1   **Title Page**

2   **Quantification of double stranded DNA breaks and telomere length as proxies for corneal**
3   **damage and replicative stress in 64 human keratoconus corneas**
4   *The role of DNA damage in the development of keratoconus*

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26   Abbreviations: KC = keratoconus, HC = healthy control, DG = decompensated graft, ROS = reactive
27   oxidative species, mtDNA = mitochondrial DNA, hTERT = humane telomerase reverse transcriptase,
28   UV = ultraviolet, CXL = corneal crosslinking, TL = telomere length

29   Keywords: keratoconus, DNA damage, Alu elements, telomere length, hTERT

30

1 **Abstract**

2 Purpose:

3 The pathogenesis of keratoconus (KC) is multifactorial and associated with oxidative stress and

4 subsequent DNA damage. The aim of this study was to investigate differences in DNA damage and

5 replicative stress in patients with KC, and in both healthy and diseased controls.

6 Methods:

7 Sixty-four corneal buttons were obtained from 27 patients with KC after corneal transplant surgery,

8 21 patients with a decompensated graft (DG), and 16 healthy controls (HC). The amount of intact

9 Alu elements per genome copy as measured by qPCR was used to quantify intact DNA. Telomere

10 length was measured as a proxy for replicative stress. In addition, telomerase reverse transcriptase

11 (*hTERT*) gene expression level was assessed.

12 Results:

13 Mean (±SD) DNA damage was similar between the KC (5.56 ±14.08), DG (3.16 ±8.22), and HC (3.51

14 ±6.66) groups (P=0.807). No associations were found between DNA damage and patient age

15 (P=0.523), atopic constitution (P=0.240), or contact lens wear (P=0.393). Telomere length differed

16 (P=0.034), most notably in the KC group, and *hTERT* was not detected in any corneal sample. Three

17 cross-linked (CXL) KC corneas did not contain significant more DNA damage (2.6x, $P = 0.750$).

18 Conclusions:

19 Based on these findings, differences in actual corneal DNA damage in KC could not be identified,

20 and the longer telomere length in KC did not support replicative stress as a major etiological factor

21 in the pathogenesis of KC. Future longitudinal investigations on KC etiology should assess

1   progressive early cases to better comprehend the cellular and molecular processes preceding the

2   archetypical morphological changes.

3

4   **Precis**

5   Oxidative stress is allegedly linked with the development of keratoconus. Whether these stressors

6   actually lead to persisting DNA damage and replicative stress is debated. DNA damage was

7   comparable with control samples, and a shortened telomere length was not identified.

8

1 **Introduction**

2 Keratoconus (KC) is a corneal condition that can lead to refractive myopia, irregular astigmatism,

3 corneal thinning, and poor visual acuity due to the hallmark "cone-like" shape of the cornea and—in

4 advanced cases—corneal scarring.[1] Both environmental (e.g., eye-rubbing, atopic constitution, etc.)

5 and genetic factors have been linked to hypersensitive oxidative stress responses at the ocular

6 surface.[2-4] Currently, crosslinking (CXL; a combination of local ultraviolet (UV)-radiation and

7 riboflavin photosensitizing eye-drops) is regarded an established treatment to prevent disease

8 progression and much research is devoted to study UV-related treatment effects.[5] However, DNA

9 damage induced by UV radiation has also been suggested as a possible causative factor in the

10 development of KC.[6,7] UV radiation can damage DNA, leading to breaks in the double stranded DNA,

11 and the ocular surface is heavily exposed to the potentially detrimental effects of UV radiation on

12 DNA integrity.[7,8] Consequently, the cornea has several robust intrinsic defense systems against UV-

13 induced damage and reactive oxidative species (ROS) in particular. Indeed, studies have found

14 altered activity of several enzymes in the corneas of patients with KC, including the enzymes

15 superoxide dismutase[9], aldehyde dehydrogenase[10], catalase[11], cathepsin[11], glutathione reductase,

16 transferase, and peroxidases[10]; this altered enzyme activity may therefore contribute to oxidative

17 stress and accumulation of damage.

