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Age and gender effects on DNA strand break repair in peripheral blood mononuclear cells

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Summary

Exogenous and endogenous damage to DNA is constantly challenging the stability of our genome. This DNA damage increase the frequency of errors in DNA replication, thus causing point mutations or chromosomal rearrangements and has been implicated in aging, cancer, and neurodegenerative diseases. Therefore, efficient DNA repair is vital for the maintenance of genome stability. The general notion has been that DNA repair capacity decreases with age although there are conflicting results. Here, we focused on potential age-associated changes in DNA damage response and the capacities of repairing DNA single-strand breaks (SSBs) and double-strand breaks (DSBs) in human peripheral blood mononuclear cells (PBMCs). Of these lesions, DSBs are the least frequent but the most dangerous for cells. We have measured the level of endogenous SSBs, SSB repair capacity, γ-H2AX response, and DSB repair capacity in a study population consisting of 216 individuals from a population-based sample of twins aged 40–77 years. Age in this range did not seem to have any effect on the SSB parameters. However, γ-H2AX response and DSB repair capacity decreased with increasing age, although the associations did not reach statistical significance after adjustment for batch effect across multiple experiments. No gender differences were observed for any of the parameters analyzed. Our findings suggest that in PBMCs, the repair of SSBs is maintained until old age, whereas the response to and the repair of DSBs decrease.

Key words: aging; double-strand break repair; gender; peripheral blood mononuclear cells; single-strand break repair; γ-H2AX.

Introduction

Our genome is vulnerable to injury inflicted by various exogenous and endogenous damaging agents. Accumulation of DNA damage can lead to errors in DNA replication causing point mutations or chromosomal rearrangements, and this is thought to play a critical role in aging, cancer, and neurodegenerative diseases (Jeppesen *et al.*, 2011). Depending on the specific type of lesion, the DNA damage is repaired by one of the four major DNA repair pathways: base excision repair (BER), nucleotide excision repair (NER), mismatch repair or double-strand break repair (DSBR).

Base excision repair is the main guardian against DNA lesions derived from endogenous cellular metabolism, because it removes base adducts resulting from attack by reactive oxygen species, methylation, deamination, and hydroxylation. Due to the constant and immense attack of the genome from reactive oxygen species, BER is very important for stability of the genome and is involved in repair of both nuclear and mitochondrial DNA (Gredilla, 2010). One of the prominent theories of aging, 'The free radical theory of aging', states that free radicals (generating base lesions and single-strand breaks) are involved in the intrinsic aging process (Harman, 1992). Accordingly, several studies in yeast and animal models suggest that defects in BER enzymes shorten chronological lifespan and are associated with aging or age-related diseases (Maclean *et al.*, 2003; Marusyk & DeGregori, 2008). The BER pathway is initiated by the removal of the modified base by a DNA glycosylase, which leaves an apurinic/apyrimidinic (AP) site. AP sites are incised generating single-strand breaks (SSBs). The subsequent steps in BER are similar to SSB repair (SSBR). SSBs arise from various forms of damages including AP sites, direct breaks, and intermediates of the BER pathway. After end processing, the final steps of BER/SSBR pathway include filling in the gap and subsequently ligation (Maynard *et al.*, 2009).

DNA double-strand breaks (DSBs) are particularly hazardous to the cell, because they can lead to genome rearrangements. Phosphorylation is a molecular switch of DSBR, and immediately after DSB formation, the histone protein H2AX is phosphorylated at serine 139 (γ-H2AX). γ-H2AX molecules accumulate in the chromatin around the break during the first hour after DSB formation, and γ-H2AX is involved in the DSB signaling response (Muslimovic *et al.*, 2008). Together with γ-H2AX, several other DSB damage response proteins accumulate in foci around DSBs and send signals via signal transducers to a set of downstream effectors, which affect events like DNA repair, cell cycle checkpoints, telomere maintenance and transcription. Two major mechanisms exist to repair DSBs: nonhomologous end joining (NHEJ) and homologous recombinational repair (HRR). The steps involved in NHEJ include recognition of DSBs, end processing, and finally ligation. HRR uses homologous sequences in sister chromatids to repair DSBs, especially those formed at collapsed replication forks. The main difference between these two major DSBR pathways is the error-prone nature of NHEJ in contrast to the error-free HRR. The NHEJ pathway takes place throughout the cell cycle, whereas HRR repairs DSBs during S and G_2 phases of the cell cycle (Shrivastav *et al.*, 2008). DSBR is considered to have a fast and a slow component, primarily represented by NHEJ and HRR, respectively (Jeggo *et al.*, 2011).

Previous studies investigating SSBR in peripheral blood mononuclear cells (PBMCs) from donors of different age suggested minor or no effect

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on age (Muller *et al.*, 2001; Zana *et al.*, 2006; Trzeciak *et al.*, 2008, 2012). Some reports have suggested an age-related increase of endogenous SSBs in human PBMCs (Mutlu-Turkoglu *et al.*, 2003; Moller, 2006; Humphreys *et al.*, 2007), whereas others have suggested no age effect (Slyskova *et al.*, 2011). An age-related decline of NHEJ functions in brain tissue has been suggested from animal studies (Ren & Pena de Ortiz, 2002; Vyjayanti & Rao, 2006). In line with these results, PBMCs from elderly humans showed reduced nuclear localization and DNA binding of the NHEJ-specific Ku70/80 complex when compared to young individuals (study population: 24 subjects, aged 20–89 years) (Frasca *et al.*, 1999). An age-related decline of Ku70 levels was observed in a study of PBMCs from 48 subjects aged 20–80 years (Ju *et al.*, 2006), and another study reported declining levels of Ku80 with age (Scarpaci *et al.*, 2003). Together these studies suggest changes in DNA repair with age, although powerful functional and epidemiological studies are missing.

