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*Article*

# Radiation-Induced Lymphocyte Apoptosis and Chromosomic Aberrations for Prediction of Toxicities in Patients Treated by Hypofractionated Radiotherapy for Breast or Prostate Cancers

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**Abstract: Background:** In trials using normofractionated regimen, the radiation-induced lymphocyte apoptosis (RILA) and the chromosomal damage assays (CDA) have shown prognostic roles of radiation-induced adverse events. The main objectives here were to validate RILA and CDA in extreme and moderate hypofractionation regimens for breast (FAST) and prostate (CHHiP) cancers. **Methods:** Blood samples were collected from 400 volunteers included in FAST and CHHiP trials. The primary endpoints (PE) were first change in photographic breast appearance and first grade ≥2 RTOG bladder or bowel toxicity (BBT) in FAST and CHHiP, respectively. The secondary endpoints was first grade ≥2 breast clinical changes (BCC) in FAST and BBT using different scales in CHHiP. **Results:** 103 FAST and 297 CHHiP patients with lab and clinical data were included. In FAST trial, no significant association of RILA with the primary endpoint was observed. A significant association of higher RILA levels with lower risk of any RIAE was found. The risk of developing grade ≥2 RIAE decreased significantly for patients with RILA≥24% compared to those with RILA≤16% with a HR of 0.50 (95%CI 0.25-1.00, p=0.012). Concerning chromosomic aberrations, no significant associations were found with change in photographic breast appearance nor with the secondary endpoint of any RIAE. In CHHiP trial, a decreased risk of grade≥2 RTOG bladder or bowel RIAE was observed for increasing values of RILA (HR 0.97, 95%CI 0.94-1.01, p=0.11) but did not reach statistical significance. Concerning chromosomic aberrations, we found significant association of higher levels of micronuclei per cell with lower risk of gr2+ RTOGAE (p=0.021 for trend test across tertiles; p=0.023 for upper vs lower tertile). **Conclusions:** This study is the first to evaluate RILA and CDA as predictors of late effects after breast and prostate RT in the "hypofractionation" era. Development of toxicity biomarkers whatever the RT fractionation are urgently needed.

**Keywords:** apoptosis; lymphocyte; chromosomic aberrations; radiotherapy; breast fibrosis; pelvic toxicities

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## 1. Introduction

Severe but also moderate toxicities after curative-intent radiotherapy (RT) following breast and prostate cancers can have a negative impact on quality of life, including psychological outcome [1–3]. The risk of severe radiation-induced side effects is low [4] but because of the large number of breast and prostate cancers treated annually by RT worldwide [5] and the high efficacy of treatment, the cumulative number of survivors with radiation sequela is increasing year on year [6].

A number of factors are known to increase the risk of radiation toxicity including individual radiosensitivity [4]. While clinical toxicity risks for populations of patients are known, the determination of an individual's normal tissue radiosensitivity is seldom possible before treatment. Therefore, current practice standards commonly prescribe radiation dose according to clinical scenarios, without regard to the genotype or cellular phenotype of the individual being irradiated.

In that context, we have developed prospectively a rapid (72 h) radiosensitivity assay based on flow cytometric assessment of radiation-induced CD8 T-lymphocyte apoptosis (RILA) [7–10]. An excellent negative predictive value was found in case of high RILA value and low late toxicity after breast [9] and prostate RT [10]. The majority of moderate or severe side-effects (grade ≥2) were observed in patients with low values of RILA.

In addition, the metaphase chromosome damage assay (dicentric assay) in lymphocytes has been used for several decades as the gold standard for radiation biodosimetry. More recently, an association between chromosomal aberrations and grade 2-3 toxicities have been reported in small retrospective cohorts of breast and head and neck patients [11–13].

The two main objectives of the present translational study (called Trans-FAST/CHHiP) were i) to validate an apoptosis assay identifying patients at low risk of adverse effects (AE) and ii) to validate a chromosomal damage assay identifying groups at high risk of AE in patients in two published randomised trials evaluating extreme and moderate hypofractionation regimen for early breast (FAST) and prostate (CHHiP) cancer, respectively [14,15].

## 2. Materials and Methods

### *2.1. Selection of Patients in the Prospective Clinical Studies*

Blood samples were collected from 400 volunteers attending annual follow up appointments specified in FAST [14] and CHHiP [15] trial protocols. Eligible patients included all trial participants attending The Royal Marsden NHS Foundation Trust, Sutton, UK, for annual follow up post-randomisation who offered written informed consent to a blood sample for the stated research.

CHHiP and FAST trials tested the hypothesis that fewer, larger radiotherapy doses (fractions) than delivered by standard regimens was non-inferior in terms of local control or late adverse effects, respectively. Both trials collected prospective clinician scores of late adverse effects. In addition, breast photographs were registered in FAST protocol and patient self-assessed scores were collected in CHHiP trial.

Briefly, FAST (ISRCTN62488883) was a prospective randomized clinical trial that compared whole breast radiotherapy using 50 Gy in 25 fractions in 5 weeks with 30 Gy or 28.5 Gy in 5 once-weekly fractions of 6 or 5.7 Gy. The primary endpoint was change in photographic breast appearance at 2 and 5 years; secondary end points were physician assessments of normal tissue events and local tumour control [14].

CHHiP (ISRCTN97182923) was a randomized, phase 3, non-inferiority trial that recruited men with localised prostate cancer and compared conventional (74 Gy delivered in 37 fractions over 7.4 weeks with one of two hypofractionated schedules (60 Gy in 20 fractions over 4 weeks or 57 Gy in 19 fractions over 3.8 weeks). The primary endpoint was time to biochemical or clinical failure; secondary endpoints incuded both physician and patient reported toxicities assessed annually to 5 years [2,15,16].

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*2.2. Radiation-Induced CD8 T-Lymphocyte Apoptosis (RILA) Procedure*

After obtaining informed consent, all blood samples were sent on Monday s at room temperature for the RILA procedure from the Institute of Cancer Research, London to the Institute of Cancer Research of Montpellier (IRCM) under the Human tissue transfer agreement #IE-2012-621 delivered by the French Ministry of Research. RILA was performed in the Montpellier radiobiology laboratory (IRCM, INSERM U1194, Montpellier, France) on Tuesdays (one night flight) and results were concealed from the clinicians until final statistical analyses.