18

19 When DNA damage accumulates, cells usually enter apoptosis. Hence, when there is increased

20 exposure to -for instance- UV light, cells will accumulate DNA breaks rapidly. When DNA repair

21 mechanisms are not able to cope with these breaks, the cells will enter apoptosis, subsequently

22 leading to an increased cell turnover. In keratoconus, one can hypothesize that an increased

23 epithelial apoptosis influences the local homeostasis of the cornea, leading to the loss of stromal

24 collagen fibrils fundamental in the disruption of the normal architecture of the cornea. In addition,

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1   increased cellular turnover could be a sign of increased damage to corneal cells, forcing increased

2   cellular replication to replace damaged cells. A well-known measure for the rate of cellular turnover

3   and replicative senescence is telomere length. Telomeres function as a non-coding protective end

4   region of chromosomes. Due to the DNA endreplication inefficiency of polymerases, chromosomes

5   shorten every cell division. After a certain number of divisions, the threshold of attrition is reached,

6   and cells go into apoptosis or enter a senescence state. Telomeres therefore are indicative of the

7   number of divisions the cell lineage has undergone and are a measure of replicative stress. Repair

8   systems exist (i.e. *hTERT*, human telomere reverse transcriptase) and are sometimes upregulated in

9   cells that divide rapidly, such as stem cells.[12]

10

11   To answer the question whether corneal cells present in the cornea of keratoconus patients consist

12   of cells refractive to DNA-damage induced apoptosis we quantified the amount of DNA breaks in

13   these cells by measurement of intact *Alu* elements, a proxy for the amount of DNA breaks.[13-17] The

14   amount of DNA breaks represents a composite measure of the balance between DNA repair and

15   DNA damage. An increase of DNA breaks in keratoconus corneas compared to healthy or

16   decompensated graft corneas could highlight a role for either increased DNA damage, or decreased

17   damage response in this disease.

18

19   Secondly, we investigated whether these corneal cells show signs of increased replicative cell

20   turnover by measuring telomere lengths and *hTERT* gene expression as additional parameters of

21   replicative function. Hypothetically, short telomeres in corneal cells could point to a constitutional

22   increased replicative rate, preceding the disrupted morphological structure of a keratoconus

23   cornea.

24

1   To answer these questions we assessed DNA breaks, telomere length (TL), and *hTERT* expression in

2   64 human corneal buttons from 27 patients with KC who underwent corneal transplant surgery, 21

3   patients with a decompensated graft (diseased controls; DG) not related to KC, and 16 unaffected

4   (healthy) post-mortem donor corneas (HC).

5

1   **Material and Methods**

2   *Corneal samples*

3   This study was approved by The Medical Research Ethics Committee of the UMC Utrecht. It reviews

4   research protocols in accordance with the Medical Research Involving Human Subjects Act (WMO).

5   The MREC of the UMC Utrecht is accredited by the Central Committee on Research Involving Human

6   Subjects (CCMO) since november 1999. The MREC of the UMC Utrecht is also member of the Dutch

7   union of MRECs (NVMETC). None of the donors were from a vulnerable population and all donors

8   or next of kin provided written informed consent that was freely given.

9   Twenty-seven cornea samples were obtained from 27 patients who received a corneal transplant

10   for severe KC and one case of pellucid marginal degeneration (the KC group). A second group of 21

11   corneal samples was obtained from 21 patients who underwent a re-grafting procedure due to a

12   decompensated corneal graft in which the indication for the primary graft was not KC (the DG

13   group). The corneal buttons in the KC and DG groups were embedded in Tissue-Tek (Sakura

14   Finetek USA, Inc., Torrance, CA) immediately after resection and stored at -80°C.

15   Corneal samples were also obtained from ten healthy controls (the HC group); these samples were

16   obtained from the Euro Cornea Bank (Beverwijk, the Netherlands) and the Department of Anatomy,

17   University Medical Center Utrecht, Utrecht, the Netherlands. Within 24 hours of death, the corneas

18   were prepared from post-mortem tissue obtained from 16 unrelated donors, each of whom had no

19   documented history of KC, ocular inflammation, or vitreo-retinal disease. All patients provided

20   written informed consent after the explanation of the procedure. Informed consent for the post-

21   mortem donation of ocular tissue was provided under the auspices of the head of the Department of

22   Anatomy, University Medical Center Utrecht, the Netherlands. All tissues were acquired in

1   compliance with Dutch law (*Wet op de lijkbezorging*, Art 18, lid 1/ 18–06–2013) and the

2   institutional guidelines established by the University Medical Center Utrecht.

