

Circulating mitochondrial DNA increases with age and is a familiar trait: Implications for “inflamm-aging”

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Mitochondrial components, including mitochondrial DNA (mtDNA), when released extracellularly, can act as “damage-associated molecular pattern” (DAMP) agents and cause inflammation. As many elderly people are characterized by a low-grade, chronic inflammatory status defined “inflamm-aging,” we evaluated if circulating mtDNA can contribute to this phenomenon. Eight hundred and thirty-one Caucasian subjects were enrolled in the study, including 429 siblings aged 90–104 (90+ siblings). mtDNA plasma levels increased gradually after the fifth decade of life. In 90+ subjects, mtDNA values of two members of the same sibling relationship were directly correlated, suggesting a role for familiar/genetic background in controlling the levels of circulating mtDNA. The subjects with the highest mtDNA plasma levels had the highest amounts of TNF- α , IL-6, RANTES, and IL-1ra; the subjects with the lowest mtDNA levels had the lowest levels of the same cytokines. In vitro stimulation of monocytes with mtDNA concentrations similar to the highest levels observed in vivo resulted in an increased production of TNF- α , suggesting that mtDNA can modulate the production of proinflammatory cytokines. Our findings therefore show that circulating mtDNA increases with age, and can significantly contribute to the maintenance of the low-grade, chronic inflammation observed in elderly people.

Keywords: Aging · Circulating mtDNA · Inflammation · Longevity · Survival · Ultranonagenarian siblings



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Introduction

With the recent dramatic increase in human life expectancy, the immune system is facing Ag exposure several decades more than in our recent evolutionary past. Thus, it is likely that a lifelong antigenic stress is on the basis of the observed increased inflammatory status typical of old age, a condition referred to as “inflamm-aging” [1]. This peculiar chronic, low-grade inflammation is thought to be a major contributor to age-associated frailty, morbidity, and mortality. However, the precise mechanisms and molecules that cause such phenomenon have not been yet identified.

Age-related diseases such as atherosclerosis, Alzheimer’s disease, Parkinson’s disease, and type 2 diabetes, among others, are initiated or worsened by systemic inflammation [2–5]. Nevertheless, very recent evidence suggests that plasma levels of proinflammatory cytokines increase with age independently of chronic antigenic burden such as CMV [6]. It is therefore possible that stimuli different from “classical” Ags derived from viruses and bacteria can also contribute to the phenomenon of inflamm-aging. In this regard, it has been observed that intramitochondrial components, including mitochondrial DNA (mtDNA), *N*-formyl peptides, and lipids such as cardiolipin, can be released extracellularly, enter the blood flow, and act as damage-associated molecular pattern (DAMP) agents, triggering the same pathways that respond to PAMPs, and cause inflammation, traumatic shock [7], and heart failure [8]. Thus, mitochondria not only participate in danger signaling inside the cell, but are also a major source of molecule able to activate an innate immune response [9]. Consistently, it has been observed that mtDNA can bind TLR-9, a receptor able to sense unmethylated DNA with CpG motifs derived from bacteria and viruses, and activate its downstream pathway [10–12]. Accordingly, it has been shown that mtDNA plasma levels increase during HIV infection [13], a condition characterized by a chronic inflammatory status [14]. Therefore, we wondered whether circulating mtDNA could give a significant contribution to the onset of inflamm-aging.

To address this question, we have evaluated mtDNA content in plasma samples of subjects of different ages, from 1 to 104 years. In order to evaluate a possible familiar/genetic contribution to the circulating level of mtDNA, we compared such level in a large cohort of ultranongenarian (90+) siblings. We also evaluated the possible correlation between plasma level of mtDNA and proinflammatory cytokines in old subjects and 90+ siblings, and the capacity of mtDNA to stimulate the production of proinflammatory cytokines in vitro. Finally, we assessed the predictive role of mtDNA plasma levels to 4 years survival after the age of 90.

Results

mtDNA plasma levels change with age and are highly correlated in 90+ sibling pairs

We measured mtDNA content in plasma samples from 831 subjects belonging to five different age groups (Table 1), from 1 to 104

Table 1. Characteristics of the subjects enrolled in the study, by age and sex.

Sex	Group 1 (1–11 years)		Group 2 (21–41 years)		Group 3 (51–76 years)		Group 4 (sib1–proband, ≥90 years)		Group 5 (sib2, ≥90 years)	
	Number of subjects	Age (years)	Number of subjects	Age (years)	Number of subjects	Age (years)	Number of subjects	Age (years)	Number of subjects	Age (years)
	N (%)	Mean (95% CI)	N (%)	Mean (95% CI)	N (%)	Mean (95% CI)	N (%)	Mean (95% CI)	N (%)	Mean (95% CI)
Males	50 (45.9)	6.8 (6.1–7.6)	32 (51.6)	33.4 (31.4–35.3)	64 (27.7)	64.2 (62.8–65.7)	65 (29.1)	95.0 (94.3–95.7)	68 (33.0)	91.7 (91.3–92.2)
Females	59 (54.1)	7.0 (6.3–7.7)	30 (48.4)	32.0 (30.0–34.1)	167 (72.3)	61.4 (60.4–62.3)	158 (70.9)	95.2 (94.7–95.6)	138 (77.0)	92.0 (91.7–92.4)
Total	109	6.9 (6.4–7.4)	62	32.7 (31.3–34.1)	231	62.2 (61.4–63.0)	223	95.1 (94.7–95.5)	206	91.9 (91.6–92.2)

