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Age-dependent skewing of X chromosome inactivation appears delayed in centenarians' offspring. Is there a role for allelic imbalance in Healthy Aging and Longevity?

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Summary

Recently, it has been proposed that age-related X chromosome inactivation (XCI) skewing can clinically result in late-onset X-linked disorders. This observation leads to hypothesize that age-related skewed XCI might also influence lifespan in women. To investigate this issue, we employed a new experimental model of longevity and healthy aging including 55 female centenarians, 40 of their offspring, 33 age-matched offspring of both non-long-lived parents and 41 young women. Peripheral blood DNA from 169 females was screened for heterozygosity at the HUMARA locus. We confirmed that skewing of XCI is an age-dependent phenomenon. However, skewed XCI was significantly less severe and frequent in centenarians' offspring [degree of skewing (DS) = 0.16 ± 0.02] compared to age-matched offspring of both non-long-lived parents (DS = 0.24 ± 0.02) ($P < 0.05$). A second goal was to assess whether changes in XCI pattern could be a consequence of loss of methylation on X chromosome. Using a methylation array evaluating 1085 CpG sites across X chromosome and eleven CpG sites located at HUMARA locus, no differences in methylation levels and profiles emerged between all groups analysed, thus suggesting that age-associated epigenetic changes could not influence HUMARA results. In conclusion, the results presented herein highlight for the first time an interesting link between skewing of XCI and healthy aging and longevity. We speculate that the allelic imbalance produced by XCI skewing may compromise the cooperative and compensatory organization occurring between the two cell populations that make up the female mosaic.

Key words: centenarians; longevity regulation; molecular biology of aging; life span; genetics; longevity.

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Introduction

In female mammalian cells, one of the two X chromosomes is epigenetically inactivated in early embryonic life. Females are therefore a mosaic of cells with either the maternal or the paternal X chromosome inactivated (Belmont, 1996). In young women, the distribution of the two cell lines is close to normal with a mode corresponding to the 50:50 ratio. However, a marked deviation from this equivalent ratio occurs in some conditions. This event, defined as skewing of X chromosome inactivation (XCI), may be primary, due either to chance or to factors that affect the process of X inactivation during early embryo development (Minks *et al.*, 2008), or secondary to a selection process against or in favour of cells with a specific genotype (Bolduc *et al.*, 2008). Skewed XCI is differently defined by several investigators in terms of percentage of cells with the predominantly inactive X: 75%, 80% and 90% are commonly accepted cut-offs.

Several studies have shown that skewed XCI increases significantly in blood cells of female subjects during aging (Hatakeyama *et al.*, 2004; Sandovici *et al.*, 2004; Kristiansen *et al.*, 2005a; Knudsen *et al.*, 2007; Bolduc *et al.*, 2008). The reasons behind the age-dependent skewed XCI have been widely debated during last years. Some authors proposed that this age-related process could be the result of a clonal stochastic loss of haematopoietic cells (Busque *et al.*, 1996, 2009; Kristiansen *et al.*, 2005a) or of a competitive advantage for haematopoietic stem cells with a specific genotype of X-linked genes (Abkowitz *et al.*, 1998). Conversely, other investigators argued that skewing of XCI might be a technical bias because of age-associated loss of methylation at the HUMARA locus (Swierczek *et al.*, 2008). The controversy on this topic is still open and bright.

The existence of an age-dependent skewed XCI may raise important implications for understanding the physiology of haematopoiesis during aging and the pathogenesis of age-related haematopoietic malignancies (Ørstavik, 2006). Recently, it has been proposed that age-related XCI skewing can clinically manifest as late-onset X-linked disorders (X-linked sideroblastic anaemia, autoimmune diseases) (Cotter *et al.*, 1995; Ozbalkan *et al.*, 2005). Moreover, age-related XCI skewing also seems to be implicated in the pathogenesis of other diseases, such as scleroderma, autoimmune thyroid diseases (Graves disease and Hashimoto thyroiditis), primary biliary cirrhosis and several tumours (Invernizzi *et al.*, 2004; Brix *et al.*, 2005; Kristiansen *et al.*, 2005b; Medema & Burgering, 2007). Taken together, these observations lead to hypothesize that age-related skewed XCI might act at genomic level favouring the expression of multiple deleterious alleles and consequently influencing human health.

