

Original Research Article

Multi-centre technical evaluation of the radiation-induced lymphocyte apoptosis assay as a predictive test for radiotherapy toxicity



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ABSTRACT

Predicting which patients will develop adverse reactions to radiotherapy is important for personalised treatment. Prediction will require an algorithm or nomogram combining clinical and biological data. The radiation-induced lymphocyte apoptosis (RILA) assay is the leading candidate as a biological predictor of radiotherapy toxicity. In this study we tested the potential of the assay for standardisation and use in multiple testing laboratories.

The assay was standardised and reproducibility determined using samples from healthy volunteers assayed concurrently in three laboratories in Leicester (UK), Mannheim (Germany) and Montpellier (France). RILA assays were performed on samples taken prior to radiotherapy from 1319 cancer patients enrolled in the REQUITE project at multiple centres. The patients were being treated for breast (n = 753), prostate (n = 506) or lung (n = 60) cancer.

Inter-laboratory comparisons identified several factors affecting results: storage time, incubation periods and type of foetal calf serum. Following standardisation, there was no significant difference in results between the centres. Significant differences were seen in RILA scores between cancer types (prostate > breast > lung), by smoking status (non-smokers > smokers) and co-morbidity with rheumatoid arthritis (arthritis > non-arthritis).

An analysis of acute radiotherapy toxicity showed as expected that RILA assay does not predict most end-points, but unexpectedly did predict acute breast pain. This result may elucidate the mechanism by which the RILA assay predicts late radiotherapy toxicity.

The work shows clinical trials involving multiple laboratory measurement of the RILA assay are feasible and the need to account for tumour type and other variables when applying to predictive models.

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1. Introduction

Radiotherapy is an important modality for treating many cancers, but a minority of patients develop adverse reactions that may be long-lasting and lower quality-of-life. Predicting patients with increased risk for developing adverse reactions is of interest for personalised treatment because it would allow either modifica-

tion of treatment or interventions to mitigate the risk of side effects.

Research aiming to develop predictive assays for radiotherapy adverse reactions involves approaches including proteomics [1], transcriptomics [2] and genomics [3]. Radiogenomic studies identified several replicated genetic associations with late reactions to radiotherapy, but those found so far only account for a small fraction of the genetic variance [4–9].

Various assays for cellular radiosensitivity have been evaluated as predictive tests for late reactions, mainly using patient lymphocytes or skin fibroblasts [7,10–12]. The approach with the best evidence for predictive value is the radiation-induced lymphocyte

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apoptosis (RILA) assay. In a prospective study of 399 patients with various cancers, individuals with low lymphocyte apoptosis were more likely to develop late toxicity, but there was no predictive value for acute toxicity [13]. Subsequent papers confirmed the finding of an inverse correlation between lymphocyte apoptosis and toxicity in cancers of the cervix, head and neck, prostate and breast [13–21]. A recent prospective multi-centre trial recruited 502 breast cancer patients from ten clinical centres and carried out the RILA assay in a single laboratory in Montpellier [22]. Patients with low RILA scores (bottom tertile) had higher toxicity, with the test having a high negative predictive value for low toxicity in patients with middle and upper tertile scores. A key-point was that RILA significantly predicted less toxicity with increasing values when taken as a continuous variable, leading to the integration of RILA in a multifactorial nomogram [23]. Larger datasets using uniform laboratory and clinical protocols are needed to incorporate RILA assay data into statistical models including the known prognostic patient, treatment and genetic factors. A barrier to collection of larger datasets is the lack of information on the transferability of the assay between laboratories. A previous study which compared the results from two different laboratories on 25 head and neck cancer patients found good agreement, but larger studies in more centres and cancer types are needed [24].