The most abundant DNA damages are SSBs, yet DSBs are one of the most lethal kinds of DNA damage; hence, efficient repair of these specific lesions is considered essential for the cells. The high cytotoxicity of DSBs is expected to affect longevity more than other types of damages [reviewed by (Li *et al.*, 2008)]. PBMCs and other blood cells are replaced more rapidly than cells from other organs, and therefore, they may not be affected by age to the same extent; nonetheless, age-related reduction in the immune repertoire and reduced immune function have been reported at old age (Gill *et al.*, 2001).

As men and women appear to age differently, it is interesting to search for gender differences in parameters, which are affected by age. We therefore also investigated whether DNA repair parameters assayed in this study are affected by gender. Gender effects on the level of endogenous SSBs have previously been reported in human PBMCs (Hofer *et al.*, 2006; Slyskova *et al.*, 2011), and indications of gender effects on DSBR were reported in a small study of 20 study subjects (Mayer *et al.*, 1991). Several reports have suggested gender effects on other DNA repair pathways, primarily NER (Wei *et al.*, 1993; Slyskova *et al.*, 2011; Uppstad *et al.*, 2011).

In the current study, we have searched for possible age and gender effects on DNA strand break repair with more statistical power and more sensitive methodologies than in previous studies. The age span from 40 to 77 years is a period of life with substantial decrease in physical and cognitive functions (Frederiksen *et al.*, 2002; Izaks *et al.*, 2011; Jeppesen *et al.*, 2011), and we hypothesized that this decline is reflected in DNA repair parameters. This study involves a molecular investigation of repair capacity of SSBs and DSBs in isolated PBMCs from the study subjects by three molecular assays. Using the neutral comet assay and the fluorometric analysis of DNA unwinding (FADU) assay, we investigated the DNA repair capacities and the level of endogenous damage, whereas DNA damage response was investigated by flow cytometric analysis of γ-H2AX formation after DNA damage induction.

Results

Demographic and health characteristics of the study subjects

Two hundred and sixteen study subjects were selected from The Danish Twin Registry (DTR) (no selection criteria apart from being a twin pair born 1930–1969 and no exclusion criteria). The age span was from middle adulthood (40 years of age) to old age (the oldest subject being 77 years old). The study sample was a random cross-sectional sample of the Danish population, and no intrapair analysis was performed in this study. Bias due to family relations was accounted for by adjusting for nonindependence within twin pairs (see: Statistical methods). The mean age of the study population was 57.2 years at the time of sampling. Due to different pairwise participation rates at the clinic, women were over-represented with 68% vs. 32% men of the total sample size. Demographic and health characteristics of the study subjects are summarized in Table 1. A total of 15 cancer patients (six skin cancer) participated in this study, and total of 49 daily smokers were included. The study subjects gave subjective assessment of their health, and 18 individuals considered their health poorer than that of their contemporaries. None of the study subjects were diagnosed with neurodegenerative diseases. An internal control individual (48-year-old healthy woman) was included for methodological reasons.

Unchanged basal level of endogenous SSB and SSB repair capacity with increasing age

To identify possible age effects on the level of endogenous SSB and SSBR in PBMCs from the study subjects, we performed FADU assays. A subsample of the total sample size was analyzed by this assay: endogenous SSB ($N = 172$) and SSBR ($N = 166$). An example of outcome measured by the FADU assay is presented in Fig. 1A. The levels of endogenous SSBs (T-P0) normalized to the internal control as a function of age are shown in Fig. 1B. No statistically significant changes were observed in the levels of endogenous SSBs with advancing age in the study population (Table 2). As it was impossible to analyze all samples in a single experiment (batch), we have combined data from different batches. We tested all data using two regression models: the random intercept regression model adjusted for nonindependence within twin pairs and the mixed-effects regression model adjusting for batch effects (see: Experimental procedures). The capacity to repair irradiation-induced DNA damage was measured as the ratio between repaired damage (R_1-P_1) and the induced damage (P_0-P_1) (Fig. 1A). The SSBR capacity appears to be constant throughout the age span investigated (Fig. 1C). No statistically significant association between SSBR and age was observed in either regression models (Table 2). To investigate the association between SSBR capacity and the level of endogenous damage, we calculated Pearson's correlation. A statistically significant negative correlation was observed (Fig. 1D; $r = -0.31$, $P < 0.01$). In a subanalysis, it was tested whether individuals with cancer, poor health, or smokers had different mean SSBR capacity, and we found borderline statistically significant higher SSBR capacity in smokers ($N = 35$) compared with nonsmokers ($P = 0.05$, two-sample *t*-test).

Table 1 Demographic and health characteristics

Age		Men (years)		Women (years)
Mean age (gender)		58.8		55.6
Mean age (total)		57.2		
Age group	Men (*N*)	Women (*N*)	Total (*N*)	%
41–49	14	48	62	28.7
50–59	26	52	78	36.1
60–69	21	44	65	30.1
70–77	8	3	11	5.1
Total	69	147	216	100
Smokers	15	34	49	22.7
Poor health	4	13	17	7.9
Cancer	4	10	14	6.5

