

H2AX phosphorylation and survival in bladder cancer

H2AX phosphorylation level in peripheral blood mononuclear cells as an event-free survival predictor for bladder cancer

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Abstract

Bladder cancer (BC) has a typical aetiology characterized by a multistep carcinogenesis due to environmental exposures, genetic susceptibility and their interaction. Several lines of evidence suggest that DNA repair plays a role in the development and progression of BC. In particular, the study of individual susceptibility to DNA double strand breaks (DSBs) may provide valuable information on BC risk, and help to identify those patients at high-risk of either recurrence or progression of the disease, possibly personalizing both surveillance and treatment.

Among the different DSB markers, the most well-characterized is phosphorylation of the histone H2AX (γ -H2AX). We assessed any potential role of γ -H2AX as a molecular biomarker in a case-control study (146 cases and 146 controls) to identify individuals with increased BC risk and at high-risk of disease recurrence or progression. We investigated γ -H2AX levels in peripheral blood mononuclear cells before and after their exposure to ionizing radiation (IR). We did not find any significant difference among cases and controls. However, we observed a significant association between γ -H2AX basal levels and risk of disease recurrence or progression. In particular, both BC patients as a whole and the subgroup of non-muscle invasive BC (NMIBC) with high basal H2AX phosphorylation levels had a decreased risk of recurrence or progression (for all BC HR 0.70, 95% CI 0.52-0.94, $p=0.02$; for NMIBC HR 0.68, 95% CI 0.50-0.92, $p=0.01$), suggesting a protective effect of basal DSB signalling. Our data suggest that γ -H2AX can be considered as a possible molecular biomarker to identify patients with a higher risk of BC recurrence.

Summary

We analysed γ -H2AX levels in peripheral blood mononuclear cells before and after exposure to ionizing radiation in a hospital-based study on bladder cancer. We observed that γ -H2AX basal levels were significantly associated with risk of disease recurrence or progression.

Introduction

The maintenance of genome integrity is essential for human cells. The DNA damage response (DDR) pathway, which aims to restore the DNA to its original configuration, is a set of biochemical signalling and effector pathways that maintain genomic stability [1]. DNA double strand break (DSB) lesions, i.e. complete breaks of the DNA double helix, may impair DNA replication, transcription or distribution of the genetic material to daughter cells. DSBs are difficult to repair and since broken DNA ends tend to dissociate, unrelated ends may re-join thus allowing for gross loss or amplification of genomic information, as well as chromosomal rearrangements. All these kinds of damage are commonly associated with the early stages of cellular transformation and tumorigenesis [2].

Among the different DNA DSB markers, the most well-characterized is phosphorylation of the histone H2AX (γ -H2AX). Within minutes after the induction of a DSB, several thousands of H2AX molecules are phosphorylated at serine 139 near the site of damage with a crucial role in recruiting and maintaining many factors involved in DNA DSB repair [3]. Nonetheless, γ -H2AX is often used as a marker for exploring the spatial distribution and the DNA repair kinetics of cells following ionizing radiation (IR) exposure and it has also been suggested as a biomarker to predict patient response to specific radiotherapy treatments [4-8]. In the last years, γ -H2AX has become a widely used marker for DSB damage in translational studies, including areas such as carcinogenesis, radiation research, drug development and clinical assessment of DNA targeted anticancer therapies [8-10].

Bladder cancer (BC), which is the fourth most common malignancy among men in the Western world, has a typical aetiology characterized by a multistep carcinogenesis, reflecting that multiple lesions in the DNA are required for tumour development. More specifically, interaction between genetic susceptibility and environmental exposures is crucial. Among the latter, the most relevant risk factors associated with the occurrence of BC are tobacco smoking, occupational exposure to aromatic amines, IR and arsenic-contaminated drinking water [11]. Exposure to these carcinogens causes different types of DNA damage,

including DSBs. For all these reasons, the identification of susceptibility to DSB may provide valuable information about individual cancer risk [12].

