

Original Article

Dynamic γ -H2AX response in blood lymphocytes for prediction of radiotherapy induced bladder toxicity in cervical cancer patients

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Background and objectives: Radiotherapy for advanced cervical cancer (CaCx) often results in unintended genitourinary toxicities, notably bladder damage. Predicting such radiation-induced toxicity remains challenging. γ -H2AX, a marker of DNA double-strand breaks (DSBs), offers promise as a predictive biomarker for radiosensitivity. This study aimed to evaluate γ -H2AX foci kinetics in peripheral blood lymphocytes (PBLs) as a surrogate for DNA damage response and a predictor of bladder toxicity in CaCx patients undergoing pelvic radiotherapy.

Methods: In this prospective study, 43 FIGO stage IIIB CaCx patients were enrolled. Stage I (n=31) assessed γ -H2AX induction post-CT simulation (2–6 mGy); Stage II (n=34) evaluated γ -H2AX kinetics across three radiotherapy fractions (FR1, FR13, FR25) during external beam radiotherapy (50 Gy in 25 fractions \pm cisplatin). Blood samples were collected at baseline, 1-, 4-, and 24-h post-irradiation. γ -H2AX foci were quantified via flow cytometry. Bladder toxicity was graded using Radiation Therapy Oncology Group (RTOG) criteria.

Results: CT and radiotherapy both induced significant γ -H2AX foci, peaking at 1 h. Patients without bladder toxicity showed higher foci induction and faster decay (1 \rightarrow 4h: 48.9% vs. 39.4%; 1 \rightarrow 24h: 43.6% vs. 12.8%) across all fractions. Persistent foci at 24 h correlated with increased toxicity risk, indicating deficient DNA repair capacity.

Interpretation and conclusions: γ -H2AX foci kinetics effectively reflect in vivo DNA repair efficiency and predict radiation-induced bladder toxicity. This minimally invasive biomarker may guide personalized radiotherapy, enabling early identification of high-risk patients and potential use of radioprotectors or treatment modifications.

Keywords γ -H2AX; Bladder toxicity; Cancer cervix; Chemoradiotherapy; CT scan; Peripheral blood lymphocytes

Concurrent chemoradiotherapy (CCRT) is the standard treatment for advanced cervical cancer (CaCx), improving survival but often causing significant toxicity to adjacent organs like the bladder, leading to genitourinary toxicity, including haematuria, incontinence, and pain, which can affect up to 80% of patients and compromise treatment adherence and quality of life.^{1–3} Despite advances in imaging and radiation planning, predicting which patients will experience severe toxicity remains difficult.^{4,5} Several clinical studies have examined γ -H2AX as a marker of normal tissue sensitivity to radiotherapy, mainly

in breast, prostate, and head-and-neck cancers, often using single post-treatment time points or small patient groups.^{6–10}

Markers of individual radiosensitivity could allow treatment to be tailored, maximizing effectiveness while reducing side effects. The DNA damage response (DDR) plays a central role in determining cellular radiosensitivity. Inefficient repair of radiation-induced DNA double-strand breaks (DSBs) leads to persistent damage, inflammation, and long-term toxicity of the irradiated tissue. Individual differences in the DDR capacity may explain why patients receiving the

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same treatment have different toxicity outcomes.^{11,12} γ -H2AX, a phosphorylated form of histone H2AX, is a sensitive and reliable marker of DSB and declines as DNA repair progresses, reflecting both damage induction and repair efficiency.^{13–15} Persistent γ -H2AX expression indicate impaired repair and have been associated with increased radiosensitivity in both experimental and clinical studies.¹⁶

While γ -H2AX is widely used in laboratory studies, clinical data on its role as a predictive biomarker for radiotherapy toxicity are limited. Various studies assessed γ -H2AX at single time point or in very small cohorts, and few have linked longitudinal γ -H2AX kinetics in peripheral blood lymphocytes to clinically relevant bladder toxicity during fractionated pelvic radiotherapy.¹⁷ The potential of γ -H2AX as an early predictor of normal tissue radiosensitivity and acute toxicity remains unclear. Emerging evidence suggests that inter-individual differences in γ -H2AX repair kinetics may reflect radiosensitivity, highlighting its potential for tailoring of radiotherapy doses.^{17–19}

This study was designed to determine whether γ -H2AX repair kinetics in patients peripheral blood lymphocytes (PBLs) could serve as an early predictor of bladder toxicity in cervical cancer patients (CaCx) receiving pelvic radiotherapy with or without concurrent cisplatin. Our aim was not to apply γ -H2AX as a biodosimeter for absorbed dose estimation, but rather to evaluate its potential as a biomarker of DNA repair capacity and predict inter-individual radiosensitivity. By correlating γ -H2AX dynamics with clinical outcomes (toxicity), we sought to assess its utility for patient stratification and personalized radiotherapy.