The protocol was adapted from our previous studies [7]. Briefly, one blood sample was collected from each patient in a 5-ml heparinized tube. 200 µl of blood was aliquoted into a 6-well plate. All tests were carried out in triplicate for both 0 and 8 Gy. Irradiations (single dose of 8 Gy in a 25 cm × 25 cm field size at a dose rate of 1 Gy/min) were delivered after 24 hours (H24) using a linear accelerator (2100 EX, 200 UM/min, Varian, US) in the Radiation Department. Control cells were removed from the incubator and placed for the same period of time under the Linac but without radiation treatment. After irradiation, the flasks were immediately incubated at 37°C (5% $CO_2$). After a further forty-eight hours (H72), it was labelled with anti-human CD8-FITC antibody (10µL/tests, Becton Dickinson, USA). After addition of lysis buffer (Becton Dickinson, USA), propidium iodide (Sigma, France) and RNAse (Qiagen, France) was added to each tube and prepared for flow cytometry (FACS). FACS (Gallios Flow Cytometer, Beckman Coulter) was used to define cells as apoptotic based on reduced PI staining, and RILA score calculated as the difference in percentage of apoptotic cells between irradiated and non-irradiated control.

*2.3. Chromosome and DNA Damage Foci Assays Protocol*

All patients gave written informed consent to the study. 10 ml blood samples were sent by express courier to the UKHSA at Chilton for analysis of the primary biomarkers of interest (total aberrations/cell and foci/cell 4 Gy at 24h) and the secondary biomarkers of interest (dicentrics/cell, foci/cell 0.5Gy at 30 min, ratio of 4Gy/0.5Gy, micronuclei, nuclear division index). The detailed procedures for all these endpoints are described elsewhere [17]. Briefly, blood samples were aliquoted for the gamma-H2AX foci (FCA), dicentric (DCA) and micronucleus (MNA) assays. For the gamma-H2AX assay, whole blood was separated on histopaque and isolated lymphocytes suspended in medium prior to irradiation. Separated lymphocytes and whole blood samples were exposed to 0.5 and 4 Gy (FCA) and 6 and 2 Gy (DCA, MNA), respectively, of 250 kVP X-rays at 0.5 Gy/min at 37°C. Sham-irradiated controls were included for the FCA and DCA, but not for the MNA, due to the limited amount of blood available.

The 0.5 Gy lymphocyte samples were incubated for 0.5 h and the 4 Gy ones for 24 h to determine the initial and residual numbers of radiation-induced foci. Subsequently, the lymphocytes were spotted onto electrostatically charged slides, fixed with formaldehyde, permeabilised with Triton X-100, blocked with bovine serum albumin and immunostained for gamma-H2AX and 53BP1 using fluorophore-conjugated secondary antibodies. Fifty cells per sample were scored manually by one person (with the exception of 8 donors), for colocalising gamma-H2AX and 53BP1 foci [18].

For the DCA, irradiated whole blood samples were mixed with growth medium, incubated in the presence of Colcemid for 70h, then metaphases harvested using hypotonic treatment and methanol/acetic acid. For the MNA, blood/medium mixes were cultured for 72h, with cytochalasin B added at 24h. Potassium chloride-treated and methanol/acetic acid-fixed samples were dropped onto slides. Fifty Giemsa-stained metaphases were manually scored per patient for the DCA by three scorers. MNA slides were stained with DAPI and at least 500 binucleated cells analysed using the automated Metafer system with MNscore software (Metasystems, Altlussheim, Germany). The MNA was performed with and without a cut-off for cells containing >4 micronuclei. The nuclear division index was also determined, based on the number of mono- and binucleated cells.

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*2.4. Objectives and End-Points*

The aim of the Trans-FAST/Trans-CHHiP study was to validate the correlation of two blood lymphocyte assays following in vitro irradiation with late effects after curative-intent radiotherapy of breast and prostate: (i) the RILA assay which reported patients were at risk of developing radiation-induced late effects when RILA is low [7] and (ii) the chromosomal damage assay that showed a correlation between late adverse events and lethal chromosome aberrations [12].

For Trans-FAST study, the primary endpoint was the time from start of radiotherapy to date of first change in photographic breast appearance graded as 'mild' or 'marked' on a 3-point graded scale including 'no change'. Photographic assessments were made at two and five years from randomization and compared with a baseline photograph taken post-surgery but pre-radiotherapy. Patients with no 'mild' or 'marked' change were censored at date of last photographic assessment or date of death.

The secondary endpoint was the time from start of radiotherapy to date of first grade ≥2 (moderate or marked) breast shrinkage, induration (on central axis), telangiectasia or breast oedema. In addition, individual adverse events were analysed as separate secondary endpoints if there were sufficient events. Clinicians assessed late adverse events annually from randomisation using a 4-point scale: none, a little, quite a bit, very much (interpreted as none, mild, moderate, marked). Patients with no event were censored at date of last clinical assessment or date of death.

For Trans-CHHiP study, the primary endpoint was time from start of radiotherapy to date of first grade ≥2 RTOG bladder or bowel toxicity. The secondary endpoints were clinician-assessed late bowel and bladder toxicities using the RTOG, RMH and LENTSOM scales. Clinicians assessed late toxicity at 6, 12, 18, 24, 36, 48 and 60 months from randomisation. Patients with no event were censored at date of last clinical assessment or date of death.

*2.5. Sample Size Calculation*

At the time of the study initiation, 869 FAST and 2275 CHHiP patients were on follow-up in all recruiting centres in total, 80% of whom were expected to be alive with minimum 5-year follow-up at the time of the first expected correlative analysis in 2014. Only patients from RMH were eligible for participation in the Trans-FAST-CHHiP study.