Non-muscle invasive BC (NMIBC) is characterized by a high risk of recurrence after endoscopic resection and intravesical therapy, especially within the first two years after diagnosis. Therefore, surveillance with repeated cystoscopies is mandatory to rule out recurrences. Several clinical and pathologic factors have been identified as predictors of tumour recurrence and progression, including stage, grade, size, multiplicity, presence of carcinoma in situ and previous recurrences [13]. However, given the limited accuracy of these traditional prognostic factors, the discovery of novel molecular biomarkers would be most useful in the early detection of patients at higher risk of recurrence. Such an improvement would allow a better sub classification of these patients, avoiding unnecessary cystoscopies in some cases while proceeding to more aggressive therapies in some others, with non-negligible health costs savings. The same goes for muscle-invasive BC (MIBC) patients, where the risk of disease recurrence after radical cystectomy remains relevant [14]. Several biomarkers, including chromosomal markers, genetic variations or epigenetic alterations, have been investigated in terms of recurrence prediction, but the results are still inconclusive [15,16].

In a relatively large ongoing hospital-based case-control study, H2AX phosphorylation has been evaluated as a potential molecular biomarker to identify individuals with increased BC risk or at higher risk of developing BC recurrence or progression. We investigated levels of H2AX phosphorylation before and after exposure to IR in peripheral blood mononuclear cells (PBMCs), the easiest cells to obtain for γ -H2AX assessment *in vivo*. Our data highlight that γ -H2AX is not associated with BC risk as it does not differ among cases and controls. On the other hand, it can be considered as a possible molecular marker to identify patients with higher risk of BC recurrence, both in BC in general and NMIBC patients in particular.

Material and Methods*Study population*

The study population included all newly diagnosed, histologically confirmed cases of BC registered at two urology departments of A.O. U. Città della Salute e della Scienza, formerly S. Giovanni Battista hospital, in Turin (Italy), during the years 1994–2008 [17]. All subjects were men, aged 40–75 years, living in the Turin metropolitan area and were enrolled for a large hospital-based study on BC: the Turin Bladder Cancer Study (TBCS) [11,18]. Before any treatment, a trained interviewer used a detailed questionnaire to conduct a face-to-face interview. Controls were men recruited daily in random fashion from patients treated at the same urology departments for non-neoplastic disease (prostatic hyperplasia, cystitis and others) or from patients treated at the medical and surgical departments for hernias, vasculopathies, diabetes, heart failure, asthma or other benign diseases. Patients with cancer, liver or renal diseases and smoking-related conditions were excluded [19]. Cases and controls were furtherly matched for age and smoking habit. All subjects were informed and provided written consent to participate in the study and to approve the use of their biological samples for the analyses, according to the Helsinki declaration. The design of the study was approved by the local Ethics Committee. Clinical information, including the type of therapy (e.g., Bacillus Calmette-Guerin (BCG), chemotherapy and radiotherapy), was recorded through the perusal of clinical records. Patients treated with one instillation of chemotherapy immediately after transurethral resection were not considered in the chemotherapy group. Patients with BC were followed by urologists with periodic cystoscopic examinations. Clinical end-points (progression and recurrence) were abstracted from clinical and pathology records by medical doctors with a standardized chart. Progression was defined as the transition from non-muscle invasive disease to invasive or metastatic disease, while recurrence was defined as a newly identified bladder tumour after a previous negative follow-up cystoscopy. Vital status was ascertained through linkage with the local demographic office and death certificates were retrieved to identify the specific causes of death.