3

4   *Clinical data extraction*

5   Additional data was extracted from the patient records and included both the patient history and

6   the preoperative assessment, which included the results of a slit lamp evaluation, Schirmer's test,

7   and Scheimpflug corneal tomography (Pentacam HR, Oculus GmbH, Wetzlar, Germany). The data

8   available for the HC group were limited to age, gender, and cause of death. Each KC cornea was

9   graded as clear, mildly hazy, or clouded based on slit lamp biomicroscopy. All decompensated grafts

10   were considered clouded, and all healthy corneas were considered clear.

11   *Assessment of DNA damage*

12   DNA was isolated from the corneal buttons using TRIzol (Life Technologies, Thermo Fisher

13   Scientific, Grand Island, NY, USA); this approach allows for the isolation of small DNA molecules,

14   which can be lost when using column-based isolation techniques. After dissolving the cornea in

15   TRIzol reagent, the RNA was removed, and the original tubes containing the TRIzol reagent and

16   DNA were stored at -20°C. After isolation, double-stranded DNA was measured using a Qubit 2.0

17   Fluorometer (Life Technologies, Thermo Fisher Scientific). The number of intact Alu elements in

18   the DNA was measured using qPCR and was used as a proxy for quantifying intact DNA per genome

19   copy, as indicated by the single genome marker 36B4.[13] DNA damage was assessed in the first 33

20   consecutive samples, and validated in the subsequent 31 samples. Since baseline characteristics

21   and study outcomes varied only marginally, these were reported as one pooled cohort.

22   *Measurement of telomere length (TL)*

1   Corneal Telomere Length was measured by Bio-rad cfx-96 real-time qualitative polymerase chain

2   reaction (qPCR) detection system in duplicates in two separate experiments. Briefly, the length of

3   telomeres—long repetitive hexamer (TTAGGG) sequences–can be accurately determined by using a

4   calibration curve based on linear serial dilution of a synthetic 84-mer (14 consecutive TTAGGG

5   sequences) oligonucleotide (Geneworks, Adelaide, Australia) with a predetermined molecular

6   weight per reaction (60x10-12 gr of telomere oligomer or 1.36x109 oligomers). The total number

7   of base-pares in the highest standard can be calculated as ((1.36x109 molecules of oligomer) x (84

8   oligomer length) = 1.18x108 kilo base-pares). The relative telomere length per sample is

9   extrapolated from serial dilutions of the synthetic standard in each qPCR measurement. Similarly, a

10 synthetic standard was also designed for the single copy house-keeping gene 36B4. Absolute

11 telomere base-pares per genome are quantified by subdividing the total number of telomere base-

12 pares from 36B4 (which has only one copy per gene) following; Telomere length/house-keeping

13 gene = telomere length per genome. [18] TL quantification was performed in the first 33 consecutive

14 corneal samples.

15 *hTERT gene expression*

16 *hTERT* gene expression level was quantified using synthesized cDNA (Biorad iScript kit) from RNA

17 that was extracted from the corneal cells, in the first 33 consecutive cornea samples. Quantstudio

18 qPCR apparatus with TaqMan assay (Applied Biosystems, Thermo Fisher Scientific) were used to

19 perform qPCR under conditions as specified by the manufacturer. To normalize the hTERT-gene

20 expression, the housekeeping GUSB and GAPDH genes were included.