For RILA, an estimated hazard ratio of approximately 4 for a grade 2-3 long term effects between low and intermediate/high apoptosis patients has been assumed. In the first prospective trial [7], we observed a rate of 76% for long term toxicity of grade 2/3 in patients with RILA score ≤16% compared with a rate of 22% in patients with a score of >16%. For the purpose of the current study, these were converted into a relative event rate of 5.7 [log(1-0.76)/log(1-0.22)], but a conservative estimate of 4 was more realistic to allow for overestimation and potential differences.

For chromosomal damage, Hoeller et al. observed that in patients with low/intermediate sensitivity to chromosomal damage of lymphocytes, the proportion of patients who remained free of grade 2 or 3 fibrosis at three years was 96.5% compared with 88% in patients with high sensitivity, a relative risk of 3.6 [12]. The extrapolated rates at 10 years were 85% and 70%, respectively.

Based on interim FAST results, the cumulative incidence of physician-assessed breast shrinkage was 13% at 5 years and assuming that 20% of patients were in the hypersensitive group, 13% of 200 patients included in Trans-FAST would contribute approximately 26 events.

Based on CHHiP results, late onset bladder and bowel toxicities each affected around 15% of patients at 2 years. Assuming again that 20% of patients were in the hypersensitive group, 15% of 200 patients included in Trans-CHHiP would contribute approximately 30 events.

In total, approximately 56 events were expected and analyses were planned to be stratified by disease (breast/prostate) and sought to detect an overall hazard ratio of 3 for each endpoint. With 56 events, it was thought to be possible to detect a hazard ratio of 3 with more than 90% power.

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*2.6. Statistical Analysis*

FAST and CHHiP data were analysed separately. Each biomarker was categorised into tertiles (T1, T2, T3) defined from within the FAST and CHHiP datasets separately (i.e., different cut-offs for FAST and CHHiP patients). Apoptosis was also categorised as <16%, 16-24% and >24% as defined in the princeps study [7]. All biomarkers were also modelled as continuous variables in the Cox models described below. Distributions of each biomarker were summarised using descriptive statistics.

The correlation between radiation-induced lymphocyte apoptosis (CD8 8Gy – CD8 0Gy) and the ratio of foci per cell 4 Gy at 24 hours / 0.5 Gy at 30 minutes was assessed using Spearman's rank correlation coefficient. ROC curves were plotted to identify whether there was an optimal cut-off for lymphocyte apoptosis for the FAST and CHHiP primary and secondary endpoints. Positive and negative predictive values were calculated using the cut-off of >16% for apoptosis for FAST and CHHiP primary and secondary endpoints.

Associations between the biomarkers and adverse event endpoints were assessed using survival analysis methods. Kaplan-Meier estimates of cumulative incidence event-free at specific time points were obtained (with 95%CI) according to biomarker categories, and Kaplan-Meier plots presented the data graphically. Kaplan-Meier estimates of cumulative incidence event-free were calculated at 5 years 3 months, to allow for follow-up assessments occurring after due date. Hazard ratios (with 95%CI) were obtained from Cox proportional hazards (PH) regression models, with biomarkers modelled as continuous variables as well as within categories. Secondary Cox PH regression analyses for the primary adverse event endpoints adjusted for risk factors associated with adverse events (different factors according to tumour type: age, breast size, surgical deficit, randomised treatment, hormonal therapy for FAST patients and age, randomised radiotherapy schedule, haemorrhoids at baseline within the last 12 months, use of IGRT, previous pelvic surgery, baseline bowel and bladder symptoms for CHHiP patients). Use of hormones was not included in the multivariable regression models for CHHiP patients as only one patient did not receive hormones in the dataset. The adjusted analyses were only run for the Cox models including biomarkers as continuous variables, for simplicity. The log-rank test was used to assess statistical significance of the biomarkers in survival analyses (log-rank test for trend for ordered categorical variables). *P* value of 0.025 was used as a cut-off for statistical significance for the primary adverse event endpoints and 0.01 for secondary adverse event endpoints, as specified in the statistical analysis plan. All analyses were done using Stata v16.1. Snapshots of the FAST trial clinical and photographic assessments databases were taken on 17/7/2018 and 02/03/2017 respectively; median follow-up was 9.9 years (interquartile range, IQR, 8.3-10.1 years). CHHiP analyses use a database snapshot taken on 9/10/2019; median follow-up was 9.2 years (IQR 9.2-9.3). Note that late adverse effects were assessed up to 5 years in the CHHiP trial.

## 3. Results

The final Trans-FAST/CHHiP dataset included 103 FAST trial patients and 297 CHHiP trial patients with lab data and data for at least one adverse event endpoint. Denominators for individual analyses varied due to missing data for biomarkers and for primary and secondary endpoints. Baseline demographic and clinical characteristics and treatment details for patients used in the analysis are described in **Table 1**.

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**Table 1.** Baseline demographic and clinical characteristics and treatment details for (A) FAST and (B) CHHiP patients used in the analysis.

**(A).**

| | FAST (N=103) | |
|---|---|---|
| Age | No. | |
| Median (IQR), range | 63 (58, 68), 50, 79 | |
| | No. | % |
| Fractionation | | |
| 50Gy/25f | 30 | 29 |
| 30Gy/5f | 36 | 35 |
| 28.5/5f | 37 | 36 |
| Histology | | |
| Ductal | 75 | 73 |
| Lobular | 17 | 17 |
| Mixed | 7 | 7 |
| Special type | 4 | 3 |
| Tumour grade | | |
| 1 | 29 | 28 |
| 2 | 66 | 64 |
| 3 | 8 | 8 |
| Pathological tumour size (cm) | | |
| <1 | 22 | 21 |
| 1-2 | 60 | 58 |
| ≥2 | 21 | 20 |
| Axillary surgery | | |
| Axillary clearance | 41 | 40 |
| Axillary sampling | 39 | 38 |
| SNB | 15 | 15 |
| SNB & axillary sampling | 5 | 5 |
| Other | 3 | 3 |
| Adjuvant therapy | | |
| None | 10 | 10 |
| Tamoxifen | 74 | 72 |
| AI | 16 | 15 |
| Tamoxifen → AI | 3 | 3 |

f: fraction; SNB: Sentinel node biopsy; AI; Aromatase inhibitor.