The importance of X chromosome on longevity has been widely highlighted by different authors (Christensen *et al.*, 2001; Austad, 2006; Aviv, 2007). In females, the compensatory effect of the second X chromosome has been proposed to explain, at least in part, the longer life expectancy of women compared with men. While in men any deleterious allele on the X chromosome has no compensatory allele, in women, the second X chromosome could balance any potential unfavourable allele, conferring a biological advantage to the female cellular mosaicism (Austad, 2006). However, the role of skewed XCI in the modulation of longevity has not been studied yet in human females.

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Subjects able to reach extreme longevity, such as centenarians, are an extraordinary model to study human longevity and to identify its genetic determinants (Cevenini *et al.*, 2008). On the other hand, there are several disadvantages inherent in the study of centenarians: rarity, presence of frailty because of extreme age and lack of an appropriate control group. In the past, numerous biological parameters measured in centenarians have been compared to a control group of younger subjects to identify factors potentially involved in the longevity. This approach may be misleading for parameters showing a clear age influence (Terry *et al.*, 2004), like skewing of XCI. Centenarians' offspring are recognized to undergo an aging process 'better' than that of subjects of the same age and show a lower morbidity and higher survival (Terry *et al.*, 2004; Cevenini *et al.*, 2008). Therefore, centenarians' offspring appear to be a new and promising approach to identify biological parameters that contribute to human longevity and healthy aging, without the disadvantages observed in the studies of centenarians. Indeed, they are more numerous than centenarians, and it is possible to compare them with a demographically matched control group (subjects matched for age, sex, ethnicity, parent year of birth, but born from non-long-lived parents), thus avoiding cohort effects.

The aims of this study were as follows: (i) to use a new experimental model to explore the role of skewing of XCI in the modulation of longevity, investigating XCI pattern in DNA extracted from peripheral leucocytes of female centenarians, centenarians' daughters, women born from both non-long-lived parents and young women; (ii) to identify whether differences in the methylation status of the X chromosome or of HUMARA locus could underlie the age-related XCI skewing, thus influencing the results of the XCI analysis, as previously reported (Swierczek *et al.*, 2008); and (iii) to identify epigenetic features of X chromosome potentially associated with healthy aging and longevity.

Results

Study population characteristics

As indicated in Table 1, peripheral blood DNA from 169 females was screened for heterozygosity at the HUMARA locus; heterozygous status was observed in 148 samples (87.6%): 50 centenarians (age: 100.7 ± 0.2 years), 31 centenarians' offspring (age: 68.8 ± 1.1 years), 30 offspring of both non-long-lived parents (age: 71.5 ± 1.2 years) and 37 young women (age: 31.2 ± 0.9 years).

Our proposed model including female centenarians' offspring and female offspring of both non-long-lived parents seems to be a valid approach to investigate features of healthy aging and longevity. Both groups were comparable for age but extremely different in terms of probability to become long-lived. Indeed, the prevalence of several diseases (cardiovascular, skeletal, respiratory, neurologic diseases and tumours) was lower in centenarians' offspring than in offspring of both non-long-lived parents (Fig. 1).

Skewed X chromosome inactivation is less severe and frequent in centenarians offspring compared to offspring from both non-long-lived parents

As already reported, skewing of XCI was an age-dependent phenomenon as the degree of skewing (DS) evaluated in the overall population correlated positively with age ($r = 0.330$, $P < 0.0001$, Fig. 2). In centenarians, the DS (0.27 ± 0.019) was significantly higher than that observed in centenarians' offspring (0.16 ± 0.022, $P < 0.001$) and in young women (0.16 ± 0.023, $P < 0.001$). Conversely, it was not significantly different from that observed in offspring of both non-long-lived parents (0.24 ± 0.020) (Fig. 3). Interestingly, in this last group, skewed XCI appeared to be significantly higher than that observed in the centenarians' offspring ($P < 0.05$); notwithstanding, both groups were comparable for age. Furthermore, the DS of centenarians' offspring was similar to that observed in young women (Fig. 3). In addition, we calculated the prevalence of skewing in each group, defining skewed XCI on the basis of two commonly accepted cut-offs (75% and 80%) for the percentage of cells with the predominantly inactive X (Table 1). Using a cut-off of 75% for skewing, the prevalence of skewing in blood cells of centenarians' offspring (22.6%) was lower than that observed in centenarians (52%, $P = 0.006$) and in offspring of both non-long-lived parents (53.3%, $P = 0.01$), but comparable to that detected in young women (21.6%, $P = 0.6$). As shown in Table 1, similar results were obtained using 80% as cut-off value for the definition of skewed XCI.