21 *Statistical analysis*

22 The level of DNA damage (measured using the number of amplified Alu elements) was corrected for

23 input DNA and graphed in a box plot; the mean levels of DNA damage and telomere length were

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1   calculated for each study group. Statistical analyses were performed using SPSS 21.0 (IBM, Armonk,

2   NY, USA). Outlier analysis was performed by removing samples with DNA damage that exceeded >3

3   SD; these values were analyzed separately. Differences in DNA damage, telomere length and *hTERT*

4   expression were tested using the one-way independent ANOVA or Kruskal Wallis test. Multiple

5   comparisons were tested using the post hoc Tukey's test.

Results

Study population

The mean (±SD) ages of the subjects in the KC, DG, and HC groups were 42.8 ± 14.8, 67.6 ± 12.0, and 83.6 ± 8.8 years, respectively. Males were overrepresented in the KC group (63.0%), underrepresented in the DG group (33.3%), and equally distributed in the HC group (56.3%). As expected, concurrent atopic disease was more prevalent in the KC group (in 63.0% of patients) compared to the DG group (9.5%); contact lens wear was also more prevalent in the KC group compared to the DG group (77.8% vs. 57.1%, respectively). The KG and DG eyes were similar with respect to the Schirmer's test results. Four samples in the DG group, five samples in the HC group and one sample in the KC group did not yield sufficient DNA for analysis, thus reducing the effective sample size from 64 corneas to 54. The characteristics of the four DG patients with non-viable samples did not differ from the mean group (data not shown). The donors of the non-viable HC samples were among the oldest samples (the mean age of these four subjects was 87 years). The mean age of the 33 samples used for telomere length/*hTERT* assessment did not differ materially from the pooled group (KC 40.8 ± 15.2, DG 66.7 ± 11.9, HC 81,5 ± 9.8 years).

DNA damage

The mean levels of DNA damage in the pooled KC (n=26), DG (n=17), and HC (n=11) groups were 5.56±14.08 , 3.16±8.22, and 3.51±6.66, respectively. Log_{10} normalized values were -0.66±1.63, -0.59±1.54, and -1.01±1.60 respectively (P=0.807; Figure 1).

The values above represent the total number of breaks in the DNA double strands of all Alu elements per genome copy; thus, considerable variability was observed with respect to the total number of DNA breaks per sample (which ranged from $8.653×10^{-5}$ to 70.2 in this study). Therefore, we use outlier analysis to normalize the groups; using this approach, we excluded three KC

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1   samples, one DG sample, and two HC samples from the analysis (see Table 1). Removing these

2   outliers reduced the average DNA damage in the KC, DG and HC groups to 1.74 ±2.71, 1.19 ±1.24

3   and 0.71 ±1.61 respectively, but did not affect the mean differences between the groups (*P*=0.407).

4   Similar results were obtained when the results were adjusted for age (data not shown), and we

5   found no significant correlation between age and DNA damage (Spearman's ρ=−0.095, *P*=0.523).

Table 1: Summary of DNA damage in the three study groups (N=54*)						
	N	Mean age	Mean ± SD (all samples)	Multiple comparison[1]	Mean ± SD (outliers removed)[2]	Multiple comparison[1]
Keratoconus (KC)	26	42.7	5.56 ± 14.08		1.74 ±2.71	
Decompensated grafts (DG)	17*	71.4	3.16 ± 8.22	*P*=0.807	1.19 ± 1.24	*P*=0.407
Healthy controls (HC)	11*	81.4	3.51 ± 6.66		0.71 ±1.61	

[1] Independent samples Kruskall-Wallis test. [2] Three outliers were removed from the KC group, one outlier was removed from the DG group, and two outlier was removed from the HC group. * Four samples in the DG group and five samples in the HC group did not yield sufficient DNA for analysis

6

7   Both atopy and contact lens wear are known risk factors for developing keratoconus. In the KC

8   group, seventeen patients had atopy and twenty-one patients wore contact lenses; in the DG group,

9   two patients had atopy and twelve patients wore contact lenses. Therefore, we next investigated

10   the extend of DNA damage among these subgroups. The amount of DNA damage did not differ