**(B).**

| | CHHiP | (N=297) |
|---|---|---|
| Age | No. | |
| Median (IQR), range | 70 (65, 74), 53, 83 | |
| | No. | % |
| Fractionation | | |
| 74Gy/37f | 103 | 35 |
| 60Gy/20f | 96 | 32 |
| 57Gy/19f | 98 | 33 |
| NCCN risk group | | |

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| | | |
|---|---|---|
| Low Risk | 48 | 16 |
| Intermediate Risk | 231 | 78 |
| High Risk | 18 | 6 |
| Gleason score | | |
| ≤6 | 99 | 33 |
| 7 | 191 | 64 |
| 8 | 7 | 2 |
| Clinical T stage | | |
| T1a/T1b/T1c/T1x | 138 | 46 |
| T2a/T2b/T2c/T2x | 133 | 45 |
| T3a/T3x | 26 | 9 |
| Pre-hormone PSA (ng/ml) | | |
| Median (IQR), range | 10.9 (7.6, 15.2), 0.3, 29 | |
| Mean (SD) | 11.9 (5.4) | |
| Intended hormone therapy | | |
| LHRH+ short term AA | 273 | 92 |
| 150 mg Bicalutamide | 20 | 7 |
| MAB | 2 | 1 |
| None | 1 | <1 |
| Duration of hormone therapy (weeks) | | |
| Median (IQR) | 22 (18, 26) | |
| Time from start of hormone therapy to radiotherapy (weeks) | | |
| Median (IQR) | 15 (13, 18) | |

f: fraction; LHRH: Luteinizing Hormone Releasing Hormone;. *AA; Anti-androgen.*

### *3.1. Distributions of Biomarkers within FAST and CHHiP Patients*

Median RILA were 18.18 (IQR 12.13-23.65) and 18.25 (IQR 13.64-24.64) with no statistical difference between the FAST and CHHiP trials, respectively. In addition, median values of foci/cell 0.5 Gy at 30 min and nuclear division index were similar in both trials. All other chromosomal biomarkers' median values (total aberrations/cell, foci/cell 4 Gy at 24h, dicentrics/cell, ratio of 4 Gy/0.5 Gy and micronuclei) were statistically significantly different between FAST and CHHiP patients (details are presented in **Table 2**).

**Table 2.** Summary statistics for biomarkers according to trial.

| Biomarker | FAST | | | CHHiP | | | Comparison between FAST and CHHiP; *P*-value* |
|---|---|---|---|---|---|---|---|
| | N | Median (IQR) | Range | N | Median (IQR) | Range | |
| **Primary biomarkers of interest** | | | | | | | |
| Lymphocyte apoptosis | 100 | 18.18 (12.13-23.65) | 6.40-50.34 | 296 | 18.25 (13.64-24.64) | 4.55-61.87 | 0.642 |
| *Total aberrations/cell* | *103* | *6.38 (5.90-6.80)* | *4.78-7.86* | *297* | *5.86 (5.36-6.28)* | *4.12-8.40* | *<0.001* |
| *Foci/cell 4 Gy at 24h* | *98* | *9.70 (9.10-10.20)* | *7.20-12.30* | *275* | *10.00 (9.30-10.60)* | *7.60-12.80* | *0.015* |
| **Secondary biomarkers of interest** | | | | | | | |
| *Dicentrics/cell* | *103* | *3.16 (2.86-3.50)* | *1.86-3.88* | *297* | *2.86 (2.50-3.22)* | *1.94-4.28* | *<0.001* |
| Foci/cell 0.5 Gy at 30min | 93 | 8.40 (8.00-8.80) | 6.60-10.60 | 258 | 8.40 (7.90-8.90) | 5.90-10.30 | 0.900 |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Ratio of 4 Gy / 0.5 Gy* | *90* | *1.16 (1.11-1.24)* | *0.81-1.54* | *253* | *1.22 (1.12-1.29)* | *0.89-1.58* | *0.037* |
| *Micronuclei no cut-off* | *99* | *0.49 (0.23-1.08)* | *0.05-2.64* | *293* | *0.29 (0.20-0.57)* | *0.06-2.28* | *<0.001* |
| *Micronuclei >4 cut-off* | *99* | *0.34 (0.20-0.46)* | *0.05-0.73* | *293* | *0.24 (0.18-0.33)* | *0.06-0.67* | *<0.001* |
| Nuclear division index | 98 | 1.14 (1.10-1.20) | 1.04-1.40 | 285 | 1.15 (1.11-1.20) | 1.04-1.49 | 0.100 |

* Mann-Whitney test.

No correlation between RILA and the ratio of foci per cell 4 Gy at 24 hours / 0.5 Gy at 30 minutes was observed neither in FAST (n=87, r = -0.07, *P* = 0.51) nor in CHHiP (n=252, r = 0.08, *P* = 0.23).

*3.2. Associations between Biomarkers and Clinical Endpoints*

In FAST trial, no significant association of RILA with the primary endpoint of mild/marked change in photographic breast appearance was observed (**Figure 1** and Supplementary **Table S1**). A significant association of higher RILA levels with lower risk of any moderate/marked (grade ≥2) clinical adverse event (breast shrinkage, induration, telangiectasia, breast oedema) was found, see **Figure 2**. In total, 60 grade≥2 adverse events (gr2+AE) were observed out of 100 patients with available biological data and long-term follow-up (60%). There was an inverse relationship between the incidence of gr2+AE and RILA, with decreasing risk of gr2+AE for increasing values of RILA (HR, 0.96, 95%CI 0.93-1.00, p=0.027). The relative risk of developing gr2+AE decreased significantly for patients with RILA≥24% with a HR of 0.50 (95% confidence interval (CI), 0.25-1.00; *P* = 0.012). Details are presented in **Supplementary Table S2**. The corresponding Kaplan-Meier 5-year cumulative incidence event-free estimates (5y-EFS) were 43.2% (95%CI, 27.2-58.3) for patients in the sub-group of RILA<16% and 79.2% (95%CI, 57.0-90.7) for those in the sub-group of RILA≥24%. 5y-EFS were quite similar when sub-groups were divided by statistical RILA tertiles: 47.1% (95%CI, 29.8-62.5) for patients in the sub-group of RILA<15.63% and 72.6% (95%CI, 53.9-84.7) for those in the sub-group of RILA>21.68% (**Figure 2A,B**).

