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## Ulcerative colitis is a disease of accelerated colon aging: evidence from telomere attrition and DNA damage

Rosa Ana Risques<sup>1</sup>, Lisa A. Lai<sup>2</sup>, Teresa A. Brentnall<sup>2</sup>, Lin Li<sup>3</sup>, Ziding Feng<sup>3</sup>, Jasmine Gallahe<sup>1</sup>, Margaret T. Mandelson<sup>3,4</sup>, John D. Potter<sup>3</sup>, Mary P. Bronner<sup>5</sup>, and Peter S. Rabinovitch<sup>1,3</sup>

<sup>1</sup>Department of Pathology, University of Washington, Seattle, WA

<sup>2</sup>Department of Medicine and Division of Gastroenterology, University of Washington, Seattle, WA

<sup>3</sup>Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA

<sup>4</sup>Center for Health Studies, Group Health Cooperative, Seattle, WA

<sup>5</sup>Department of Anatomic Pathology, Cleveland Clinic, Cleveland, OH.

### Abstract

**Background & Aims**—Telomere shortening is implicated in cancer and aging, and might link these two biological events. We explored this hypothesis in ulcerative colitis, a chronic inflammatory disease that predisposes to colorectal cancer and where shorter telomeres have been associated with chromosomal instability and tumor progression.

**Methods**—Telomere length was measured by quantitative PCR in colonocytes and leukocytes of two different sets of ulcerative colitis patients, and compared with normal controls across a wide range of ages. For a subset of patients, telomere length was measured in epithelium and stroma of right and left colon biopsies. A third set of biopsies was analyzed for phosphorylation of histone H2AX ( $\gamma$ H2AX), a DNA damage signal, by immunofluorescence, and for telomere length by quantitative FISH. Relationships between telomere length,  $\gamma$ H2AX intensity, age, disease duration, and age of disease onset were explored.

**Results**—Colonocyte telomeres shorten with age almost twice as rapidly in ulcerative colitis patients as in normal controls. This extensive shortening occurs within approximately eight years of disease duration. Leukocyte telomeres are slightly shorter in ulcerative colitis patients than in controls, but telomeres of colon stromal cells are unaffected.  $\gamma$ H2AX intensity is higher in colonocytes of ulcerative colitis patients than in controls and is not dependent on age or telomere length.

**Conclusions**—Colonocytes of ulcerative colitis patients show premature shortening of telomeres, which might explain the increased and earlier risk of cancer in this disease. Shorter leukocyte telomeres and increased  $\gamma$ H2AX in colonocytes might reflect oxidative damage secondary to inflammation.

**Correspondence should be addressed to:** Peter S. Rabinovitch, Department of Pathology, Box 357705, 1959 NE Pacific St., K-081 HSB, University of Washington, Seattle, WA 98195-7705, Phone: 206.685.3761, Fax: 206.616.8271, E-mail: petersr@u.washington.edu.

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## Keywords

ulcerative colitis; telomere length;  $\gamma$ H2AX; colon; age

## INTRODUCTION

Telomeres protect the end of the chromosomes from end-to-end fusions, degradation, and recombination. Telomeres shorten approximately 100 base pairs in each cell division due to their incomplete replication<sup>1</sup>, and also as a consequence of oxidative damage<sup>2</sup>. Telomere attrition plays two opposing roles in cancer: as a tumor-suppressor mechanism when DNA damage checkpoints are intact<sup>3</sup>, and as a mechanism to generate chromosomal instability when DNA damage checkpoints are dysfunctional<sup>4</sup>. In addition, cross-sectional studies suggest that telomere shortening also plays an important role in aging: not only does telomere length decrease with age in most human tissues<sup>5</sup>, but shorter leukocyte telomeres are associated with mortality, diseases of aging, and factors that predispose to aging<sup>6, 7</sup>. Although the risk of most cancers increases with age<sup>8</sup>, it is still not known to what extent telomere shortening is responsible for this association. Support for the role of telomeres as a key factor linking cancer and aging comes from some premature aging syndromes, such as dyskeratosis congenita and aplastic anemia, characterized by accelerated rate of telomere attrition with age, chromosomal instability and predisposition to cancers (reviewed in<sup>9</sup>).

Critically short, uncapped telomeres lead to replicative senescence by activation of a DNA damage response that involves phosphorylation of the histone H2AX ( $\gamma$ H2AX)<sup>10</sup>. This response is normally triggered by DNA double-strand breaks to arrest cell cycle progression until the DNA damage has been repaired, and it can be visualized by fluorescence microscopy as nuclear accumulation or foci of  $\gamma$ H2AX and other DNA repair factors<sup>11</sup>. In the presence of uncapped telomeres, growth arrest is permanent and  $\gamma$ H2AX foci are persistent and a marker of cellular senescence<sup>12</sup>. Although the DNA damage response that links short telomeres, H2AX phosphorylation, and senescence is increasingly well characterized *in vitro*<sup>13</sup>, less is known of how telomere shortening translates into aging and disease in humans.

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon that predisposes to colorectal cancer<sup>14</sup>. The risk of colorectal cancer increases with duration, extent and early onset of disease<sup>15</sup>, and with the severity of inflammation<sup>16</sup>. We and others have previously demonstrated telomere shortening in colonocytes of UC patients, especially in those progressing to dysplasia and cancer<sup>17, 18</sup>. In addition, we have shown that telomere shortening is associated with chromosomal instability and anaphase bridges (a result of end-to-end chromosomal fusion), providing a mechanistic connection between telomere shortening and chromosomal damage leading to cancer<sup>18</sup>. The mechanism that triggers UC is, however, unknown, but inflammation and oxidative damage are thought to play a crucial role<sup>19</sup>. Because telomeres shorten with cell replication<sup>1</sup> as well as oxidative damage<sup>2</sup>, we hypothesized that, in UC, telomeres might shorten faster than in normal colon. We have previously demonstrated that in normal colon telomere length decreases with age until approximately age 60. Beyond that age, the average telomere length increases, presumably reflecting the selective survival of patients with longer colonocyte telomeres<sup>20</sup>. Therefore, by comparing colonocyte telomere length and  $\gamma$ H2AX damage-related foci in UC and normal individuals across a wide range of ages, we aimed to study the role of telomere attrition and DNA damage in this disease and as a potential link between cancer and aging. In addition, we analyzed telomere length in stroma adjacent to colonocytes and in leukocytes of UC patients to determine whether telomere attrition in UC patients is systemic or cell and tissue specific.

