The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences, 2023, **78**(11), 1935–1943
https://doi.org/10.1093/gerona/glad160
Advance access publication 9 July 2023
Original Article

Phenotypic Frailty Assessment in SAMP8 Mice: Sex Differences and Potential Role of miRNAs as Peripheral Biomarkers

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Decision Editor: Gustavo Duque, MD, PhD (Biological Sciences Section)

Abstract

Frailty is a geriatric syndrome characterized by age-related decline in physiological reserves and functions in multiple organ systems, including the musculoskeletal, neuroendocrine/metabolic, and immune systems. Animal models are essential to study the biological basis of aging and potential ways to delay the onset of age-related phenotypes. Unfortunately, validated animal models of frailty are still lacking in preclinical research. The senescence-accelerated prone-8 (SAMP8) mouse strain exhibits early cognitive loss that mimics the deterioration of learning and memory in the elderly and is widely used as a model of aging and neurodegenerative diseases. Here, we examined the frailty phenotype, which includes body weight, strength, endurance, activity, and slow walking speed, in male and female SAMP8 and senescence-accelerated mouse resistant (SAMR1) mice at 6- and 9-months of age. We found that the prevalence of frailty was higher in SAMP8 mice compared with SAMR1 mice, regardless of sex. The overall percentage of prefrail and frail mice was similar in male and female SAMP8 mice, although the percentage of frail mice was slightly higher in males than in females. In addition, we found sex- and frailty-specific changes in selected miRNAs blood levels. In particular, the levels of miR-34a-5p and miR-331-3p were higher in both prefrail and frail mice, whereas miR-26b-5p was increased only in frail mice compared with robust mice. Finally, levels of miR-331-3p were also increased in whole blood from a small group of frail patients. Overall, these results suggest that SAMP8 mice may be a useful mouse model for identifying potential biomarkers and studying biological mechanisms of frailty.

Keywords: Aging, Epigenetic, Frailty, Mouse model, Sex difference

Human longevity has significantly increased over the past century, resulting in a rapid aging of the population, and posing a major challenge for health and social care. Frailty, a common condition affecting approximately 1 in 10 people aged 65 years and older, has been defined by Fried et al. as "a biological syndrome of diminished reserve and resilience to stressors, resulting from cumulative declines across multiple physiologic systems and causing vulnerability to adverse outcomes" such as falls, hospitalization, disability, and death (1). Importantly, despite higher frailty scores, women live longer than men (2), raising questions about the reasons and molecular mechanisms for this difference.

Validated animal models are still lacking in preclinical research, which could make an important contribution to the study of the pathophysiology of frailty. Most preclinical studies on frailty in rodents are based on the assessment of frailty parameters in old animals, mainly using male C57BL/6J mice (3). However, this strain is predisposed to lymphatic and hematopoietic cancers, and carcinogenic mechanisms may interfere with frailty pathways. Assessment of the frailty phenotype in rodents is based on the evaluation of weight and a number of physical parameters (including physical activity, endurance, grip strength, and walking speed) by which the animals are classified as robust, prefrail, or frail (3,4). Because the percentage of naturally occurring frailty in mice is very low (7%–9%) (3,5), studies in aged mice require large numbers of animals, are expensive and time-consuming. The senescence-accelerated mouse prone-8 (SAMP8) and

Received: February 23 2023; Editorial Decision Date: June 13 2023.

its control (senescence-accelerated mouse resistant, SAMR1, characterized by normal aging) are strains spontaneously developed by breeding from the AKR/J colony (6). SAMP8 mice are widely used as a model of physical and mental aging (6,7). Indeed, these animals show musculoskeletal and cardiac aging (7), high oxidative stress (8), inflammation, as well as age-related deterioration in memory and learning abilities (6) and epigenetic alterations (9). It has been reported that the mean life span of SAMP8 mice ranges from 9.5 to 12 months, whereas the life span of SAMR1 mice ranges from 16.5 to 19.5 months, with a percent survival of SAMP8 mice ranging from 90%–100% at 6 months to 70%–90% at 9 months (10–13). Remarkably, no difference was found between male and female SAMP8 mice (13). Importantly, although SAMP8 mice have a shorter lifespan than SAMR1 mice, available data suggest that environmental factors in different laboratories may have a major impact at this level. Taken together, these data support the idea that SAMP8 mice could be used as a model for frailty, although this has never been extensively studied before.

The molecular basis of frailty, its development, and its mechanistic link to aging are still poorly understood. The most common hypotheses regarding the pathophysiology of frailty are cell senescence, mitochondrial dysregulation, inflammation, and oxidative stress (14). At the molecular level, microRNAs (miRNAs), small (20–22 nucleotides) non-coding RNAs with a key role in posttranscriptional regulation of gene expression, are important molecular mediators of cellular processes involved in both physiological and pathological aging (15,16). In addition to their presence in cells, miRNAs have also been detected in various peripheral biological matrices, including whole blood, and have been proposed as peripheral biomarkers for several diseases (16). A few studies reported miRNA changes in the blood of frail individuals compared with robust people, suggesting that miRNAs may represent potential new candidate biomarkers for frailty in old age (17,18).