Lymphocytes isolation and cryopreservation

Ten ml of heparinized venous blood were collected from all subjects. PBMCs were separated by centrifugation with FicollPaque PLUS (GE Healthcare, Milan, Italy) at 400 x g for 30 minutes at room temperature. After two washes in RPMI 1640, 1% fetal bovine serum (FBS), 25 mM EDTA (all from Gibco, Life Technologies, Paisley, UK), PBMCs were prepared for cryopreservation. They were resuspended at 10×10^6 cells/ml in freezing medium (RPMI 1640, 50% FBS, 10% DMSO), aliquoted in cryovials and slowly frozen overnight at the rate of $-1^\circ\text{C}/\text{minute}$ in isopropyl alcohol to -80°C (Mr. Frosty containers, Nalgene, Roskilde, Denmark). Cryovials were then transferred into liquid nitrogen for long-term storage.

Case-control study

Cryopreserved PBMCs from 146 cases (collected before treatment) and 146 controls matched by gender, age (± 1 year) and smoking habits, were selected from the TBCS collection and employed for the phenotypic assay to evaluate H2AX phosphorylation.

H2AX phosphorylation assay

The assay was performed as previously described [20]. Briefly, PBMCs were thawed and allowed to recover overnight in RPMI 1640 (Gibco, Life Technologies) supplemented with 10% heat inactivated FBS (Sigma-Aldrich St Luis, MO, USA), 2mM L-Glutamine, 1% kanamycin, 1% sodium pyruvate, 1% nonessential amino acids, 0.1% β -mercaptoethanol (all from Gibco, Life Technologies). Cells were x-irradiated (2 Gy) using 6 MV opposed beams with a linear accelerator Synergy Platform (Elekta, Stockholm, Sweden). They were kept on ice for 1 h, and then returned to the incubator and harvested at 1 h and 3 h. Approximately 400,000 untreated and irradiated cells were collected, fixed in cold 70% ethanol and stored at -20°C for up to 2 weeks before analysis. Cells were washed in Tris-buffered saline (TBS) pH 7.4 and then rehydrated for 10 min at 4°C in TBS containing 4% FBS and 0.1% Triton X-100 (TST) (Sigma-Aldrich Co) prior to staining with anti- γ H2AX mAb (clone JBW301, Millipore, Billerica, MA, USA) diluted at 1:250 in TST, and incubated for 2 h at 37°C . After two washes in TBS, they were resuspended

in PE-conjugated goat anti-mouse IgG1 (BD PharMingen, Becton Dickinson & Co., Franklin Lakes, NJ, USA) diluted at 1:100 in TST as a secondary antibody, and shaken for 1 h at room temperature in the dark. A minimum of 10,000 stained cells were acquired on a Cyan ADP flow cytometer (Beckman Coulter, Brea, CA, USA) and analysed with Summit 4.3 software. In order to normalize the results, aliquots of PBMCs derived from the same subject were used at each irradiation and staining step as reference control and flow cytometer setting relative to PE-fluorescence were established on the reference control untreated sample. Each experimental point was evaluated in technical triplicate.

For the analysis, the mean of the reference control at each experimental point (untreated, 2 Gy 1 h and 2 Gy 3 h) was calculated considering all the irradiation experiments. Raw data were subsequently normalized to the average signal of the reference control of each specific irradiation (Average normalization procedure). Data corresponding to the amount of γ -H2AX at 1 h were expressed as ratio of median γ -H2AX level at 1 h to the baseline γ -H2AX level of the same non-irradiated sample. Data corresponding to the dephosphorylation of γ -H2AX after 3 h of recovery were expressed for each sample as percentage of median γ -H2AX fluorescence of irradiated cells at 3 h on median γ -H2AX fluorescence at 1 h.

Statistical analyses

The patient characteristics were described as absolute frequencies for qualitative variables, and median, minimum and maximum values for quantitative variables. The analysed variables were: basal damage in untreated cells, H2AX phosphorylation at 1 h (γ -H2AX ratio), and percentage of dephosphorylation after 3 h. Eleven samples encountered technical problems during the assay performance, therefore they were excluded from further analysis together with their relative matched counterpart. Fisher exact test was used to ensure no under/over-representation of cases or death in the excluded samples.