## MATERIAL AND METHODS

### Patients and samples

Three different sets of UC patients and normal colon controls were analyzed (Table 1). The first set included fresh frozen biopsies from 79 normal colon controls (ages 2–79 years) and 85 UC patients (ages 14 to 79 years) and was analyzed for telomere length using quantitative PCR (Q-PCR). The second set included leukocyte DNA from 45 normal controls (ages 33–79 years) and 102 UC patients (ages 31–80 years) and was analyzed for telomere length using Q-PCR. UC patients in set 2 are only 2% overlapping with set 1, but the normal controls are 77.8% overlapping with set 1. The third set included paraffin-embedded, lightly paraformaldehyde-fixed blocks from 53 normal colon controls (ages 31 to 79 years) and 43 UC patients (ages 31 to 78 years) and was analyzed for in situ  $\gamma$ H2AX immunostaining and for telomere length by quantitative FISH (Q-FISH). Set 3 shares most of the UC cases with set 1 (83.7%), but only a small percentage of normal controls (9.4%). Data from the 3 sets were analyzed independently. To study the potential relationship between biopsy telomere length and colon location, and to add an additional cell type (stroma) as a control, we analyzed telomere length by Q-PCR in a subset of biopsies from 14 UC cases and 14 age-matched normal controls from set 1 (ages 30–59). One right colon biopsy and one left colon biopsy were analyzed for each UC patient. For the normal controls, only one biopsy was analyzed.

Normal colon was obtained from individuals with no history of colorectal malignancy and included patient diagnoses of trauma, appendicitis, diverticular disease, solitary rectal ulcer syndrome, focal active colitis (infectious), and benign lymphoid tissue. Clinical information available for UC patients includes: disease duration and age of onset, which was assessed by the first appearance of symptoms; disease activity, categorized as inactive, mild or severe; and medication use, including 5-aminosalicylate (5-ASA) drugs, vitamins and folic acid (Table 1). UC samples were obtained during colonoscopy or from surgically resected colons. Only biopsies graded as negative for dysplasia were included in this study; however, about half of the UC patients (set 1: 44%, set 2: 52%, set 3: 44%) had dysplasia or cancer at a distant site. Multiple biopsies were analyzed for 32% of the UC cases in set 1 and 56% of the UC cases in set 3. For those cases, the average of multiple biopsies was taken. For the rest of UC cases and for all normal-colon controls, only one biopsy was analyzed. Samples were collected at the University of Washington Medical Center and the Group Health Cooperative in Seattle, WA, and at the Cleveland Clinic in Cleveland, OH. These studies were approved by the Human Subjects Review Boards of each institution, with annual renewals.

### Epithelial cell isolation and DNA extraction

Epithelial cells from frozen biopsies were isolated using epithelial shake-off as previously described<sup>21</sup>. Cytokeratin staining confirmed that at least 90% of the cells were epithelial. DNA was extracted from epithelium, stroma, and blood using Qiagen DNA extraction kits (Qiagen, Valencia, CA), following manufacturer's instructions.

### Telomere measurements by Q-PCR

Telomere length was measured by Q-PCR, as previously described<sup>22, 23</sup>. Telomeric DNA and a single-copy internal control gene (36B4, acidic ribosomal phosphoprotein PO) were amplified in each sample. A four-point standard curve (2-fold serial dilutions from 5 to 0.625ng of DNA) was used to transform cycle threshold into nanograms of DNA. All samples were run in triplicate and the median was used for subsequent calculations. The amount of telomeric DNA (T) was divided by the amount of single-copy internal control gene DNA (S), producing a relative measurement of the telomere length (T/S ratio). Two control DNA samples were included in each run to allow for normalization between experiments and periodic

reproducibility experiments were performed to guarantee accurate measurements. The intra- and inter-assay variability (coefficient of variation) for Q-PCR was 6 and 7%, respectively.

### **$\gamma$ H2AX immunostaining and telomere Q-FISH**

Paraffin-embedded, lightly paraformaldehyde-fixed slides were processed using a modification of previous protocols<sup>13, 18</sup>. Slides were incubated in 10 mM sodium citrate pH 6.5 for 40 minutes at 90°C for antigen retrieval. Slides were blocked in 3% BSA (Sigma) and incubated at 4°C overnight with mouse monoclonal anti-phospho-histone H2AX antibody (Ser139) clone JBW301 (Upstate Biotech, Charlottesville, VA) diluted 1:1000 in blocking buffer. Slides were washed with 5% goat serum/PBS and incubated with 1:1000 secondary antibody (goat anti-mouse Alexa 568 IgG<sub>1</sub>; Molecular Probes, Carlsbad, CA). Antibody was fixed with 4% paraformaldehyde for 20 minutes followed 0.25 mM glycine for 20 minutes. Slides were dehydrated through serial ethanol baths, then digested with 1 mg/ml pepsin (Sigma), dehydrated in ethanol series, and incubated with 10 mg/ml RNase A. Slides were hybridized with a 1:250 dilution of PNA-FITC (5'ATTCGTTGGAAACGGGA3') as described<sup>18</sup>. Nuclear DNA was counterstained with 10 mg/ml DAPI. Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Images were taken on a Zeiss LSM Meta 510 microscope at 63X with excitation at 543 nm for Alexa 568, 488 nm for FITC, and 405 nm for DAPI, using sequential scans at constant settings for all slides. Both telomere spots and  $\gamma$ H2AX foci images were quantitatively analyzed<sup>20, 24</sup>.