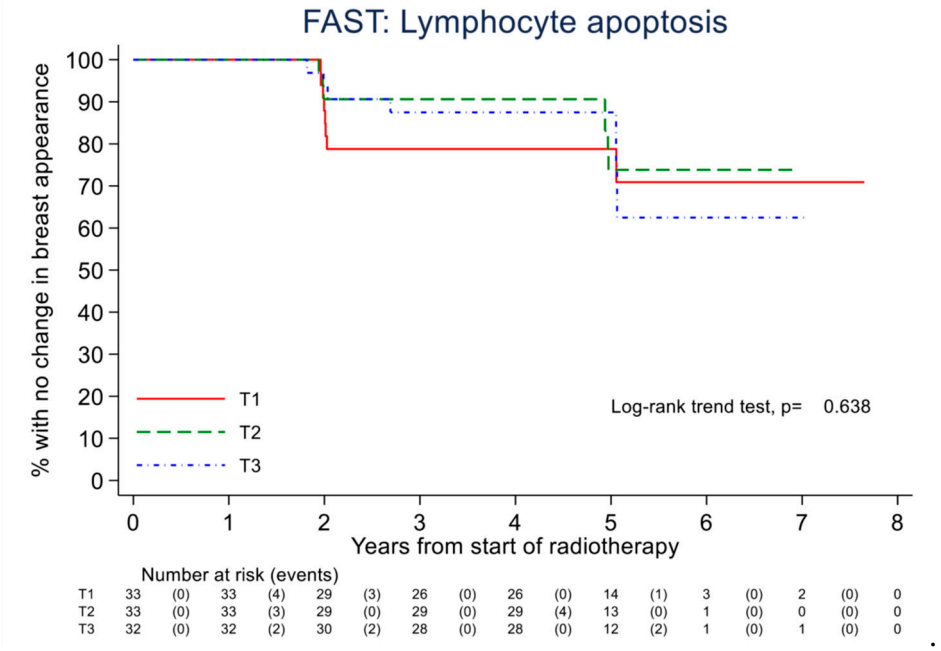


**Figure 1.** Kaplan-Meier plot for FAST trial primary endpoint of any change in photographic breast appearance. Sub-groups of patients using cut-offs determined by RILA statistical tertiles (T): T1<15.63%, T2:15.63-21.68% and T3>21.68%.

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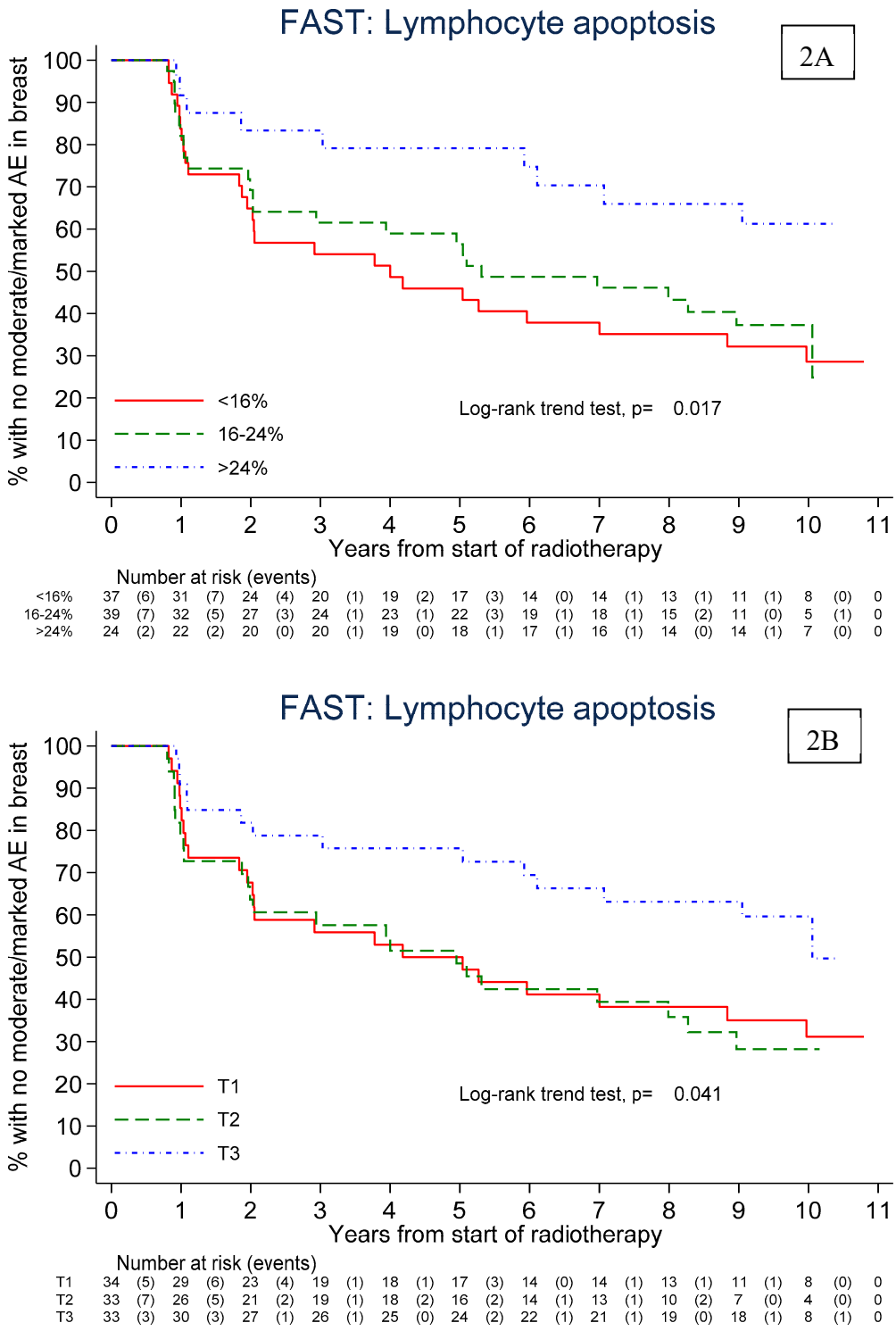