years of age. Data are reported in Table 2, after logarithmic (\log_{10}) conversion. The mtDNA mean plasma level was 10.99 (± 0.41) copies/mL in group 1 (children), 10.84 (± 0.36) copies/mL in group 2 (young adults), 11.42 (± 0.65) copies/mL in group 3 (old adults), 11.69 (± 0.56) copies/mL in group 4 (90+ probands), and 11.66 (± 0.55) copies/mL in group 5 (90+ younger siblings). No difference was present between group 1 and 2, while a significant increase was observed from group 2 onwards. The smallest variability in mtDNA level was observed in younger adults in comparison to all the others age groups.

Next, we divided subjects according to gender, and no difference in mtDNA content was observed between males and females considering both the whole population and the single age groups (Table 2).

By linear regression analysis (Fig. 1), we did not find a correlation between mtDNA content and age when group 1 and 2 were considered, but we could identify a direct correlation considering groups 2, 3, and 4 ($p < 0.001$; $r^2 = 0.1980$), that is, we found a significant increase after the age of 20 years.

We next wondered whether circulating mtDNA level had a familiar/genetic component. For this, we took advantage of the fact that groups 4 and 5 were composed of pairs of siblings. This allowed us to compare members of the same sibling relationship. We thus performed a linear regression analysis comparing the values measured in the probands (sib1) with those measured in the younger siblings (sib2).

As shown in Fig. 2, linear regression analysis showed a significant correlation in mtDNA plasma level between siblings of the same sibling relationship (i.e., the proband versus his/her younger sibling, $p < 0.0001$, $r^2 = 0.1975$). The same analysis was performed according to the gender of the siblings. Thus, we identified three combinations: male pairs, opposite sex pairs, and female pairs. A statistically significant correlation was found when the pair included one female ($p = 0.001$, $r^2 = 0.1589$ for opposite sex pairs; $p < 0.001$, $r^2 = 0.2980$ for females pairs). Such correlations were further confirmed by random permutation analysis, which showed that the correlations were lost when sibling pairs were shuffled in couples where the younger sibling was substituted with an unrelated subject but sharing gender, year, and place of birth with the younger sibling of the proband ($p = 0.367$, $r^2 = 0.0048$; data not shown). An additional confirmation was obtained by a linear regression analysis, which showed that the correlations in mtDNA level were missing when the 90+ probands were compared with the spouse of his/her offspring sharing the house and the daily habits with the old sibling ($p = 0.262$, $r^2 = 0.030$; data not shown).

mtDNA plasma levels correlate with proinflammatory cytokine levels

In order to understand if the level of circulating mtDNA was correlated with pro- or anti-inflammatory molecules, we quantified the levels of representative cytokines (TNF- α , IL-1 β , IL-6, IL-1ra, RANTES) in the plasma samples of the twenty 90+ subjects with

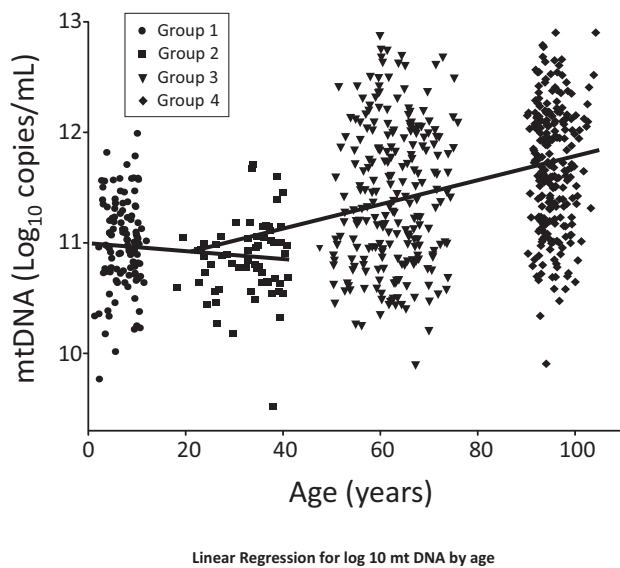
Table 2. Circulating mtDNA plasma levels in donors of different age, that is, from children to the oldest old.