Chromosome X and HUMARA methylation profile

No differences in average methylation values and profiles over the X-linked 1085 CpG sites emerged between all groups analysed (Fig. 4A,C). In addition, we investigated variation in methylation profile of HUMARA by assessment of methylation levels in 11 different CpG sites located at this locus. Average methylation values of these CpG sites were comparable in all groups (Fig. 4B,D).

To identify epigenetic signatures of X chromosome potentially involved in healthy aging and longevity, a locus-by-locus analysis through the 1085 X-linked CpG sites has been performed in centenarians' offspring and offspring of non-long-lived parents. We identified 13 CpG loci significantly ($q < 0.05$) hypermethylated and 15 CpG sites significantly

Table 1 Genotypic frequencies of subjects enrolled in the study and prevalence of skewed X inactivation

Group	Total no. of women enrolled in the study	No. of women heterozygotes at HUMARA locus	No. of skewed individuals ≥ 75%	% of skewing ≥ 75%	ORs	IC	*P*-value	No. of skewed individuals ≥ 80%	% of skewing ≥ 80%	ORs	IC	*P*-value
Centenarian females	55	50	26	52.0	0.27	0.08–0.80	0.006*	24	48.0	0.16	0.03–0.56	0.001*
Centenarian offspring	40	31	7	22.6	–	–	–	4	12.9	–	–	–
Offspring of both non longlived parents	33	30	16	53.3	0.26	0.07–0.86	0.01*	11	36.7	0.26	0.05–1.05	0.04*
Young females	41	37	8	21.6	1.05	0.26–3.89	0.6	6	16.2	0.76	0.14–3.64	0.7

Considered skewed if inactivation of the predominant allele ≥75% or 80%, ORs and *P* values have been calculated vs. Centenarians' Offspring group.
*Significant *P* values.

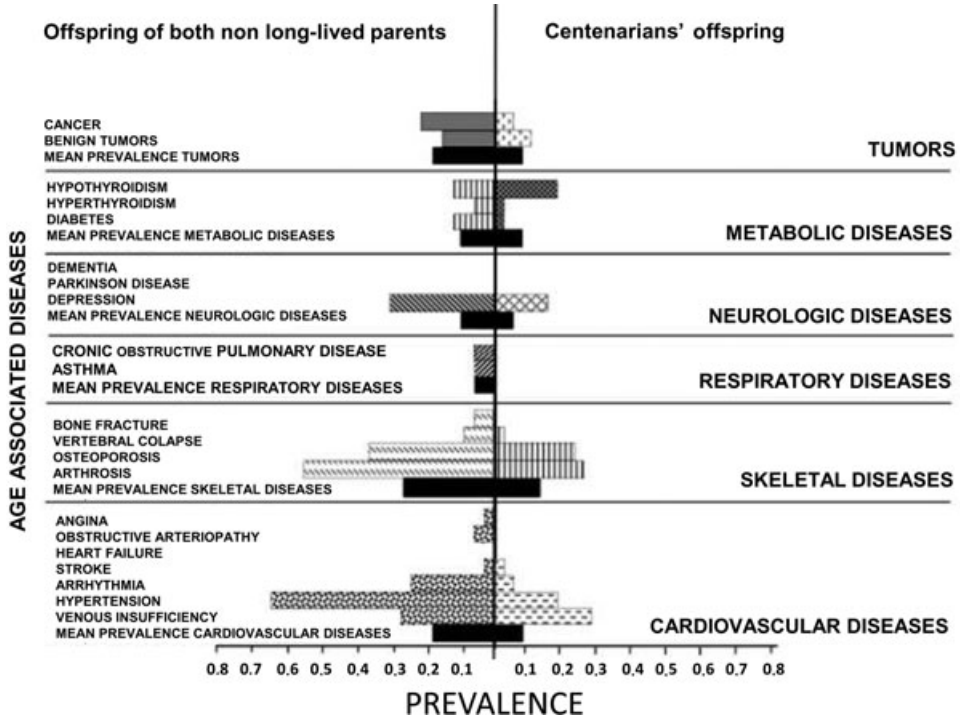

Fig. 1 Prevalence in aging-associated diseases. Pathological conditions have been grouped in categories as function of the system involved.