**Figure 2.** Kaplan-Meier plots for FAST trial secondary endpoint of any grade≥2 clinical adverse events (breast shrinkage, induration, telangiectasia, breast oedema) including all annual assessments. (2A) Sub-groups of patients using RILA cut-offs of <16%, 16-24% and ≥24%. (2B) Sub-groups of patients using cut-offs determined by RILA statistical tertiles (T): T1<15.63%, T2:15.63-21.68% and T3>21.68%.

Concerning chromosomic aberrations, no significant associations were found with change in photographic breast appearance nor with the secondary endpoint of any clinical adverse event (breast shrinkage, induration, telangiectasia, breast oedema). Details are presented in the **Supplementary Tables S1 and S2**.

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Distribution of endpoint events and biomarker descriptive statistics by fractionation for FAST patients used in the analysis are presented in **Supplementary Table S3**.

In CHHiP trial, a decreased risk of grade≥2 RTOG bladder or bowel adverse events (gr2+ RTOGAE) was observed for increasing values of RILA (HR 0.97; 95%CI 0.94-1.01; *P* = 0.11) but did not reach statistical significance (**Figure 3**). The relative risk of developing RTOG gr2+ RTOGAE decreased for patients with RILA≥24% with a HR of 0.63 (95%CI 0.33-1.23, *P* = 0.148). Details are presented in **Supplementary Table S4**.

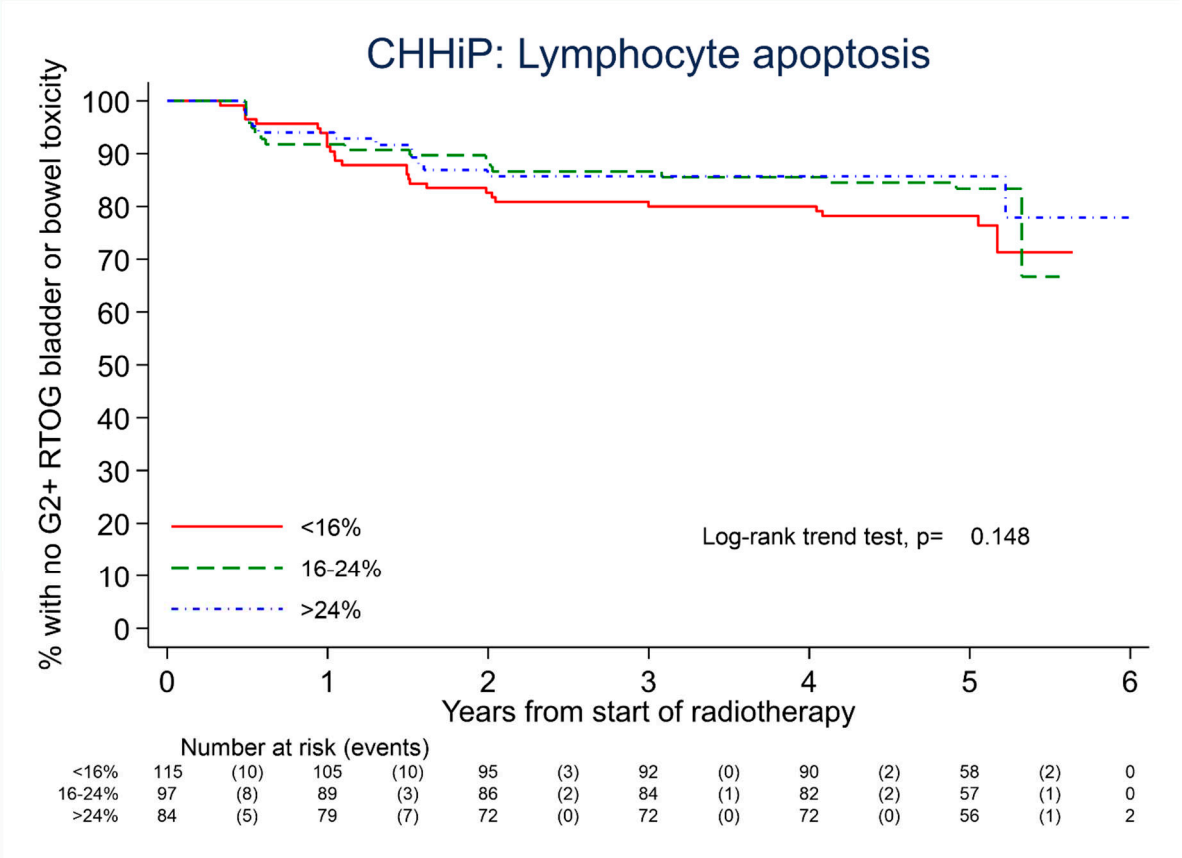


**Figure 3.** Kaplan-Meier plot for CHHiP trial of any grade≥2 RTOG bladder or bowel toxicity. Sub-groups of patients using RILA cut-offs of <16%, 16-24% and ≥24%.

Concerning chromosomic aberrations (**Supplementary Table S4**), we found some evidence of association of higher levels of dicentrics per cell with lower risk of gr2+ RTOGAE, although not statistically significant (*P* = 0.058 for trend test across tertiles; p=0.038 for upper vs lower tertile). We also observed some evidence of association of higher levels of ratio of foci per cell 4Gy at 24 hours/0.5Gy at 30 minutes with lower risk of gr2+ RTOGAE, although not statistically significant (p=0.051 for trend test across tertiles; *P* = 0.053 for upper vs lower tertile. Finally, we found significant association of higher levels of micronuclei per cell (without cut-off) with lower risk of gr2+ RTOGAE (*P* = 0.021 for trend test across tertiles; *P* = 0.023 for upper vs lower tertile, **Supplementary Table S4** and **Figure 4**).