Groups	Group 1 log ₁₀ mtDNA (copies/mL)	Group 2 log ₁₀ mtDNA (copies/mL)	Group 3 log ₁₀ mtDNA (copies/mL)	Group 4 (sib1—proband) log ₁₀ mtDNA (copies/mL)	Group 5 (sib2) log ₁₀ mtDNA (copies/mL)
Sex	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)
Males	10.99 (10.88–11.09)	10.79 (10.66–10.93)	11.56 ^{a),b)} (11.41–11.71)	11.57 ^{a),b)} (11.43–11.71)	11.64 ^{a),b)} (11.51–11.78)
Females	11.00 (10.88–11.11)	10.90 (10.78–11.03)	11.36 ^{a),b)} (11.26–11.46)	11.74 ^{a),b),c)} (11.65–11.82)	11.67 ^{a),b),c)} (11.58–11.76)
Total	10.99 (10.92–11.07)	10.84 (10.75–10.93)	11.42 ^{a),b)} (11.33–11.50)	11.69 ^{a),b),c)} (11.62–11.76)	11.66 ^{a),b),c)} (11.59–11.74)

^{a)} $p < 0.001$ versus group 1, ANOVA test with Bonferroni correction.

^{b)} $p < 0.001$ versus group 2, ANOVA test with Bonferroni correction.

^{c)} $p < 0.001$ versus group 3, ANOVA test with Bonferroni correction.



Groups	Number of obs.	R-squared	Beta Co eff.	Age p	95% CI
Group 1 and 2	171	0.0215	-0.0045	0.055	-0.0091 0.0001
Group 2, 3 and 4	516	0.1590	0.0115	<0.001	0.0092 0.0138

Figure 1. Age-related increase of circulating mtDNA plasma level in the different age groups. Data are generated from 831 samples, and are expressed as \log_{10} mtDNA copies/mL of plasma.

the highest levels of mtDNA and of the twenty 90+ subjects with the lowest levels. The results are reported in Fig. 3. The levels of TNF- α , IL-1 β , and IL-6 were similar to those observed in previous studies on healthy centenarians [15]. The levels of TNF- α , IL-6, RANTES, and IL-1ra were significantly higher in the group of subjects with the highest levels of mtDNA; such difference was not present for IL-1 β .

Monocytes are activated by high concentrations of mtDNA

In order to evaluate whether mtDNA (at the same plasma concentrations we have found) was able to induce the release of proinflammatory molecules, we purified peripheral blood monocytes (which express TLR-7 and TLR-9) from seven healthy young donors and stimulated them *in vitro* with the highest and the lowest concentration of mtDNA measured *ex vivo* in plasma from 90+ subjects, in combination or not with LPS. Then, we quantified the concentration of TNF- α present in the culture medium after 2 or 16 h of treatment. As expected, mtDNA alone was not able to induce any significant variation of TNF- α production by monocytes. After 2 h of treatment, no difference was observed among samples treated with LPS, or LPS in combination with mtDNA (Fig. 4, upper panel). However, the highest, but not the lowest, concentration of mtDNA was able to increase the response to LPS after 16 h of treatment (lower panel). The same amounts of nuclear DNA are not able to induce any increase in the production of TNF- α , either in the presence of LPS or not (not shown). As far as mRNA levels are concerned, TNF- α showed a significant

increase after 2 h of treatment with LPS. The highest concentration of mtDNA caused a slight, but not significant, increase in the effect of after 2 h of treatment (Fig. 5, upper panel). After 16 h, mRNA returned to basal levels in all samples (lower panel).

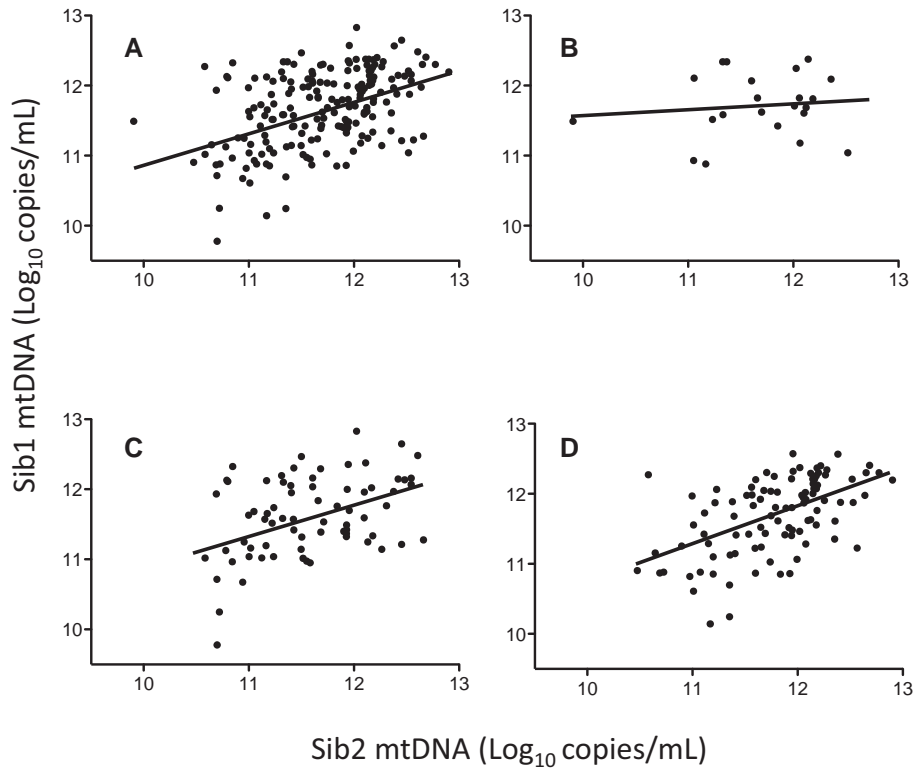
To confirm that release of TNF- α is mediated by triggering of TLR-9, we performed the same experiment in six additional donors, using the highest concentration of mtDNA alone, in combination with LPS, and in combination with LPS and ODNTT-1, an inhibitor of TLR-9. Again, mtDNA alone was not able to induce any activation, while it was able to increase the effect of LPS after 16 h of treatment (Supporting Information Fig. 1). This effect is completely abolished by the presence of ODN-TT1, a well-known inhibitor of TLR-9. The use of ODN-2395, an agonist of TLR-9, mimics the effects of mtDNA, further suggesting that mtDNA is able to trigger the pathway downstream of TLR-9 (Supporting Information Fig. 1). No effects were observed as far as the expression of IFN- α is concerned (not shown).