11   significantly between the atopic (2.26 ± 2.97) and non-atopic patients (1.26 ± 1.30; *P*=0.240) or

12   between the patients who wore contact lenses prior to surgery and the patients who did not wear

13   contact lenses (*P*=0.393). We found that the level of DNA damage did not differ significantly from

14   the clear corneas to hazy corneas (*P*=0.418). Clear corneas showed a comparable amount of DNA

15   damage (1.18 ± 2.47) to the mildly hazy corneas (1.80 ± 2.57), and the clouded corneas (1.18 ±

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1   1.16). Three KC cases underwent CXL prior to a grafting procedure. DNA-damage increased two-

2   fold compared to overall outcomes, though this difference was not significant (P= 0.750).

3   *Telomere length and hTERT expression*

4   Telomere length (TL) is represented as total number of base pairs per genome. Four samples did

5   not yield sufficient DNA for analysis. Mean telomere length in KC cases (n=11) was 3.92×10^9 ±

6   6.61×10^9 ($\log_{10}$ 8.88 ±0.945), 2.09×10^9 ± 5.78×10^9 ($\log_{10}$ 7.22 ±1.83) in decompensated grafts

7   (n=10), and 5.83×10^9 ± 1.46×10^{10} ($\log_{10}$ 8.02±1.49) base-pairs in healthy controls (n=8). These

8   differences were statistically significant (P= 0.034). Correction for age and gender did not affect

9   these findings. TL was $\log_{10}$ normalized to perform these analyses. *hTERT* did not reach detectable

10   levels in any corneal sample.

11   **Discussion**

12   In this study, we hypothesized that increased DNA damage could be a feature of keratoconus

13   corneal cells, caused either by increased DNA damage or decreased DNA damage responses. In

14   addition, we hypothesized that increased damage could lead to increased replicative senescence or

15   that increased replication of corneal cells on itself could underlie the epithelial remodeling in

16   keratoconus. In this particular large sample, we found that the level of DNA damage and telomere

17   length in the corneas of patients with keratoconus was similar to two control groups (patients with

18   a decompensated graft not related to KC and healthy donor subjects). Telomerase reverse

19   transcriptase (*hTERT*) was not expressed in any corneal sample. In addition, we found no

20   significant correlation between DNA damage, telomere length and either age, gender, atopic

21   constitution, or the use of contact lenses. Telomere length and *hTERT* expression levels have not

22   been assessed previously in corneal tissue.

Page 13

1 Several lines of evidence support the notion that keratoconic eyes have altered anti-oxidant

2 function and/or an inadequate DNA repair system.[19] DNA breaks are a feature of severe DNA

3 damage and can be caused by various agents such as UV light, ROS or chemicals. When these breaks

4 occur, cells initiate DNA repair responses. After this response there are three possible outcomes;

5 the break is repaired, there is too much damage and the cell goes into apoptosis or, as recently

6 discovered, the break persists and the cell goes into a senescent state.[20] Taking this into mind, the

7 DNA breaks assessed in our study both represent recent non-repaired breaks as well as

8 accumulated unrepaired breaks that did not lead to apoptosis. Following our results, we do not

9 have an indication of increased DNA damage in corneal cells of keratoconus patients compared to

10 healthy and diseased controls.

11 This does not rule out a contribution to KC development by more specific mechanisms. For

12 example, Atilano et al. reported increased damage to mitochondrial DNA (mtDNA) in the corneas of

13 patients with KC. However in another study, the corneal epithelium of KC patients did not appear to

14 have increased DNA damage.[6,21]

15

16 The majority of DNA sampled in our specimens should be regarded of epithelial origin and these

17 short lived mitotic highly active cells are replenished form the stem cell niche, located at the corneal

18 limbus. The limbal region of the cornea is not explanted in routine corneal transplant surgery,

19 though the epithelial cells should represent the genomic make-up of their progenitor stem cells.[22]

20 This has been well studied and established for telomere attrition; due to the DNA end-replication

21 inefficiency of polymerases, chromosomes shorten every cell division. In line with this, the

