The prognostic value of basal DNA damage level in peripheral blood lymphocytes of patients affected by bladder cancer

The prognostic value of basal DNA damage level in peripheral blood lymphocytes of patients affected by bladder cancer.

Word count: 3,136

Alessandra Allione, PhD, Italian Institute for Genomic Medicine (IIGM), Via Nizza 52, Turin, Italy; e-mail: alessandra.allione@iigm.it; phone: +390116709545; fax: +390112365601

Barbara Pardini, PhD, Italian Institute for Genomic Medicine (IIGM), Via Nizza 52, Turin, Italy; e-mail: barbara.pardini@iigm.it

Clara Viberti, PhD, Italian Institute for Genomic Medicine (IIGM), Via Nizza 52, Turin, Italy; e-mail: clara.viberti@iigm.it

Marco Oderda, MD, Division of Urology – Dept of Surgical Science, A.O. Città della Salute e Della Scienza di Torino – Molinette, University of Turin, Corso Dogliotti 14, Turin, Italy; e-mail: marco.oderda@libero.it

Marco Allasia, MD, Division of Urology – Dept of Surgical Science, A.O. Città della Salute e Della Scienza di Torino –Molinette, University of Turin, Corso Dogliotti 14, Turin, Italy; e-mail: marc.allasia@gmail.com

Paolo Gontero, MD, Division of Urology – Dept of Surgical Science, A.O. Città della Salute e Della Scienza di Torino –Molinette, University of Turin, Corso Dogliotti 14, Turin, Italy; e-mail: paolo.gontero@unito.it

Paolo Vineis, MD, MRC-HPA Centre for Environment and Health, School of Public Health, Imperial College London, South Kensington London, SW7 2AZ London, United Kingdom; e-mail: p.vineis@imperial.ac.uk

Carlotta Sacerdote, MD, PhD, Unit of Cancer Epidemiology, Città della Salute e della Scienza University-Hospital and Center for Cancer Prevention (CPO), Via Santena 7, Turin, Italy; e-mail: carlotta.sacerdote@cpo.it

Giuseppe Matullo, PhD, Italian Institute for Genomic Medicine (IIGM – FKA HuGeF), Via Nizza 52, Turin, Italy; Dept of Medical Sciences, University of Turin, Via Santena 19, Turin, Italy; e-mail: giuseppe.matullo@unito.it

The prognostic value of basal DNA damage level in peripheral blood lymphocytes of patients affected by bladder cancer.

Alessandra Allione[a,b#,*], Barbara Pardini[a,b,#], Clara Viberti[a,b], Marco Oderda[c], Marco Allasia[c], Paolo Gontero[c], Paolo Vineis[a,d], Carlotta Sacerdote[e], Giuseppe Matullo[a,b]

[a] Italian Institute for Genomic Medicine (IIGM), Via Nizza 52, Turin, Italy

[b] Department of Medical Sciences, University of Turin, Via Santena 19, Turin, Italy

[c] Division of Urology – Department of Surgical Science, A.O. Città della Salute e Della Scienza di Torino – presidio Molinette, University of Turin, Corso Dogliotti 14, Turin, Italy

[d] MRC-HPA Centre for Environment and Health, School of Public Health, Imperial College London, South Kensington London, SW7 2AZ London, United Kingdom

[e] Unit of Cancer Epidemiology, Città della Salute e della Scienza University-Hospital and Center for Cancer Prevention (CPO), Via Santena 7, Turin, Italy

# Authors equally contributed to the work

*Corresponding author:

Alessandra Allione,

IIGM - Italian Institute for Genomic Medicine

Via Nizza 52, Torino, Italy.

Phone: +390116709545

Fax: +390116709546

e-mail: alessandra.allione@iigm.it

Abstract

Background: Bladder cancer (BC) is one of the most aggressive malignancies of the urinary tract, with the highest lifetime treatment costs per patient of all cancers, due to the high rate of recurrences requiring continuous surveillance. An early diagnosis is essential to improve survival of BC patients. Non-invasive and sensitive molecular biomarkers are needed to improve current strategies for the detection and monitoring of BC. Previous studies suggested that elevated DNA damage levels and suboptimal nucleotide excision DNA repair (NER) may be associated with BC.

Methods: In the present study, we investigated basal DNA damage and DNA repair capacity in peripheral blood mononuclear cells (PBMCs) from 146 newly diagnosed BC patients and 155 controls using a modified comet assay able to evaluate NER activity after challenging cells by benzo(a)pyrene diolepoxide (BPDE).

Results: We found an association between DNA damage levels in PBMCs of BC cases and patients' outcomes. Basal DNA damage at diagnosis was significantly increasing with tumor grades (trend test p=0.02) and risk classes (trend test p=0.02). The overall survival analysis showed that DNA damage in patients at BC diagnosis was significantly higher in subjects with a shorter survival time (HR=3.7, 95% CI 1.3–10.6, p=0.02).

Conclusions: Based on these data, we suggest that DNA damage levels measured in PBMCs of BC patients may potentially represent a prognostic marker associated with poor survival; further validation is needed to better stratify BC patients for clinical trials.

Key words: comet assay; bladder cancer; DNA damage; DNA repair

1. Introduction

Bladder cancer (BC) is one of the most frequent cancers of the urinary tract with the highest lifetime treatment costs per patient of all cancers [1]. In Europe, BC is the fourth most common cancer in men and the eighth most common cause of cancer-specific mortality [2].