REQUITE is an EU-funded multi-centre observational study that has recruited >4400 patients with breast, lung or prostate cancer and is following them for at least two years to score radiotherapy side effects [25]. Three of the centres involved in REQUITE (ICM, Montpellier; UMM Mannheim and University of Leicester) carried out the RILA assay on recruited patients. The aims of the work described here were to: standardise the RILA protocol across three laboratories in different countries; identify experimental factors affecting RILA results; identify clinical/patient factors affecting data obtained using a standardised assay; and analyse relationships with acute toxicity.

2. Methods

2.1. Patient recruitment and ethics

Breast, prostate and non-small cell lung cancer patients were recruited according to inclusion and exclusion criteria defined by the REQUITE project [25]; further documentation available from

www.requite.eu). All patients were to undergo radiotherapy with curative intent with no prior treatment except hormone therapy in some prostate cancer patients. Venepuncture was carried out prior to radiotherapy and peripheral blood collected in lithium heparin blood tubes (Becton Dickinson, 367526). [Supplementary Table 1](#) summarises patient characteristics. Ethical approval was gained from regulatory authorities in each country and written informed consent received from each patient. [Table 1](#) shows frequencies of acute radiotherapy side effects.

2.2. Radiation-induced lymphocyte apoptosis (RILA) assay

The initial protocol was from the ICM Montpellier as described elsewhere [13,22,26]. The final standardised operating procedure used is available as form RQ7 on request.

In brief, the protocol involves incubating whole blood in tissue culture medium for one day, followed by X-irradiation or sham treatment and a further two days of culture. Red cells were then lysed and cytotoxic T lymphocytes labelled with CD8 antibody and propidium iodide. Flow cytometry 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. More detail is available in the [Supplementary Methods](#), together with details of the pre-experiment inter-laboratory standardisation.

2.3. Statistics

Statistical analysis was performed using IBM SPSS Statistics 22. RILA assay scores were expressed as mean \pm standard deviation. Correlation analyses were either performed using the Pearson product-moment correlation (r) or Spearman's rank correlation (ρ) tests. Regression analyses were logistic, multinomial or linear as appropriate. Mean differences were analysed using Student's t or Kruskal-Wallis tests as appropriate.

3. Results

3.1. Effect of protocol time parameters

Using the standardised assay, data were collected for 1319 patients enrolled in the REQUITE study. RILA data on 23 patients

Table 1
Frequency of acute radiotherapy toxicity. Change from baseline by two grades or higher at follow-up six weeks after end of radiotherapy, except where noted as G1 which is one grade higher than baseline. German patients were not asked questions on sexual health.

Cancer site/end-point	End-point	Leicester	Mannheim	Montpellier	Combined
Breast	N	180	142	382	635
	Pain	G1 33.5%	G1 24.8%	G1 21.5%	G1 24.8%
	Oedema	0%	0.7%	1.6%	0.9%
	Ulceration	0%	2.8%	7.2%	4.5%
	Erythema	18.1%	15.9%	17.6%	17.4%
Prostate	Hyperpigmentation	G1 1.1%	G1 48.3%	G1 10.3%	G1 15.5%
	N	196	52	216	430
Rectal	Proctitis	4.6%	8.2%	4.7%	4.9%
	Diarrhoea	9.7%	8.3%	8.3%	8.8%
Urinary	Rectal bleeding	1.0%	0%	0.5%	0.6%
	Flatus	2.6%	0%	0%	1.1%
	Urinary obstruction	0.5%	0%	5.6%	2.8%
	Urinary incontinence	2.6%	0%	2.3%	2.1%
	Urinary frequency	12.2%	6.3%	3.2%	7.5%
	Urinary urgency	12.2%	8.2%	0.9%	6.4%
	Urinary retention	15.3%	0%	2.8%	7.7%
Sexual	Erectile dysfunction	0%	N/A	13.1%	7.1%
	Ejaculation disorder	0%	N/A	16.4%	8.7%
	Orgasmic dysfunction	0%	N/A	19.2%	10.1%
	Libido	0%	N/A	18.1%	9.5%
Skin	Radiation dermatitis	2.0%	2.9%	0.5%	1.3%

Table 2
Length of time intervals for different stages of the RILA protocol achieved in practice. All times in hours.