For each of the considered variables we tested whether they were normally distributed by the Shapiro-Wilk test. Since the variables were not normally distributed, non-parametric paired test (two-sided Wilcoxon Rank Sum test) was employed to verify differences between cases and controls.

Overall Survival (OS) in patients was evaluated as the time from diagnosis using the date of death (regardless of the cause) or the date of follow up termination as the end point. Event-free survival (EFS) was defined as the time from diagnosis to the occurrence of any event such as recurrence, progression or death, whichever came first. Investigated variables were considered as continuous. The survival curves for OS and EFS were derived by the Kaplan–Meier (KM) method and statistical significance was determined using the log-rank test (R version 3.0.3, Survival package). The relative risk of death or of recurrence/progression was estimated as hazard ratio (HR) using Cox regression. Multivariate survival analyses were adjusted for matching variables, risk category (based on the six most significant clinical and pathological factors: number of tumours, tumour size, prior recurrence rate, T category, presence of concurrent CIS, and tumour grade (WHO1973) as described in [21,22]), and therapy for the whole group of patients. The investigated variables that resulted significantly associated with OS or EFS were also considered as categorical. Several cut-off values for the categorization of continuous variables were considered, and the one maximizing the area under the ROC curve (AUC) was chosen as the best cut-off. The study population was then divided according to the best cut-off into 2 groups: 1) the “Fast recognition of the damage” group, which included patients with the basal H2AX phosphorylation above the best cut-off value, and the “Slow recognition of the damage” group, which have the basal H2AX phosphorylation under the generated best cut-off value.

To evaluate the improvement in the performance of EFS prediction when including the H2AX assay variables, discrimination analysis comparing two nested models was carried out: the first one included matching variables, risk category, and therapy; the second one included also H2AX assay variables. Discrimination analysis was conducted comparing the area under ROC curve (AUC) of the two nested

models by the De Long test [23]. All the analyses were performed with the open source R (version 3.0.3; *survival package*).

Results

Study population

The study included 146 males newly diagnosed, previously untreated BC patients and 146 control subjects, matched by age, gender and smoking habits. The epidemiological data, tumour histological classification and therapy details for the whole cohort are summarized in **Table I**. For 121 patients tumours were classified as NMIBC (Tis,Ta,T1), while 13 patients were MIBC (>T2). Thirty-one cases had grade G1 cancer, while 56 had G2 and 40 G3. Fifty cases were classified as high risk, 41 as intermediate and 30 as low risk. Most of the patients (69) were not treated with any therapy, whereas part of them were treated with chemotherapy (24 patients) and/or BCG (39 patients).

During H2AX phosphorylation assay, technical problems were encountered for four controls and seven cases. Since no additional aliquots of cryopreserved PBMC were available, the experiments could not be repeated. Therefore, these samples were excluded from the analysis together with their relative matched samples. Finally, 135 matched couples were included in the case-control study and 139 cases were included in the overall and event-free survival analyses.

Case-control study

H2AX phosphorylation assay results are reported in **Table II**. We considered the basal H2AX phosphorylation as the basal individual level of DSB signalling, the H2AX phosphorylation after 1 h irradiation as the peak of DNA damage recognition and percentage of H2AX dephosphorylation after 3 h irradiation as the individual capacity to repair the induced damage. No significant differences were observed between cases and controls (**Table II**). Similarly, among all subgroups stratified for smoking habit, no significant differences were observed between cases and controls. There was only a significant difference in the percentage of H2AX dephosphorylation between cases and controls in smokers ($p=0.05$) (**Supplementary Table I**); however, these data refer a very small population ($n=22$) and for this reason the result has to be considered with caution.