Non-muscle-invasive bladder cancer (NMIBC), which represent the 75-80% of newly diagnosed BC, recurs in 50 to 70% of cases and progress to muscle-invasive disease in 1-2% of them [3]. Muscle-invasive bladder cancer (MIBC) patients have a 5-year survival rate of <50% [3].

Increased levels of DNA damage and ineffective repair mechanisms are the underlying molecular events in the pathogenesis of most of the life-threatening diseases like cancer and degenerative diseases. The sources of DNA damage can be either exogenous or endogenous. BC is strongly linked to occupational and environmental exposure to chemicals [4], suggesting that DNA lesions can play an important role in its onset and development.

Persistent basal DNA damage may reflect higher exposure to carcinogens and/or deficient DNA repair [5]. In recent studies we evaluated DNA repair and DNA damage levels, potentially caused by occupational and/or environmental exposure, in BC patients and healthy controls. We found significantly increased frequencies of micronuclei (MN) and nuclear buds (NBUD) in BC cases compared to controls [6] and an association between the basal levels of phosphorylation of histone variant H2AX (γ-H2AX) and risk of disease recurrence or progression [7]. These data confirmed the presence of a high genetic instability in cryopreserved lymphocytes of in BC patients. In particular, in Turinetto et al. [7] we evaluated the damage connected to double strand break repair (DSBR) mechanism, which recovers complete break of the DNA double helix. This kind of breaks /damage impairs DNA replication, transcription, or distribution of the genetic material to daughter cells [8]. Similarly to this last, also nucleotide excision repair (NER) pathway alterations can drive tumor behaviour and response to treatment [9].

To address the question whether DNA damage and NER capacity could be used as a prognostic factor in BC, we investigated basal DNA damage and DRC in peripheral blood mononuclear cells (PBMCs) from a cohort

of BC patients and healthy controls already described [6, 7], using a modified comet assay able to evaluate NER activity [10, 11].

The comet assay is a relatively simple, sensitive, rapid and inexpensive method that has already been employed in several DNA damage/repair clinical studies [12, 13]. Several studies have shown that basal DNA damage is increased in PBMCs of patients with different types of cancer, while other studies have also exposed PBMCs from cancer patients (usually prior to any kind of therapy) to DNA-damaging agents to assess if susceptibility to DNA damage and subsequent repair capacity differs from control samples [14].

2. Materials and Methods

2.1. *Study population.*

The study population included newly diagnosed, previously untreated, histologically confirmed males with BC recruited in the Turin Bladder Cancer Study [15, 16]. Controls were men recruited during the same period as cases in a random fashion from patients treated at the same urology departments for non-neoplastic disease or from patients treated at the medical and surgical departments for various problems (hernias, vasculopathies, diabetes, heart failure, asthma or other benign diseases). All subjects were informed and provided written informed consent according to the Helsinki declaration. None of the patients received any treatment at the time of blood sampling. The design of the study was approved by the local Ethics Committees. Details of the study population were previously described in Pardini et al. [6]. All tumors were evaluated and classified in blind by at least two urologists of our internal department. Clinical information and risk category for NMIBC were registered through the perusal of clinical records.

2.2. Isolation and storing of PBMCs.

The isolation and storing of blood cells was performed as previously described [6, 7]. Briefly, PBMCs were separated from heparinized venous blood by centrifugation with FicollPaque PLUS (GE Healthcare, Milan, Italy) at 400 × g for 30 min at room temperature. After two washes in RPMI 1640 (Invitrogen, Paisley, UK),

1% FBS (Invitrogen), 25 mM EDTA (Invitrogen), PBMCs were prepared for cryopreservation. They were resuspended at $10x10^6$ cells/ml in freezing medium (RPMI 1640, 50% FBS, 10%DMSO), aliquoted in cryovials and slowly frozen overnight at the rate of –1 °C/min in isopropyl alcohol to –80 °C (Mr Frosty containers, Nalgene, Roskilde, Denmark). Cryovials were then transferred into liquid nitrogen for long-term storage.

2.3. Aphidicolin-block NER comet assay

Cryopreserved PBMCs were thawed quickly in a 37 °C water bath and suspended in 5 ml of cold medium containing 50% FBS, 49% RPMI 1640 and 1% dextrose. Treatment with aphidicolin (APC) or/and benzo[a]pyrene diol epoxide (BPDE) was performed as previously described [11]. Briefly, cells were spun down by centrifugation and treated with 2.5 μg/ml phytohaemoagglutinin (PHA) (Sigma-Aldrich Co, St Louis, MO). Twenty-four hours later samples were centrifuged and treated in the following ways: A) 2.5 μg/ml APC (Sigma-Aldrich Co), 30 min at 37 °C, 5% CO_2; B) 0.5 μM BPDE (NCI Chemical Carcinogen Reference Standards Repository, Midwest Research Institute, Kansas City, MO, USA), 2 h at 37 °C, 5% CO_2; C) pretreatment with 2.5 μg/ml APC (30 min) followed by 0.5 μM BPDE (2 h). At the end of the treatment, cells were centrifuged and pelleted cells were processed for comet assay.