Cohort	Time interval	Mean (h)	Median (h)	Min (h)	Max (h)	Interquartile range (h)	Break-down (h)
Leicester (n = 415)	Storage	15.4	20.1	0.3	47.1	2.9–25.3	0–12 h n = 182 12–36 h n = 227 36–48 h n = 6
	Pre-rad incubation	18.9	17.8	0.5	25.3	16.5–21.3	0–12 h n = 1 12–36 h n = 414
	Post-rad incubation	49.1	49.2	45.7	50.2	48.8–49.5	
Mannheim (n = 194)	FACS lag	3.6	3.6	0.2	5.5	3.1–4.1	
	Storage	22.0	23.5	0.2	34.5	22.4–24.8	0–12 h n = 13 12–36 h n = 181 36–48 h n = 0
	Pre-rad incubation	12.6	3.0	0.3	28.3	2.0–25.7	0–12 h n = 107 12–36 h n = 87 36–48 h n = 4 >48 hr n = 3
Montpellier (n = 678)	Post-rad incubation	43.7	44.0	19.6	69.0	43.5–44.3	
	FACS lag	1.5	1.3	0.3	4.5	1.0–1.9	
	Storage	10.4	5.5	0	32.8	2.5–24.5	0–12 h n = 498 12–36 h n = 180 36–48 h n = 0
	Pre-rad incubation	20.1	20.0	16.8	28.7	18.3–21.2	
	Post-rad incubation	45.5	45.8	38.3	49.3	44.3–47.3	
	FACS lag	4.4	4.4	0.3	8.8	3.5–5.2	

were excluded from analysis because they failed quality control, with the data on the remaining 1296 patients being analysed. Four time intervals were recorded for each sample: storage time (time from blood draw to setting up cultures), pre-irradiation incubation (time from setting up of cultures to irradiation), post-incubation (time from irradiation to cell lysis) and FACS lag (time from cell lysis to loading on FACS machine). Table 2 shows variation in timings at the three centres.

For 85 samples Mannheim performed the assay with both 0 h and 24 h pre-irradiation incubation (0/48 and 24/48 protocols). RILA scores were a mean 10.7% (range –14.7% to 32.7%) higher for the 24/48 protocol (or 0.45%/hour). The wide range of scores hampers application of a standard correction factor for comparison of results obtained using different protocols, but the correlation between 0/48 and 24/48 RILA scores was high ($r = 0.71$, $p < 0.001$).

The effect of variation in protocol timings on RILA scores was investigated (Table 2 and Fig. 3). Different factors significantly affected RILA score in each cohort (Supplementary Table 2). Combining data across all three centres shows that RILA scores increased with total time from venepuncture to FACS (Supplementary Table 2 and Fig. 3).

3.2. Results by cancer type and centre

At the time of analysis full patient data were available on 1247 of the 1296 patients: 52 lung, 724 breast and 471 prostate cancer patients. The distributions of RILA scores for each cancer type were broadly similar (Fig. 1). There was a significant difference in RILA score between the three cancer types: breast cancer samples had mean RILA = $19.2 \pm 11.8\%$, prostate samples $22.1 \pm 12.0\%$ and lung cancer samples $15.1 \pm 9.5\%$ (Kruskal-Wallis $p < 0.001$). Two-way comparisons were also significant: breast vs prostate $p < 0.001$, lung vs breast $p = 0.008$, prostate vs lung $p < 0.001$ (Mann-Whitney).

RILA scores on all REQUITE samples were significantly different between the three centres (Kruskal-Wallis $p < 0.001$) (Fig. 2 and Table 3). Comparing RILA scores between the centres for each of the three cancer types showed significant differences for breast and prostate (Kruskal-Wallis $p < 0.001$) but only a trend for lung ($p = 0.07$).