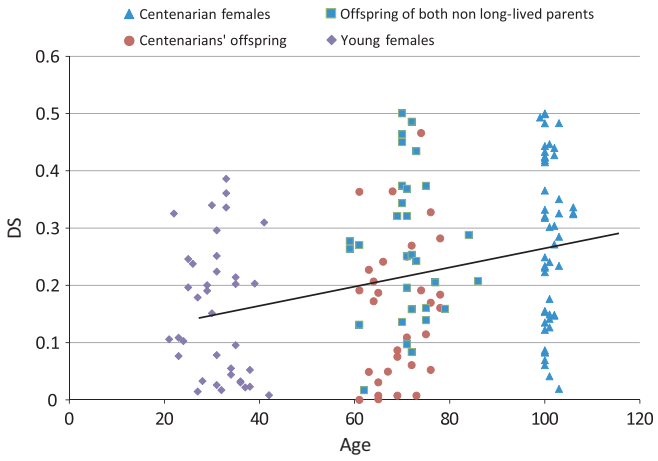

Fig. 2 Scatterplot of age and degree of skewing (DS) observed in centenarian women (n = 50), centenarians' offspring (n = 31), offspring of both non-long-lived parents (n = 30) and young women (n = 37). The DS correlated positively with age (r = 0.330, P < 0.0001).

hypomethylated in centenarians' offspring compared with offspring of both non-long-lived parents (Table S1).

Discussion

Age-related skewed XCI appears to be a natural consequence of aging, as evidenced in longitudinal studies (Sandovici *et al.*, 2004). This phenomenon starts around 55 years of age and continues to increase until 100 years of age (Kristiansen *et al.*, 2005a), and it is not restricted to the blood compartment (Knudsen *et al.*, 2007; Bolduc *et al.*, 2008). Concordance of skewing documented in elderly monozygotic vs. dizygotic twins supported also a genetic component to the trait with a hereditability of 0.6 (Kristiansen *et al.*, 2005a). Moreover, a recent large study of XCI in

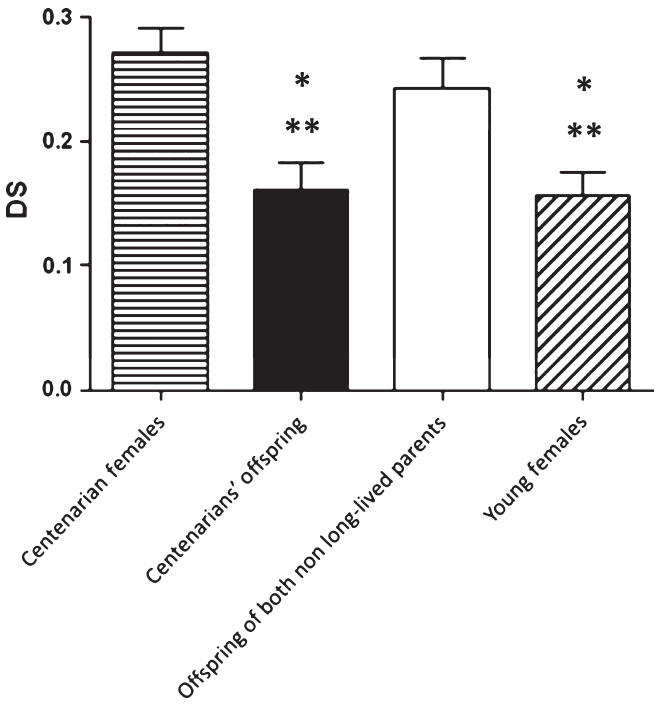

Fig. 3 Degree of skewed X chromosome inactivation (degree of skewing) observed in centenarian women (n = 50), centenarians' offspring (n = 31), offspring of both non-long-lived parents (n = 30) and young women (n = 37). *P < 0.001 vs. centenarian females; **P < 0.05 vs. offspring of both non-long-lived parents

450 neonates and their mothers revealed no correlation between XCI in the mother–child pairs (Bolduc *et al.*, 2008), suggesting that in humans, the XCI pattern observed at birth does not reflect a single heritable genetic locus (Bolduc *et al.*, 2008).