Correlation among mtDNA plasma level, health status, and mortality in old subjects and 90+ siblings

Within the age groups of old subjects and 90+ siblings, we evaluated the possible correlation between mtDNA content and the presence of diseases (actual and previous cancer, actual diabetes, cardiovascular diseases, and dementia), as well as between mtDNA level and body mass index (BMI). Circulating mtDNA level resulted not correlated with present or past diseases, with the exclusion of previous stroke/cerebral thrombosis/hemorrhage in old subjects (Supporting Information Table 1). A possible correlation between mtDNA content and the main hematobiochemical parameters (i.e., blood cell counts, erythrocyte sedimentation rate, C-reactive protein, glycaemia, albumin, creatinine, sodium, potassium) was also assessed. A weak association between mtDNA level and inflammatory parameters such as erythrocyte sedimentation rate, albumin, and C-reactive protein was found only in 90+ siblings. Interestingly, a stronger association was found with blood leukocytes subpopulations, such as neutrophil count, in both old subjects and 90+ siblings (Supporting Information Table 2). We then evaluated the correlation between plasma mtDNA content and mortality in the group of 90+ siblings. Siblings were divided in two groups, that is, those who died within 4 years after the enrolment: 230 subjects; \log_{10} mtDNA mean content: 11.67 (± 0.52) copies/mL, and those who were still alive after 4 years: 199 subjects; \log_{10} mtDNA mean content: 11.69 (± 0.58) copies/mL. Plasma levels of mtDNA were almost the same in the two groups, even when the age at the recruitment time was taken into account (Table 3). Finally, we observed that in this group age and gender, but not plasma mtDNA levels, were risk factors for the probability of survival (Table 4).

Discussion

In this study, we analyzed mtDNA content in plasma samples from a total of 831 subjects from 1 to 104 years, investigated the role of



Linear Regression for Sib1 mtDNA by Sib2 mtDNA

Groups	Number of pairs	R-squared	Beta Coeff.	p	95%CI
All pairs	191	0.1975	0.4558	<0.001	0.3240 0.5877
Males Pair	22	0.0167	0.1702	n.s.	-0.4384 0.7789
Opposite sex Pairs	72	0.1589	0.3980	0.001	0.1797 0.6162
Females Pairs	97	0.2980	0.5300	<0.001	0.3644 0.6958

Figure 2. Correlation of circulating mtDNA plasma level between 90+ sib1 and sib2 of the same sibling relationship. Data are generated from 831 samples, and are expressed as \log_{10} mtDNA copies/mL of plasma, and show correlations between (A) all pairs, (B) male pairs, (C) opposite sex pairs, and (D) female pairs.

the familiar/genetic background, and studied the correlations with different hematochemical parameters. Furthermore, we analyze the capacity of mtDNA to induce the production of cytokines in purified monocytes.

The first striking observation is that the number of copies of circulating mtDNA was significantly increased with age after the fifth decade of life, when compared with that in younger subjects, reaching the maximum value in the group of 90+ subjects. This observation fits the evolutionary hypothesis that during the first four to five decades of life the rate of aging is negligible in order to preserve the reproductive capability of the organism, and that afterward the aging process accelerates, as the force of natural selection fades. Moreover, the increase in plasma mtDNA can contribute to the onset and maintenance of proinflammatory status observed in elderly people that we indicated as “inflammaging” [1]. Accordingly, we observed that the highest concentrations of circulating mtDNA were associated to elevated levels of proinflammatory cytokines. Therefore, our observations identify a new source of inflammatory stimuli potentially relevant for aging.

Despite a strong interindividual variability, we were able to show that plasma levels of mtDNA were highly correlated in two members of the same family. In particular, almost 20% of the variability of mtDNA plasma level, a trait displaying a strong individual variability, is explained by a familiar component. This result indicates that, at a certain degree, the mechanisms that regulate the levels of a molecule capable of triggering inflammation are genetically determined.

When the whole population of siblings was divided in subgroups according to the gender of the pair, the correlation was still present when a female was part of the pair. It is well known that the number of female centenarians largely overwhelms those of males, that the male half of the population live approximately 10% shorter lives than the female half, and that in developed countries the female/male ratio of centenarians is usually 5–6:1 [16]. Thus, the absence of correlation in male pairs was probably due to the relatively low number of older male siblings in this study.