In this study, to challenge the hypothesis that SAMP8 can be used as a model of frailty, we investigated the frailty phenotype in 6- and 9-months-old male and female SAMP8 mice, together with measuring blood levels of selected miRNAs as putative peripheral biomarkers of frailty.

Materials and Methods

Animals

Male and female SAMP8 and SAMR1 mice were purchased from ENVIGO RMS RSL (San Pietro al Natisone, UD, Italy). Mice were housed under standard conditions (20–22° C, 12 h light/dark cycle, light on at 7 a.m.), with water and food *ad libitum*. Animal husbandry and experimental procedures were performed in accordance with European Community Council Directive 2010/63/UE and approved by the Italian legislation on animal experimentation (Decreto Legislativo 26/2014, authorization N 951/2018-PR). All the animals were weighed and analyzed for behavior at 6- and 9-months of age. Mice were sacrificed at the end of behavioral testing at 9-months of age. All efforts were made to minimize the stress on mice and reduce the number of animals used in this study.

Behavioral Analysis

At the age of 6 and 9 months, mice were exposed to a battery of tests (one per day) in the following order: Open Field Test (OFT), hanging test, grip strength test, and rotarod test. Each day, the animals were moved into the behavioral room and allowed to acclimatize for at least 30 minutes before the beginning of behavioral tests. Male and female mice were tested on separate days to account for sensitivity to pheromones during behavioral testing.

Open Field Test

Mice were individually placed in the center of a square arena (44 × 44 × 30 cm) and allowed to explore the apparatus for 10 minutes (19,20). The test was conducted in a poorly lit room. To evaluate central exploratory behavior, a central (20 × 20 cm) area and a periphery area were defined. Total distance traveled and time spent in the center were analyzed by a video-tracking system (Any-Maze purchased by Ugo Basile, Varese, Italy). The open field maze was cleaned with a 70% ethyl alcohol solution at the end of each trial. OFT was used to evaluate both frailty parameters (total distance traveled and walking speed) and the anxious-like phenotype (time spent in the center of the arena).

Hanging Test

Hanging test was used to measure muscle strength and endurance (21). A single mouse was placed on a wire mesh (30 × 30 cm), which was then carefully turned upside down. The wire mesh was held at a height of 50–60 cm above a soft surface to prevent failing injuries. Duct tape was placed around the perimeter of the wire mesh to prevent the mouse from walking over the edge. Latency to fall was measured, with an elapsed time of 60 seconds. Each mouse was tested 3 times with an intertrial interval of 5–10 minutes. The average of the 3 measurements was used for the statistical analysis.

Grip Strength

A grip strength meter (Ugo Basile) was used to measure grip strength (5). A single mouse was positioned on the top of the grid and allowed to grasp the grid with all 4 paws. Then the mouse tail was pulled back slowly and steadily, keeping the mouse torso in a horizontal position, until it released its grip. Each mouse was tested 5 times with an intertrial interval of 1–2 min. Average of the 5 measurements was used for the statistical analysis.

Rotarod Test

The rotarod test is a performance test based on a rotating rod with forced motor activity widely used for determining overall motor function in mice (3,21). Mice were initially placed on the rod and habituated to the apparatus (Ugo Basile) by walking at 4 rpm for 60 seconds. Then mice were subjected to three accelerated trials with an intertrial interval of 30 minutes. During the accelerated rotarod test, the rod cylinders accelerate from 4 rpm to 40 rpm over a period of 5 minutes. Rotation speed and latency time were recorded when the mouse fell. Average of the 3 measurements was used for the statistical analysis.

Mouse Frailty Phenotype Evaluation

The mouse frailty phenotype assessment was determined as previously described (3,4,21) with minor modifications (Table 1), considering the following parameters: activity (total distance measured in the OFT), derived endurance score (average of the latencies measured in rotarod and hanging tests), weakness (grip strength), derived slowness

Table 1. Frailty Criteria Applied for the Classification of the Animals in Robust, Prefrail, and Frail

Frailty Criteria		
Human Frailty Criteria Fried et al. (2021)	**Mouse Frailty Criteria**	**Cutoff Values Mean 1.5*SD***
Low activity	Total distance traveled	Males: 4.68 m Females: 16.02 m
Poor endurance	Rotarod (latency) + hanging	Males: 76.42 s Females: 121.03 s
Weakness	Grip strength	Males: 157.77 g/f Females: 161.71 g/f
Slowness	Velocity (OFT) + rotarod (RPM)	Males: 0.19 m/s Females: 0.29 m/s
Unintentional weight loss	Body weight	Males: 35.38 g Females: 24.65 g

Note: OFT = open field test; RPM = revolutions per minute; SD = standard deviation.

score (average of the maximum speeds measured in the OFT and rotarod test), and weight loss. To deem the animals as frail, prefrail, or robust, the performance of each mouse was assessed in each of these criteria based on previous publications (3,4,21). Briefly, a cutoff was set at 1.5 *SD* below the control group mean for each parameter, and animals with 3 or more parameters below the cutoff were defined as frail, animals with 2 parameters below the cutoff were defined as prefrail, although the others were considered robust. Cutoff points for male and female mice were calculated separately (Table 1).