The NER comet assay was performed according to the methods previously described [10], with slight modifications [11]. After treatment, cells were mixed with low-melting-point agarose (0.75%; Sigma-Aldrich Co) and layered on 85 x 100 mm GelBond films (Lonza, Basel, Switzerland). Each GelBond film comprised eight 19 x 23 mm agarose gels. The GelBond films were immersed in lysis solution (2.5 M NaCl, 0.1 mM Na_2EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1.5 hours at 4 °C, then placed in an electrophoresis tank for 40 minutes, submerged into electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, approximately pH 13), and finally ran at 30 V (0.8 V/cm) and 300 mA for 20 min. After neutralization with 0.4 M Tris-HCl (pH 7.5), gels were fixed in ethanol and dried at room temperature. For scoring, slides were stained with YOYO-1 iodide (1 mM solution in DMSO, diluted 1:250 in PBS; Life Technologies Italia, Monza, Italy) and nuclei were visualized by a Leica fluorescence microscope at 40X magnification. Two gels of 50 nuclei for each

sample treatment were scored with Comet IV software (Perceptive Instrument, Suffolk, UK). The median tail moment (TM) of 100 nuclei was used as a measure of DNA damage. The TM is defined as the product of the tail length and the fraction of total DNA in the tail (Tail moment=tail length x % of DNA in the tail). TM is calculated automatically by the computer software system as an average for the 50 cells selected for measurement. For each electrophoresis run, a human K562 erythroleukemia cell line was included as reference standard and used to normalize results [11]. Since in our previous study we demonstrated that APC-treated and untreated cells did not differ in DNA damage levels [11], in this study we directly used APC-treated cells endpoint as representative of basal DNA damage. DRC (NER capacity) was calculated for each subject as: TM $DNA_{APC+BPDE}$ – TM DNA_{BPDE} - TM DNA_{APC}.

2.4. Statistical analysis.

Patient characteristics were described as absolute frequencies for qualitative variables, and with mean, standard deviation (SD) and range values for quantitative variables. The analysed variables were basal DNA damage in APC-treated cells and DRC, obtained as described above.

The influence of possible explicative variables on the basal DNA damage or DRC was evaluated by multivariate analysis and/or non-parametric statistical hypothesis test, since variables did not follow a normal distribution (Shapiro–Wilk normality test).

Wilcoxon or Kruskal-Wallis Rank Sum tests were applied to compare the distribution between groups of basal DNA damage or DRC.

Overall survival (OS) was evaluated calculating the time (in years) between the date of BC diagnosis and the date of death or follow up termination as the endpoint for each patient. Event-free survival (EFS) was calculated as the time (in years) between the date of BC diagnosis until date of relapse, death or censorship, whichever came first. The relative risk of death or recurrence against each of the basal DNA damage or DRC endpoints was estimated as hazard ratios (HR) using Cox regression (R 3.2.4, Survival package). Basal DNA damage or DRC were all considered as categorical (above/below the median value). Multivariate survival analyses were adjusted for age, smoking, T stage and therapy variables for the whole

group of patients. The prognostic role of DNA damage or DRC on survival was also evaluated using Kaplan Meier curves and log-rank test.

All the analyses were performed with the open source R (R 3.2.4).

3. Results

3.1. Study population.

The study included PBMCs from 146 cases and 155 controls. For 61 subjects technical problems were encountered in one or more of the three points (i.e., slides were not scorable), therefore basal DNA damage or DRC were not evaluable. Since no additional aliquots of cryopreserved PBMCs were available, these subjects were excluded from the analysis. Finally, 133 BC patients and 141 control subjects were included in the basal DNA damage analysis, while 121 BC patients and 119 controls subjects were included in the DRC analysis. The characteristics of the whole cohort are summarized in Table 1. For 131 patients, tumors were classified as NMIBC, whereas 15 patients resulted MIBC at diagnosis. As current grading classifications in BC (WHO 2004/2016) are suboptimal, and as the 1973 system (WHO 1973) identifies more aggressive tumors [17], we took into consideration both classifications in the statistical analysis. Thirty-eight BC cases presented grade G1 cancer, while 60 and 48 had G2 and G3 grade, respectively. Fifty-five cases were classified as high-risk, 41 as intermediate and 35 as low-risk. Fifty-two BC patients developed one or more recurrences, whereas 94 did not.

3.2. Comet assay endpoints in relation to clinical outcomes.

DRC was evaluated by a modified comet assay that measures the capacity of PBMCs to resolve DNA damage after *in vitro* challenging with BPDE in the presence or absence of APC, a potent and specific inhibitor of DNA polymerases α and σ [18]. No differences between cases and controls were observed by Wilcoxon Rank Sum test, neither considering only NMIBC or MIBC cases (Table 2). Same results were obtained with a generalized linear model, controlling for age and smoking habits.

Basal DNA damage levels and DRC correlation with grading, risk classes and recurrences were evaluated, either in all cases or NMIBC only (Table 3). Although DRC did not show any significant correlation with clinical characteristics of tumors, basal DNA damage at diagnosis was significantly different between tumor grading (all BC: WHO 1973 G3 vs G1 adjusted linear regression p=0.02; WHO 2004/2016 adjusted linear regression p=0.04, Fig 1A and 1B, respectively; NMIBC: WHO 1973 G3 vs G1 adjusted linear regression p=0.01; WHO 2004/2016 adjusted linear regression p=0.03, Supplementary Fig S1A and S1B, respectively). We also observed a significant positive trend of correlation between basal DNA damage and tumor grading (all BC: p=0.02; NMIBC: p=0.01), suggesting that DNA damage increases with invasive progression. Moreover, basal DNA damage was significantly different also between risk classes (all BC: High vs Low risk adjusted linear regression p=0.03; Fig. 1C; NMIBC: High vs Low risk adjusted linear regression p=0.02; Supplementary Fig S1C), with a significant positive trend in NMIBC with the increase of risk (p=0.02).