Methods

This prospective exploratory study was undertaken by the radiation Biology Lab, department of Radiation oncology, ACTREC, Navi Mumbai, India after obtaining approval from the hospital Ethics and Scientific Review Committee, comprising two stages (**Supplementary Fig. 1**). The study was conducted between January 2013 and December 2015, during which data on γ -H2AX foci kinetics were acquired. Patients were followed up for bladder toxicity until June 2023.

Patient population: The study population comprised FIGO stage IIIB CaCx patients scheduled to receive radical pelvic radiotherapy using External Beam

Radiation Therapy (EBRT) to a dose of 50 Gy in 25 fractions (2 Gy/fraction). Eligibility required fitness for radical pelvic radiotherapy per institutional guidelines. Exclusion criteria included inability to provide consent, or prior X-ray/CT scans within one week of enrolment. Eligible patients were recruited following written informed consent. Relevant clinical data, including disease staging, CT simulation parameters, and radiotherapy dosimetry, were documented in a secure, access-restricted, anonymised database.

Sample size: The cohort size was determined by feasibility during the study period. A convenience sample of 50 patients was initially planned for enrolment during the study period. Seven patients were deemed screen failures as they withdrew their consent before the start of the study; hence, the blood samples were not taken. Only 43 patients who had given their consent were analysed. Of the 43 patients, the toxicity data of nine patients could not be captured as the patients did not complete the planned treatment. Hence, the final analysis included 34 patients in whom the γ -H2AX foci kinetics were correlated with bladder toxicity.

Study design:

Stage I: Initial γ -H2AX foci assessment in patients undergoing Computed Tomography (CT) simulation for radiotherapy planning

Thirty-one CaCx patients undergoing pelvic CT simulation were enrolled. Blood samples were drawn pre-CT and at 1-, 4-, and 24-h post-CT to assess γ -H2AX foci levels. Residual foci were evaluated at each time point. Blood CT doses (2–6 mGy) were estimated using body mass, scanned volume, and CT slice thickness as described by Prins *et al.*²⁰ The body dose report is generated by most commercial scanners at the end of the CT scanning procedure. This stage assessed whether low-dose CT exposure induced detectable DSBs and whether DSB repair could be monitored.

Stage II: γ -H2AX foci analysis in patients undergoing pelvic radiotherapy

Thirty-four CaCx patients (**Supplementary Fig. 1**) treated with EBRT, with or without weekly cisplatin, were included in the analysis. Patients treated with radiation therapy (RT) alone (n=17) and CT+RT alone (n=17). Blood samples were collected before and 1-h post-2 Gy fraction, followed by samples at 4- and 24-h post-irradiation. Additional samples were taken after 24 Gy and at the completion of 50 Gy. This stage evaluated DSB repair kinetics during CCRT.

Table I. γ -H2AX foci levels before and after CT simulation and radiotherapy (Wilcoxon signed-rank test/ Paired t-test). Association of γ -H2AX Foci with radiation-induced bladder toxicity

| Characteristics | Pre | Post | Difference (95% C.I) | P value |
|-------------------------|-------------|--------------|-----------------------|---------|
| CT | | | | |
| 1HR, median (IQR) | 0.75 (0.71) | 0.94 (1.04) | -0.25 (-0.475, -0.06) | 0.006 |
| 4HR, median (IQR) | 0.9 (1.15) | 1.12 (1.67) | -0.24 (-0.77, -0.005) | 0.044 |
| 24HR, median (IQR) | 1.22 (0.93) | 1.47 (1.63) | -0.21 (-0.62, 0.05) | 0.124 |
| RT | | | | |
| 1HR_FR1, mean (SD) | 0.99 (1.76) | 10.43 (5.74) | -9.44 (-11.62, -7.26) | <0.0001 |
| 4HR_FR1, median (IQR) | 1.01 (0.65) | 5.26 (4.98) | -4.12 (-5.67, -2.95) | <0.0001 |
| 24HR_FR1, median (IQR) | 0.92 (0.69) | 1.24 (1.07) | -0.38 (-0.81, -0.10) | 0.005 |
| 1HR_FR13, median (IQR) | 0.45 (0.50) | 6.31 (5.9) | -5.53 (-7.15, -4.19) | <0.0001 |
| 4HR_FR13, median (IQR) | 1.15 (1.14) | 2.61 (2.99) | -1.76 (-2.91, -1.15) | <0.0001 |
| 24HR_FR13, median (IQR) | 2.0 (11.85) | 2.59 (3.06) | -0.57 (-1.30, 0.005) | 0.053 |
| 1HR_FR25, median (IQR) | 0.61 (0.49) | 5.64 (4.63) | -3.77 (-5.10, -3.04) | <0.0001 |
| 4HR_FR25, median (IQR) | 2.31 (1.61) | 3.67 (2.68) | -1.60 (-2.34, -0.98) | <0.0001 |
| 24HR_FR25, mean (SD) | 3.15 (2.32) | 3.52 (1.84) | -0.37 (-1.05, 0.31) | 0.276 |