### **Statistical analysis**

The association between telomere length and  $\gamma$ H2AX vs. age, disease duration and age of onset was analyzed by both simple and two-segment linear regression models. Permutation tests were used to select the best fitted model and to estimate the confidence interval of slopes and change point, if the two-segment model was chosen<sup>25</sup>. Lowess smoothing curves were also fitted to the data with a confidence interval band from bootstrap analysis. Simple linear regression was used to assess the association between telomere length and  $\gamma$ H2AX. The comparisons of telomere length and  $\gamma$ H2AX by age decades were done by Wilcoxon Rank Sum test. Paired t-tests were used to compare telomere length between epithelium and stroma, and unpaired t-tests were used to compare left UC biopsies, right UC biopsies and normal colon biopsies.

## **RESULTS**

### **Accelerated telomere shortening in the colon of UC patients**

The relationships between telomere length and age in normal control and UC colonocytes are shown in Figure 1. We previously reported the association between Q-PCR measured telomere length and age in a subset of 47 normal colons<sup>20</sup>. In that set, however, only 4 cases were younger than 40 years old. To explore colonocyte telomere dynamics more broadly in younger patients, we have added 32 additional individuals, most of them between 2 and 40 years of age. Figure 1A shows the expected decrease of telomere length until age 59 (95% CI: 48.5 to 67), followed by a significant increase in average patient telomere length thereafter, consistent with our previous report. A two-segment model fitted the data significantly better than a single linear model ( $p=0.002$ ). The slope for the first segment was  $-0.025$  (95% CI:  $-0.033$  to  $-0.020$ ) and for the second was  $0.012$  (95% CI:  $-0.003$  to  $0.046$ ).

In UC, a two-segment model also fitted the data significantly better than a single linear model ( $p=0.002$ ) but, in these patients, the change point was much earlier, corresponding to an age of 33 years (95% CI: 25.5 to 48.5) (Figure 1B). The slope for the first segment was  $-0.041$  (95% CI:  $-0.087$  to  $-0.018$ ) and for the second was  $-0.001$  (95% CI:  $-0.005$  to  $0.008$ ). A 95% bootstrap confidence interval (dashed lines) for the smoothed curve (light continuous line) supported the fitted two-segment models and suggested a steep decrease in telomere length at

young ages in UC patients. The two-segment association between telomere length and age was statistically significantly different between normal and UC subjects ( $p=0.005$ ). The change point for UC patients was estimated to be 26 years younger than that for normal controls (95% CI of 17 to 40.5 years). In addition, the difference between the slopes of the first segment in UC and normal colon approached significance ( $-0.016$ , 95% CI:  $-0.033$  to  $0.001$ ,  $p=0.065$ ), suggesting an approximately two fold faster decrease of telomere length with age in UC than in normal colon. When compared by decades (Figure 1C), UC patients showed significantly shorter colonocyte telomeres than normal individuals in age decades 30–39 and 40–49 ( $p<0.001$  and  $p=0.02$ , respectively). After age adjustment, telomere length in UC patients was not significantly associated with the disease activity or medication use.

### **Shorter telomeres are associated with longer disease duration**

We analyzed telomere length as a function of age of onset and duration of disease (Figure 2). Age of onset was not related to colonocyte telomere length either in a simple linear or a two-segment model (Figure 2A). Two subsets of UC patients were observed: early onset (age of disease onset under 50 years) and late onset (above 50 years). As seen in Figure 2B, late onset patients generally had short telomeres in spite of recent clinical onset of their disease. However, in early onset patients, telomeres were related to disease duration by a two-segment rather than a single linear model (Figure 2B,  $p=0.028$ ), with an estimated change point at 8.5 years (95% CI: 1.5 to 30). Except for one case, UC patients with long telomeres were young individuals (less than 30 years of age) who had the disease for less than 8.5 years.

### **Shorter telomeres are found in both the right and left colon of UC patients**

One right and one left colon biopsy were analyzed in a subset of 14 UC patients, ages 30 to 59. Figure 3 shows that UC telomeres from both left and right biopsies were significantly shorter than age-matched normal colon biopsies ( $p=0.02$  and  $p=0.04$ , respectively), and were not significantly different from each other ( $p=0.96$ ).

### **Stromal telomere length is not shortened in UC colons**

Telomere length was analyzed in the paired stromal cells obtained after epithelial shake-off (see methods) in right and left biopsies of the same 14 patients as above. Epithelial telomere length was significantly shorter than stromal telomere length in both left ( $p=0.004$ ) and right ( $p=0.004$ ) UC biopsies, but right and left UC stromal telomere lengths did not differ from each other ( $p=0.95$ ) or from those of normal colon ( $p=0.39$  and  $p=0.37$  for left and right UC colon, respectively).

### **Leukocyte telomeres are significantly shorter in UC patients than in normal controls**

Leukocyte telomere length significantly decreased with age, both in UC patients and in normal controls ( $p=0.01$  and  $p<0.001$ , respectively; Figure 4). Since these two populations were not matched for age and gender, we used a linear regression model to compare leukocyte telomere length in both groups after age and gender adjustments. This showed slightly, but significantly shorter telomeres in leukocytes from UC patients compared to normal individuals ( $p=0.046$ ). The adjusted leukocyte telomere length means calculated at age 50 were  $1.22\pm0.24$  for control and  $1.14\pm0.23$  for UC, a 6.6% difference. There were no significant associations between age-adjusted leukocyte telomere length and disease activity or medication use, although there was a non-significant trend ( $p=0.08$ ) for longer telomeres in patients that used 5-ASA.

### **Higher levels of $\gamma$ H2AX in UC patients than in normal controls**

We compared the mean intensity of  $\gamma$ H2AX in the colonocytes of UC patients and normal individuals as a measure of the activation of the DNA damage response elicited by DNA double strand breaks and critically short telomeres. In normal colon specimens, we observed that levels



of  $\gamma$ H2AX increased linearly with age until approximately age 69 years (95% CI: 63 to 74.5 years) (Figure 5A). After that age,  $\gamma$ H2AX intensity dropped, producing a two-segment regression that was nearly the direct inverse of what we observed for telomere length ( $p=0.001$  for a two-segment model vs. a single linear model). The slope for the first segment was 0.014 (95% CI: 0.009 to 0.018) and for the second segment was  $-0.051$  (95% CI:  $-0.153$  to  $-0.024$ ). In UC cases, there was no significant association between  $\gamma$ H2AX levels and age (Figure 5B), but  $\gamma$ H2AX levels were statistically significantly higher in UC cases than in normal controls between ages 30 and 59 (Figure 5C).  $\gamma$ H2AX intensity was not related to duration, age of onset of UC, disease activity or medication use (data not shown).