A regression analysis was performed to compute a RILA score adjusted for the effect of incubation times and patient factors identified below (rRILA). This rRILA score was not significantly different between the three centres (Kruskal-Wallis $p = 0.08$), indicating that much of the difference between centres can be explained by these factors.

3.3. Effect of clinical patient factors

The characteristics of the patients for each cohort studied are shown in Supplementary Table 1.

In the combined data two patient factors were associated with RILA scores in a univariate analysis: smoking status and rheumatoid arthritis. Comparing never and ex-smokers (mean RILA 20.5 ± 12.2 , $n = 1025$) against recent quitters and current smokers (mean RILA 18.1 ± 10.1 , $n = 222$) gave a significant difference in RILA scores (Mann-Whitney p value = 0.02). Patients with rheumatoid arthritis had a significantly higher RILA (mean = 26.2 ± 16.4 , $n = 29$) than those without (mean = 20.0 ± 11.7 , $n = 1218$) (Mann-Whitney $p = 0.04$).

In a hierarchical linear regression analysis, smoking status ($B = -2.1$, $p = 0.01$) and rheumatoid arthritis ($B = 8.2$, $p = 0.001$) were both significant predictors of lymphocyte apoptosis. Including all the factors that significantly affect RILA score into a single hierarchical linear regression model showed that in the final model they explained 7.6% of the variation in RILA (by R squared) (Supplementary Table 3).

3.4. Analysis with acute toxicity

Acute radiotherapy toxicity endpoints were recorded six weeks after radiotherapy and the pre-radiotherapy baseline score deducted (Table 1). The endpoints were studied for association with rRILA score by regression analysis. rRILA did not predict any prostate cancer toxicity acute end-point, either singly or when combined into STAT scores for urinary, rectal and sexual dysfunction, or overall acute toxicity.

For breast cancer, rRILA did predict acute pain (Mann-Whitney $p = 0.008$), but none of the other end-points or a STAT score for overall acute toxicity. Patients with a grade 1 acute pain had a