IQR, inter quartile range; CI, confidence interval; SD, standard deviation; CT, computed tomography; RT, radiotherapy

CT Simulation protocol: CT simulation (Siemens SOMATOM Sensation 16-slice™) was conducted without contrast, adhering to institutional protocols. Scanning parameters were standardised: slice thickness 5 mm, from L4-L5 to mid-thigh.

Radiotherapy treatment protocol: Patients received EBRT to a dose 50 Gy in 25 fractions over five weeks. High-energy photon beams (linear accelerator, Siemens Primus™) targeted the pelvis (L4-L5 to lower border of the obturator foramen) using two-field or four-field techniques. PTV and OARs (urinary bladder and rectum) were delineated on CT simulation scans. Treatment planning used Varian Eclipse TPST™, with 3D dose distribution calculated for Planning Target Volume (PTV) and Organs at Risk (OARs).

Isolation of PBL for γ -H2AX assay: Venous blood was collected before and 1-h post-CT or post-2 Gy radiation. Samples in EDTA tubes were transported at room temperature. PBLs were isolated using Ficoll-Hypaque density gradient centrifugation. Lymphocytes were washed with PBS and resuspended in 2.5 per cent Haes-Steril in PBS for further analysis.²¹

Estimation of γ H2AX foci in PBLs by flow cytometry: PBLs were fixed in 4% paraformaldehyde, permeabilized for 15 min, and blocked with 3% BSA. Cells were incubated overnight at 4°C with rabbit monoclonal anti- γ -H2AX (phospho-Ser139) antibody [Cell Signaling Technology, (1:200 in PBS + 1% FCS)], then with goat anti-rabbit secondary antibody

(1:400) for 90 min. Cells were washed with PBS + 1% FCS. γ -H2AX phosphorylation was analysed by flow cytometry as per Huang *et al*¹. Quantitative γ -H2AX data were used to assess DSBs.²²

Toxicity assessment: Bladder toxicities were evaluated using radiation therapy oncology group (RTOG) criteria.²³ All grades were used to correlate with γ -H2AX foci decay.

Statistical data analysis: At least 100 PBL nuclei were scored per time point. γ -H2AX foci were summarized as mean (SD) or median (IQR) based on the Shapiro-Wilk normality test. Paired t-test or Wilcoxon signed-rank test with the Hodges–Lehmann estimator for 95% confidence interval was reported to assess differences in foci counts across time points for CTRT and RT-alone groups. Patients with normal repair capacity retained foci within 5% of baseline. A two-sided $P < 0.05$ was considered significant. Analysis was done using IBM SPSS v25 (IBM Corp, Armonk, New York) and R Statistical Software v4.2.0 (R Core Team, 2022).

Results

γ -H2AX foci induction following CT simulation: PBLs were analysed before and after CT simulation to assess DNA DSB formation. A significant increase in γ -H2AX foci was observed at 1-h post-CT. At 4 h post-CT, γ -H2AX foci levels remained elevated. At 24 h post-CT, the increase was not statistically significant, suggesting partial DNA repair within 24 h (**Table I**).