Overall and event-free survival analyses

Results from the survival analysis are reported in **Table III**. There was no association between overall survival in patients and γ -H2AX assay results. On the other hand, when analysing the EFS, there was a significantly decreased risk of recurrence/progression in patients with an increased basal H2AX phosphorylation level both for all BC patients and also when stratified only for NMIBC (HR 0.70, 95% CI 0.52-0.94, $p=0.02$ and HR 0.68, 95% CI 0.50-0.92, $p=0.01$, respectively).

We then repeated the analysis considering the significant variable as categorical (**Table IV**). Instead of separating samples according to the median value, we used the optimal cut-off value based on the AUC (see Methods). Two categories were generated: those individuals with the basal H2AX phosphorylation above the best cut-off value were included in the “Fast recognition of the damage” group, while those under the best cut-off value correspond to the “Slow recognition of the damage” group. A similar association between decreased risk of recurrence and basal H2AX phosphorylation above the calculated best cut-off was observed for all BC and NMIBC subjects: patients with high basal DSB signalling had a better EFS (HR 0.35, 95% CI 0.20-0.63, $p=0.0005$ and HR 0.32, 95% CI 0.17-0.62, $p=0.0006$, respectively).

The results on EFS were confirmed also in the univariate KM analysis for the same model (log rank test p -value=0.004 and 0.002, respectively for all BC and NMIBC, for the basal H2AX phosphorylation; **Figure 1 A, B**). For EFS, the best cut-offs obtained to perform the analyses for the basal H2AX phosphorylation were 6.33 (considering all BC cases) and 6.29 (considering NMIBC cases).

Finally, to evaluate the improvement in the prediction accuracy given by the H2AX assay, we assumed 2 models, including respectively: i) matching variables (age and smoking habit), risk category and therapy as EFS predictors; ii) model i) plus H2AX assay variable resulted associated to EFS, i.e. basal damage in untreated cells. Comparing the AUC of the 2 models we found a statistically significant improvement in discrimination (AUC model 1 = 0.55; AUC model 2 = 0.63, DeLong's test $p = 0.03$).

Discussion

DNA damage pathways have been shown to be implicated in BC risk [24]. To our knowledge there is a lack of studies evaluating the relationship between H2AX phosphorylation levels and the clinical outcome. In the present study we observed a significant association between H2AX basal phosphorylation level and BC recurrence risk. In particular, individuals with higher basal H2AX phosphorylation had a significantly decreased risk of recurrence, suggesting a protective effect of high basal DSB signalling in terms of preventing BC recurrences. One may speculate that individuals expressing high basal levels of γ -H2AX are capable of recruiting crucial DNA repair elements towards areas of DSBs and effectively activating the DNA repair pathway. Therefore, having a high basal H2AX phosphorylation implicates a constitutional activated response system in recognizing the damage, while those individuals having a low basal H2AX phosphorylation are in general slower in recognizing the basal damage. Interestingly, a study on H2AX phosphorylation in non-invasive low grade urothelial carcinoma (LG-URC) of the bladder demonstrated a significant higher recurrence rate in γ -H2AX negative tumour tissues, suggesting a protective effect of γ -H2AX expression in terms of preventing LG-URC recurrences [25]. Surprisingly, we did not observe any association between the 1 h H2AX phosphorylation peak and recurrence risk, although a borderline association was evidenced (for all BC HR 1.67, 95% CI 0.97-2.88, $p=0.06$; for NMIBC HR 1.79, 95% CI 0.88-3.67, $p=0.11$). One possible explanation for this partially missed observation could be that 2 Gy IR activates massive DSB signalling that might mask subtle individual differences.

Additionally, considering the H2AX dephosphorylation capacity after 3 h from IR, individuals with higher dephosphorylation capacity had a decreased risk of recurrence, although not reaching a statistical significance. These findings suggest a protective effect of a rapid response in recognizing (basal H2AX phosphorylation) and repairing (3 h H2AX dephosphorylation) DNA damage, possibly preventing BC recurrence.