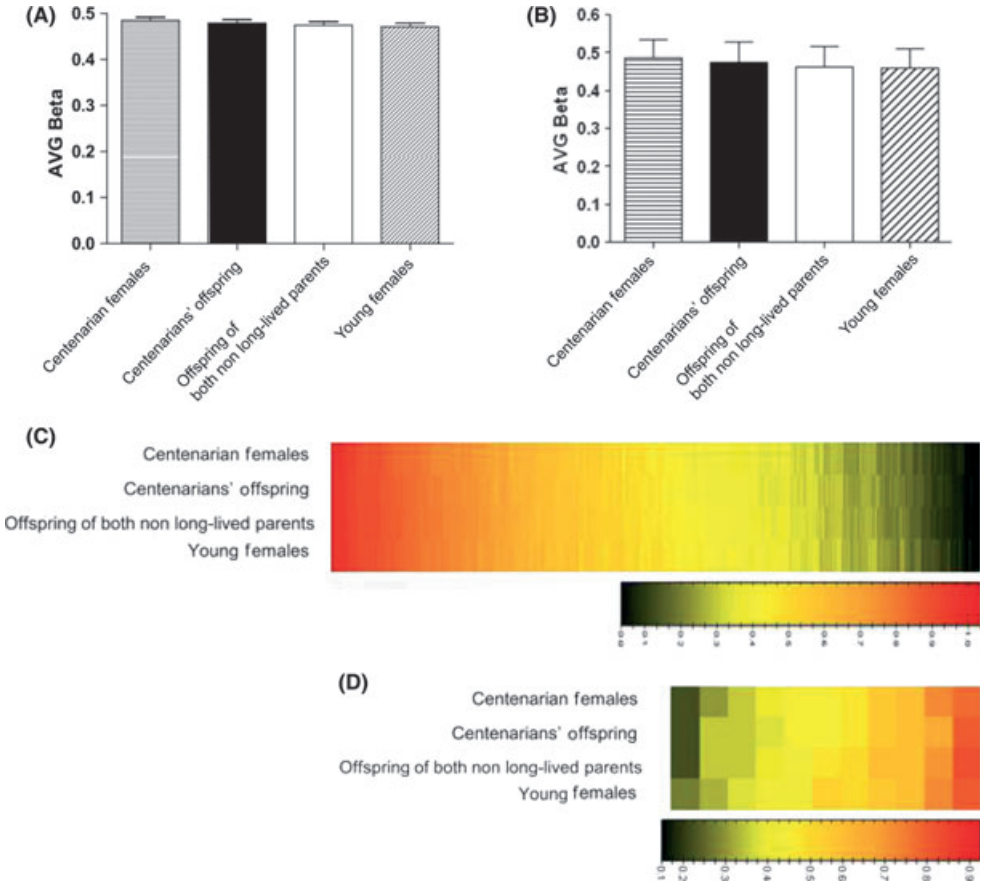

Fig. 4 Distribution of average beta methylation values (A, B) and heatmap (C, D) of the X-linked 1085 CpG sites (A, C) and of 11 different CpG sites located in HUMARA locus (B, D).

Notwithstanding the nature of this phenomenon remains for many aspects still unclear, different studies suggested that age-associated XCI skewing may also contribute to the development of several diseases, such as cancer and autoimmune diseases (Cotter *et al.*, 1995; Invernizzi *et al.*, 2004; Brix *et al.*, 2005; Kristiansen *et al.*, 2005b; Ozbalkan *et al.*, 2005; Medema & Burgering, 2007). Despite these suggestive evidences, there are no clear results in literature about the role of XCI in the modulation of human healthy aging and longevity.