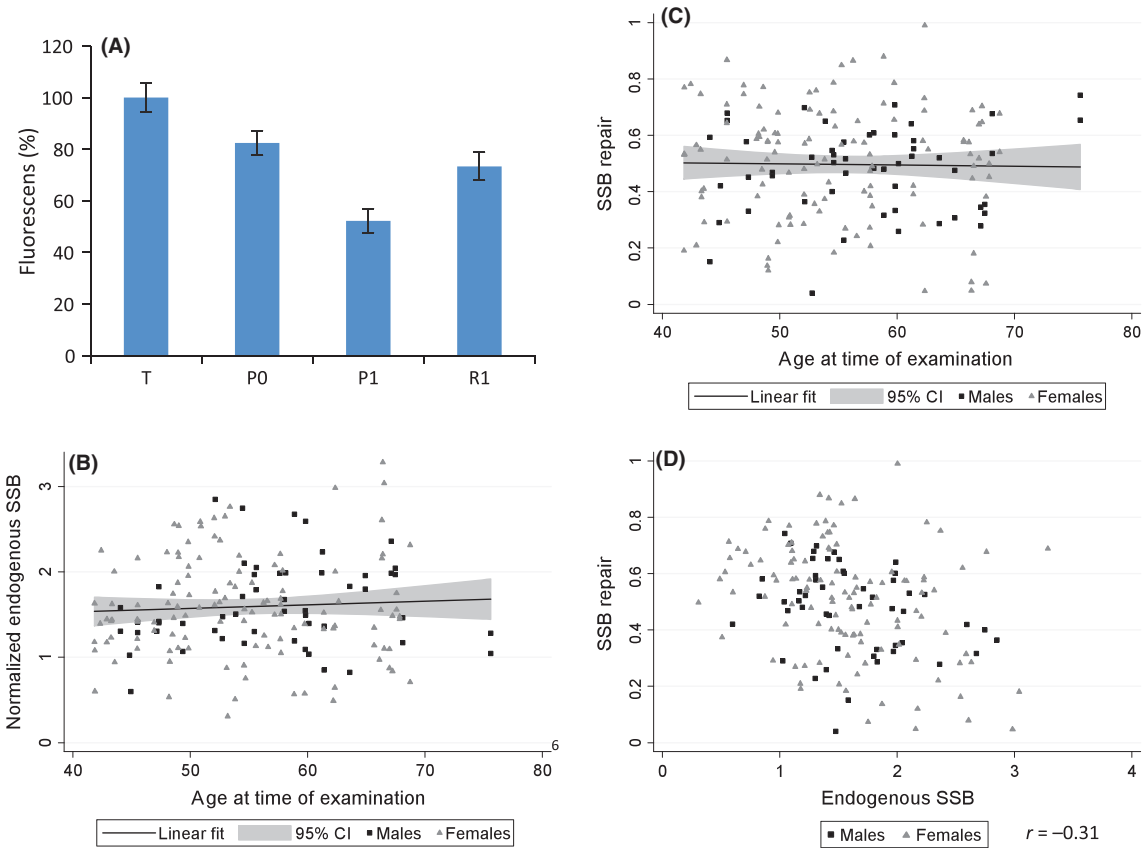

Fig. 1 No age or gender effects on endogenous single-strand break (SSB) level and SSB repair capacity. (A) Representative diagram of the outcome variables from the fluorometric analysis of DNA unwinding (FADU) assay: T, P_0, P_1, and R-values. The T samples represent the total amount of double-stranded DNA (dsDNA). P_0 samples represent the amount of dsDNA remaining after alkaline unwinding of DNA from cells under physiological conditions. P_1 and R_1 samples were X-irradiated (3.8 Gy), but only R_1 samples were allowed to recover (40 min at 37°C); hence, P_1 represents the amount of dsDNA remaining after alkaline unwinding of DNA from cells after damage induction, and R_1 represents the same after damage and repair. From these values, the endogenous level of strand breaks and the capacity of strand break repair were calculated (see Experimental Procedures for details). All bars represent average of four individual measurements. Error bars represent standard deviations. (B) The level of endogenous strand breaks as a function of age measured by the FADU assay (normalized to the control cells). Men are presented as black squares and women as gray triangles. Regression line (blue) and 95% confidence intervals (gray) are presented. (C) Strand break repair capacity as a function of age. Gender, regression line, and confidence intervals are presented as in B. (D) Negative correlation (Pearson) between endogenous SSB levels and the SSB repair capacity ($r = -0.31$, $P < 0.01$).

Table 2 Multiple regression models for the effects of age and gender on DNA repair variables

	Endogenous SSB		SSB repair		DSB repair		γ-H2AX response	
Regression model	A	B	A	B	A	B	A	B
Age, 10 year (adj. gender)	0.04	−0.01	0.00	0.00	−0.09	−0.04	−0.13	0.00
P-value (age)	0.51	0.90	0.95	0.697	<0.01*	0.11	<0.01*	0.91
Gender, ref. group male (adj. age)	−0.01	−0.02	0.03	0.03	0.02	0.02	−0.06	−0.01
P-value (gender)	0.94	0.83	0.34	0.38	0.70	0.60	0.57	0.90
Intercept	1.37	1.65	0.48	0.43	1.33	1.06	2.04	1.27
N	172	172	166	166	205	205	209	209

DSB, double-strand break; SSB, single-strand break.
*$P < 0.01$.