### Association between $\gamma$ H2AX and telomere length in normal colon, but not in UC

For normal colon controls, telomere length measurements by Q-FISH were inversely correlated with higher levels of  $\gamma$ H2AX (slope= $-0.483$ ,  $p=0.009$ , Figure 6A). In UC colonocytes the high levels of  $\gamma$ H2AX show no significant association with telomere length ( $p=0.73$ , Figure 6B), just as there was no association of  $\gamma$ H2AX and age (Figure 5B).

## DISCUSSION

We have demonstrated an accelerated shortening of colonocyte telomere length with age in UC patients compared to normal controls. On average, by age 33 years, UC patients have acquired very short colonocyte telomeres, comparable to that reached in normal individuals by age 60 years. Moreover, this rapid attrition occurs within the 8 first years of the disease, although patients with late-onset disease have short colonocytes telomeres irrespective of disease duration. These findings have a parallel in clinical practice: colon cancer risk becomes elevated in UC only after 8 years disease duration<sup>26</sup>, which marks the beginning of colorectal cancer screening and, interestingly, the acquisition of short colonocytes telomeres. Similarly, in the general population, colorectal cancer screening starts at age 50 years, which is concordant with the age at which short colonocyte telomere length is reached in normal individuals. Thus, rapid telomere shortening in UC colonocytes might reflect accelerated colon aging of these patients, predisposing them to colorectal cancer at an earlier age. Indeed, the average age of colorectal cancer incidence in UC patients (age 47) is more than two decades earlier than in the general population (age 70)<sup>27</sup>, and the lifetime risk is 19 times higher<sup>26</sup>.

It is well known that telomere length decreases with age in most human tissues, including colon<sup>20</sup>, and it has been hypothesized that short telomeres might partially explain the connection between cancer and aging<sup>8,9</sup>. When telomeres become critically short, and in the absence of proficient DNA damage checkpoints, anaphase bridges are formed in mitosis as a consequence of end-to-end chromosome fusions, which initiates cycles of breakage-fusion-bridge. These cycles facilitate the accumulation of genetic changes and chromosomal instability. To emerge from this crisis cells need to acquire mechanisms of telomere maintenance, which usually consist on reactivation of telomerase. Telomerase prevents further accumulation of chromosomal instability and confers unlimited replicative potential, setting the stage for cancer progression<sup>4</sup>. We previously demonstrated that, in UC, colorectal cancer progression is associated to shorter colonocyte telomeres, chromosomal instability and anaphase bridges<sup>18</sup>. In this expanded cross-sectional study, we demonstrate that, in UC, age-related telomere shortening is accelerated. Our results also suggest that there is a minimal viable length of colonocyte telomeres, consistent with data from both human cell lines<sup>28</sup> and telomerase deficient mice<sup>29</sup>. When this critical length is reached, UC colonocytes defective for DNA damage checkpoints can continue to proliferate, but with increased chromosomal instability. Since p53 and p16 are frequently altered in UC non-neoplastic epithelium<sup>30,31</sup>, this could be a common pathway of tumor progression in this disease. Further research is needed,

however, to characterize the mechanisms of activation and abrogation of senescence in UC, and to determine the timing of these events.

An important question that arises in the light of our results is whether accelerated telomere shortening in UC is due to an inherent telomere maintenance defect or is a consequence of the chronic inflammation characteristic of this disease. If the former was true, then we would expect shorter telomeres not only in colonic epithelium, but also in other tissues. Our data shows that shorter telomeres are observed only in the epithelium, but not in the adjacent stroma of UC colon biopsies. On the other hand, leukocyte telomere length is on average 6.6% shorter in UC than in normal controls. While this difference is significant, it is considerably smaller than the telomere reduction observed in syndromes with an inherent telomere maintenance defect, such as dyskeratosis congenita<sup>32</sup>. The small shortening observed in leukocytes of UC patients is more similar to that previously reported in cardiovascular diseases, where affected patients have average telomere length reductions of 3–12% compared to normal controls<sup>33–35</sup>. These small differences require of a large number of cases to be detected, especially given the large variability of telomere length between individuals, which might explain why shortened leukocyte telomere length was not reported in a previous study that included 19 UC patients and 18 controls<sup>36</sup>. However, lymphocytes from UC patients were found to have higher frequency of telomeric associations and chromosome aberrations than normal controls<sup>37</sup>, which may be a consequence of the telomere attrition observed here. Multiple associations between short leukocyte telomere and diseases of aging have been reported<sup>6, 7</sup> and, although this could reflect a predisposition of individuals with constitutionally short telomeres to age-related diseases, recent results suggest that these associations are more likely due to the ability of leukocyte telomere lengths to reflect the history of inflammation and oxidative stress of the individual<sup>23, 38</sup>. We thus hypothesize that inflammation and proliferation are responsible for telomere shortening in UC: the largest shortening is observed in colonocytes, which are highly proliferative cells located in the areas of inflammation; second to this, leukocytes, which are also proliferative and frequently travel through the inflamed colon, experience moderate shortening; lastly, stroma cells, which have very low proliferative rates, seem to be protected from shortening.

Another way to study the role of telomere shortening in the colon of UC patients is to compare right and left biopsies. In UC, inflammation can be confined to the rectum (proctitis) or extend throughout the entire colon (pancolitis). We found comparable telomere shortening in both right and left colon epithelium, probably due to the fact that most of the patients analyzed had pancolitis. Interestingly, the only patient that had exclusively left disease showed longer telomeres in the right than in the left colon. Kinouchi et al. analyzed telomere length in rectal and cecal mucosa of 17 UC patients, 7 of which had left disease, and found significantly shorter telomeres in the rectal mucosa, especially in those patients with dysplasia<sup>17</sup>. Regarding telomerase activity, Usselman et al. reported a decrease along the colon of UC patients and a negative correlation with inflammation<sup>39</sup>. However, two other studies reported no such association<sup>40</sup>, but rather an increase in activity corresponding to closer proximity to dysplasia<sup>41</sup>. In spite of the conflicting results, these studies suggest a possible link between the extent and severity of inflammation, the presence of dysplasia, and colonocyte telomere dynamics, that is worth investigating.