Several authors proposed that the female mosaic organization may play an important role in longevity and may be considered a winning strategy adopted by women (Dobyns, 2004; Migeon, 2006; Aviv, 2007; Ørstavik, 2009). The advantage provided by the mosaic organization has also been underlined in many physiological and pathological conditions by elegant studies demonstrating the existence of several cooperative and interactive mechanisms occurring between the two cell populations of the female mosaic (Dobyns, 2004; Migeon, 2006). An emblematic example is given by the Hunter syndrome, a pathological condition induced by the mutation in the iduronate sulfatase gene located on the X chromosome. In heterozygous females, it has been observed a cooperative mechanism in which wild-type cells are able to sustain cells carrying the mutation by providing the functional enzyme by mannose-6-phosphate-mediated endocytosis (Dobyns, 2004).

On the other hand, the absence of any cooperative strategy in men has been proposed to explain their greater biological vulnerability and their shorter lifespan compared to women (Dobyns, 2004; Austad, 2006; Migeon, 2006; Aviv, 2007). Based on these observations, we speculate that XCI skewing may have a detrimental effect on the cellular cooperative model and may deeply influence survival and healthy aging in women.

Our proposed model, including female centenarians' offspring and female offspring of both non-long-lived parents, seems to be a valid approach to validate this theory. Both groups are comparable for age and extremely different in terms of probability to become long-lived (Terry *et al.*, 2004; Cevenini *et al.*, 2008). Indeed, we confirmed that the prevalence of several diseases such as cardiovascular, skeletal, respiratory, neurologic diseases and cancer is significantly lower in centenarians' offspring than in offspring of both non-long-lived parents. Interestingly, hypothyroidism was more prevalent in centenarians' offspring than in offspring of both non-long-lived parents. This evidence is not surprising; indeed, it has been reported that a decreased thyroid function might be beneficial in the elderly through its lowering effects on basal metabolic rate and oxidative metabolism with a consequent reduction of DNA damage reactive oxygen species (Peeters, 2009). High levels of TSH (Gussekloo *et al.*, 2004) and low levels of FT4 (Van den Beld *et al.*, 2005) have been associated with a better survival in elderly subjects. In addition, centenarians and centenarians' offspring showed higher levels of TSH compared to controls (Atzmon *et al.*, 2009). These evidences seem to be confirmed also in our population, because the resting metabolic rate (basal energy expenditure), calculated with the Harris–Benedict equation (Harris & Benedict, 1919), resulted significantly lower in centenarians' offspring compared to offspring of both non-long-lived parents (data not shown).

The results reported herein confirm that skewing of XCI is an age-related phenomenon. Although the cause–effect relation is difficult to be assessed in a cross-sectional study, the lower prevalence of skewed XCI in female centenarians' offspring compared to age-matched females born from both non-long-lived parents may suggest for the first time the

existence of an interesting association between skewing of XCI, healthy aging and life expectancy in women. The prevalence of skewing of XCI in centenarians and young controls resulted in line with that reported in previous studies (Sullivan *et al.*, 2003; Kristiansen *et al.*, 2005a,b; Bolduc *et al.*, 2008). In contrast, we cannot compare the prevalence of XCI skewing in centenarians' offspring and in offspring of both non-long-lived parents with previously reported data because this is the first study that has investigated these particular cohorts of subjects.

A second goal of this study was to assess whether changes in XCI were a consequence of loss of methylation on X chromosome at the HpaII sites, used to assay XCI at the HUMARA locus, as suggested by Swierczek *et al.* (Swierczek *et al.*, 2008). We studied this particular issue for two main reasons: i) to exclude that our results might be influenced by epigenetic changes and ii) to verify whether the HUMARA method used to asses XCI can be considered a valid approach. In agreement with Busque *et al.*'s (Busque *et al.*, 1996) study, our data exclude that potential age-associated changes in methylation patterns could influence HUMARA results.

Methylation analysis of the X chromosome through a genome-wide methylation assay identified a small number of CpG sites that resulted differently methylated between centenarians' offspring and offspring of both non-long-lived parents. These genes, potentially important for healthy aging and longevity, resulted scattered among different biological functions, but gene ontology analysis failed to identify any significant molecular process enrichment. These findings lead to speculate that allelic imbalance produced by XCI skewing rather than differences in DNA methylation might have a predominant influence on healthy aging and longevity.