Trend toward an age-related decline in DSB repair (NHEJ) capacity

Neutral comet assays were performed to measure DSBR capacity among the study subjects. DSBR capacity was calculated as the amount of repaired DSBs during a 3-h period after DNA damage induction. DSBR capacity appears to be negatively associated with age as depicted by decreasing regression line in Fig. 2A. Images of representative comets before and after damage induction and repair are shown in Fig. 2B. In the random intercept regression model adjusted for nonindependence within twin pairs, a statistically highly significant association is found between DSBR capacity and age ($P < 0.01$; Table 2). Due to variance observed between

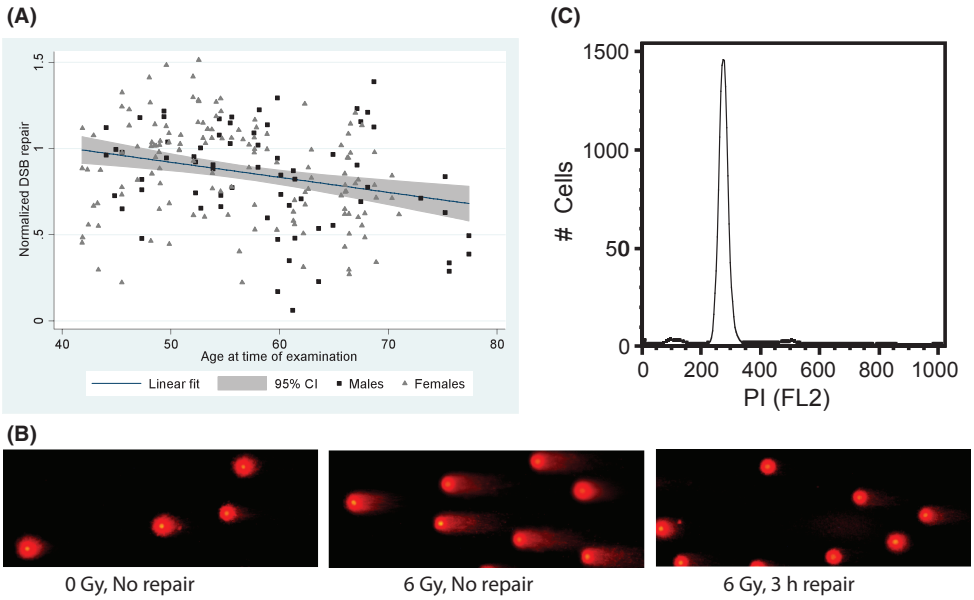

Fig. 2 Tendency of decline in double-strand break repair (DSBR) capacity with increasing age for men and women. (A) DSBR capacity as a function of age (regression line in blue and 95% confidence intervals in gray) and presented by gender (men: black squares, women: gray triangles). The repair capacity is the percentage of repaired DNA damage relative to total induced damage (calculated as shift in mean comet tail moment). Data are normalized to an internal control. (B) Representative comet assay images of single cells: Undamaged cells (left), gammairradiated (6 Gy), no repair (middle), and after damage induction and 3-h repair incubation (right). (C) Cell cycle analysis of control peripheral blood mononuclear cells shows that the majority of cells are in G_0/G_1 (90%) and only a minor part in S phase (4%) and G_2 (3%).

results from different batches, we used a regression model adjusting for batch effect, which indicates a weaker association ($P = 0.11$). In a subanalysis, it was tested whether the observed decrease was due to cancer, smoking, or poor health. After exclusion of smokers, cancer patients, or individuals with poor health condition, respectively, the age-related decrease remained the same (data not shown). A lower mean DSBR was observed for skin cancer patients ($N = 6$) vs. nonskin cancer subjects, but only with borderline significance using two-sample *t*-test ($P = 0.08$).

Despite the fact that the most stringent analysis did not reach statistical significance, the association suggests a decline in DSBR with increasing age. The batch size was on average 20 for the comet assay, which is considered low to filter batch effects accurately (Johnson *et al.*, 2007; Leek *et al.*, 2010). Hence, the mixed-effects regression model may overadjust the data due to relatively small batch sizes.

As DSBs are repaired by either NHEJ or HRR, we wanted to determine which of the pathways were responsible for the repair activity measured by the neutral comet assay. Therefore, cell cycle analysis was performed. Previous studies have identified the NHEJ pathway to take place throughout the cell cycle, whereas HRR takes place during S and G_2 phases (Shrivastav *et al.*, 2008). Due to these differences between NHEJ and HRR, simple determination of cell cycle analysis of the PBMCs was informative to determine which of the DSBR pathways were responsible for the repair activity measured by the comet assay. Results from a representative cell cycle analysis of PBMCs from the internal control individual are shown in Fig. 2C. Most cells (91%) were in G_0/G_1, and only a minor part was in S phase (4%) and G_2 phase (3%). Therefore, our results strongly indicate that the DSBR capacity measured in this study must primarily be NHEJ, which is also the major repair pathway for the fast component (0–3 h) of DSBR after gamma-irradiation (Jeggo *et al.*, 2011).

Tendency toward a declining γ-H2AX response with increasing age

The response to DNA damage induction was measured as the maximum formation of γ-H2AX foci after 6 Gy γ-irradiation. PBMCs were stained with fluorescent antibody against γ-H2AX, and we observed a shift in fluorescence intensity, which corresponds to the γ-H2AX signal. Results from γ-H2AX phosphorylation assay are exemplified in Fig. 3A, representing a histogram with peaks for unirradiated (red) and irradiated (blue) cells. The distance between the two peaks represents the amount of γ-H2AX phosphorylation after induction of damage. The flow cytometric analysis was performed on a uniform population of PBMCs, represented in the SSC–FSC plot in Fig. 3A. The best-fitting random intercept model revealed a negative association between the γ-H2AX response and age ($P < 0.01$) when adjusting for effects of gender and nonindependence within twin pairs (Fig. 3B; Table 2). However, after adjusting for batch effects, applying the mixed-effects regression model, the association no longer reached statistical significance ($P = 0.91$; Table 2). Again, it should be noted that the relatively small batch size (on average 12 samples per assay) could potentially cause overadjustment for batch effects. Similarly to the results on the comet assay, a subanalysis was performed and demonstrated that the decline was not due to smoking, cancer or poor health condition.