Blood Collection, RNA Isolation, Reverse Transcriptase, and qPCR

Whole blood was collected into sodium citrate from anesthetized mice by cardiac venipuncture as previously described (22) and immediately mixed with an equal volume of TRIFAST reagent (Euroclone, Milan, Italy). Total RNA was obtained using the Direct-zol RNA MiniPrep (Zymo Research, purchased by Euroclone, Milan, Italy) according to the manufacturer's instructions. Reverse transcription of miRNAs was carried out using the miRCURY LNA RT kit (Exiqon) according to the manufacturer's instructions. qPCR analysis was performed on a CFX Connect Real-Time System (Bio-Rad, Hercules, CA) using the iTaq Universal SYBR Green supermix (Bio-Rad) with specific primers (miRCURY LNA miRNA PCR assay, Exiqon). Experimental miRNAs were normalized to the levels of miR-16a (23). The relative expression of miRNAs was calculated by the comparative C_t ($\Delta\Delta C_t$) method and expressed as fold change (24).

Patients' Recruitment, Clinical Assessment, and RNA extraction

This study was approved by the local Ethical Committee, registration number "91 A/CESC 16/10/2018" and performed following the Declaration of Helsinki Principles. Patients' recruitment, clinical assessment, and RNA extraction from human blood were described in Carini et al. (18). All participants were gathered at ULSS 3 "Dolo" (Venezia) and were clinically examined by specialists in geriatric. Age > 70 years and both genders were considered as inclusion criteria, although life expectancy of less than 12 months and the presence of acute or chronic diseases that might affect the study's outcomes (eg, NYHA class 3–4 heart failure, severe renal or hepatic failure, dementia, major depression, or other relevant neurological/psychiatric diseases) were considered exclusion criteria. After having obtained the informed consent, a questionnaire was completed about their recent and past medical histories and current medications. A physical examination was then performed, including measurements of height and weight.

Patients have been divided into robust and fragile categories using the Fried's frailty phenotype criteria suggested by (1) ie, (a) unintentional weight loss > 5% in the last year; (b) weakness, as measured by handgrip strength; (c) slow gait speed over 4 m of walking; (d) exhaustion, determined by asking the participant on the 30-item Geriatric Depression Scale, "Do you feel full of energy?," and (e) low energy expenditure, as determined by the Physical Activity Scale for the Elderly (PASE) (25) defined as weekly physical activity below 383 kilocalorie/week for men and 270 kilocalorie/week for women. Individuals with at least 3 of these criteria were defined as frail (1). Nineteen frail and 22 robust subjects were age- and sex-matched.

Whole blood from robust and frail subjects was collected in the PAXgene Blood RNA Tube (PreAnalytiX GmbH, Hombrechtikon, Switzerland), and total RNA enriched of small RNAs (smRNAs) was extracted using the PAXgene Blood miRNA kit (PreAnalytiX GmbH) according to the manufacturer's protocol. RNA quality control was assessed on an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA).

Statistical Analysis

Statistical analysis of the data was carried out using GraphPad Prism9 (GraphPad Software Inc., Boston, MA). Results are presented as mean ± standard error of the mean (SEM). All the data were analyzed using unpaired *t*-test or one-way ANOVA followed by Tukey's post hoc multiple comparison test, when appropriate.

Results

Body Weight was Reduced Selectively in Male SAMP8 Mice

Male SAMP8 mice had lower body weight compared with SAMR1 mice at both 6 (unpaired *t*-test, $p < .0001$;

Supplementary Figure 1A) and 9 months of age (unpaired *t*-test, $p < .0001$; Figure 1A). In female mice, there was no significant difference in body weight between SAMP8 and SAMR1 mice at either 6 or 9 months of age (Supplementary Figure 1E; Figure 1I).

SAMP8 Mice Showed Higher Locomotor Activity and Anxiety-Like Behavior

Total locomotor activity and maximum speed measured in the OFT were higher in SAMP8 males compared with SAMR1 at both 6 and 9 months of age (unpaired *t*-test, $p < .0001$) (Supplementary Figure 1B–C; Figure 1B–C respectively). In addition, 6-month-old male SAMP8 mice, but not 9-month-old male SAMP8 mice, spent less time in the center of the OFT (unpaired *t*-test, $p < .05$) (Supplementary Figure 1D; Figure 1D respectively), suggesting an anxious-like phenotype compared with SAMR1 mice at the younger age. In contrast, female SAMP8 mice showed only a significant increase in total locomotor activity at 9 months of age (unpaired *t*-test, $p < .05$) (Figure 1J), with no other significant differences (Figure 1K–L; Supplementary Figure 1F–H).