Table II. γ -H2AX foci profiles stratified by bladder toxicity. Foci levels were assessed at 1-, 4-, and 24-h following radiotherapy fractions FR1 (initial), FR13 (mid-treatment), and FR25 (end of treatment). Percentage decay from 1HR to later time points was calculated to assess DNA repair kinetics. Grade 0 = no bladder toxicity; Any Grade = any level of bladder toxicity

| | γ -H2AX Foci Profile | | | % Decrease in γ -H2AX Foci | | |
|--|-----------------------------|----------------------|------------------------|---------------------------------------|-------------|---------------|
| | Time point | Grade 0-Median (IQR) | Any Grade-Median (IQR) | Time interval | Grade 0 (%) | Any Grade (%) |
| Analysis at FR1 (initial response) | | | | | | |
| FR1 | POST_RT_1h | 11.07 (6.46) | 8.83 (4.22) | 1h \rightarrow 4h (Early decay) | 48.9 | 39.4 |
| | POST_RT_4h | 5.66 (3.82) | 5.35 (3.07) | 1h \rightarrow 24h (Overall decay) | 86.0 | 87.1 |
| | POST_RT_24h | 1.55 (1.13) | 1.14 (0.48) | | | |
| Analysis at FR13 (Mid-treatment response) | | | | | | |
| FR13 | POST_RT_1h | 7.22 (6.73) | 4.57 (5.05) | 1 h \rightarrow 4h (Early decay) | 65.4 | 44.6 |
| | POST_RT_4h | 2.50 (3.81) | 2.53 (2.16) | 1 h \rightarrow 24h (Overall decay) | 65.6 | 45.9 |
| | POST_RT_24h | 2.48 (2.85) | 2.47 (4.81) | | | |
| Analysis at FR25 (Late-treatment response) | | | | | | |
| FR25 | POST_RT_1h | 6.01 (2.66) | 4.13 (4.74) | 1h \rightarrow 4h (Early decay) | 32.1 | 27.1 |
| | POST_RT_4h | 4.08 (3.24) | 3.01 (2.65) | 1h \rightarrow 24h (Overall decay) | 43.6 | 12.8 |
| | POST_RT_24h | 3.39 (1.70) | 3.60 (1.84) | | | |

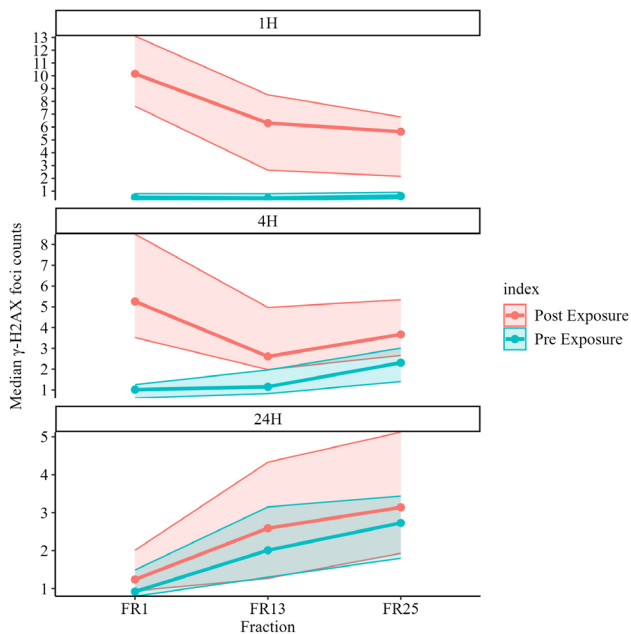


Figure. Median γ -H2AX Foci Counts with Q1-Q3 Confidence Intervals in Peripheral Blood Lymphocytes at 1 h, 4 h, and 24 h Post-Irradiation Across Fractions FR1, FR13, and FR25 in Carcinoma Cervix Patients Undergoing Radiotherapy.

γ -H2AX foci kinetics in patients undergoing radiotherapy:

γ -H2AX foci levels were assessed pre- and post-radiotherapy (RT) (Table II). A significant increase in γ -H2AX foci was observed at 1 h post-RT for FR1, FR13 and FR25, and foci remained elevated at 4 hours. By 24 h, partial resolution was evident in FR1 and FR13, whereas FR25 showed no statistically significant change.

Figure depicts γ -H2AX foci kinetics in carcinoma cervix patients across FR1, FR13 and FR25 at 1-, 4- and 24-h post-RT, with pre-RT γ -H2AX foci levels as baseline. Foci peaked at \sim 1 h post-RT, declined by 4 h, and in FR25 approached near-baseline by 24 h. A progressive decrease in mean γ -H2AX induction across treatment fractions (FR1 \rightarrow FR13 \rightarrow FR25) was observed.