To identify potential association between DSBR capacity and the induced γ-H2AX response, we used Pearson's correlations and found no statistically significant correlation ($r = 0.06$, $P = 0.38$).

No gender influence on DNA repair parameters

In the regression analysis for age effects, we adjusted for possible gender effects, because men and women are known to age differently. Given that aging is affected by gender, and that we have observed tendencies of age effects on some of the DNA repair parameters, we next analyzed for possible gender effects on DNA repair. No statistically significant differences were observed for any of the four DNA repair parameters between men and women in the study population (Table 2). Data for men and women are plotted separately in Figs 1, 2, and 3, illustrating similar trends in men and women. Women were over-represented in the study population, but nevertheless, the size of each group exceeds previous studies analyzing gender effects on DNA repair (147 women and 67 men, Table 1).

Discussion

The mechanism of aging is very complex, and numerous health outcomes are affected by aging. We hypothesized that DNA repair of

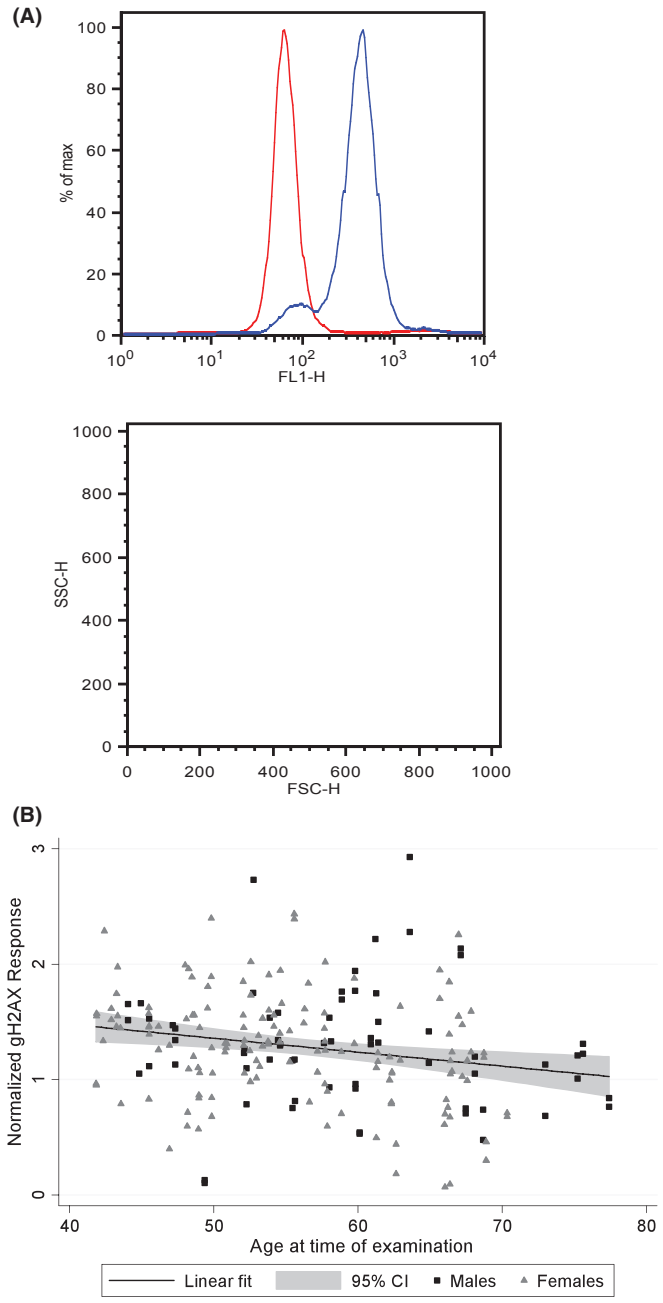

Fig. 3 γ-H2AX response shows a tendency to decline with increasing age for men and women. (A) Representative results showing the γ-H2AX response in unstressed cells (red) and 1h after irradiation (blue) measured by flow cytometry. The shift in fluorescence between unstressed and stressed cells corresponds to the γ-H2AX response. The SSC–FSC plot shows the uniform population of cells investigated, and colors depict amount of cell (blue: single cells, red: multiple cells). (B) Scatter plot of γ-H2AX response and age. The tendency of age-related decline in γ-H2AX response is illustrated with a regression line (blue) and 95% confidence intervals (gray).

two important types of DNA lesions, SSBs and DSBs, is involved in the aging process.