3.3. Overall survival, recurrence rate and event-free survival analyses

Results from the survival analysis are reported in Table 4 and shown in Fig. 2. Patients were stratified into two categories, above and under the median value of DNA damage or DRC, to calculate Kaplan–Meier OS curves.

Values above the median value of basal DNA damage corresponded to the “High DNA damage” group, those under the median value corresponded to the “Low DNA damage” group. There was a significantly decreased survival in patients with an increased basal DNA damage level both in all BC patients and also when stratified only for NMIBC (HR=3.7, 95%CI 1.3–10.6, p=0.02 and HR=4.4, 95%CI 1.1–17.3, p=0.03, respectively). There was no association between DRC and OS, neither between EFS and both comet assay endpoints (Table 4).

Patients that developed recurrences at follow-up showed a significantly lower basal DNA damage compared to patients that did not (all BC, adjusted linear regression p=0.03, Fig. 1D and Table 3; NMIBC, adjusted linear regression p=0.01, Supplementary Fig S1D). This suggests that patients having a higher basal

DNA damage probably presented an induced increased DNA repair response resulting in a lower risk of recurrences.

4. Discussion

In the present study, we observed a significant increased basal DNA damage in PBMCs from BC patients when stratified for grade, risk class or recurrence rate. Moreover, a lower DNA damage in patients at diagnosis was associated with longer survival time.

Morphologic and pathologic criteria (e.g. histology, stage, grade) used for conventional diagnosis of BC [19] have inadequate power to predict patient outcome precisely, and there remains significant variability in the prognosis of patients with similar characteristics [20]. Thus, new biomarkers that predict clinical behaviour in patients with BC is needed especially regarding non-invasive tissues, like blood or urine [21].

Elevated DNA damage levels and suboptimal NER, the major DNA repair mechanism for repairing bulky DNA damage generated by most environmental factors [22], may be associated with BC, as reported in previous studies [23, 24, 25]. We hypothesized that a quantification of the DNA damage and DRC might serve as BC risk and prognostic biomarkers. The comet assay modified method used in this study allows in fact to measure the accumulation of DNA breaks, as incision events, by blocking repair synthesis, and is adequate to detect DRC inter-individual differences in the context of NER pathway [26].

In a previous study, baseline levels of DNA damage measured by a similar assay was significantly higher in BC patients than in controls [27]. The main difference with our study consisted in the type of cells: while Schabath et al [27] quantified DNA damage on fresh whole blood , we measured DNA damage on cryopreserved PBMCs. Moreover, we compared BC cases with hospitalised controls, instead of healthy subjects: this could be an issue when measuring disease markers, as the ideal control group would comprise a random sample from the general population that gave rise to the cases. However, as in our case, this is not always possible in practice. We applied in a previous study on DNA repair NER comet assay on 122 healthy subjects [26]. Unfortunately these subjects were different from BC cases both in age (mean age 24.5 years) and in gender distribution (39 males, 83 females) and could not be used as controls in the

present study. However, we observed basal DNA damage levels lower than in controls from BC study (0.022 in subjects included in [26] vs 0.21 in BC study controls; data not shown). These data may explain why we could not observe a significant difference between BC cases and controls.

In the present study, we found that the basal DNA damage was significantly associated with a worse patient prognosis. The DNA damage increase in PBMCs of high grade (WHO 2004/2016) or G3 (WHO 1973) BC, could be interpreted as an altered status in the DNA damage repair system or a consequence of an unknown past exposure. Tumor grade is an important predictor of cancer prognosis [28]. However, histopathological classifications are known for their inter- and intra-observer variability which may have profound limitations in prognosis [29]. Hence, basal DNA damage levels in PBMCs may serve as potential predictive biomarkers for a better stratification of BC patients.

The comet assay used to measure DRC is specific for NER, but it detects a wide range of type of DNA damages at basal levels. Alkaline comet assay, in fact, is capable of detecting DNA double-strand breaks, single-strand breaks, alkali-labile sites, DNA-DNA/DNA-protein cross-linking, and incomplete excision repair sites, with no specific assignment of the DNA repair pathways responsible for that. We hypothesize that increased DNA damage in patients with worse outcome is associated with alterations in different DNA repair pathways that subsequently may lead to carcinogenesis. However, the exact mechanism explaining this phenotype remains to be elucidated.

We previously observed a significant association between increased γ-H2AX basal phosphorylation level and a decreased BC recurrence risk [7]. The results suggested a protective effect of high basal DSBR signalling in terms of preventing BC recurrences. On the contrary, a more general assessment of DNA damage as determined by comet assay revealed an association between higher basal DNA damage at the time of BC diagnosis and a decreased risk of recurrences at follow-up. One possible explanation could be that DNA repair pathways are involved in tumor chemoresistance [30], decreasing therapy benefits. However, further analyses are needed to elucidate this conflicting results. The relationship between DNA damage and repair is complex; no single pathway efficiently repairs all types of DNA lesions, and some of

them are substrates for more than one pathway. Moreover, there is an increasing evidence of extensive interactions among proteins involved in distinct pathways.