To further explore the effects of telomere shortening in UC, we also measured the intensity of  $\gamma$ H2AX, which is an indication of the degree of phosphorylation of the histone H2AX, an event that occurs as a response to DNA double-strand breaks and critically short, uncapped telomeres. Phosphorylation of H2AX is known to be one of the triggers in the cascade of events that leads to senescence<sup>10</sup>. We showed that the levels of  $\gamma$ H2AX were higher in the colons of UC patients than in normal individuals, across all decades of age. This confirms previous reports of higher levels of DNA damage in UC colonocytes compared to normal controls, especially in UC cases

with dysplasia and cancer<sup>42, 43</sup>. It is important to note that the normal individuals in this study include some diagnoses that involve colonic inflammation (e.g., diverticular disease or infection) and possible increased oxidative damage, so the  $\gamma$ H2AX signal in truly healthy colon might be even lower. Surprisingly, in UC there was neither an age-related pattern, nor a correlation between  $\gamma$ H2AX foci and telomere length, contrary to what was observed in normal colon. Although short telomeres in UC colon are likely to contribute to high levels of  $\gamma$ H2AX signal, our data indicate that there must be other large sources of DNA damage. Double strand breaks caused by reactive oxygen and nitrogen species secondary to chronic inflammation are a very likely cause<sup>19</sup>. While activation of DNA damage pathways that lead to senescence acts as a tumor suppressor mechanism, it is also a hallmark of premalignant tissues and a sign of potential cancer progression, as tumors can eventually bypass senescence and acquire proliferative ability in the presence of high levels of DNA damage<sup>44</sup>.

Secondary to our main goal, but highly relevant to the field of aging, we found that normal colonocytes not only shorten telomeres with age, but they also accumulate  $\gamma$ H2AX foci, up to approximately age 67 years, after which the trend reverses. This two-segment model for age-dependent increase in DNA damage foci in normal colon is the inverse of the age-dependent changes in telomere length previously reported<sup>20</sup>, and there is a significant correlation between these variables: the shorter the telomeres, the higher the mean intensity of  $\gamma$ H2AX foci. Older individuals (above 60 years old) tend to have longer telomeres and less  $\gamma$ H2AX signal, which, as previously hypothesized, probably reflects the selective survival of individuals with constitutionally longer telomeres, better antioxidant defense, or less cumulative exposure to inflammation and oxidative stress in their lifetimes.

In summary, we have shown accelerated telomere shortening and accumulation of DNA damage in the colon of UC patients, which might be secondary to inflammation, increased cell turnover, and oxidative stress. In light of these results we postulate that UC could be considered a disease of premature aging of the colon, which might explain the high and early incidence of colorectal cancer in these patients. Further studies are needed to clarify the role of senescence in UC and to determine the specific contribution of colonocyte turnover and oxidative damage in the shortening of telomeres.

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## Abbreviations

$\gamma$ H2AX, phosphorylated H2AX; Q-FISH, quantitative fluorescence in situ hybridization; Q-PCR, quantitative PCR; UC, ulcerative colitis..