Interestingly, no statistically significant changes were identified in our study population for the level of endogenous SSB and the SSBR capacity (Fig. 1B and C; Table 2). These data suggest that the ability to repair SSB is generally maintained up to age 77 in human PBMCs. The younger age group below 70 years of age consists of more individual than the old group above 70 years of age (Table 1), resulting in more statistical strength up to age 70. The observed lack of significant age effects on SSBR in PBMCs is in agreement with several previous studies. However, those studies analyzed fewer subjects (N = 25–50) (Muller *et al.*, 2001; Zana *et al.*, 2006). The subanalysis indicated higher SSBR capacity in smokers compared with nonsmokers (P = 0.05, two-sample t-test), which could be pursued in future studies although it could also be a chance finding among the multiple testings. The level of endogenous SSBs is expected to increase with age, assuming that the SSBR capacity is not sufficient to cope with the load of SSB lesions. The current study, however, shows no statistically significant age-related changes in endogenous SSBs, and the regression line indicates a minimal increase with age (Fig. 1B), whereas other reports indicate positive association with age (Mutlu-Turkoglu *et al.*, 2003; Humphreys *et al.*, 2007). The discrepancy between previous and the current study may be due to different assays for analysis of endogenous SSB levels. We employed the FADU assay due to reported lower coefficient of variation compared with the alkaline comet assay used in previous studies (Forchhammer *et al.*, 2010; Moreno-Villanueva *et al.*, 2011). Compared with the FADU assay, the alkaline comet assay requires more manual handling, which is potentially more stressful to frail cells from old study subjects. Consequently, there may be a greater risk of overestimating the endogenous level of SSBs when using the alkaline comet assay on cells from old individuals. On the other hand, the FADU assay has proven to have high reproducibility on PBMCs (Moreno-Villanueva *et al.*, 2009, 2011). A statistically significant age-related decline was observed in DSBR and γ-H2AX response in the age span investigated (Figs 2B and 3B; Table 2). However, we observed batch effects during data analysis, deriving from experimental variation across multiple experiments, and we adjusted the regression model accordingly. As mentioned above, it is problematic to adjust for batch effects when batch sizes are small (< 25), as in this study, and hence, our adjustment may overestimate the batch effect (Johnson *et al.*, 2007). When adjusting for batch effects, we found no statistically significant age-associated change in DSBR or γ-H2AX response within the investigated age span.

Double-strand breaks are the more harmful lesions; hence, we anticipated a more pronounced age effect in γ-H2AX response and DSBR compared with the SSB parameters. Indeed, our data indicate a tendency of negative association of DSBR capacity and γ-H2AX response with age (Figs 2A and 3B). If this tendency is an effect of significant age-related decrease in a whole population, it can explain a part of the complex process of aging. At ages over 70 years, this tendency may be increased, which is beyond the limits of this study. In the current DSBR analysis, we predominantly measured NHEJ activity (Fig. 2). Despite the error-prone nature of NHEJ, it is considered important for the fast component of DSBR and therefore important for preventing DNA rearrangements. A recent study investigated DNA rearrangements in PBMCs in a cohort study, and they identified more DNA rearrangements in a group of elderly subjects ($\geq$ 60 years) compared with a group of younger subjects ($\leq$ 55 years) (Forsberg *et al.*, 2012). These findings indicate an age-related increase in DNA rearrangements, which could be explained by the age-related decrease in NHEJ activity and γ-H2AX response, reported here. Consequently, reduced survival and function of PBMCs are expected to follow the increase in DNA rearrangements at old age. If the capacity to repair DSBs is maintained throughout life, which the adjusted regression model implies, it may nonetheless be important for the aging process. The load of DSB damage may exceed a constant capacity to repair them, resulting in DSB accumulation and cellular senescence. The subanalysis suggested lower DSBR capacity in skin

cancer patients compared with nonskin cancer patients (borderline significant). This finding could inspire future studies on the role of DSBR in skin cancer.

Indications of age-related decline in other DNA repair pathways, such as NER, have been reported (Moriwaki *et al.*, 1996; Qiao *et al.*, 2002). The age effect on DNA strand break repair capacity might be too weak to be identified by the methods employed, when considering the statistical power of the study and the variance of the measurements. In this case, future studies on the subject will need even more refined methods and/or additional samples. A longitudinal approach following the same individuals might be another advantageous approach; however, it implicates considerable practical issues, although it is not impossible in a long term using registries such as the DTR.

The age span of the subjects was 40–77 years in this study, which is a period of life with great decline in numerous physical measures, including the function of several inner organs, and hence, we expected an effect of age on DNA repair. Healthy PBMCs are critical for the immune system and thus important for all organs. However, PBMCs have a higher turnover compared with the cells of solid organs and are potentially healthier and may not be affected by age until very old age. Slow or nonproliferating cells may be influenced by age to a greater extent.

Higher levels of endogenous SSBs have been reported in PBMCs from men compared with women (Hofer *et al.*, 2006; Slyskova *et al.*, 2011). Men had higher levels of DNA damage than women in these studies including approximately 100 study subjects. An early publication including only 20 study subjects indicated gender effects on DSBR, suggesting less efficient DSBR in women (Mayer *et al.*, 1991). Gender differences have also been reported for the NER pathway investigating blood samples or human cell lines (Wei *et al.*, 1993; Uppstad *et al.*, 2011). But recently, a powerful epidemiological study investigating NER activity in PBMCs from 100 healthy individuals identified no statistically significant gender effect ($P = 0.86$) (Slyskova *et al.*, 2011). Similarly, none of the DNA repair parameters investigated in our study revealed significant gender differences (Table 2). The aforementioned indications of gender effects on SSB and DSBR parameters were concluded from statistically weaker studies compared with the current study and might well have been due to chance findings or bias. Potential gender effects could however exist at very old age, which is beyond the limitations of this study.

In the current study, SSBR capacity and endogenous SSB levels were negatively correlated, $r = -0.31$, $P < 0.01$ (Fig. 1D), suggesting a statistically significant lower level of endogenously derived SSBs in cells with efficient SSBR capacity. The correlation was moderate, which can be due to generally efficient SSBR in the study subjects. The SSBR capacity probably exceeds the amount of endogenous SSBs to be able to cope with a sudden dramatic increase in SSB levels (e.g., after physical exercise). The reliability of the data may be confirmed by the observation that the SSB parameters correlated as expected.