We are aware of some limitations of the present study. The potential use of the comet assay for DNA damage and repair activity associated with cancer was reviewed by McKenna et al. [14]. Recent studies showed that high levels of DNA damage in PBMCs were associated with different types of cancer, including breast [31, 32], cervix [33], Hodgkin's disease [34], and oesophageal cancer [35]. On the other hand, these outcomes were not observed in other type of cancer such as lung [36] and prostate [37]. Noteworthy, the observed associations between DNA damage and disease established in case–control studies do not allow to conclude whether the elevated DNA damage is a cause or a consequence of the disease. To establish causality, prospective studies need to be conducted. Finally, in our study we recruited only men affected by BC, thus we can only hypothesise that DNA damage could be a good prognostic biomarker also in BC women. Møller et al. [38] reported that gender is one of the factors that influence the level of DNA damage detected by the comet assay in biomonitoring occupational studies, confirming that further analyses should be done in female patients affected by BC.

5. Conclusions

We have shown an association between DNA damage levels and BC patients' outcomes.

Our data suggest that basal DNA damage levels in BC patients may potentially represent an important prognostic marker associated with poor OS and after further validation could be used for a better stratification of BC patients for therapy, decreasing progression rate and improving patients' outcomes. Hence, reliable methods for detecting DNA damage levels in PBMCs in cancer patients may improve and amplify the diagnostic and prognostic tools. Moreover, targeting DNA damage repair pathways may contribute to improving conventional therapy regimens.

Funding: This work was supported by: the Italian Institute of Genomic medicine (IIGM) and Compagnia di San Paolo Torino, Italy (PV, GM); Fondazione Umberto Veronesi "Post-doctoral fellowship Year 2014, 2015,

2016, and 2017" (BP); Fondazione Umberto Veronesi "Grant 2013" (GM); and Associazione Italiana per la Ricerca sul Cancro -AIRC (Italian Association for Cancer Research) - IG 17464 (GM).

References

1. Sievert KD, Amend B, Nagele U, Schilling D, Bedke J, Horstmann M, et al. Economic aspects of bladder cancer: what are the benefits and costs? World J Urol. 2009;27 3:295-300; doi: 10.1007/s00345-009-0395-z. http://www.ncbi.nlm.nih.gov/pubmed/19271220.
2. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127 12:2893-917; doi: 10.1002/ijc.25516. http://www.ncbi.nlm.nih.gov/pubmed/21351269.
3. Smith ZL, Guzzo TJ. Urinary markers for bladder cancer. F1000Prime Rep. 2013;5:21; doi: 10.12703/P5-21. http://www.ncbi.nlm.nih.gov/pubmed/23864929.
4. Shirai T. Etiology of bladder cancer. Semin Urol. 1993;11 3:113-26. http://www.ncbi.nlm.nih.gov/pubmed/8210833.
5. Friedberg EC, Aguilera A, Gellert M, Hanawalt PC, Hays JB, Lehmann AR, et al. DNA repair: from molecular mechanism to human disease. DNA Repair (Amst). 2006;5 8:986-96. http://www.ncbi.nlm.nih.gov/pubmed/16955546.
6. Pardini B, Viberti C, Naccarati A, Allione A, Oderda M, Critelli R, et al. Increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of bladder cancer. Br J Cancer. 2017;116 2:202-10; doi: 10.1038/bjc.2016.411. http://www.ncbi.nlm.nih.gov/pubmed/27959887.
7. Turinetto V, Pardini B, Allione A, Fiorito G, Viberti C, Guarrera S, et al. H2AX phosphorylation level in peripheral blood mononuclear cells as an event-free survival predictor for bladder cancer. Mol Carcinog. 2016;55 11:1833-42; doi: 10.1002/mc.22431. http://www.ncbi.nlm.nih.gov/pubmed/27439749.
8. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet. 2001;27 3:247-54; doi: 10.1038/85798. http://www.ncbi.nlm.nih.gov/pubmed/11242102.
9. Jeggo PA, Pearl LH, Carr AM. DNA repair, genome stability and cancer: a historical perspective. Nat Rev Cancer. 2016;16 1:35-42; doi: 10.1038/nrc.2015.4. http://www.ncbi.nlm.nih.gov/pubmed/26667849.
10. Vande Loock K, Decordier I, Ciardelli R, Haumont D, Kirsch-Volders M. An aphidicolin-block nucleotide excision repair assay measuring DNA incision and repair capacity. Mutagenesis. 2010;25 1:25-32; doi: 10.1093/mutage/gep039. http://www.ncbi.nlm.nih.gov/pubmed/19843590.
11. Allione A, Russo A, Ricceri F, Vande Loock K, Guarrera S, Voglino F, et al. Validation of the nucleotide excision repair comet assay on cryopreserved PBMCs to measure inter-individual variation in DNA repair capacity. Mutagenesis. 2013;28 1:65-70; doi: 10.1093/mutage/ges054. http://www.ncbi.nlm.nih.gov/pubmed/23042048.
12. Galardi F, Oakman C, Truglia MC, Cappadona S, Biggeri A, Grisotto L, et al. Inter- and intra-tumoral heterogeneity in DNA damage evaluated by comet assay in early breast cancer patients. Breast. 2012;21 3:336-42; doi: 10.1016/j.breast.2012.02.007. http://www.ncbi.nlm.nih.gov/pubmed/22406214.
13. Herrera M, Dominguez G, Garcia JM, Pena C, Jimenez C, Silva J, et al. Differences in repair of DNA cross-links between lymphocytes and epithelial tumor cells from colon cancer patients measured in vitro with the comet assay. Clin Cancer Res. 2009;15 17:5466-72; doi: 10.1158/1078-0432.CCR-08-3268. http://www.ncbi.nlm.nih.gov/pubmed/19690199.
14. McKenna DJ, McKeown SR, McKelvey-Martin VJ. Potential use of the comet assay in the clinical management of cancer. Mutagenesis. 2008;23 3:183-90; doi: 10.1093/mutage/gem054. http://www.ncbi.nlm.nih.gov/pubmed/18256034.
15. Russo A, Modica F, Guarrera S, Fiorito G, Pardini B, Viberti C, et al. Shorter leukocyte telomere length is independently associated with poor survival in patients with bladder cancer. Cancer Epidemiol Biomarkers Prev. 2014;23 11:2439-46; doi: 10.1158/1055-9965.EPI-14-0228. http://www.ncbi.nlm.nih.gov/pubmed/25234236.
16. Sacerdote C, Guarrera S, Ricceri F, Pardini B, Polidoro S, Allione A, et al. Polymorphisms in the XRCC1 gene modify survival of bladder cancer patients treated with chemotherapy. Int J Cancer. 2013;133 8:2004-9; doi: 10.1002/ijc.28186. http://www.ncbi.nlm.nih.gov/pubmed/23553206.