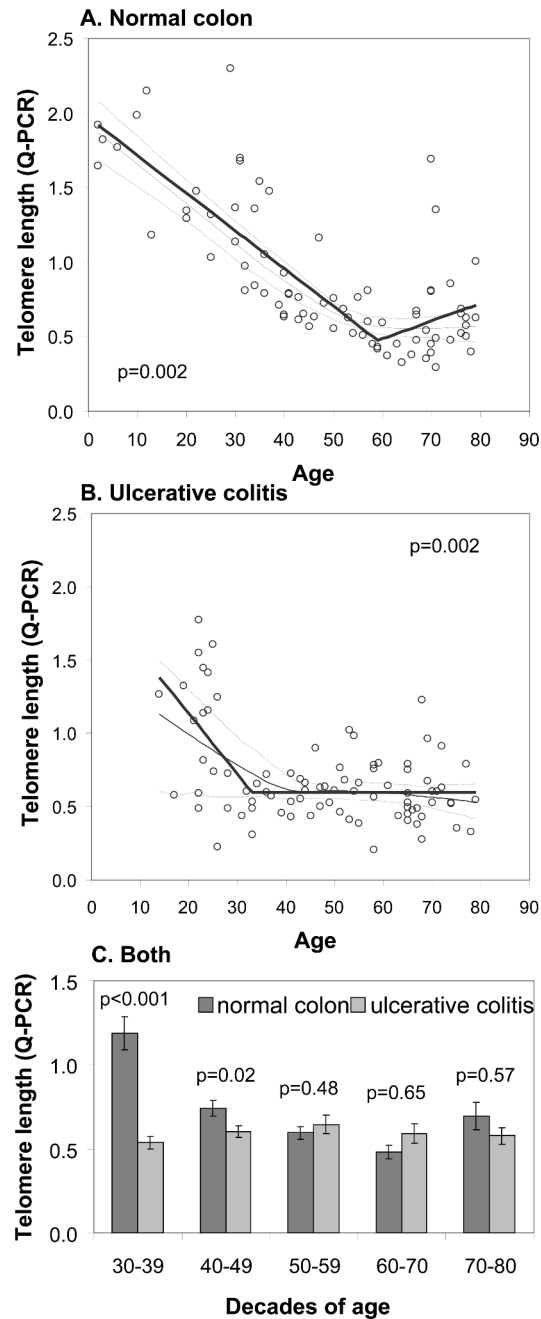
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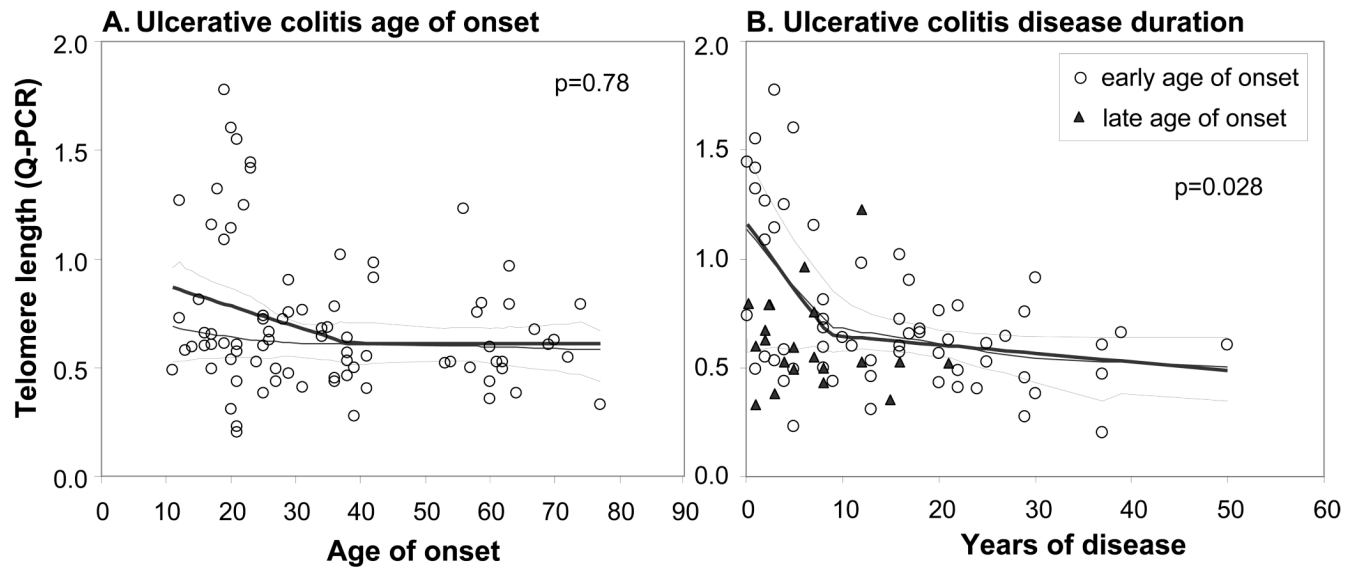
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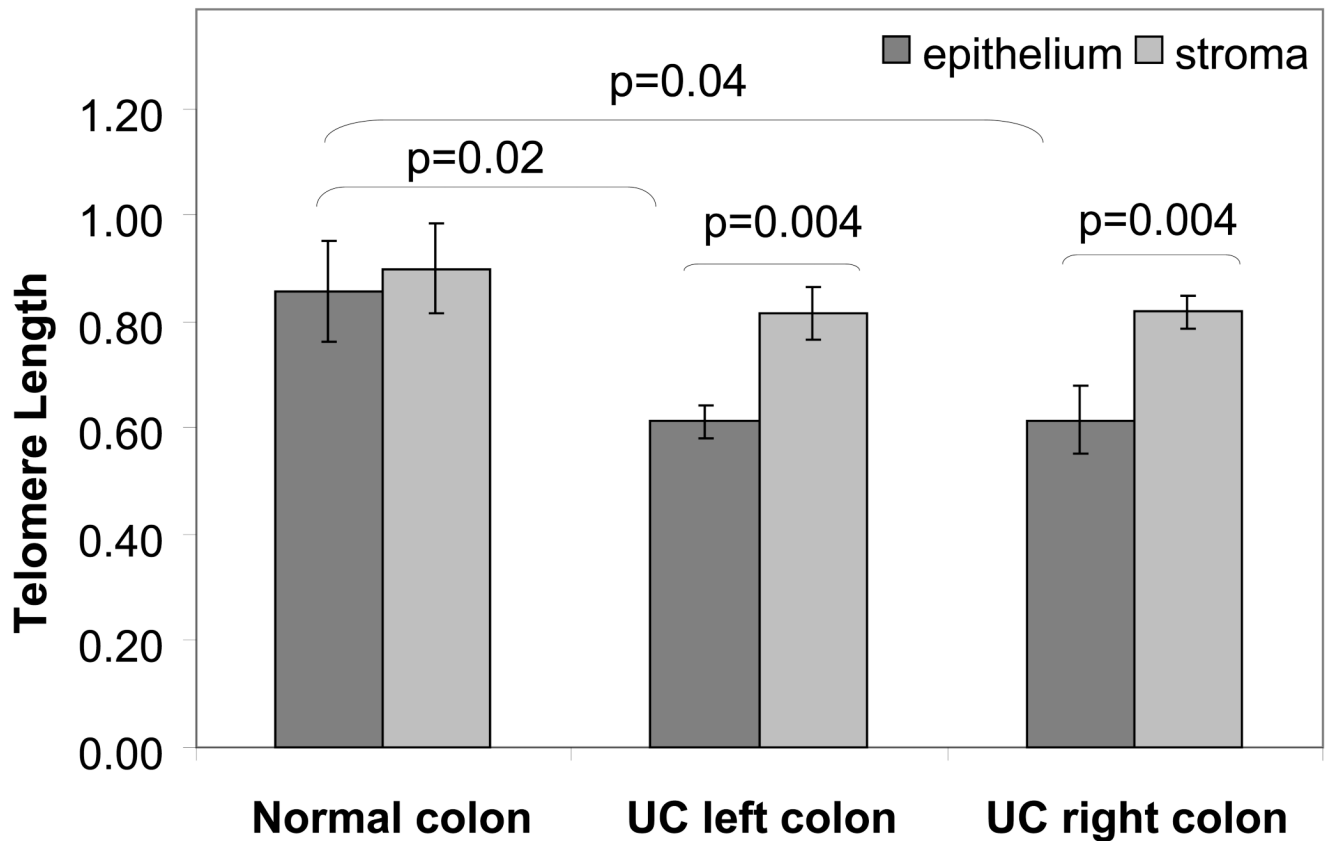
**Figure 1.**

Associations between colonocyte telomere length and age in normal colon (A) and ulcerative colitis (B). Both the two-segment model (dark continuous line) and the smoothed curve with bootstrap confidence interval (light continuous line and dashed lines) are indicated. The p-values correspond to the comparison of the two-segment model vs. a single linear model. (C) Comparison of telomere length (mean  $\pm$  sem) in normal colon and ulcerative colitis by decades of age. The p-values correspond to Wilcoxon Rank Sum tests for each age group.