Muscle Strength and Rotarod Latency are Reduced in SAMP8 Mice

To investigate skeletal muscle function, SAMP8 and SAMR1 mice were subjected to hanging and grip strength tests. In the hanging test, 9-month-old SAMP8 mice hung on wire mesh for a shorter time than SAMR1 mice, regardless of sex (unpaired *t*-test, males: $p < .0001$; females: $p < .0001$) (Figure 1E and M). Similar results were observed in younger animals (see Supplementary Figure 2A and D). Grip strength was also reduced in both male (unpaired *t*-test, $p = .0001$) and female ($p < .0001$) SAMP8 mice compared with SAMR1 at both 9 months (Figure 1F and N) and 6 months of age (see Supplementary Figure 2B and E).

Similarly, as for latency measured in the rotarod test, we found that both male and female SAMP8 mice performed worse compared with SAMR1 mice, regardless of age (unpaired *t*-test, $p < .0001$) (Figure 1G and O; Supplementary Figure 2C and F).

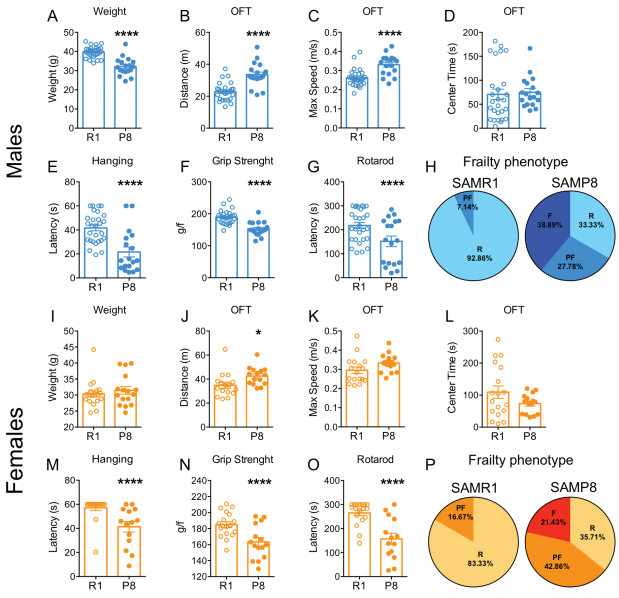

Figure 1. SAMP8 mice displayed reduced muscle strength and coordination. A and I: Body weight measured in 9-month-old male (A) and female mice (I). B–D and J–L: Open Field Test (OFT). Total distance (B, J), maximum speed (C, K), and total time spent in the center measured in 10 min OFT (D, L), in 9-month-old male (B–D) and female mice (J–L). E and M: hanging test in 9-month-old male (E) and female mice (M). F and N: grip strength in 9-month-old male (F) and female mice (N). G and O: rotarod test in 9-month-old male (G) and female mice (O). Unpaired *t*-test, *$p < .05$; ****$p < .0001$. H and P: Percentage of prefrail and frail mice estimated by the mouse frailty phenotype criteria in 9-month-old male (H) and female mice (P). Males: $n = 18–28$ mice/ group. Females: $n = 14–18$ mice/ group. R1: SAMR1; P8: SAMP8.

Most SAMP8 Mice Displayed a Prefrail/Frail Phenotype, With Some Sex Differences

To identify frail mice, we applied the 5 criteria used to define the frailty phenotype: low activity, poor endurance, weakness, slowness, and reduced weight; see Methods for more details (3,4,21; Table 1). A cutoff was set at 1.5 *SD* below the control group mean for each parameter, and animals with 3 or more parameters below the cutoff were defined as frail, animals with 2 parameters below the cutoff were defined as prefrail, and the others were considered robust. The cutoff points for male and female mice were calculated separately (Table 1).

At 6 months of age, 14.29% of male SAMP8 mice were classified as prefrail, whereas 61.9% were considered frail (Supplementary Figure 3). None of the male SAMR1 mice were prefrail or frail (Supplementary Figure 3). Conversely, 60% of 6-month-old female SAMP8 mice were prefrail, whereas 13.33% were frail. Although no female SAMR1 mice were frail, 5.56% were classified as prefrail (Supplementary Figure 3).

At 9 months of age, 7.14% of male SAMR1 mice were classified as prefrail, whereas none were frail (Figure 1H, left pie chart). Instead, 27.78% of male SAMP8 mice were classified as prefrail, whereas 38.89% were frail (Figure 1H, right pie chart). Differently, 16.67% of female SAMR1 mice were classified as prefrail, whereas none were classified as frail (Figure 1P, left pie chart). Finally, 42.86% of female SAMP8 mice were prefrail, whereas 21.43% were frail (Figure 1P, right pie chart). It is however important to mention that from 6 to 9 months of age, 3 male and 1 female SAMP8 mice died.

Overall, our data show that the majority of SAMP8 mice (both male and female) exhibit a frail/prefrail phenotype at as early as 6 months and at 9 months of age, although SAMR1 mice remain largely robust up to 9 months of age.