17. Soukup V, Capoun O, Cohen D, Hernandez V, Babjuk M, Burger M, et al. Prognostic Performance and Reproducibility of the 1973 and 2004/2016 World Health Organization Grading Classification Systems in Non-muscle-invasive Bladder Cancer: A European Association of Urology Non-muscle Invasive Bladder Cancer Guidelines Panel Systematic Review. Eur Urol. 2017; doi: 10.1016/j.eururo.2017.04.015. http://www.ncbi.nlm.nih.gov/pubmed/28457661.
18. Speit G, Schutz P, Hoffmann H. Enhancement of genotoxic effects in the comet assay with human blood samples by aphidicolin. Toxicol Lett. 2004;153 3:303-10; doi: 10.1016/j.toxlet.2004.04.047. http://www.ncbi.nlm.nih.gov/pubmed/15454306.
19. Comperat E, Varinot J. Immunochemical and molecular assessment of urothelial neoplasms and aspects of the 2016 World Health Organization classification. Histopathology. 2016;69 5:717-26; doi: 10.1111/his.13025. http://www.ncbi.nlm.nih.gov/pubmed/27353436.
20. Zhao M, He XL, Teng XD. Understanding the molecular pathogenesis and prognostics of bladder cancer: an overview. Chin J Cancer Res. 2016;28 1:92-8; doi: 10.3978/j.issn.1000-9604.2016.02.05. http://www.ncbi.nlm.nih.gov/pubmed/27041931.
21. Talukdar S, Emdad L, Das SK, Sarkar D, Fisher PB. Noninvasive approaches for detecting and monitoring bladder cancer. Expert Rev Anticancer Ther. 2015;15 3:283-94; doi: 10.1586/14737140.2015.989838. http://www.ncbi.nlm.nih.gov/pubmed/25494295.
22. Neumann AS, Sturgis EM, Wei Q. Nucleotide excision repair as a marker for susceptibility to tobacco-related cancers: a review of molecular epidemiological studies. Mol Carcinog. 2005;42 2:65-92; doi: 10.1002/mc.20069. http://www.ncbi.nlm.nih.gov/pubmed/15682379.
23. Wu X, Gu J, Grossman HB, Amos CI, Etzel C, Huang M, et al. Bladder cancer predisposition: a multigenic approach to DNA-repair and cell-cycle-control genes. Am J Hum Genet. 2006;78 3:464-79; doi: 10.1086/500848. http://www.ncbi.nlm.nih.gov/pubmed/16465622.
24. Qiu J, Wang X, Meng X, Zheng Y, Li G, Ma J, et al. Attenuated NER expressions of XPF and XPC associated with smoking are involved in the recurrence of bladder cancer. PLoS One. 2014;9 12:e115224; doi: 10.1371/journal.pone.0115224. http://www.ncbi.nlm.nih.gov/pubmed/25535740.
25. Gu J, Zhao H, Dinney CP, Zhu Y, Leibovici D, Bermejo CE, et al. Nucleotide excision repair gene polymorphisms and recurrence after treatment for superficial bladder cancer. Clin Cancer Res. 2005;11 4:1408-15; doi: 10.1158/1078-0432.CCR-04-1101. http://www.ncbi.nlm.nih.gov/pubmed/15746040.
26. Allione A, Guarrera S, Russo A, Ricceri F, Purohit R, Pagnani A, et al. Inter-individual variation in nucleotide excision repair pathway is modulated by non-synonymous polymorphisms in ERCC4 and MBD4 genes. Mutat Res. 2013;751-752:49-54; doi: 10.1016/j.mrfmmm.2013.08.005. http://www.ncbi.nlm.nih.gov/pubmed/24004570.
27. Schabath MB, Spitz MR, Grossman HB, Zhang K, Dinney CP, Zheng PJ, et al. Genetic instability in bladder cancer assessed by the comet assay. J Natl Cancer Inst. 2003;95 7:540-7. http://www.ncbi.nlm.nih.gov/pubmed/12671022.
28. Sylvester RJ, van der Meijden AP, Oosterlinck W, Witjes JA, Bouffioux C, Denis L, et al. Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. Eur Urol. 2006;49 3:466-5; discussion 75-7; doi: 10.1016/j.eururo.2005.12.031. http://www.ncbi.nlm.nih.gov/pubmed/16442208.
29. van Rhijn BW, van Leenders GJ, Ooms BC, Kirkels WJ, Zlotta AR, Boeve ER, et al. The pathologist's mean grade is constant and individualizes the prognostic value of bladder cancer grading. Eur Urol. 2010;57 6:1052-7; doi: 10.1016/j.eururo.2009.09.022. http://www.ncbi.nlm.nih.gov/pubmed/19765886.
30. Pan ST, Li ZL, He ZX, Qiu JX, Zhou SF. Molecular mechanisms for tumour resistance to chemotherapy. Clin Exp Pharmacol Physiol. 2016;43 8:723-37; doi: 10.1111/1440-1681.12581. http://www.ncbi.nlm.nih.gov/pubmed/27097837.
31. Santos RA, Teixeira AC, Mayorano MB, Carrara HH, Andrade JM, Takahashi CS. Basal levels of DNA damage detected by micronuclei and comet assays in untreated breast cancer patients and healthy