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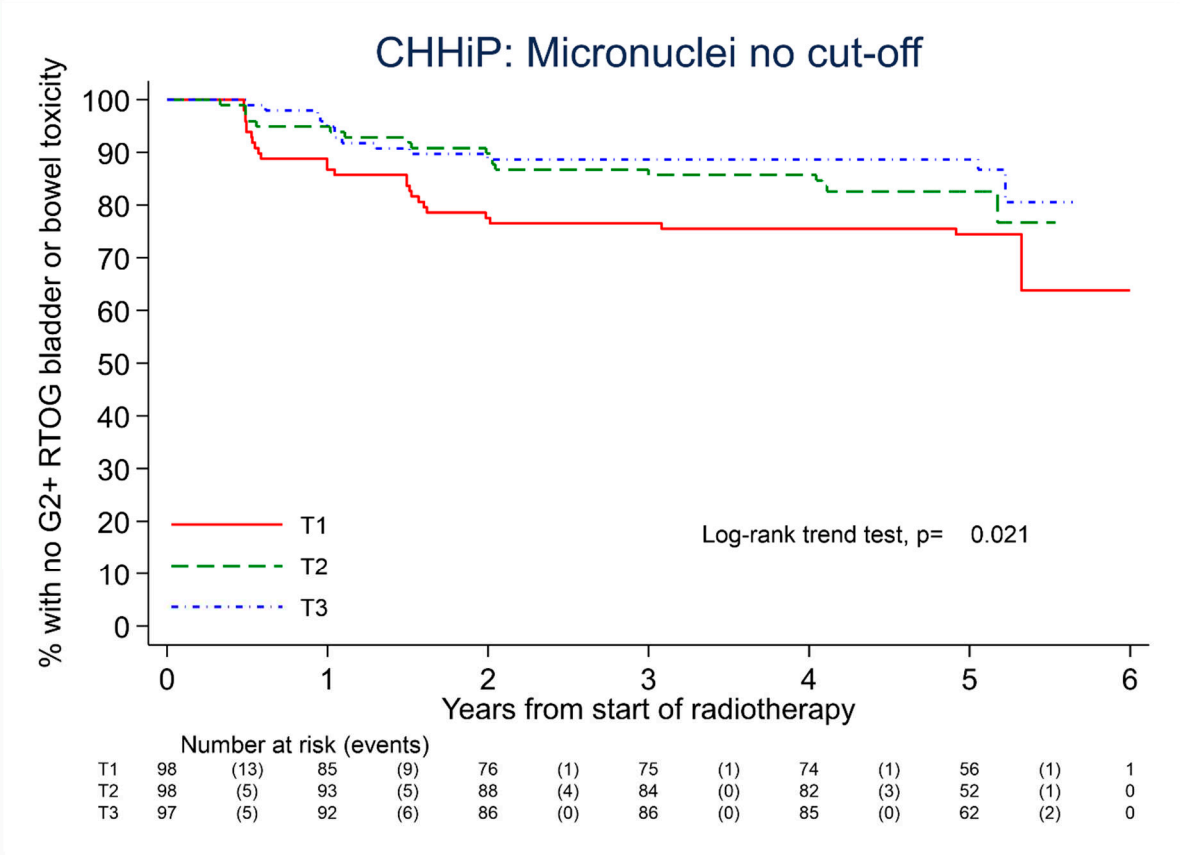


**Figure 4.** Kaplan-Meier plot for CHHiP trial of any grade≥2 RTOG bladder or bowel toxicity. Sub-groups of patients using cut-offs determined by micronuclei statistical tertiles (T): T1<0.230, T2:0.230-0.469 and T3>0.469.

Distribution of endpoint events and biomarker descriptive statistics by fractionation for CHHiP patients used in the analysis are presented in **Supplementary Table S5**.

Details of all data are presented in **Supplementary Table S6 and S7** considering bladder or bowel individually as secondary endpoints.

## 4. Discussion

In the present study, we confirmed in FAST trial that a decreased risk of grade ≥2 clinical breast adverse events (shrinkage, induration, telangiectasia, and oedema) was observed for increasing values of RILA. That association was not found when mild/marked change in photographic breast appearance was used as an evaluation criterion. Nevertheless, higher levels of foci per cell 4 Gy at 24 hours were associated with lower risk of mild/marked change in photographic breast appearance. This finding was contrary to our expectations based on previous findings for a small, highly selected group of breast cancer patients with no or very severe changes in photographic breast appearance [13].

In CHHiP trial, a decreased percentage of gr2+ RTOGAE was observed for increasing values of RILA but did not reach statistical significance. Concerning chromosomic aberrations, we found some evidence of association of higher levels of dicentrics per cell and ratio of foci per cell 4 Gy at 24 hours/0.5Gy at 30 minutes with lower risk of gr2+ RTOGAE as well as a significant association of higher levels of micronuclei per cell (without cut-off) with lower risk of gr2+ RTOGAE. While consistent with the results obtained for the FAST trial participants, these findings directly contradict our original hypothesis that systemic inefficient DNA repair, resulting in excess residual foci, chromosome aberrations and micronuclei, may contribute to late normal tissue toxicity.