Whole Blood Levels of Selected miRNAs Were Altered in SAMP8 Mice

To determine whether behavioral changes and/or the prefrailty/frailty phenotype in SAMP8 mice were associated with changes in blood miRNA levels, we measured by qPCR the expression of a panel of miRNAs previously reported to be involved in frailty (miR-1-3p, miR-34a-5p, miR-101-3p, miR-133a-3p, miR-142-5p), aging (miR-1-3p, miR-26b-5p, miR-34a-5p, miR-101-3p, miR-142-5p, miR-331-3p), senescence (miR-26b-5p), and cognitive deficits (miR-26b-5p, miR-34a-5p, miR-101-3p, miR-142-5p, miR-331-3p; 17,18,26–28).

First, we compared the levels of the selected miRNAs between SAMR1 and SAMP8 mice in male and female animals separately. In male mice, the expression of miR-26b-5p ($p < .01$; Figure 2B), miR-34a-5p ($p < .01$; Figure 2C), and

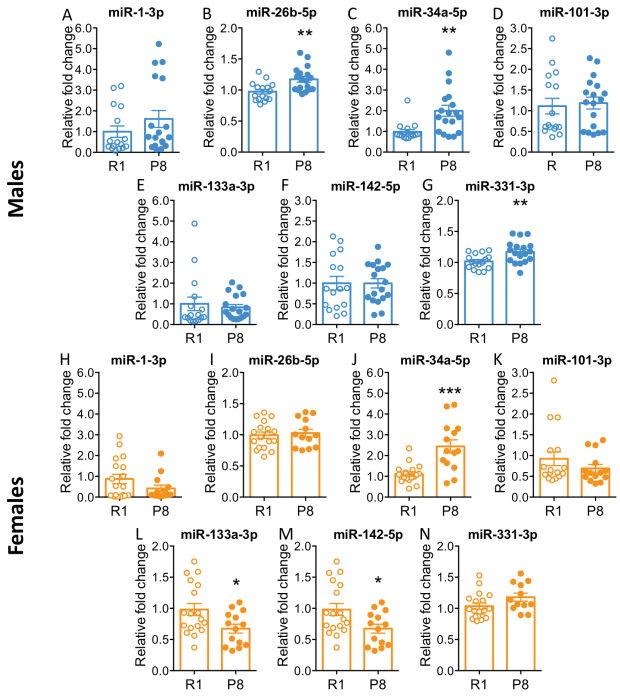

Figure 2. MiRNAs are differentially expressed in the whole blood of male and female SAMR1 and SAMP8 mice. qPCR analysis of expression levels of selected miRNAs in the whole blood of 9-month-old SAMR1 (R1) and SAMP8 (P8) male (A–G) and female mice (H–N). Unpaired *t*-test, **p* < .05; ***p* < .01; ****p* < .001. Males: *n* = 12–18 mice/group. Females: *n* = 12–18 mice/ group.

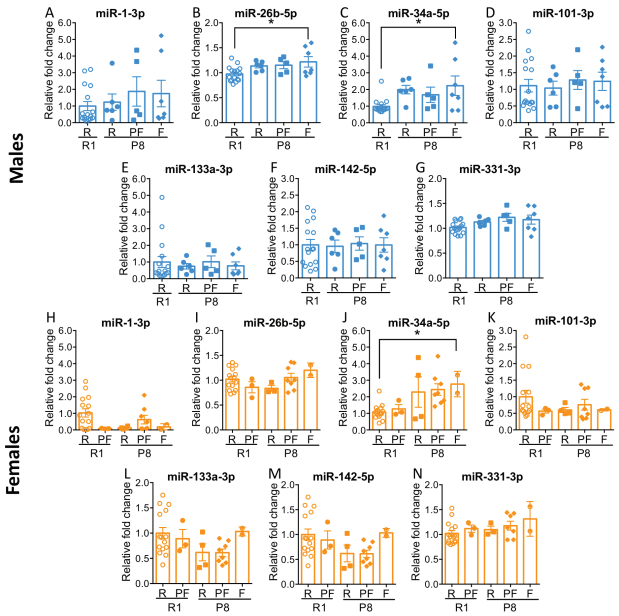

Figure 3. MiRNAs are differentially expressed in the whole blood of SAMR1 and SAMP8 prefrail and frail male and female mice. qPCR analysis of the expression levels of selected miRNAs in the whole blood of 9-month-old SAMR1 (R1) and SAMP8 (P8) (A–G) and female mice (H–N), after classification in robust, prefrail, and frail. One-way ANOVA followed by Tukey's post hoc analyses, **p* < .05. Males: *n* = 3–16 mice/ group. Females: *n* = 2–15 mice/group. F = frail; PF = prefrail; R = robust;.

miR-331-3p ($p < .01$ Figure 2G) were significantly increased in the whole blood of SAMP8 mice compared with SAMR1 mice. In female mice miR-34a-5p was also increased in SAMP8 mice compared with SAMR1 ($p < .001$; Figure 2J), although miR-133a-3p and miR-142-5p levels were significantly reduced ($p < .05$; Figure 2L and M, respectively).