Double-strand break repair capacity and γ-H2AX response did not correlate ($r = 0.06$, $P = 0.38$), which may be explained by the somewhat dissimilar nature of the two parameters. Moreover, recent studies have suggested that a γ-H2AX-independent NHEJ pathway may exist (Yuan *et al.*, 2010)**.** The PBMCs investigated here were predominantly in G_0 or G_1 phase of cell cycle (Fig. 2C), meaning that the observed DSBR capacity reflects mainly NHEJ. Therefore, it is likely that the NHEJ capacity is partly independent of the phosphorylation state of H2AX, and this may account for the observed lack of correlation between the DSBR and γ-H2AX response parameters in this study. We consider the γ-H2AX response a valuable marker for DSB response in this study, because it is involved in both NHEJ and HRR pathways; and interestingly, we identified similar tendencies of age-related decline for both DSBR and the γ-H2AX response. Also, H2AX deficiency in mice leads to a reduced DSBR capacity (Bassing *et al.*, 2002; Xie *et al.*, 2004). As the DSB damage response protein H2AX sends signals via signal transducers to a set of downstream effectors, which, in addition to DSBR, also affects cell cycle checkpoints, telomere stability, and transcription, we did not expect a high correlation.

In conclusion, we identified a tendency of decline in DSBR and γ-H2AX response with aging; however, none of the associations of the investigated SSBR or DSBR parameters with age were statistically significant after adjusting for batch effects. No influence of gender on the investigated parameters was observed. Endogenous SSB levels and SSBR correlated negatively, suggesting an expected link between these two parameters. At ages over seventy years, DNA damage levels may increase and/or DNA repair may decline more than the observed trends; however, it is beyond the limits of this study to approach that question.

Experimental procedures

Study population

The study sample ($N = 216$) was comprised of subjects enrolled in *The Danish Twin Registry*. The Danish Twin Registry is a nationwide, population-based registry established in 1954 and includes data from over 80 000 twin pairs born between 1870 and 2004 (Skytthe *et al.*, 2011). Since the 1960s, selected cohorts from the registry have participated in questionnaire and survey studies.

In the period 2008–2011, twins born in Denmark between 1931 and 1969 were invited to a clinical investigation in one of the five centers in Denmark. The subjects in the current study were selected randomly among twin pairs participating in one of these centers. The Science Ethics Committee of Southern Denmark approved this study (Project number S-VF-19980072).

Isolation and storage of peripheral blood mononuclear cells

Whole blood was collected from each subject in BD Vacutainer® CPT™ tubes. PBMCs were isolated according to the manufacturer's protocol (BD, Europe, Vacutainer® CPT™, REF 362761), and cells were suspended stepwise in cryopreservation RPMI 1640 medium at a final concentration of 20% FBS and 10% DMSO. Freezing to −80°C was carried out gradually, followed by storage in liquid nitrogen. Prior to the different assays, cells were thawed in a stepwise fashion to maintain maximum cell viability. After thawing the PBMCs, they were allowed to recover for 30 min at 37°C in 5% CO_2 in RPMI 1640 medium before each experiment.

Fluorimetric detection of alkaline DNA unwinding

Levels of endogenous strand breaks and capacity to repair-induced strand breaks were measured by the automated and modified version of the FADU assay. This assay measures both SSBs and DSBs, but in the view of the much larger numbers of SSBs, FADU data predominantly reflect SSBs (Moreno-Villanueva *et al.*, 2009, 2011). Briefly, strand breaks were induced in PBMCs by X-irradiation at 3.8 Gy, followed by 40-min repair incubation in RPMI (without FBS supplementation) at 37°C. The repair rate of SSBs was in the linear range after 40 min, and approximately 50% of the induced strand breaks were repaired, enabling detection of possible differences (Fig. 1C illustrates on average

≈ 50% repair). This assay is based on progressive DNA unwinding under highly controlled conditions of alkaline pH, time, and temperature. The starting points for the unwinding process are DNA 'open sites' such as DNA strand breaks. For monitoring DNA damage, we used SybrGreen® (Life Technologies Europe BV), a fluorescent marker for double-stranded DNA (dsDNA), and a decrease in the fluorescence intensity indicates an increase of DNA unwinding, and consequently, a greater number of DNA strand breaks present at the time of cell lysis. Five different parameters were determined in 4 replicates for each study subject: T (total amount of DNA), P_0 (amount of dsDNA at physiological conditions), P_1 (amount of dsDNA after damage induction), R_1 (amount of dsDNA after repair), and B (background fluorescence). T, P_0, P_1 and R_1 measurements were corrected for background fluorescence deriving from SSBs and autofluorescence (B), and log10 transformation was performed. Endogenous level of SSBs was calculated as the difference between total dsDNA (T) and dsDNA at physiological conditions (P_0). The level of endogenous SSBs was normalized to the internal control. SSBR capacity was calculated by using the following equation: $SSBR = (R_1-P_1)/(P_0-P_1)$. An SSBR value of 1 represents 100% repair efficiency.

Neutral comet assay

Peripheral blood mononuclear cells from each study subject and an internal control were analyzed by neutral comet assay. Untreated cells and γ-irradiated cells (6 Gy, Cs-137 source) with and without recovery (3 h, 37°C in 5% CO_2, complete RPMI) were mixed with 0.5% low melting point agarose and cast on a plastic film (Lonza). Gels were submerged for 90 min in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% sodium lauroyl sarcosinate, 0.01% Triton X-100, pH 9.5). Irradiation dose and recovery time were optimized with respect to linear damage response, avoidance of high numbers of apoptotic cells, and incomplete repair to detect possible differences (internal control cells repaired approximately 75% of the strand breaks induced). The neutral comet assay is not specific for detecting DSBs, and we therefore optimized damage induction to induce DSBs, which also occur when SSBs are in close approximation. Induction of SSBs was carried out in control experiments with H_2O_2 treatment (50 μM for 10 min) and did not induce significant increase in Olive tail moment (data not shown). Induced SSBs are repaired markedly faster than the 3-h period we used to incubate the cells for DSBR (described for FADU assay). Based on these considerations, we report neutral comet results as a measure of predominantly DSBR.