Among the possible mechanisms that may influence the levels of circulating mtDNA, there can be (i) those controlling the

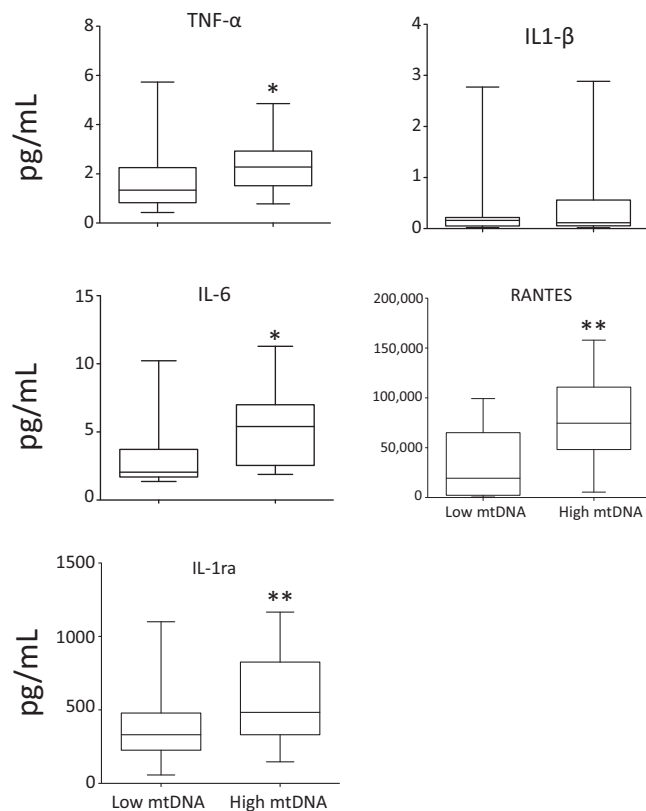


Figure 3. Plasma levels of TNF- α , IL1- β , IL-6, IL1-ra, and RANTES in the twenty 90+ subjects with the highest levels of plasma mtDNA (high mtDNA) and in twenty 90+ subjects with the lowest levels (low mtDNA). Data are expressed as pg/mL and shown as mean, interquartile range, and SD of 20 samples. * $p < 0.05$ versus low mtDNA; ** $p < 0.01$ versus low mtDNA, Student's t-test.

intracellular mtDNA copy number; (ii) the immune mechanisms for capturing bacteria; and (iii) the propensity to cell death, which increases with aging [17], among others. Moreover, the role of endothelial cells, which undergo striking changes during the aging process [18], and of the mitochondrial alterations that can occur in such a large compartment have also to be taken into account.

Concerning the regulation of intracellular mtDNA copy number, little is known about the genetic mechanisms at the basis of such process, and the possible differences existing among subjects. Despite the identification of many genes involved in the regulation of mtDNA replication, only a few such as POLG, TFAM, Twinkle, and Lon protease have been deeply investigated [17, 19, 20], no studies have evaluated the expression of these genes in association with the levels of circulating mtDNA, nor during aging and longevity. However, mutations or drugs [21, 22] that affect the functionality of these genes and effect severe reductions in intracellular mtDNA are well known [23, 24]. The study of circulating mtDNA levels in patients with different mutations in genes regulating mtDNA is therefore an attractive new topic.

Concerning immune mechanisms, it has been reported that, in response to inflammatory triggers, eosinophils eject DNA of mitochondrial origin in an instantaneous catapult-like fashion, producing a sticky network that can capture bacteria and pro-

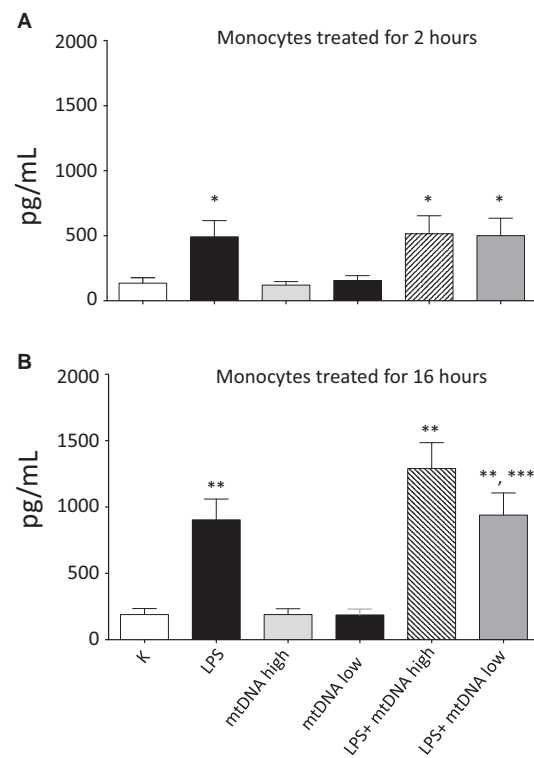


Figure 4. TNF- α concentrations in the supernatants of monocytes treated for (A) 2 h or (B) 16 h with different combinations of LPS and mtDNA. mtDNA high and mtDNA low are the highest and lowest measured mtDNA plasma levels, respectively. Data are expressed as pg/mL and shown as mean \pm SD of seven donors. * $p < 0.05$ versus K; ** $p < 0.01$ versus K; *** $p < 0.05$ versus LPS + mtDNA high, ANOVA test for repeated measures with Tukey's post test.