Whole Blood miRNAs Levels Were Altered in Prefrail and Frail Mice

We then analyzed the changes in miRNA levels in male and female mice after deeming the animals into robust, prefrail, or frail (Figure 3). In males, one-way ANOVA analysis revealed a significant difference among groups for miR-26b-5p ($F_{(3,\ 30)} = 3.785$; $p = .026$; Figure 3B) and miR-34a-5p ($F_{(3,30)} = 4.69$; $p = .0385$; Figure 3C). Tukey's post hoc analysis revealed that both miR-26b-5p ($p < .05$) and miR-34a-5p ($p < .05$) were upregulated in SAMP8 frail mice compared with SAMR1 robust mice. In females, one-way ANOVA analysis revealed a significant difference only for miR-34a-5p ($F_{(4,27)} = 10.11$; $p = .0019$; Figure 3J), which, as for males, was upregulated in frail SAMP8 mice compared with robust SAMR1 mice (Tukey's post hoc analysis $p < .05$).

Finally, we analyzed miRNA levels by pooling all mice according to whether they were robust, prefrail, or frail, regardless of genotype and sex (Figure 4). One-way ANOVA analysis revealed a significant difference for the levels of miR-26b-5p ($F_{(2,62)} = 4.009$; $p = .023$; Figure 4B), miR-34a-5p ($F_{(2,63)} = 6.113$; $p = .004$; Figure 4C), and miR-331-3p ($F_{(2,61)} = 4.957$; $p = .001$; Figure 4G). Notably, the levels of miR-26b-5p were increased only in frail compared with robust mice ($p < .05$; Figure 4B), although the levels of miR-34a-5p and miR-331-3p were increased in both prefrail ($p < .05$) and frail ($p < .05$) compared with robust mice (Figure 4C and G respectively).

MiR-331-3p Levels Were Elevated in the Whole Blood of Human Frail Subjects

Having found that the levels of miR-26b-5p, miR-34a-5p, and miR-331-3p were increased in whole blood from prefrail/frail mice, we asked whether these changes were also detectable in a small group of frail patients (19 frail patients and 22 robust age- and sex-matched controls) (18), with the aim of verifying whether blood changes of these miRNAs could be considered translational biomarkers of frailty. Unfortunately, the levels of miR-26b-5p and miR-34a-5p were very low and not detectable by qPCR in our samples but, in line with the results obtained in the animal model, we measured a significant increase of miR-331-3p in the frail group compared with the robust group (Figure 4H; $p < .05$).

Discussion

In the present work, we investigated sex differences in the frailty phenotype between SAMP8 and SAMR1 mice and measured whole blood levels of selected miRNAs as possible peripheral biomarkers of frailty. We found that both male and female SAMP8 mice displayed higher frailty scores than the SAMR1 control line, showing that SAMP8 mice can be used to study frailty at the preclinical level. In addition, we collected evidence for genotype- and frailty phenotype-dependent sex differences in the whole blood levels of selected miRNAs.

Frailty is a geriatric syndrome characterized by a diminished ability to cope with stressors and a general decline in physiological functions (14,29). Although the clinical relevance of frailty has increased in recent years, its etiology

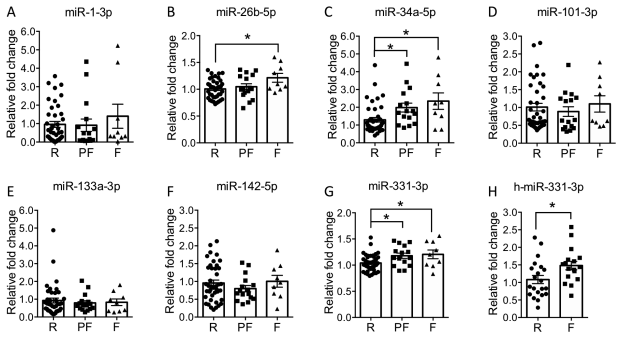

Figure 4. MiRNAs are differentially expressed in the whole blood of prefrail and frail mice. A–G: qPCR analysis of the expression levels of selected miRNAs in the whole blood of 9-month-old mice based on frailty phenotype only, regardless of genotype and sex. One-way ANOVA followed by Tukey's post hoc analyses. $N = 6$-34 mice/group. *$p < .05$. R = robust; PF = prefrail; F = frail. H = MiR-331-3p expression is increased in the whole blood of frail humans. qPCR analysis of the expression levels of miR-331-3p in the whole blood of robust and frail human subjects. Unpaired *t*-test, *$p < .05$. Robust: $n = 22$ subjects/group. Frail: $n = 19$ subjects/group. F = frail; R = robust.

remains poorly understood, and preclinical models of frailty could be of great help in this context. In particular, a reliable frailty mouse model would allow testing and developing of potential new therapies for the treatment and possible prevention of frailty. Unfortunately, only a few mouse models of frailty have been described in the literature to date, based largely on natural aging (3,4,30). However, because the percentage of naturally occurring frailty in mice is very low, studies using aged mice require a large number of animals and thus are very expensive and time-consuming. An interesting alternative could be genetically modified mice such as interleukin-10 (31) and copper/zinc superoxide dismutase (32) knock-out mice. Unfortunately, although these 2 models are potentially attractive frailty models because they exhibit reduced grip strength, physical activity, and endurance, frailty itself has never been measured in either model (19).