There are few limitations that need to be underlined and addressed: (i) the number of subjects analysed is relatively small. However, this is a pilot study, and notwithstanding the extreme rarity of the subjects enrolled, to the best of our knowledge, we investigated the greatest cohort of centenarians reported in literature. (ii) Because of the difficulties in obtaining tissue samples from old subjects, we analysed XCI only in blood cells. We lack data regarding XCI status in other tissues, but previous evidence indicating that age-associated XCI status is not only restricted to the blood compartment (Knudsen *et al.*, 2007; Bolduc *et al.*, 2008) support the assumption that the mechanism described herein might be valid also at systemic level. (iii) Relevant epigenetic changes are apparently not emerging between the group analysed, although it has to be considered that this is a pilot study and that we focussed our attention only on X chromosome limiting our analysis at methylation level. For these reasons, we cannot exclude that whole-genome DNA methylation changes and other kind of epigenetic alterations, such as histone modifications and chromatin remodelling, may have a role in the processes involved in healthy aging.

In conclusion, this new model, including centenarians' offspring and offspring of both non-long-lived parents, highlights for the first time a new interesting issue in the biology of aging, underling the potential role of skewing of XCI in the modulation of women lifespan and healthy aging. These findings might have important prognostic consequences in medicine improving the ability to describe the health status and to better estimate disease risk factors in females. Further studies in larger cohorts of women and in different tissue samples are mandatory to support the usefulness of XCI status as a predictor of healthy aging.

Experimental procedures

Subjects recruitment

In this study, we enrolled 169 females living in northern Italy: 55 centenarians (born between the 1900–1908); 40 centenarians' offspring; 33 age-matched offspring of both non-long-lived parents, with both parents born between 1900 and 1908 and dead before the average life expectancy calculated at 15 years of age (67 years if male and 72 years if female) by the Italian mortality tables (see website 'Human Mortality Database' of the Max Plank Institute for Demography, Rostock, Germany: http://www.mortality.org/); and 41 young women, randomly recruited, with an age ranging between 21 and 41 years (Table 1). The study protocol was approved by the Ethical Committee of the Sant'Orsola-Malpighi University Hospital (Bologna, Italy).

DNA extraction and HUMARA analysis

Genomic DNA was extracted from peripheral blood with the Wizard genomic DNA purification kit (PROMEGA, Madison, WI, USA). Peripheral blood was processed for DNA isolation as follows: 3–5 mL of blood was lysed with lysis solution provided and digested with proteinase K in sodium dodecyl sulphate (SDS) buffer at 37 °C for 1 h, and then DNA was extracted by salting-out and resuspended in Tris–EDTA(TE)buffer. X inactivation patterns were determined in blood DNA using a modification of the methylation analysis of the HUMARA locus as previously described (Busque *et al.*, 1996; Gale *et al.*, 1997). Briefly, 250 ng of DNA was digested at 37 °C for 2 h with 10 U HpaII and 10 U HhaI (CELBIO), and a no-enzyme control digest was also set up for each sample; moreover, a male DNA was used in each experiment as negative control. Digested and undigested DNAs were then amplified in duplicate PCRs using primers, amplifying the highly polymorphic CAG repeat region in exon 1 of the AR gene at Xq12. The sequences of the primers used were as follows: forward; 5′-GCT GTG AAG GTT GCT GTT CCT CAT-3′ labelled with 5′-phosphoamidite dye, reverse; 5′-TCC AGA ATC TGT TCC AGA GCG TGC-3′. The samples were amplified for 35 cycles comprising of 15 s at 95 °C, 30 s at 62 °C and 30 s at 72 °C with an initial denaturation at 95 °C for 5 min. The PCR products were separated on an ABI 310 automated sequencer. The size of PCR product from each allele was analysed by Genescan software for the quantification by peak height. A representative experiment of Genescan electropherogram patterns obtained from DNAs with different DS is showed in Fig. S1.