mote their extracellular killing [25, 26]. A similar, but distinct mechanism is present in neutrophils, called neutrophil extracellular traps [27, 28]. Since granulocytes represent the largest part of circulating blood cells, we can suppose that these mechanisms can give a relevant contribution to the levels of mtDNA in the plasma. Indeed, we found that the correlation between mtDNA and neutrophil count showed a positive trend. Further studies in larger cohorts will clarify if the number of neutrophils can influence circulating mtDNA levels, while studies on the capacity of other cells (endothelial cells, fibroblasts, etc.) to exert the same function could add further information.

Concerning the propensity to undergo cell death, it is known that not only necrotic process but also other more physiological mechanisms of cell death, such as pyroptosis, can release cytoplasmic content outside the cell [29]. Interestingly, pyroptosis is induced by the inflammasome-mediated activation of caspase-1 in response to infection by intracellular pathogens [30]. It is therefore conceivable that high levels of circulating mtDNA can correlate with the activation of innate immune responses toward chronic viral infections, such as CMV. In this respect, it is worth noting that almost all old people in Southern Europe display CMV seropositivity [31].

The correlation between proinflammatory cytokines (in particular IL-6 and TNF- α) and mtDNA levels suggests a strong

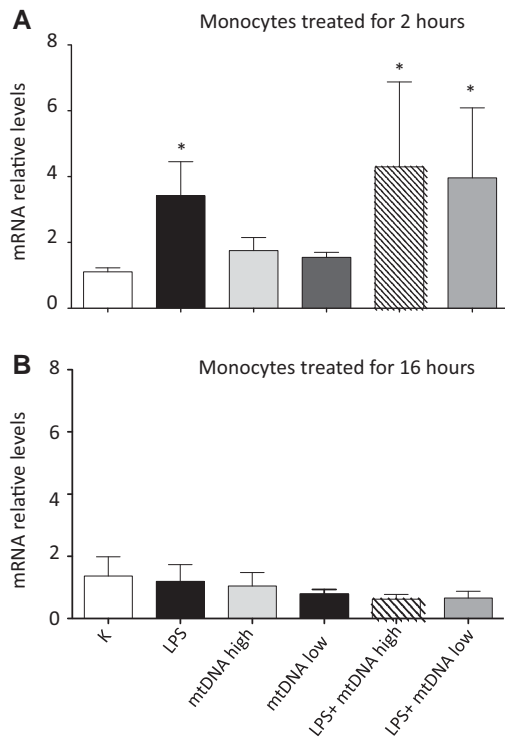


Figure 5. Relative expression of TNF- α messenger RNA in monocytes treated for (A) 2 h or (B) 16 h with different concentrations of LPS and mtDNA. High mtDNA and low mtDNA are the highest and lowest measured mtDNA plasma levels, respectively. Data are expressed as relative changes with respect to untreated sample, arbitrarily set to 1, and shown as mean \pm SD of seven donors. * $p < 0.05$ versus K, ANOVA test for repeated measures with Tukey's post test.

association between mtDNA and inflammatory status. As by the hypothesis of a balance between inflamm-aging and anti-inflamm-aging [32], we also found a positive correlation between mtDNA and IL-1ra. Our data also provide direct evidence that mtDNA increases an inflammatory response of LPS-activated monocytes. On the whole, the in vivo and in vitro data suggest that circulating mtDNA is not a mere epiphenomenon associated with inflammation, but likely plays an active role in this process. It has been previously shown by many authors that several immune cells, including monocytes and granulocytes, express TLR-9, and that triggering of TLR-9 in these cells by nonmethylated CpG DNA, like mtDNA, induces the release of type I IFNs and TNF- α , through

the activation of IRAK-1, IRAK-2, and IRAK-4 and the phosphorylation of p38 MAP kinase. Our data fit perfectly with these scenarios, and indicate that the amount of mtDNA that we observe in elderly subjects is sufficient to enhance such well-known pathways [7, 8, 11, 33–40]. High levels of circulating mtDNA, as those observed in elderly subjects, polarize adaptive immune responses toward Th1, by activating monocytes/macrophages and possibly other APCs. However, negative feedback mechanisms may exist, which warrant further investigation.

Finally, the apparent absence of correlation between mtDNA plasma levels and the health status and mortality in the 90+ subjects could be related to the fact that this group was composed of subjects in relatively good health status for their age, and largely cognitively intact. As the standardized mortality ratio of this population was lower than that of the general population, confirming their optimal health status, we have probably underestimated the possible negative role of mtDNA levels. As a consequence, it can be envisaged that studies in frail 90+ subjects, which are actually in progress should be more informative.