Before the current study, we and others presented promising results of correlation between RILA and clinical breast late side-effects in retrospective and prospective manner with long-term

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follow-up [8,9,19]. The level of evidence of RILA was strengthened [20] with the validation of the correlation of high RILA and low late breast fibrosis in 648 patients treated by adjuvant breast radiotherapy in the REQUITE multicentre observational study [21]. Here, the key-point of our present study was the RILA validation in case of hypofractionation regimen as it is now currently used in daily practice in case of low-risk breast cancers [22,23]. Compared to usual moderate hypofractionated regimen, the extreme hypofractionated FAST protocol was of particular interest [14] as 30 Gy or 28.5 Gy in 5 once-weekly fractions are not used in France due to the perceived risk of late side-effects. Indeed, it has been previously suggested that dose per fraction was one of the most important parameters influencing radiation-induced toxicities over time [24]. The $\alpha/\beta$ estimates from FAST are consistent with the 10-year analysis of the START-A trial, which generated estimates around 3-4 Gy for late effects in the breast [23]. This consistency supports the validity of the linear-quadratic model for fraction sizes as high as 5-6 Gy [14]. Total dose and dose per fraction increased significantly any clinical breast complications with HR of 1.79 and 1.45 when 30 Gy or 28.5 Gy were used, respectively [14]. Similarly data from the CHHiP trial supports an $\alpha/\beta$ estimate for rectal toxicity of 3 Gy or less [25] but may be lower at 0.6-2.0 Gy for bladder side-effect (manuscript submitted). We showed here that individual radiosensitivity, in addition to total dose and dose per fraction, may impact clinical tolerance results whatever fraction doses used in FAST randomized arms. Surprisingly, we did not find evidence for an association between RILA and breast appearance measured by photographs. In fact, marked changes in photographic breast appearance in FAST were rare. Maybe, specificity of foci per cell 4 Gy at 24 hours would be greater than specificity of RILA but direct comparisons would only be feasible in large prospective and dedicated clinical trials, given the overall low incidence of adverse effects now achieved in breast radiotherapy.

Breast is of course not the only site for which RILA is useful. In our first clinical trial [7], many tumor sites were included and RILA predicted late rectal, bladder, or cervical toxicities after RT. It was then confirmed by others in prostate [26], head and neck [27], cervix [28], and Merckel Cell Carcinoma [29]. We recently presented first results of the PHRC trial 2 (NCT00893035) studying prostate cancer [10]. In 205 patients with localized prostate cancer (out of 383 included patients), a significant increase in the risk of grade ≥2 pelvic toxicities was found if patients had pre-treatment urinary symptoms (sHR, 1.90; $P = 0.016$) and decreased if patients presented a RILA ≥14% (sHR, 0.58; $P = 0.04$). In the present Trans-CHHiP study, this did not reach statistical significance probably due to the small sample size and the small irradiated volume. Nevertheless, we confirmed a lower relative risk of developing RTOG gr2+ RTOGAE for patients with RILA≥24% with a HR of 0.63 that was in a similar range to the French PHRC trial 2.

The mechanisms underlying the predictive role of RILA are still unknown. In lymphocytes from 26 patients with locally advanced breast carcinoma, an inverse correlation was found between initial damage to DNA and RILA [30]. We found here significant association of higher levels of micronuclei per cell (without cut-off) with lower risk of gr2+ RTOGAE. Recently, Chaouni et al. showed a significant lower increase of lymphocyte micronucleus frequency after irradiation in the most radiosensitive patients [29]. This could be related to a misrepair of DNA damage leading to fewer micronuclei but bringing potentially stochastic effects. Lymphocytes of the least radiosensitive patients, presenting more micronuclei resulting from DNA damage and repair, could continue to progress in the cell cycle with less G2-M arrest for DNA repair, possibly inhibited by the p21 pathway, to finally undergo apoptosis as shown by sub-G1 cell percentage analysis [29]. In contrast, lymphocytes from the most radiosensitive patients have fewer micronuclei perhaps due to a persistent G2-M arrest or the elimination by mitotic death of the most damaged cells.

One last point perhaps to consider in future RILA clinical use is that RILA may be better for predicting subcutaneous fibrosis observed when superficial skin is irradiated (breast, Head and Neck, skin tumors…) rather than vascular damage that may occur in the rectum or bladder with prostate irradiation.

Ongoing works within a European consortium [31] are trying to identify if some genetic polymorphisms [21,32–35] may explain the incorrect healing of the irradiated tissue after breast and

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prostate radiotherapy. Research to understand how lymphocytes can predict late RT-toxicity is ongoing in our INSERM U1194 radiobiology team in Montpellier.

The clinical utility of RILA is high as mentioned in our European REQUITE consortium propositions [36]. For example, patients identified as low risk of late effects might be offered a higher dose with modern techniques with the aim of improving local control. In some cases, radiotherapy may be avoided completely for individuals with a high risk of toxicity, provided that an effective alternative exists. For example, for breast cancer, surgery and immediate reconstruction could be offered instead of conserving-surgery and radiotherapy. Reduced volume as in partial breast RT could also be proposed in case of low-risk tumors to protect healthy tissues without compromising carcinologic outcomes [37]. For prostate cancer, other appropriate management strategies using surveillance or radical prostatectomy could be considered and new radiotherapy techniques such as MRI-RT using daily adaptive methods would be of particular interest to obtain optimal healthy tissues protection [38].

## 5. Conclusions

This study is the first to evaluate RILA and chromosomal damage as predictors of the risk of late effects after breast and prostate RT in the "hypofractionation" era. We confirmed in FAST trial that a decreased risk of grade ≥2 clinical breast adverse events was observed for increasing values of RILA. In CHHiP trial, a decreased percentage of gr2+ RTOGAE was observed for increasing values of RILA but did not reach statistical significance.

We are now entering a new era of personalized treatment [39] and radiation oncology is surely a good example with the development of a toxicity biomarker whatever the fractionation used, particularly in prostate and breast cancers.

**Author Contributions:** D.A., C.G., D.P.D., K.R., and J.Y. were involved in the conception and design of the study. D.A. and D.P.D. were involved in the provision of patients. D.A., J.S.H, C.G., D.P.D., J.M., S.B., K.R., M.B., and J.Y. were involved in data acquisition and helped to draft the report. D.A. and J.Y. supervised the study. J.S.H. and C.G. were responsible for statistical analyses. D.A., J.S.H, C.G., D.P.D., J.M., S.B., K.R., M.B., and J.Y. had access to the raw data and analysed and interpreted the data. D.A., J.S.H, D.P.D., J.M., S.B., K.R., M.B., and J.Y. were involved in writing the report, which was corrected and approved by all authors.

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**Conflicts of Interest:** David Azria participated in the creation of the start-up NovaGray. All other authors declare no potential conflicts of interest.

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