XCI ratios were calculated using P_{sup} score obtained using the equation below (Bolduc *et al.*, 2008):

$$P_{sup} = 1 - \left[\frac{\frac{(A/A+a)}{(A'/A'+a')}}{\frac{(A/A+a)}{(A'/A'+a')} + \frac{(a/A+a)}{(a'/A'+a')}} \right]$$

P_{sup} score indicates the proportion of cells harbouring the longer HUMARA allele on the active X chromosome. The longer allele is defined as that which has the greater number of CAG repeats. *A* and *A′* represent the peak heights of the longer HUMARA allele from the digested and undigested samples, respectively. The peak heights of the shorter HUMARA allele for the digested and undigested samples are represented by *a* and *a′*, respectively.

To evaluate the magnitude of the XCI skewing, we used two measures:

1 Degree of skewing, which designates the percentage of the preferentially active allele and does not take into account the direction of skewing but only the degree of deviation from a 50% XCI pattern. DS is calculated using the formula $|P_{sup} - 0.5|$ and represents a continuous variable that ranges between 0% and 50%, where 0% indicates a random X inactivation pattern and 50% a completely skewed inactivation pattern (Bolduc *et al.*, 2008).
2 Prevalence of skewing (% of skewing) that calculates the prevalence of skewed XCI in each group, defining skewed XCI on the basis of two different cut-offs used for the percentage of cells with the pre-

dominantly inactive X: ≥75% (Allen *et al.*, 1992; Gale & Linch, 1994; Gale *et al.*, 1997; Brown, 1999; Buller *et al.*, 1999; Sullivan *et al.*, 2003; Bolduc *et al.*, 2008) and ≥80% (Sharp *et al.*, 2000; Kristiansen *et al.*, 2005a).

X chromosome methylation profile

In 84 DNA samples (21 samples randomly selected from each group), genome-wide methylation profile of chromosome X was evaluated through Infinium HumanMethylation27 BeadChip (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. In brief, genomic DNA was treated with a bisulphite conversion kit (EZ DNA MethylationTM Kit; Zymo Research, Irvine, CA, USA) to convert unmethylated cytosines into uracils. After bisulphite conversion, each sample was whole-genome amplified (WGA) and enzymatically fragmented. The bisulphite-converted WGA-DNA samples were purified and applied to the BeadChips. The level of methylation was determined at each locus by the intensity of the two possible fluorescent signals, specific for the methylated and unmethylated alleles. DNA methylation values, described as beta values, were recorded for each locus in each sample by the GenomeStudio software. DNA methylation beta values are continuous variables between 0 and 1, representing the ratio of the intensity of the methylated bead type to the combined locus intensity. Background-corrected methylated and unmethylated values, beta values (as generated from the GenomeStudio software) and built-in controls were used to evaluate the quality of individual array.

Statistical analysis

The distribution of continuous variables was tested using the Kolmogorov–Smirnov test. Comparison between groups was performed by ANOVA and Kruskal–Wallis test for parametric and nonparametric data, respectively. Newman–Keuls and Dunn's tests were used as post-tests for parametric and nonparametric data, respectively. Fisher's exact test was used to analyse categorical variables. Pearson's test was used for correlation analysis. Differential methylation analysis was performed using GenomeStudio software. The Illumina Custom error model with false discovery rate correction was applied to data sets after normalizing by average and background separately. Differences in methylation levels between probes were considered statistically significant when Q-value <0.05.

Data were reported as means and SE. Probability <0.05 was considered as statistically significant.

Author contributions

Davide Gentilini designed the research, performed some experiments, analysed data and wrote the manuscript; Davide Castaldi performed some experiments and revised the manuscript; Daniela Mari contributed new reagents and DNA samples and revised the manuscript; Daniela Monti contributed DNA samples and revised the manuscript; Claudio Franceschi contributed DNA samples and revised the manuscript; Anna Maria Di Blasio designed the research, analysed data and wrote the manuscript; and Giovanni Vitale designed the research, analysed data and wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 A representative experiment showing Genescan electropherogram patterns obtained from DNAs with different degree of skewing and a male DNA used as negative control.

Table S1 List of 28 CpGs resulted significantly (q-value < 0.05) hypermethylated and hypomethylated in centenarians'offspring compared with offspring of non long-lived parents. q-values has been calculated vs. Centenarians' Offspring group.

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