As a future perspective, the identification of the role of mitochondrial DAMPs could be of importance not only to identify possible new markers of aging or of disease progression, but also in designing new therapeutic strategies against circulating mtDNA, mitochondrial DAMPs, or the receptors they use. As an example, molecules that can inhibit formyl peptides receptor 1, as well as molecules blocking TLR-9, have been described [41]. Thus, in diseases characterized by excessive inflammation, targeting formyl peptides receptor 1 and TLR-9 and/or interfering with soluble mitochondrial DAMPs could become a novel strategy to reduce a harmful immune activation.

Materials and methods

Study subjects

Eight hundred and thirty-one Caucasian subjects born in Northern Italy and belonging to four different age groups were enrolled for the study: (i) 109 young subjects (50 males, 59 females) aged 1–11; (ii) 62 adults (32 males, 30 females) aged 21–41; (iii) 231 old subjects (64 males, 167 females) aged 51–76; (iv) 223 ultranonenarians (90+, 65 males, 158 females) aged

Table 3. Circulating mtDNA plasma level and vital status of 90+ siblings after 4 years of follow-up.

Age at recruitment	Death before 4 years of follow-up		Survival after 4 years of follow-up		p Value
	Number of subjects n	log ₁₀ mtDNA (copies/mL) Mean (95% CI)	Number of subjects n	log ₁₀ mtDNA (copies/mL) Mean (95% CI)	
90–93 years	100	11.63 (11.51–11.74)	141	11.70 (11.61–11.79)	0.3205
94–97 years	86	11.72 (11.60–11.84)	50	11.67 (11.53–11.82)	0.6407
≥98 years	44	11.66 (11.49–11.82)	8	11.61 (11.19–12.04)	0.8531
Total	230	11.67 (11.59–11.74)	199	11.69 (11.61–11.77)	0.6592

Table 4. Odds ratios and 95% confidence intervals for the 90+ siblings estimated by Cox regression models.

	OR	(95% CI)		p Value
log ₁₀ mtDNA (univariate analysis)	1.08	0.78	1.49	0.637
log ₁₀ mtDNA (adjusted)	1.08	0.77	1.54	0.635
Age	0.79	0.72	0.85	<0.001
Gender (females)	1.57	1.01	2.42	0.043

OR: odds ratio; CI: confidence interval.

90–104 (including 18 ultracentenarians); and (v) 206 younger, but still 90+, siblings of group 4 subjects (68 males, 138 females) aged 90–100 (including two ultracentenarians), as reported in Table 1. The group of old subjects (group no. 3) and the groups of 90+ siblings (group nos. 4 and 5) were recruited within the framework of the European Project GEHA—Genetics of Healthy Ageing [42, 43], which was approved by the local Ethical Committee of Policlinico Sant'Orsola-Malpighi (Bologna University Hospital). Within the group of old subjects (group no. 3), we included, whenever possible, the spouses of 90+ sibling's offspring (43 subjects), who were genetically unrelated to the 90+ sibling, but were of the same ethnicity and geographical background and lived for the greatest part of their life in the same house. The enrichment in females of the study population is due to the fact that almost half of the donors were 90+, an age category in which males/females ratio is about 3 in Northern Italy (www.istat.it/it/files/2011/06/italiaincifre2011.pdf).

All the participants signed an informed consent form for blood collection; if underage, the consent was obtained from a parent. The groups of young and adult subjects were healthy, while the groups of old subjects and 90+ siblings underwent a standard questionnaire to collect information regarding the health status, clinical anamnesis, and life style. BMI calculation was also performed.

The evaluation of the main hematobiochemical parameters was performed on freshly collected whole blood samples. The resulting plasma samples were stored at -80°C and used for the quantification of circulating mtDNA.

Vital status for the total cohort of 90+ siblings was assessed after 4 years from the enrolment, by contacting the official local population registry.

DNA extraction from plasma samples

DNA was extracted from plasma according to the “blood and body fluid protocol” of the QIAamp blood reagent set (QIAGEN, Hilden, Germany); 400 μL of plasma was applied to each column, DNA was eluted in 200 μL of supplied buffer and stored at -20°C until use.

Quantification of circulating mtDNA

Plasma content of mtDNA was measured by quantitative real-time PCR using a technique developed in our laboratory [13]. mtDNA was quantified using a sequence-specific TaqMan probe carrying a fluorophore (FAM 490 at the 5' end) and a quencher moiety (BLACK HOLE at the 3' end).

In the reaction, mtDNA was measured using a mix containing 5 μL DNA sample, 12.5 μL SuperMix 2 \times (Invitrogen, CA, USA), 0.8 μL MTDdir primer (5'-CACAGAAGCTGCCATCAAGTA-3'), 0.8 μL MTrev primer (5'-CCGGAGAGTATATTGTTGAAGAG-3'), 0.1 μL MTprobe (5'-CCTCACGCAAGCAACCGCATCC-3'), and distilled water up to a final volume of 25 μL . One cycle at 50°C for 2 min was performed to reduce PCR carry-over products, then one cycle of denaturation (95°C for 3 min) was performed, followed by 40 cycles of amplification (94°C for 10 s, 60°C for 35 s). Real-time PCR was carried out in an iCycler Thermal cycler (BioRad, Hercules, CA, USA). Each reaction was monitored by using a negative control (no template) and a positive control (DNA extract from cells of HepG2 cell line and stored at -80°C in small aliquots); the efficiency of all reactions was $>98\%$. All the analyses were performed in triplicate.

