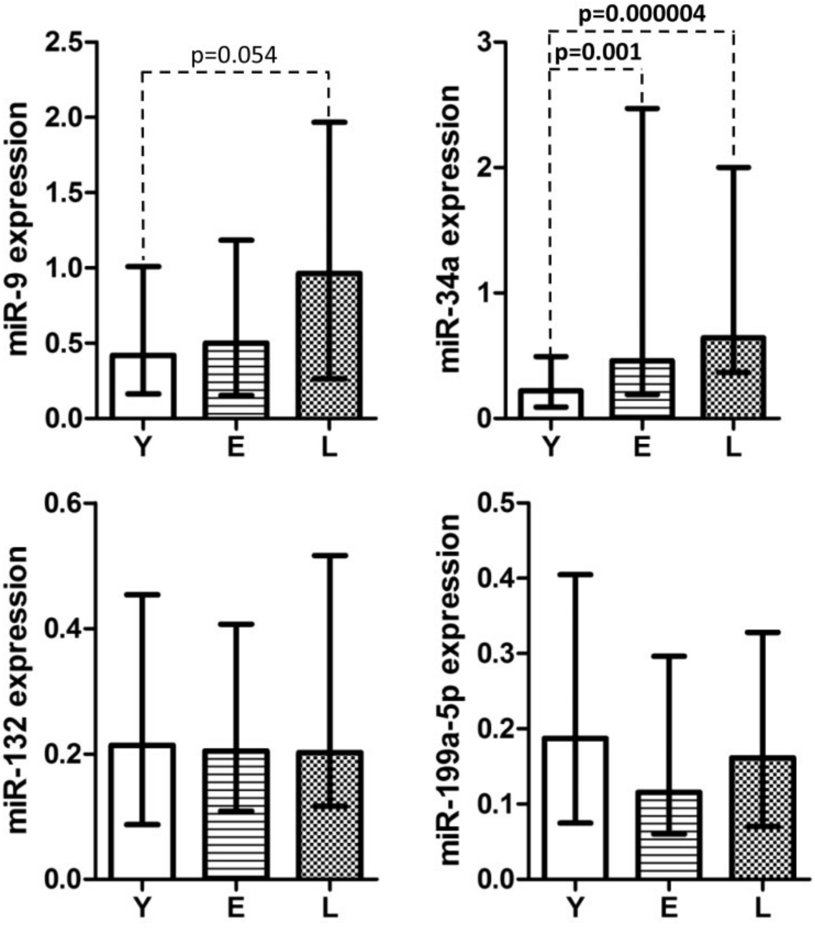

Figure 3 The median miR-9, miR-34a, miR-132, and miR-199a-5p expressions in PBMC of individuals of various ages. Results are presented in arbitrary units (specific for each miRNA) as median (25th, 75th percentile) values. Statistical analyses were performed with the Kruskal–Wallis test. The level of significance was established at 0.05.
Y: young, E: elderly, L: long-lived study participants. PBMC: peripheral blood mononuclear cells

and enhances the resolution phase of inflammatory response.[4,48] In addition, by deacetylating Agt5 and Agt7, SIRT1 induces autophagy, a mechanism removing damaged cellular organelles.[49] Alltogether, SIRT1 is a strong inhibitor of the oxidative stress and inflammatory response.[48,50] Therefore, aging-associated decrease of SIRT1 can contribute to the occurence of chronic oxidative stress and chronic inflammation, both being hallmarks of inflammaging, and can be associated with the accumulation of damaged organelles (including mitochondria), further impeding the function of immune cells.

SIRT3, the major mitochondrial deacetylase, by deacetylating a number of key mitochondrial proteins promotes urea cycle, fatty acid β-oxidation, and oxidative phosphorylation, as well as increases expression and activity of manganese superoxide dismutase, thus reducing ROS production and promoting ROS scavenging.[19,22,43,51–54] Thus, SIRT3 reduces oxidative stress, increases stress defenses, and counteracts the development of aging-associated diseases.[22,55,56] Therefore, its decreased expression in immune cells of aged humans can result in an increase in the level of ROS, and in oxidative stress and damage. This, in turn, can activate the NF-κB system.

In conclusion, the decreased expression of SIRT1 and SIRT3 might contribute to the occurence of aging-associated chronic oxidative stress and inflammaging, also present in successfully aging individuals.[19,50,56–58] Notably, the fact that the decreased expression of *SIRT* genes was detected in PBMC of our oldest study subjects indicates that the effect of their downregulation on the function of immune cells is not dominant, could be overcome by other factors and, therefore, does not contribute to the increased morbidity and mortality of successfully aging individuals who are genetically predisposed to longevity.[59] It would be highly relevant, however, to establish whether downregulation of *SIRTs* in PBMC of individuals with less favorable genetic/environmental context is also of minor importance, or indeed contributes to the increased morbidity and mortality of the aging population.