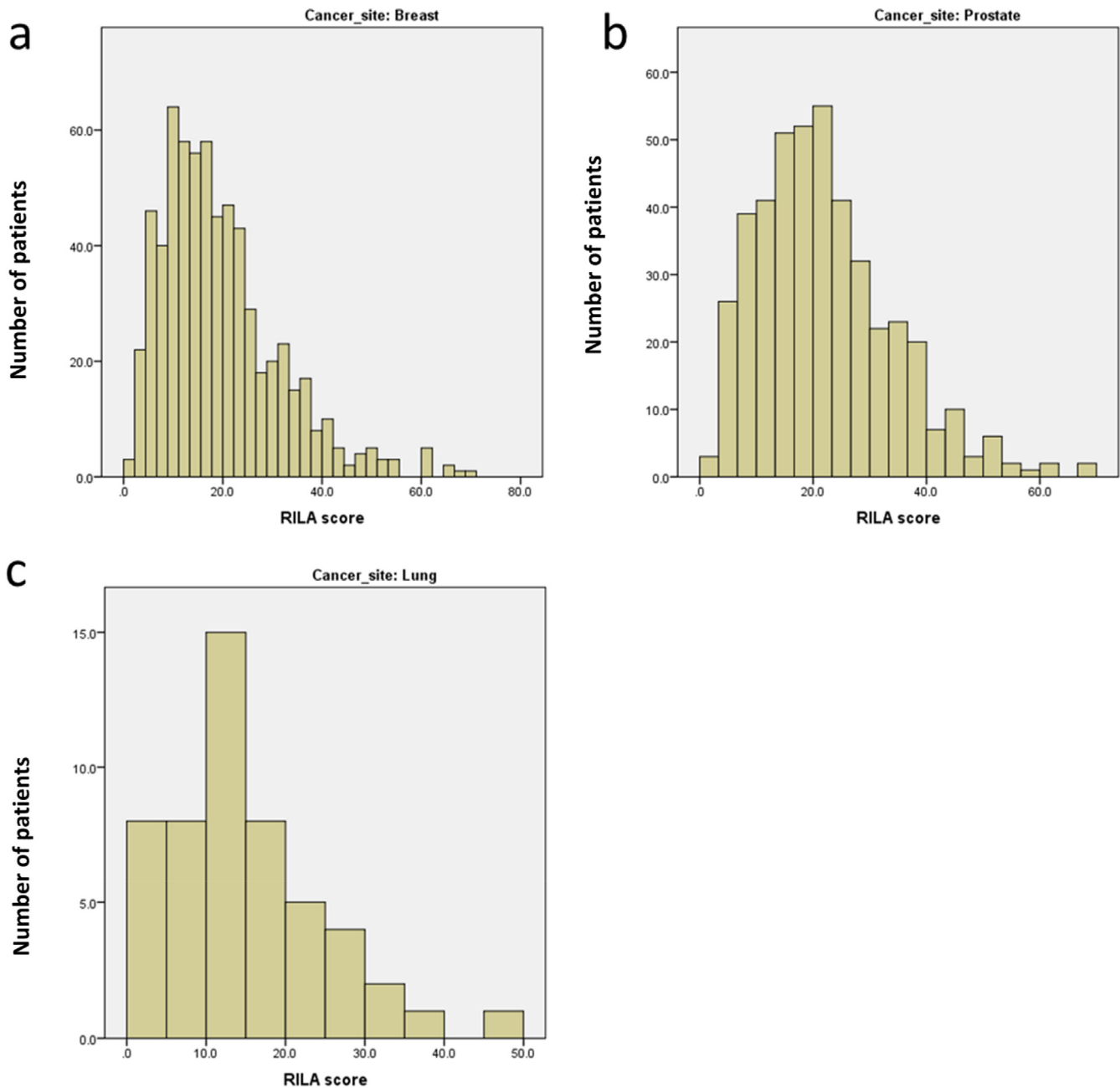


Fig. 1. Distributions of RILA score (%) by cancer site. Panel A breast, B prostate, C lung.

mean RILA score of 17.5%, while those with no increase in pain had mean 20.3%. The patients in the lowest tertile of rRILA score had a prevalence of acute breast pain of 27.4%, compared with 19.2% for the highest tertile group (OR 0.63 95% CI 0.40–0.99).

4. Discussion

The aim of the work presented here was to investigate the potential of the RILA assay for standardisation and use in multiple testing laboratories prior to attempting to validate its use as a predictive assay for late radiotherapy toxicity in a multi-centre study. We identified the steps required to generate comparable data in three laboratories across Europe. Despite the assay standardisation, mean RILA scores generated in a large multi-centre study differed across laboratories. However, the differences were lost by

adjusting scores for cancer site, incubation times and patient factors. Our work shows that clinical trials involving multi-centre measurement of the RILA assay are feasible but its implementation in predictive models needs to account for tumour type and other variables.

4.1. Variability

The data show that the incubation times affects RILA scores, with time before cell lysis increasing the score by on average 0.17%/hour, but the time from lysis to flow cytometry (FACS lag) decreasing it by 1.0%/hour. Keeping all incubations strictly controlled presents practical difficulties since the blood samples are drawn at variable times during the day, but here we show that incubation time can be included as an adjustment factor.