women. Clin Exp Med. 2010;10 2:87-92; doi: 10.1007/s10238-009-0079-4. http://www.ncbi.nlm.nih.gov/pubmed/19902326.
32. Synowiec E, Stefanska J, Morawiec Z, Blasiak J, Wozniak K. Association between DNA damage, DNA repair genes variability and clinical characteristics in breast cancer patients. Mutat Res. 2008;648 1-2:65-72; doi: 10.1016/j.mrfmmm.2008.09.014. http://www.ncbi.nlm.nih.gov/pubmed/18977234.
33. Gabelova A, Farkasova T, Gurska S, Machackova Z, Lukacko P, Witkovsky V. Radiosensitivity of peripheral blood lymphocytes from healthy donors and cervical cancer patients; the correspondence of in vitro data with the clinical outcome. Neoplasma. 2008;55 3:182-91. http://www.ncbi.nlm.nih.gov/pubmed/18348650.
34. Lorenzo Y, Provencio M, Lombardia L, Diaz R, Silva J, Herrera M, et al. Differential genetic and functional markers of second neoplasias in Hodgkin's disease patients. Clin Cancer Res. 2009;15 15:4823-8; doi: 10.1158/1078-0432.CCR-08-3224. http://www.ncbi.nlm.nih.gov/pubmed/19622580.
35. Vasavi M, Vedicherala B, Vattam KK, Ahuja YR, Hasan Q. Assessment of genetic damage in inflammatory, precancerous, and cancerous pathologies of the esophagus using the comet assay. Genet Test Mol Biomarkers. 2010;14 4:477-82; doi: 10.1089/gtmb.2010.0006. http://www.ncbi.nlm.nih.gov/pubmed/20632893.
36. Sigurdson AJ, Jones IM, Wei Q, Wu X, Spitz MR, Stram DA, et al. Prospective analysis of DNA damage and repair markers of lung cancer risk from the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial. Carcinogenesis. 2011;32 1:69-73; doi: 10.1093/carcin/bgq204. http://www.ncbi.nlm.nih.gov/pubmed/20929901.
37. Kosti O, Goldman L, Saha DT, Orden RA, Pollock AJ, Madej HL, et al. DNA damage phenotype and prostate cancer risk. Mutat Res. 2011;719 1-2:41-6; doi: 10.1016/j.mrgentox.2010.11.005. http://www.ncbi.nlm.nih.gov/pubmed/21095241.
38. Moller P, Knudsen LE, Loft S, Wallin H. The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors. Cancer Epidemiol Biomarkers Prev. 2000;9 10:1005-15. http://www.ncbi.nlm.nih.gov/pubmed/11045781.

Table 1. Demographic and clinical characteristics of BC patients and healthy controls

Covariates	Controls (%)	Cases (%)
N	155	146
Non-muscle invasive BC	-	131 (89.7)
Muscle invasive BC	-	15 (10.3)
Age (years)		
Mean ± SD	62.46 ± 8.63	62 ± 7.86
Range	40.31 – 74.59	39.99 – 74.10
Smoking Status		
Never	25 (16.1)	23 (15.8)
Former	94 (60.7)	89 (60.9)
Current	36 (23.2)	34 (23.3)
T stage*		
Ta		78 (53.4)
T1		47 (32.2)
≥T2		15 (10.2)
Tis		3 (2.1)
Tx		3 (2.1)
Grading (WHO 1973)*		
G1		38 (26)
G2		60 (41.1)
G3		48 (32.9)
Grading (WHO 2004/2016)*		
Non-high grade		72 (49.3)
High grade		74 (50.7)
Risk*		
Low-risk		35 (24)
Intermediate Risk		41 (28.1)
High-risk		55 (37.7)
Muscle invasive		15 (10.2)
Recurrences		
No		94 (64.4)
Yes		52 (35.6)
Number of recurrences		
1		31 (59.6)
2		16 (30.8)
≥3		5 (9.6)
Progression		
No		140 (95.9)
Yes		6 (4.1)
Therapy		
No		43 (29.5)
Yes		103 (70.5)
Cystectomy		

No		119 (81.5)
Yes		27 (18.5)
BC patient' status at follow up		
Alive		122 (83.6)
Died		24 (16.4)

*For reference see Babjuk M et al, Eur Urol. 2017.