The linearity of the quantitative assay was assessed with use of the template cloned into a plasmid DNA serially diluted to prepare a series of calibrators with known concentrations.

Analysis of cytokine plasma levels

The plasma levels of different cytokines (TNF- α , IL-1 β , IL-6, IL-8, IL-1ra, RANTES) were quantified by Quantikine HS ELISA Kit (R&D Systems, Minneapolis, MN, USA), following instructions provided by manufacturer. IFN- α were quantified by using Verikine Human IFN Alpha ELISA Kit (Piscataway, NJ, USA) following provided instructions.

Isolation of mtDNA and nuclear DNA

mtDNA was isolated by using mtDNA isolation kit (Abcam, Cambridge, UK) following instructions provided by manufacturer, starting from 5×10^7 human HepG2 cells. mtDNA was checked for purity and integrity, quantified, divided in aliquots, and stored at -20°C until use for stimulating monocytes. Nuclear DNA was extracted by isolated nuclei of HepG2 cells by using DNeasy Blood Midi Kit (QIAGEN) following provided instructions, and stored and -20°C until use.

Isolation of monocytes and stimulation with mtDNA

Monocytes were isolated from PBMCs of seven healthy subjects aged 27–41 (mean \pm SD: 35.1 ± 5.5) by negative selection, using MACS monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity was checked by flow cytometry analysis by

labeling cells for CD14, CD16, and CD3. Monocytes (CD14⁺ CD16⁺ CD3⁻) were always >97% pure. Purified monocytes were plated in RPMI complete medium in the presence of LPS from *Escherichia coli* 0127:B8 (LPS, 0.25 µg/mL, from Sigma Aldrich, St. Louis, MO, USA), and/or purified mtDNA (1.46 × 10⁹–1.46 × 10¹⁴ copies/mL) and ODNTT-1 (Alpha Diagnostic International, San Antonio, TX, USA) 20 µg/mL for 2–16 h. ODN 2395, a known agonist of human TLR9, has been used as a positive control, at the final concentration of 2 µg/mL. Supernatants were collected and used for quantification of TNF-α and IFN-α; cells were harvested, washed with PBS, centrifuged at 700 g for 10 min at 4°C, and used for RNA extraction.

RNA extraction and mRNA quantification

RNA has been extracted by purified monocytes by using RNeasy minikit (QIAGEN) following provided instructions; the levels of mRNA for TNF-α were measured by real-time PCR. Quantification was performed using a mix containing 5 µL of cDNA sample, 12.5 µL SYBR Green SuperMix 2× (Invitrogen), 0.5 µL direct primer (5'-CCCAGGGACCTCTCTAATCA-3'), 0.5 µL reverse primer (5'-GCTACAGGCTGTCACTCGG-3'), and distilled water up to a final volume of 25 µL. One cycle at 50°C for 2 min was performed to reduce PCR carry-over products and then one cycle of denaturation (95°C for 3 min) was performed, followed by 40 cycles of amplification (94°C for 10 s, 60°C for 30 s). Data were normalized to the expression levels of two housekeeping genes (HBP and GAPDH), and expressed as relative variation respect to untreated sample, arbitrarily set to 1.

Statistical analysis

The skewed distribution of mtDNA plasma level had required the logarithmic transformation of values. Bonferroni test was carried out to compare mtDNA plasma level in the four different age groups.

Linear regression models were used to highlight possible correlations: (i) mtDNA plasma level and age of the donors, according to gender; (ii) mtDNA plasma level between siblings of the same sibship. For this last correlation, the probands were first compared to their second sibs, then the pairs were shuffled and the probands were compared to unrelated subjects with the same gender, year, and place of birth as their real sibs used in the first analysis. Additionally, the probands were compared with the spouse of his/her offspring, sharing the house and the daily habits with the 90+ sibling. These permutation procedures, minimizing the role of environment, enabled us to verify if the observed correlation in mtDNA plasma level in sib pairs was due to the kinship between siblings or to the exposure to the same environment; (iii) mtDNA plasma level and the presence of diseases, BMI, and hematobiochemical parameters.

Student's *t*-test was used to compare mtDNA plasma level in 90+ siblings who were alive or not after 4 years of follow-up, by

dividing the subjects in three groups according to their age at the interview.

Cox regression analysis was run for 90+ siblings to evaluate the role of mtDNA plasma level, age, and gender in the survival of 90+ siblings after 4 years from the enrolment. Odds ratios with 95% confidence intervals were calculated, adjusted for family cluster, age at the interview, and gender.

All statistical analyses were performed by using Prism 5.0 (GraphPad, La Jolla, CA, USA) or Stata version 9.0 (Stata Corp., College Station, TX, USA) software; a *p*-value < 0.05 was considered significant.

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Abbreviations: BMI: body mass index · DAMP: damage-associated molecular pattern · mtDNA: mitochondrial DNA

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