Gel electrophoresis was performed at 25 V for 20 min at 4°C in the dark using consistent conditions for every assay [½ × TBE buffer (pH 8.0)], followed by dehydration until the next day. DNA was stained using SYBR Gold (Invitrogen, Life Technologies Europe BV, Naerum, Denmark), and analysis was performed using a fluorescent microscope (Leica DMLB, Leica Microsystems, Denmark) equipped with camera (Leica DC 200). Live quantification was performed on 60–100 cells from each gel using Perceptive Instruments IV software. Apoptotic and necrotic cells were excluded during analysis to avoid bias. As a measure of DNA damage, we used Olive tail moment, because it is considered independent of comet shape and the best-established parameter for this assay, providing smaller variability compared with tail length and tail DNA (Lee *et al.*, 2004).

H2AX phosphorylation assay

The response to induced DSB was measured by a flow cytometric method, which is described in details previously (Muslimovic *et al.*, 2008). Briefly, approximately 2×10^6 PBMCs were split into nontreated and γ-irradiated (6 Gy, Cs-137) cells. Subsequently, the cells were immediately incubated in RPMI 1640 media supplemented with 10% FBS for 1 h at 37°C in 5% CO_2. Then, cells were fixed in 70% EtOH overnight at −20°C. The following day, cells were incubated in blocking buffer (PBS supplemented with 8% FBS, 0.1 g L^{-1} RNase A, 10mM NaF, 1mM $NaVO_4$, 0.25 g L^{-1} salmon sperm DNA, and 0.2% Triton-X100) for 1 h prior to staining. Staining for phosphorylated H2AX was performed with anti γ-H2AX mouse antibody (Millipore JBW301, Merck Millipore, Billerica, Massachusetts, USA) diluted 1:500 in blocking buffer and incubated for 1 h at room temperature. Cells were washed twice in TBE buffer and incubated for 1 h in Alexa Fluor 488 (Invitrogen). Negative controls (without secondary antibody) were included as a background reference. A minimum of 50 000 stained cells were acquired using a BD FACS Calibur (BD Europe). Cytometer settings were maintained for analysis on different days (SSC: voltage 387, AmpGain 1, Mode lin, FL1: voltage 535, AmpGain 1, Mode log). γ-H2AX response was measured as the difference between fluorescence signal in treated and nontreated samples (Fig. 3A). An internal control was included for each flow cytometric experiment as a reference standard to be able to correct for interexperimental variability. FlowJo V6.4.7 software was used for flow cytometric analysis (Tree Star Inc., Ashland, OR, USA).

Cell cycle analysis

Peripheral blood mononuclear cells from the internal control were collected and fixed as described above. Fixed cells were resuspended in 0.5 mL PBS containing 0.1% triton X-100, 1.0 mg mL^{-1} RNase A, and 2.5 mg mL^{-1} propidium iodide (PI) for 30 min at room temperature. Fluorescent signals were measured using a BD FACS Calibur, and the proportion of cells in G_0/G_1, S, and G_2 were estimated using FlowJo cell cycle analysis program using the 'Dean-Jett-Fox' model.

Method variability

In molecular epidemiological studies, it is important to minimize method variability, which was of major concern. The ability of an assay to detect differences in DNA repair capacity is influenced by interexperimental variability. The FADU assay has a reported average interassay coefficient of variation (CV) of 13.3% for PBMCs (Moreno-Villanueva *et al.*, 2011). Based on these results, the FADU assay is considered to provide valid measures for endogenous SSB and SSBR. Numerous studies have reported different CVs for the comet assay, and therefore, an interlaboratory validation test was performed (Forchhammer *et al.*, 2010), which reports large CVs for some laboratories. To reduce interexperimental variability, we included an internal control. Repetitive measurements of reference cells by the neutral comet assay in this study resulted in a CV of 12.9%, which we consider to reflect the day-to-day variation. By normalizing results from all test samples to the reference sample, we corrected for this variation. Similarly, the day-to-day variations of the H2AX phosphorylation assay (CV = 28.3%) were corrected for by including reference cells. The disadvantage performing this normalization is the dependency of precise measurement of internal control cells, which were measured in duplicate. Additionally, we modified the methods for higher throughput, which enabled us to analyze a large number of study subjects in a relatively small number of separate experiments. Despite our precautions, we identified batch effects in data from the H2AX phosphorylation assay and the neutral comet assay. In contrast to many studies analyzing DNA repair, we corrected for

batch effects in the current study by the statistical methods described below.

Statistical methods

Multiple regression analysis was used to evaluate whether DNA repair variables were associated with age and gender. The residuals were normally distributed, and the assumptions for multiple regression analysis were not violated. The random intercept regression model is considered to account for nonindependence within twin pairs (cluster). The mixed-effects regression model was used to correct for batch effects. Stata 11 (Stata Corp., College Station, TX, USA) was used for all statistical analysis. *P*-values < 0.05 were considered statistically significant.

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Author contributions

CG contributed by generating the study design, sampling, performing all molecular biological assays, conducting data cleanup and data analysis, interpreting data, and drafting the manuscript. TS, KC, and VAB contributed by generating the study concept and study design, supervising the research, and contributing to writing of the manuscript. IP contributed with statistical support. MMV and AB contributed by helping with acquisition and interpretation of the FADU data and critical reading of the manuscript.

Conflict of interest

The authors have declared that no conflict of interest exists.

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