Table 2. Basal DNA damage and nucleotide excision DNA repair capacity in PBMCs from BC cases and healthy controls

		Background DNA damage					
	Controls	**BC**	**p-value***	**NMIBC**	**p-value***	**MIBC**	**p-value***
N	141	133		119		14	
Mean[a] (SD)	0.21 (0.19)	0.19 (0.18)	0.64	0.19 (0.18)	0.53	0.22 (0.19)	0.66
		DNA Repair Capacity					
	Controls	**BC**	**p-value***	**NMIBC**	**p-value***	**MIBC**	**p-value***
N	119	121		108		13	
Mean[b] (SD)	12.28 (9.56)	14.15 (10.89)	0.21	14.01 (11.05)	0.29	15.34 (9.80)	0.25

[a] *Mean of comet tail moment*

[b] *Mean DRC calculated as described in Materials and methods section*

** Wilcoxon Rank Sum test*

Table 3. Basal DNA damage and nucleotide excision DNA repair capacity in PBMCs from all BC cases or NMIBC stratified for grade, risk and recurrence rate

	ALL CASES	**DNA damage (mean±sd)**	**p***	**adj p****	**trend p*****	**ALL CASES**	**DRC (mean±sd)**	**p***	**adj p****	**trend p*****
Tumor Type	NMI (n=119)	0.19 ± 0.18	0.38	ref	nd	NMI (n=108)	14 ± 11.1	0.72	ref	nd
	MI (n=14)	0.22 ± 0.19		0.62		MI (n=13)	15.3 ± 9.8		0.99	
Grading (WHO 1973)	G1 (n=36)	0.13 ±0.11	0.1	ref	**0.02**	G1 (n=33)	13.1 ±10.2	0.67	ref	0.36
	G2 (n=51)	0.2± 0.17		0.08		G2 (n=45)	15.7± 12.1		0.81	
	G3 (n=46)	0.23 ±0.22		**0.02**		G3 (n=43)	13.3 ±10.1		0.36	
Grading (WHO 2004/2016)	Non high grade (n=65)	0.16 ±0.14	0.11	ref	nd	Non high grade (n=58)	14 ±11.1	0.89	ref	nd
	High grade (n=68)	0.22± 0.20		**0.04**		High grade (n=63)	14.3 ±10.8		0.58	
Risk	Low (n=33)	0.13±0.11	0.39	ref	0.11	Low (n=30)	14.4±10.2	0.89	ref	0.55
	Intermediate (n=36)	0.19±0.17		0.25		Intermediate (n=31)	14.1±11.9		0.6	
	High (n=50)	0.22±0.21		**0.03**		High (n=47)	13.7±11.2		0.26	
	MI (n=14)	0.22±0.19		0.16		MI (n=13)	15.3±9.8		0.62	
Recurrences	No (n=88)	0.21 ±0.19	**0.03**	ref	nd	No (n=80)	14.5 ±10.9	0.67	ref	nd
	Yes (n=45)	0.15 ±0.15		**0.03**[#]		Yes (n=41)	13.5 ±10.9		0.86[#]	
	NMIBC	**DNA damage (mean±sd)**	**p***	**adj p****	**trend p*****	**NMIBC**	**DRC (mean±sd)**	**p***	**adj p****	**trend p*****
Grading (WHO 1973)	G1 (n=36)	0.13± 0.11	0.14	ref	**0.01**	G1 (n=33)	13.1± 10.2	0.69	ref	0.35
	G2 (n=50)	0.20 ±0.17		0.09		G2 (n=44)	15.5± 12.1		0.95	
	G3 (n=33)	0.23± 0.23		**0.01**		G3 (n=31)	12.9± 10.5		0.35	
Grading (WHO 2004/2016)	Non high grade (n=65)	0.16± 0.14	0.17	ref	nd	Non high grade (n=58)	14± 11.1	0.96	ref	nd
	High grade (n=54)	0.22± 0.21		**0.03**		High grade (n=50)	14.1± 11.1		0.56	
Risk	Low (n=33)	0.13 ±0.11	0.34	ref	**0.02**	Low (n=30)	14.4 ±10.2	0.81	ref	0.25
	Intermediate (n=36)	0.19 ±0.17		0.24		Intermediate (n=31)	14.1 ±11.9		0.57	
	High (n=50)	0.22 ±0.21		**0.02**		High (n=47)	13.7 ±11.2		0.25	
Recurrences	No (n=74)	0.21± 0.19	**0.04**	ref	nd	No (n=67)	14.3± 11.2	0.7	ref	
	Yes (n=45)	0.15 ±0.15		**0.01**[#]		Yes (n=41)	13.5 ±10.9		0.90[#]	

* Wilcoxon Rank Sum or Kruskal-Wallis Rank test

** Generalised linear model adjusted for age and smoking habits (ref = reference group)

*** Trend test adjusted for age and smoking habits

[#] Generalised linear model adjusted for age, smoking habits and therapy

Table 4. Basal DNA damage and nucleotide excision DNA repair capacity affecting overall survival (OS) and event free survival (EFS) in all BC and NMIBC.

All BC	**Overall Survival**			**Event-Free Survival**		
	HR	**95% CI**	**p-value***	**HR**	**95% CI**	**p-value***
DNA damage[#]	**3.7**	**1.3-10.6**	**0.02**	1	0.6-1.7	0.9
DRC [#]	0.8	0.3-2.1	0.6	0.9	0.5-1.6	0.78
NMIBC	**Overall Survival**			**Event-Free Survival**		
	HR	**95% CI**	**p-value****	**HR**	**95% CI**	**p-value****
DNA damage[#]	**4.4**	**1.1-17.3**	**0.03**	0.9	0.5-1.5	0.58
DRC [#]	0.4	0.1-1.5	0.18	0.9	0.5-1.6	0.65

*Adjusted for age, smoking habits, T stage and therapy

**Adjusted for age, smoking habits and therapy

[#] DNA damage and DRC variables were categorized below/upon the median values (reference group: "below")

1