Dynamic γ -H2AX foci expression and decay kinetics and its association with bladder toxicity: Dynamic changes in γ -H2AX foci were evaluated across radiotherapy fractions to compare DNA damage induction and resolution patterns between patients without bladder toxicity (Grade 0) and those with any grade of bladder toxicity. Patients without bladder toxicity showed higher initial γ -H2AX levels and faster clearance, indicating efficient repair. Those with toxicity had lower induction and slower resolution, suggesting impaired repair. **Tables I and II** compare γ -H2AX levels in Grade 0 *versus* any grade bladder toxicity patients at FR1, FR13, and FR25 across 1-, 4-, and 24-h time points.

γ -H2AX foci kinetics and reduction profiles across radiotherapy fractions in patients with and without bladder toxicity:

Analysis of γ -H2AX dynamics at FR1 (Initial Response to first fraction of radiotherapy): At 1h post RT time point, patients without toxicity (Grade 0) consistently displayed higher γ -H2AX levels than those with any grade of toxicity, indicating a faster and more

active repair process. Over subsequent time points, a progressive decline in γ -H2AX foci was observed in both groups.

Rate of γ -H2AX reduction over time further highlights repair differences. Differences in the temporal reduction pattern between the groups were evident during the early post-irradiation interval. Early clearance of γ -H2AX level (1–4 h post-RT) was faster in the non-toxicity group, reflecting efficient repair, whereas slower reduction in the toxicity group indicates a delay in resolving DNA double-strand breaks. Although both groups showed substantial overall decay by 24 h, the early kinetics suggest that delayed repair in susceptible patients may contribute to later toxicity (**Table II**).

Analysis of γ -H2AX dynamics at FR13 (Mid-Treatment Response): At mid-treatment, overall γ -H2AX foci levels at all post-irradiation time points were lower than those observed at the initial fraction. Nevertheless, the Grade 0 patients maintained higher levels of γ -H2AX levels at the early time point compared with patients with toxicity reflecting preserved damage response.

Differences in repair rates were more informative than absolute foci count, highlighting persistent impairment in DNA repair capacity among patients prone to toxicity. These data suggest that patients without toxicity retain a stronger repair capacity, whereas those with toxicity exhibit slower repair and may accumulate unresolved damage over time (**Table II**).

Analysis of γ -H2AX dynamics at FR25 (late/end of treatment response): At FR25, measured γ -H2AX levels were further reduced across all post-irradiation time points relative to earlier fractions, which may reflect an attenuated DDR rather than a reduction in DNA damage. Grade 0 still showed higher levels at 1HR compared to the toxicity group. Post FR25 kinetics, patients who developed toxicity retained higher residual γ -H2AX levels compared to the patient without toxicity who demonstrated relatively faster resolution of γ -H2AX levels.

Across all fractions, γ -H2AX foci demonstrated a time-dependent decline following irradiation in both patient groups. The magnitude and temporal pattern of γ -H2AX reduction differed consistently between patients without bladder toxicity and those with toxicity, with fraction-dependent divergence in early and late post-irradiation intervals. These differences became more pronounced at mid- and late-treatment fractions (**Table II**).

Discussion

This study demonstrates that γ -H2AX foci kinetics in PBLs provide a sensitive indicator of DDR and predict bladder toxicity in CaCx patients undergoing Radiation Therapy. Patients without toxicity exhibited robust induction and rapid clearance of γ -H2AX, reflecting efficient DDR mechanism, whereas those with toxicity showed lower early γ -H2AX induction and delayed repair followed by accumulation of residual foci, indicative of impaired repair capacity (**Supplementary Fig. 2**). These patterns persisted from fraction 1 (FR1) and became more pronounced by fraction 13 (FR13) and fraction 25 (FR25). By (FR25), residual γ -H2AX remained comparatively higher in the toxicity group, indicating persistent DNA damage and increased radiosensitivity.

A gradual reduction in mean γ -H2AX induction across fractions (FR1 \rightarrow FR13 \rightarrow FR25) was observed. This reduction may reflect an adaptive response of circulating lymphocytes to repeated radiation exposure, leading to altered DDR signalling or stress responses over time, resulting in reduced γ -H2AX induction over time. Similar trends in γ -H2AX kinetics during fractionated radiotherapy have been reported previously¹⁰, and repair kinetics have been linked to normal-tissue toxicity risk.²⁴ Mechanistic studies on the ATM/ γ -H2AX signalling axis support the biological plausibility of such responses^{17,25,26}, and altered H2AX phosphorylation after repeated or fractionated irradiation has been demonstrated in preclinical models.²⁷ However, adaptive responses vary widely depending on dose, fractionation, assay methodology, and cell type, and systematic reviews highlight substantial heterogeneity across models and clinical settings.^{28,29} Therefore, this interpretation should be viewed as hypothesis-generating rather than definitive. Nonetheless, the observed kinetics support further evaluation of γ -H2AX foci as a potential biomarker of radiosensitivity for personalized radiotherapy.^{8,10,24}