**Figure 2.**

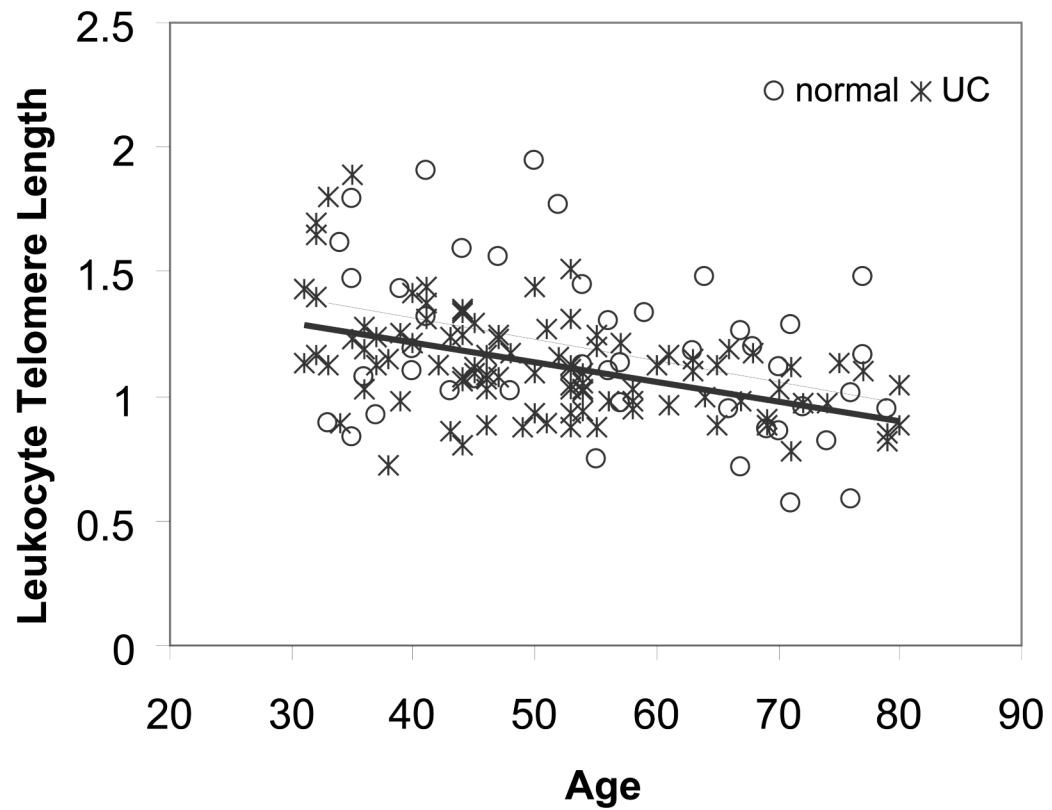
Association between telomere length and age of onset (A) and disease duration (B) in ulcerative colitis patients. Both the two-segment model (dark continuous line) and the smoothed curve with bootstrap confidence interval (light continuous line and dashed lines) are indicated. The p-values correspond to the comparison of the two-segment model versus a single linear model. In (B) patients are coded by age of onset: circles, early age of onset (<50 years); triangles, late age of onset (>50 years).



**Figure 3.**

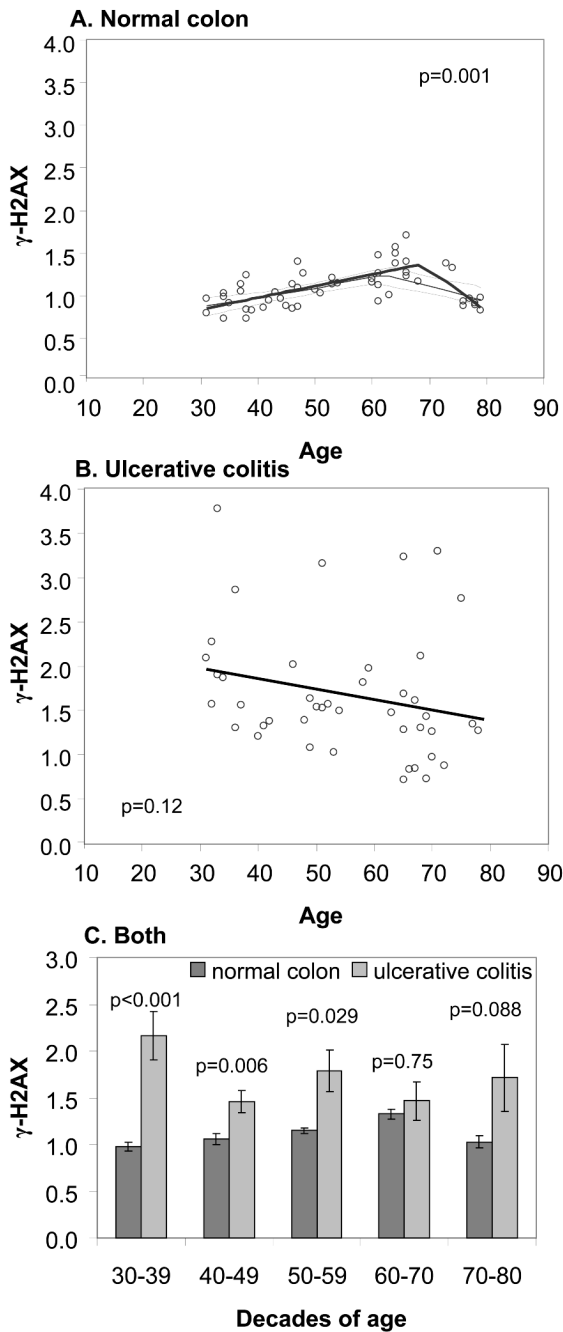
Comparison of telomere length in epithelium and matched stroma from normal colon and left and right UC colon biopsies. Mean telomere lengths are compared by paired t-tests for epithelium and stroma and by unpaired t-test for left UC biopsies, right UC biopsies and normal colon biopsies. Only significant p-values are indicated.