Aging is associated with epigenetic drift.[2,3] One of its features is the overexpression of miR-34a: this was detected in the cardiac muscle and endothelial cells of aged mice, as well as in the liver and kidneys of aged rats, among others.[60,61] Therefore, the overexpression of this miRNA in PBMC of aging humans described in this work complements existing data. miR-34a exerts its function by downregulating *SIRT1* exression, which leads to the described above alterations in the function of immune cells. It has also been shown that miR-34a knockout results in the reduced proliferation and activation of lymph nodes T cells.[62] On the other hand, overexpression of miR-34a blocked programmed cell death 1 (PD1) and programmed cell death 1 ligand 1 (PD-L1)-specific apoptosis of T lymphocytes.[63] In macrophages, miR-34a promotes M2 polarization linked to immunosuppression.[64] Therefore, age-related overexpression of miR-34a seems to be an important pro-inflammatory immunoaging-contributing factor. However, our findings also show that the negative effect of excess miR-34a in PBMC of aged humans does not depend mainly on its inhibitory effect on *SIRT1*, as downregulation of *SIRT1* was detected also in long-lived study subjects who did not suffer from aging-related diseases. Therefore, the genetic/environmental context and/or the inhibition of other factors by miR-34a might play a negative role in human aging.

Microarray analysis showed that miR-9 regulates the expression of a significant number of genes associated with the function of the immune system. It increases the expression of the interferon-induced genes and major histocompatibility complex class I molecules genes, as well as increases the IL-7 and IL-2 expression, and decreases IL-1 and IL-6 expression.[65,66] In activated monocytes, NF-κB rapidly increases the expression of miR-9, which in turn reduces the expression of NF-κB, thus closing the negative feedback loop.[67] In contrast to miR-34a, miR-9 induces M1 polarization of macrophages, which is associated with the production of pro-inflammatory cytokines.[64,68] Therefore, in addition to its role in immunoaging exerted *via SIRT1*, other miR-9 mechanisms are involved in this process. Nevertheless, its overexpression in PBMC of long-lived humans suggests that it is not detrimental, at least in individuals who age successfully.

PBMC is a mixture of various T and B lymphocyte subtypes, natural killer cells, and monocytes. In humans, even though healthy aging is not associated with significant changes in the absolute numbers of cell types, it is associated with changes in the percentage of cell sub-types, such as a decrease in the number of naive T and B cells and an increase in the number of memory and effector cells, as well as with alterations of their function.[69,70] Since aging of various immune cells might be characterized by different expression profiles of *SIRTs* and miRNAs, it is plausible that the observed expression changes reflect age-related changes in the PBMC composition. Therefore, the analysis of each cell type separately might yield more probing results. According to the literature data, other potential cause for differences in the expression of SIRT1 and miR-34a may be a severe cognitive impairment.[71–73] However, we did not test if there were any differences in the levels of SIRT1, miR-34a, and miR-9 in PBMC of our study subjects since our young and elderly study subjects were cognitively normal, and long-lived group was composed of individuals with largely normal cognition or MCI. In addition, we have not examined age-related changes in the expression of *SIRTs* at the protein level. Such an examination would support the reliability of our results, especially in the case of miR-9. Possibly due to the lack of full complementarity between miR-9 and its binding site, blockage and sequestration of the *SIRT1* mRNA were much more likely than degradation, resulting in the lack of correlation between the miR-9 and *SIRT1* mRNA levels.[74] However, there may be a negative correlation between this miRNA and SIRT1 protein. Unfortunately, performing both fractionation and protein analyses in PBMC was not possible in this study due to the limited amount of blood obtained from the elderly and long-lived study participants.

To sum up, in individuals who healthily age, the sustained expression of the SIRT genes in PBMC is not a prerequisite to longevity. It might however affect the process of immunosenescence.

Conclusion

The fact that downregulation of *SIRTs* and overexpression of miR-34a and miR-9 were detected in PBMC of long-lived individuals suggests that, at least in these individuals, they are not risk factors for morbidity and mortality but might affect the function of the immune system by accelerating or exacerbating immunosenescence. Therefore, interventions that increase the level of SIRT1 such as CR or resveratrol supplements could improve the function of the aging immune system.

Author contributions: All authors analyzed data and participated in interpretation of the studies and review of the manuscript. MO conducted the experiments, performed the statistical analysis, MB, ADS, JB, JP, and MG conducted the experiments, PS performed the statistical analysis, MPK designed the study, wrote manuscript. All authors read and approved the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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