SAMP8 is a natural mouse model of accelerated aging that has been used primarily in cognitive and neurodegenerative studies (9,33). Here, we tested this strain as a model of frailty. We found that although only male SAMP8 mice (and not females) exhibited significant weight loss compared with SAMR1 mice, both male and female SAMP8 mice showed decreased muscle strength, endurance, and motor function compared with SAMR1 mice as early as 6 months of age. Our results are consistent with previous studies showing a more rapid decline in muscle function and locomotor activity in male SAMP8 mice compared with SAMR1 mice (34) and extend these changes to female SAMP8 mice. In male SAMP8 mice, decreased muscle strength has been previously associated with decreased phosphocreatine levels, decreased muscle mass and contractility, atrophy of II-type fibers (35), and dysregulation of sarcopenia-related genes (36).

Using the criteria of frailty phenotype in mice (3,4,21), we detected frail mice at 6- and 9-months of age in the SAMP8 strain but not in the SAMR1 line (in which only a few prefrail animals were identified). Considering that previous studies have identified the onset of frailty starting at 17 months of age in both male and female C57Bl/6 and NIH Swiss mice (4,5,37), our model is the mouse model with the earliest onset of frailty in mice ever reported.

Our results showed that the overall level of frailty (prefrail and frail mice combined) was very similar in male and female SAMP8 mice at both 6- and 9-months of age, although the percentage of frail mice was higher in males than females. On the other hand, the percentage of female prefrail mice was higher in both SAMP8 and SAMR1 mice (in which no male prefrail animals were found at 6 months of age). Previous studies in humans have reported that the prevalence of frailty is generally higher in females than in males, although this difference appears to be age-dependent and becomes more pronounced after the age of 80 years (38). Studies in animal models are less consistent, with some showing no sex differences (30) and others showing higher frailty scores in female C57BL/6 (4) and NIH Swiss mice (37), although this was reported to be age-dependent and transient. The contrast between the results of the present study in SAMP8 mice and previous evidence in both humans and mice could be due to the age of the animals used here (previous studies used older animals and other mouse strains), the limited sample size, and/or differences between humans and mouse models.

Interestingly, from 6 to 9 months of age, we observed a decrease in males (from 61.9% to 38.89%) and an increase in females (from 13.33% to 21.43%) in the percentage of frail SAMP8 animals. However, it must be noted that 3 male SAMP8 mice died during the experiment, which affected the overall ratio of robust to prefrail/frail mice. In addition, we only studied the mice at 6- and 9-months of age and used a limited number of mice. We used the frailty phenotype in mice to be consistent with our clinical study, in which we used the same criteria to identify frail humans. It has been reported that the frailty phenotype and frailty index do not completely overlap in identifying frail mice, probably because the frailty phenotype assesses only activity capacity and weight loss, whereas the frailty index is used to assess the accumulation of age-related diseases in multiple organs (39). Therefore, it is possible that by using the frailty phenotype, we underestimated the total number of frail mice and overlooked the presumably increasing prevalence of frailty with age. Future longitudinal studies across the life span with a larger number of animals and eventually considering both the frailty phenotype and the frailty index are needed to determine the specific timing of the onset of frailty in male and female SAMP8 mice and to determine whether the prevalence of frailty in SAMP8 mice increases with age in both sexes. Nevertheless, our data support the use of SAMP8 mice as an animal model of frailty.

Although the increased locomotor activity measured in both male and female SAMP8 mice compared with SAMR1 is not easy to explain, the anxious phenotype of 6-months old SAMP8 males confirms that the SAMP8 strain displays not only premature physical aging but also alterations in brain functions (6,7). Given the recent interest in cognitive frailty, operationally defined as the simultaneous presence of physical frailty and cognitive impairment without concurrent dementia (40), future studies should address whether SAMP8 mice could be used as an animal model of cognitive frailty as well.

Identification of biomarkers with diagnostic and prognostic capabilities would be a major challenge in classifying frailty risk and prefrailty status. Among the various putative biomarkers, miRNAs have gained interest in recent years (16–18). miRNAs have been shown to play important roles in systemic and cellular processes involved not only in physiological