Using the measured variables as categorical with a best cut-off analysis strategy, we obtained that the best cut-off to use were different from the median, allowing to hypothesize application of this

newly determined value on other BC populations and to propose the H2AX phosphorylation assay for clinical validation on a large scale. With this approach we obtained a similar result for basal DSB signalling and risk of recurrences. Moreover, in the present study a significant improvement in the sensitivity and specificity of EFS prediction can be achieved when considering basal cell damage as an independent risk factor, thus supporting the role of H2AX assay as a prognostic biomarker.

Interestingly, we obtained analogous results restricting our analysis to NMIBC patients. These data are particularly relevant, considering that NMIBC is the most prevalent BC (75% of all BC) that recurs more frequently and often progresses to muscle-invasive bladder cancer, a deadly disease.

As nearly half of patients with BC experiences recurrence, reliable predictors of this phenotype are needed to guide surveillance and treatment. The identification of γ -H2AX as a new molecular biomarker related to BC recurrence is particularly relevant. A risk assessment calculator that is based on several clinical-pathological parameters is available for NMIBC, but it has been reported to have potential flaws. In the last two decades, great effort has been made to evaluate the prognostic and predictive roles of several molecular markers in MIBC and, even more, in NMIBC, where a precise risk stratification is urgently needed. Several tissue- and blood-based biomarkers have been identified (reviewed in [15,26]). Although none of these biomarkers have reached the clinical validation level, for some of them (e.g., p53, pRb, p21, and survivin) the predictive value has been proved in studies that included a homogeneous patient population treated with standard therapy, and, therefore, they are probably ready for clinical validation on a larger scale and could help clinicians to provide individualized prognosis and allow risk-stratified clinical decision.

DNA repair pathways may be also involved in the pathogenesis, progression, and treatment response of BC [27]. Many studies analysed predictive value of different DDR factors by immunohistochemical tissue analysis, but no data are available on molecular biomarkers in PBMCs to predict recurrence risk. Besides the previously described work that analysed H2AX phosphorylation in BC tissue [25], to the best of our knowledge there are no other studies investigating the role of γ -H2AX in the determination of BC survival and/or clinical outcome. The present study is the first one employing an

assay able to measure not only the peak of DNA damage recognition after irradiation, but also the individual capacity to recover and repair the induced damage. Another study analysed protein expression of MRE11, RAD50, NBS1, ATM, and H2AX by immunohistochemistry in pre-treatment bladder tumour specimens from three different cohorts to evaluate if predictive markers could be identified [28]. Authors demonstrated that patients with increased MRE11 expression levels had longer survival in comparison to patients with lower values. In other studies investigating DNA repair gene expression, decreased levels of *hMSH2* and *hMLH1* mismatch repair genes were associated with high grade bladder tumour recurrence [29]. It must be pointed out that previously published data regarding DNA damage factors related to BC referred mainly to immunohistochemical techniques on bladder tissues, while our results are based on the detection of a basal damage that could affect patient specific BC progression. The importance of patient's basal genetic background and consequently of his damage repair capacity in predicting BC recurrence is highlighted by different studies where genetic variants in DNA repair genes that could modify BC prognosis were identified, including polymorphisms in *XRCC1*, *XRCC4*, *XPF*, *XPB* and *XPC* [30-33].

In BC, it is highly improbable that a single marker can accurately segregate tumours into precise prognostic categories. Therefore, as already shown by different investigators, combination of independent, complementary biomarkers may provide a more accurate prediction of outcome compared with any single markers [34,35]. Future investigations should focus on promising biomarker combinations that encompass a variety of different pathways to increase the predictive value and to improve their possible application for targeted therapy. H2AX phosphorylation could be proposed as another molecular biomarker to be evaluated in recurrence risk analysis.