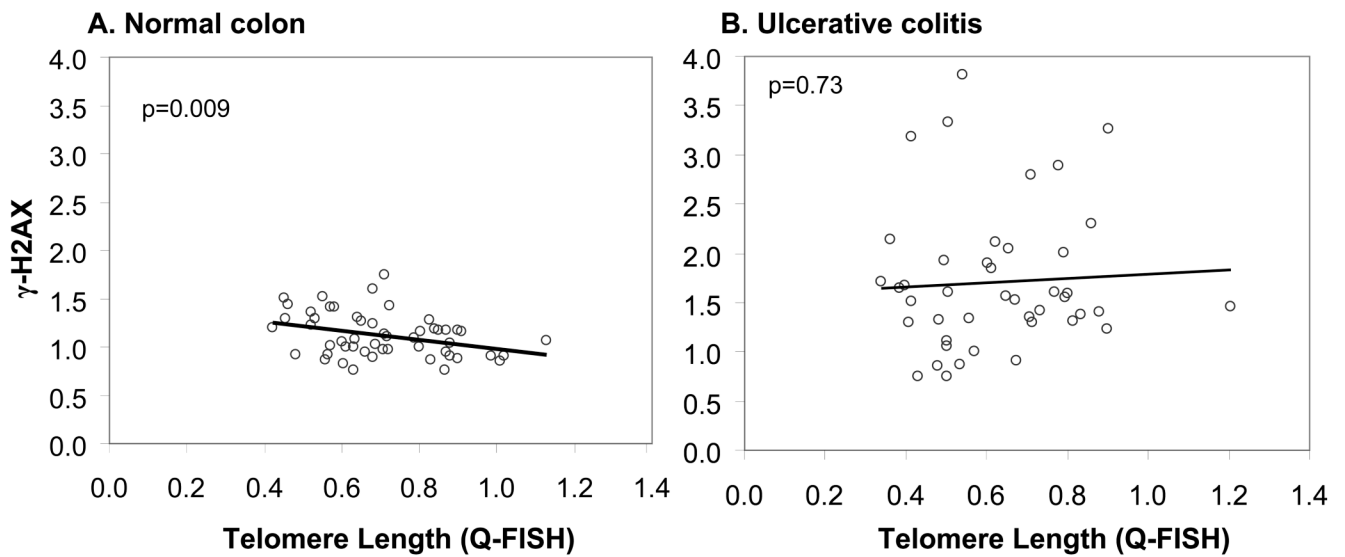
**Figure 4.**

Association between leukocyte telomere length and age in UC patients (asterisks) and normal individuals (circles). Simple regression lines are shown for UC patients (dark line) and normal controls (light line).



**Figure 5.**

Associations between colonocyte  $\gamma$ H2AX intensity and age in normal colon (A) and ulcerative colitis (B). The two-segment model (dark continuous line) and the smoothed curve with bootstrap confidence interval (light continuous line and dashed lines) are indicated in (A), and the p-value corresponds to the comparison of the two-segment model vs. a single linear model. In (B) the two-segment model was not significant and only the simple linear model is depicted for clarity. The p-value corresponds to the linear regression of telomere length vs. age. (C) Comparison of  $\gamma$ H2AX intensity (mean  $\pm$  sem) in normal colon and ulcerative colitis by decades of age. The p-values correspond to Wilcoxon Rank Sum tests for each age group.



**Figure 6.**

Associations between  $\gamma$ H2AX intensity and telomere length measured by Q-FISH in normal colon (A) and ulcerative colitis (B) colonocytes. The p-value corresponds to the simple linear regression of both parameters.

**Table 1**  
Demographics of ulcerative colitis patients and controls

	SET 1	SET 2	SET 3
<b>Description set</b>			
Samples	Colon biopsies	Blood	Colon biopsies
Parameter	Telomere length	Telomere length	H2AX and telomere length
Technique	Quantitative-PCR	Quantitative-PCR	Quantitative-FISH
<b>Normal colon</b>			
Cases	79 <sup>†</sup>	45 <sup>†</sup>	53
Age (mean±sd)	48.9 ± 21.4	55.5 ± 14.6	54.7 ± 14.9
Gender			
Male	27 (34.2%)	14 (31.1%)	28 (52.8%)
Female	52 (65.8%)	31 (68.9%)	25 (47.1%)
<b>Ulcerative colitis</b>			
Cases	85	102	43
Age (mean±sd)	48.3 ± 18.3	51.2 ± 12.8	54.8 ± 14.6
Gender			
Male	50 (58.8%)	60 (58.8%)	22 (51.2%)
Female	35 (41.2%)*	42 (41.2%)*	21 (48.8%)*
Disease duration	12.9 ± 11.1*	20.8 ± 11.4*	13.6 ± 10.7*
Age of onset	35.3 ± 18.2*	28.9 ± 12.8*	40.4 ± 20.1*
Disease activity			
Inactive	11/80 (13.8%)	32/83 (38.6%)	4/37 (10.8%)
Mild	22/80 (27.5%)	32/83 (38.6%)	11/37 (29.7%)
Severe	47/80 (58.8%)	19/83 (22.9%)	22/37 (59.5%)
Medication use			
5-ASA	43/62 (69.4%)	24/44 (54.5%)	20/25 (80%)
Vitamins	25/62 (40.3%)	8/43 (7.8%)	10/25 (40%)
Folic acid	15/61 (24.6%)	9/43 (8.8%)	5/25 (20%)

<sup>†</sup> a subset of this data was previously published in O'Sullivan, 2006

\* excludes patients for whom this information was missing (3, 28 and 1 cases in set 1, 2, and 3 respectively)