The pattern of γ -H2AX induction and resolution provides insight into individual DNA repair capacity. Rapid early γ -H2AX induction followed by timely decline reflects effective activation and resolution of DNA DSBs, whereas reduced early induction or delayed clearance indicates inefficient repair and persistence of damage.^{30–37} Persistent γ -H2AX foci have been consistently associated with increased radiosensitivity and a higher risk of normal-tissue toxicity.^{8,24,38} Across treatment, patients without bladder toxicity consistently showed more efficient γ -H2AX resolution, whereas toxicity-prone patients

शोध-संदेश

इस अध्ययन में यह देखा गया की गर्भावस्था की ग्रीवा के कैंसर (cervical cancer) के मरीजों में रेडियोथेरेपी के कारण मूलाशय को होने वाले नुकसान की पहले से पहचान कैसे की जा सकती है। रेडियोथेरेपी से डीएनए को होने वाली क्षति को मापने के लिए γ -H2AX नामक बायोमार्कर का उपयोग किया गया। शोध से पता चला कि रक्त की कोशिकाओं में γ -H2AX की गतिविधि शरीर की डीएनए मरम्मत क्षमता को दर्शाती है और इससे यह अनुमान लगाया जा सकता है कि मरीज में मूलाशय से जुड़ी रेडियेशन विषाक्तता होगी या नहीं। यह तरीका सरल और कम नुकसानदायक है, जिससे उच्च जोखिम वाले मरीजों की जल्दी पहचान कर उनके उपचार में समय रहते बदलाव किया जा सकता है।

demonstrated delayed clearance and greater persistence of residual foci, indicating sustained impairment in DNA repair capacity. Supporting the use of γ -H2AX kinetics as a measure of inter-individual repair capacity and radiosensitivity (**Supplementary Table**). These differences were detectable early and remained evident with continued fractionation, supporting the presence of a stable, patient-specific repair phenotype rather than a transient treatment-related effect.^{10,39,40} By later fractions, overall γ -H2AX induction was lower, likely reflecting reduced signalling efficiency under repeated radiation exposure rather than a decrease in DNA damage itself.^{8,41,42} Despite this overall attenuation, patients who developed bladder toxicity retained higher residual γ -H2AX foci at later time points, consistent with impaired long-term repair and accumulation of unresolved DNA damage.^{6,8,9,41} Residual γ -H2AX foci are recognized markers of unrepaired DNA lesions and have been linked to radiation sensitivity and treatment-related side effects.^{9,43} The inability to effectively clear γ -H2AX foci therefore suggests ongoing genomic instability that may contribute directly to bladder toxicity. Although interpretation at high cumulative doses may be influenced by assay dynamic-range limitations or signal saturation^{13,37} the persistence of elevated residual γ -H2AX foci in patients who developed toxicity supports a biological basis for these associations rather than an assay-related artifact, consistent with prior clinical evidence.^{6,9,24,41–43}

Strengths of the study include its prospective design with repeated sampling at clinically relevant time points and standardized toxicity scoring. Use of PBLs provides a minimally invasive surrogate for systemic DDR that is feasible for clinical workflows.^{13,37} Limitations include the relatively small, single-centre cohort, reliance on a single biomarker, and technical variability in foci quantification. Assay saturation at high dose also remains a constraint, underscoring the need for harmonized protocols and multicentre validation.

γ -H2AX assay is minimally invasive, requiring only a small blood sample (~2 mL), and results can be

obtained within 24 h using flow cytometry, enabling early risk assessment and longitudinal monitoring.⁴⁴ Supporting evidence indicates that slower γ -H2AX clearance or persistent foci correlate with higher risk of late toxicity, similar to observations from the validated RILA assay in breast cancer.^{8,24} This approach aligns with international initiatives (RENEB, IAEA) to standardize molecular biodosimetry.^{7,45} Establishing clinical utility will require multicentre validation with standardized protocols, integration with DVH, NTCP models, and genomic predictors, and inclusion of long-term and patient-reported outcomes to support personalised radiotherapy, along with assay standardization, potentially incorporating AI-based scoring.^{46–49}

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