In the present study, there was no association between overall survival in patients and γ -H2AX assay results. This result may be due to the fact that in the population study only 24 patients deceased with a consequent not enough power to detect a prognostic effect. Moreover, we did not observe any differences between cases and controls using the H2AX phosphorylation assay. In contrast, other case-control studies reported differential γ -H2AX levels in PBMCs as a risk predictor for BC or other

neoplastic diseases, such as lung cancer and oesophageal adenocarcinoma, demonstrating higher IR-induced γ -H2AX levels in cases than in control [36,37]. In particular, a study evaluating γ -H2AX levels on BC cases and controls found that a higher susceptibility to induction of DSBs was associated with an increased risk for BC [38]. This study presented a similar population study size (n=174 of which 140 were males). However, the formation of γ -H2AX foci was assayed on lymphocytes subset in whole blood cultures. The discrepancy of our results with those observed in [38] could be attributed to the different cultures employed (whole blood and cryopreserved lymphocytes) or to inter and intra-individual differences in the relative abundance of the different subpopulations of lymphocytes (as observed by [39]).

However, there are some important differences between previous studies and the present one. First, we used unstimulated PBMCs, whereas in previous studies 72 h PHA-stimulated PBMCs were employed. In this sense, the different results could be ascribed to the different responses in quiescent vs proliferating cells. Second, we detected γ -H2AX by flow cytometry, while in other works a laser scanning cytometer-based immunocytochemical method was used; these two techniques could have a different sensitivity. Finally, controls were recruited by us in a random fashion from patients treated at the same urology department for benign diseases, mainly prostate hyperplasia and cystitis (all newly diagnosed), or from patients treated at the medical and surgical departments for hernias, vasculopathies, diabetes, heart failure, asthma, or other benign diseases. In contrast, in previous studies most of the controls were enrolled during work annual health check-up.

PBMCs have been used as a surrogate tissue in several studies evaluating DNA damage and can be considered as the current standard [39-41]. In addition to its simple collection from widely available blood samples, they provide favourable settings for basal DNA damage measurement before exposure to any mutagenic agent due to their quiescent nature, avoiding interference by DNA damage occurring during S-phase [38].

There is a need to implement the identification of patients at risk for disease recurrence and/or progression. The current guidelines from the European Association of Urology (EAU) stated that

“more work is required to determine the role of molecular markers in improving the predictive accuracy of the currently existing risk tables” [22]. Notably, also in the present study the AUC model including several of the known epidemiology and clinical risk factors showed a quite limited overall prediction power. However, with the addition of the γ -H2AX biomarker, we observed an improvement in the prediction model. We are aware that the model is still limited since it is based on males only and the model presents still some limitations in the prediction power. However, it is important to note that the assay is based on a non-invasive methodology for the patients that could improve if applied on larger groups of patients. BC is almost three times more common in men than in women, therefore it is relatively “easier” to collect males patients. Performing the assay only on men reduced the variability. Moreover, it has been recently demonstrated that women with BC have poorer survival times than men. Some factors associated with a history of cystitis may contribute to or explain the poorer outcome, regardless of treatment and after adjustment for a range of other prognostic variables [42].

In conclusion, our data suggest that the H2AX phosphorylation assay applied on cryopreserved PBMCs can be considered as a possible useful molecular marker to identify BC patients with a higher risk of recurrence or progression, thus deserving clinical validation on a large scale. In small studies testing urine-based markers it has been shown a benefit in terms of survival for screened men with cost-effective consequences when implemented in high-risk populations, e.g. heavy smokers [43,44]. Our results support the inclusion of the DNA H2AX phosphorylation assay in future screening trials focused on high-risk cohorts in order to further assess its prognostic value in BC patients.

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Figure legend

Fig. 1. Kaplan-Meier event-free survival (EFS) curves according to the basal H2AX phosphorylation for all bladder cancer patients (A) and for non-muscle invasive bladder cancer patients (B). Patients were stratified into 2 categories: those with the basal H2AX phosphorylation above the best cut-off value correspond to the “Fast recognition of the damage” group, those under the best cut-off value correspond to the “Slow recognition of the damage” group. MST, median survival time.