aging but also in frailty and age-related diseases, including inflammation, cellular senescence, maintenance of skeletal muscle and energy metabolism, and maintenance of brain and neuronal functions (18,26). Here, we measured the levels of selected miRNAs that have been previously associated with frailty (miR-1-3p, miR-34a-5p, miR-101-3p, miR-133a-3p, miR-142-5p), aging (miR-1-3p, miR-26b-5p, miR-34a-5p, miR-101-3p, miR-142-5p, miR-331-3p), senescence (miR-26b-5p), and cognitive deficits (miR-26b-5p, miR-34a-5p, miR-101-3p, miR-142-5p, miR-331-3p) (17,18,26–28). We found sex-, genotype-, and frailty phenotype-dependent changes in blood miRNA levels. Considering frailty-dependent and genotype- or sex-independent alterations, we found that miR-26b-5p was increased only in frail mice, whereas miR-34a-5p and miR-331-3p were increased in both prefrail and frail mice compared with robust ones. However, considering the differences between sex, male mice appeared to have a main contribution to the increase of miR-26b-5p and miR-331-3p levels in frail animals. In contrast, the increase in miR-34a-5p was found in SAMP8 mice compared with SAMR1 mice, and specifically in frail mice compared with robust mice in both sexes, suggesting that an increase in blood miR-34a-5p levels could be considered as a biomarker for frailty in preclinical models, regardless of sex. Interestingly, we found that the levels of miR-331-3p were also elevated in the whole blood of a small group of frail individuals. This is a very preliminary result, and further studies in a larger group of frail patients are needed to assess whether increased blood levels of miR-331-3p could be used as a translational biomarker of frailty. Conversely, the levels of miR-101-3p and miR-142-5p, which we have previously found reduced in the human blood of frail individuals compared with the robust group (18), were not significantly different in frail mice compared with robust ones, suggesting that these miRNAs are biomarkers of frailty in humans only.

Interestingly, miR-26b-5p, miR-34a-5p, and miR-331-3p were previously associated with age-related processes and diseases.

miR-26b-5p belongs to the miR-26 family, which also includes miR-26a, miR-26b, miR-1297, and miR-4465, and is located on chromosome 2. miR-26b-5p regulates several important cellular mechanisms such as proliferation, differentiation, apoptosis, and autophagy, and its expression was found to be altered in Alzheimer's disease, sarcopenia, and cardiovascular diseases (28,41,42). miR-26b regulates microglial inflammation in hypoxia/ischemia (43), its dysregulation alters macrophage inflammatory response and may be involved in age-related inflammatory diseases (44). The expression of miR-26b was increased in the brain of Alzheimer's disease patients, and its upregulation in neuronal cells promotes tau phosphorylation and apoptosis (45).

miR-34a-5p is a member of the miR-34 family, which includes miR-34a, miR-34b, and miR-34c, and is located on chromosome 1. It is expressed in almost all tissues and is particularly abundant in the brain (46). miR-34a-5p regulates several important cellular functions (growth, proliferation, death, survival) and has been associated with various age-related conditions (frailty, skeletal and muscular disorders, neurological, cardiovascular, and metabolic diseases, cancer) (17,26,28,46). miR-34a-5p was found to be increased in the skeletal muscle of old people and was suggested to play a role in cellular senescence via the activation of the MAPK pathway (47). miR-34a-5p expression was also reported to be increased in the heart and spleen of mice with advancing aging, and its overexpression promoted senescence in endothelial cells through the regulation of the anti-aging SIRT1 protein (48). Levels of miR-34a-5p were also increased in the brain of aged rats and correlated with the reduction of SIRT1 (49).

There are a few studies on miR-331 that have mainly focused on cancer research. miR-331-3p, a member of the miR-331 family localized on chromosome 12q22n, regulates cell cycle progression, migration, and invasion in various cancers (50). At the mechanism level, miR-331-3p has been reported to regulate cell apoptosis, inflammation, oxidative stress, and specific autophagic pathways (51). In addition, upregulation of miR-331-3p has been described in the plasma of older (27) and patients with Parkinson's disease (52). Circulating miR-331-5p levels have been identified as a potential biomarker for osteoporosis and subsequent bone fractures (53).

Overall, the miRNAs that we have found to be associated with the frailty phenotype were previously implicated in the regulation of cellular processes involved in aging, such as apoptosis, autophagy, inflammation, and oxidative stress. This suggests that these miRNAs may not only be considered as peripheral biomarkers of frailty but could also have a mechanistic role in both physical and mental frailty. Considering the lack of knowledge about the etiopathological mechanisms of frailty, the possible functional roles of these miRNAs should be explored in more detail in future studies.

In conclusion, this is the first study to determine frailty in SAMP8 mice using the frailty phenotype approach. Our results show that the prevalence of prefrail/frail mice is higher in SAMP8 mice than in SAMR1 mice, and that frailty is accompanied by increased levels of miR-26b-3p, miR-34-p, and miR-331-3p in the blood. Although future studies are needed to better characterize the onset and prevalence of frailty across the life span in SAMP8 mice, our study suggests that SAMP8 mice may be a useful preclinical animal model to investigate the mechanisms underlying frailty, identify potential biomarkers, and test therapeutic approaches.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

Funding

This work was supported by research grants from Cariplo Foundation (grants: 2017-0620; 2018-0525).

Conflict of Interest

None.

Author Contributions

Conceptualization: A.I., L.M., A.B., and M.P. Formal analysis: A.I. Investigation: A.I., G.C. Writing—original draft preparation: A.I. Writing—review and editing: L.M., G.C.,

S.S.B; S.M., N.V., M.P., and A.B. Funding acquisition: S.S.B; M.P., and A.B. All authors have read and agreed to the published version of the manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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