22 epithelial cells will reflect the number of cell divisions made by their progenitor cells. Since the

23 differences we found between telomere lengths points towards longer telomeres in KC patients, we

24 assume that there is not a clear indication of increased regeneration of corneal epithelial cells in

25 keratoconus. Naturally, biologically younger samples are expected to have longer telomeres, and
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1   statistical correction for age effects was hampered by the poor age distribution among the three

2   sample sets. Since this is the first report on telomere length in corneal tissue, no comparison with

3   younger healthy subjects is available. Mallet et al. demonstrated that corneal epithelial cells have

4   very efficient DNA repair mechanisms, and are less prone to UV-induced apoptosis. Possibly the

5   need to remove highly damaged cells is hereby reduced.[23] Overall, it seems that the morphological

6   characteristics of keratoconus are not mediated through accumulated DNA damage, aberrant DNA

7   repair systems, or aberrant telomerase systems. Should the peculiar pre-clinical epithelial

8   remodeling rather be attributed to inflammation in the corneal micro-environment?[24,25]

9

10 Interestingly, three patients with KC in our study underwent an epithelium-off corneal crosslinking

11 procedure with UV-A irradiation years prior to the grafting procedure, and these patients only

12 showed 2.6x higher level of DNA damage compared with the other samples. Therefore our data do

13 not support a putative link between induced UV-A exposure and DNA damage, which was suggested

14 in a recent report on a case in which crosslinking was associated with intraepithelial neoplasia.[26]

15 Finally, the current study investigated samples derived from patients that generally suffered from

16 more advanced stages of corneal disease, which are inherently more eligible for corneal transplant

17 surgery. To circumvent this, further investigations should preferably take into account early stages

18 of KC and a longitudinal study design. This could be facilitated by studying corneal epithelium

19 harvested at a CXL procedure, a promising tissue that we are currently exploiting in our laboratory

20 to further explore the dynamics of KC pathology.

1   **Conclusions**

2   This study is the first to quantify DNA damage using intact *Alu* elements and assess telomere length

3   and *hTERT* expression in corneal tissues. In summary, we found no signs of increased DNA damage

4   or replicative stress in corneal samples obtained from patients with keratoconus compared to

5   healthy controls and patients with decompensated grafts. Thus, the link between DNA repair

6   system dysfunction or accumulated genetic changes due to oxidative stress or UV radiation in the

7   development of progressive KC is unclear. Future longitudinal research should be aimed at younger

8   samples of progressive KC. A thorough analysis of local and systemic auto-inflammatory changes

9   should further elucidate our understanding of KC development.

1   **Acknowledgments**
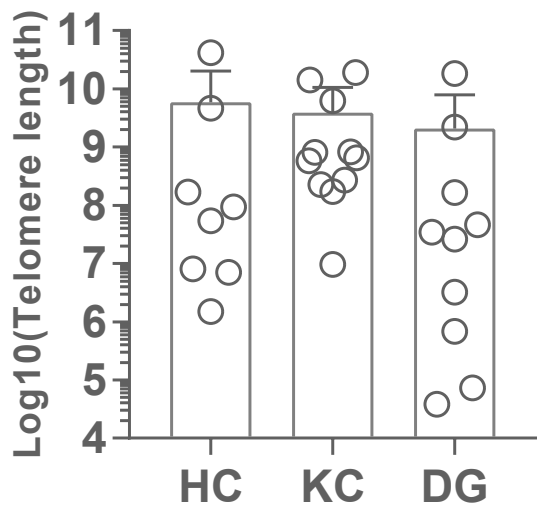
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5   **Figure Legend**

6   Figure 1: Box plots with overlying scatter plots summarizing the mean and median DNA damage

7   and telomere length (TL) measured in the three study groups. DNA damage was measured as the

8   number of intact *Alu* elements per genome copy. TL is represented as total number of base pairs per

9   genome. Top: Telomere length. Bottom: DNA damage.

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Mean(SD)　　Median(95%CI)