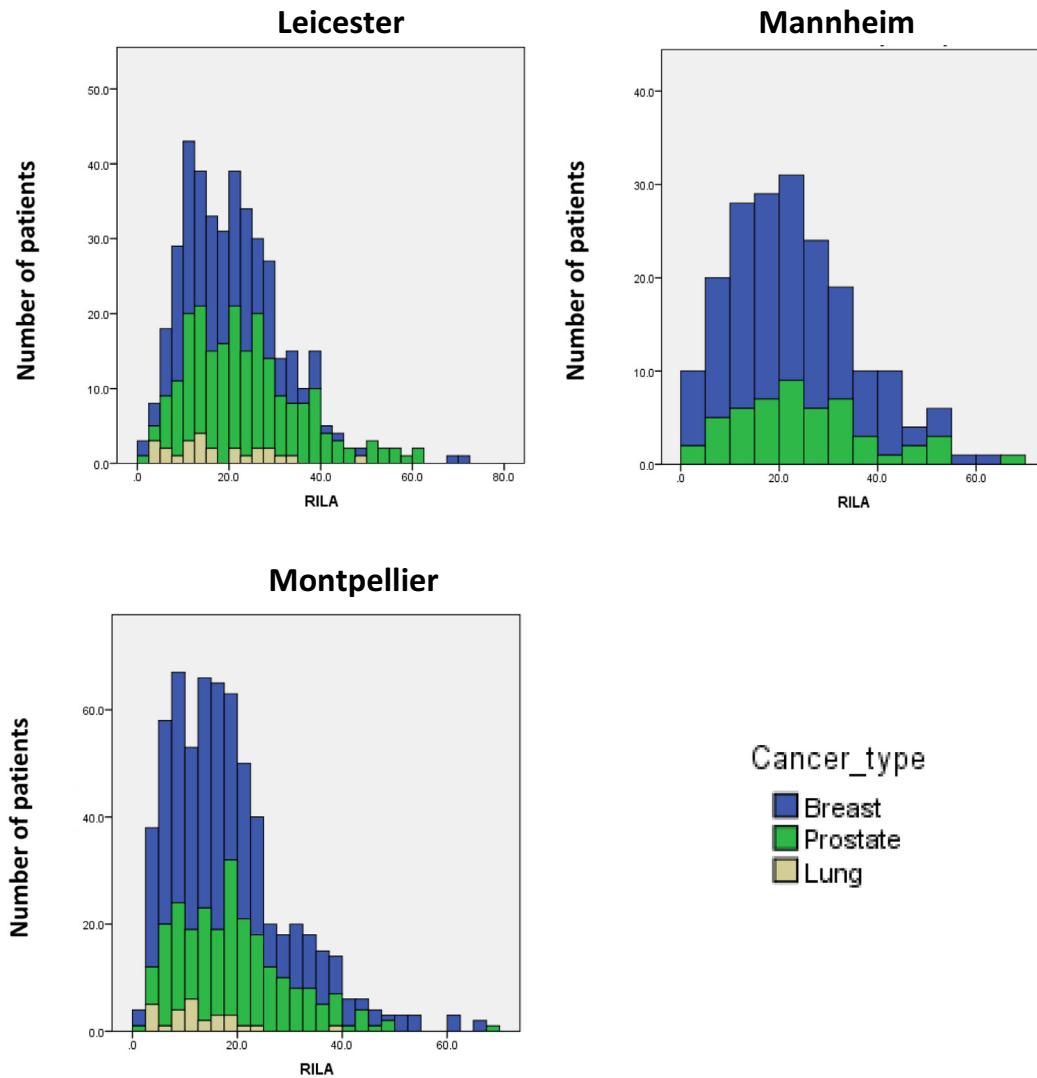


Fig. 2. Distribution of RILA scores (%) by centre and cancer type (Blue: breast cancer, Green: prostate cancer, Yellow: lung cancer). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.2. Use for prediction

Previous studies defined the group at risk of radiotherapy side effects as the lower tertile of RILA scores. An important question to address is whether a common RILA score threshold can be used across cancer types and laboratories.

We showed that RILA scores varied across the three cancer types. Although previous studies have provided some evidence [18,22], this is the first direct demonstration of a difference in lymphocyte apoptosis between cancer types, but the reason is currently unknown. Possible explanations are differences in age and sex of the three patient groups. However, our analysis showed no associations between RILA scores and age within any of the cancers and sex in lung cancer patients. Differences in treatment prior to radiotherapy might play a role: the breast tumours were resected prior to enrolment in this study, some of the prostate cancer patients had hormones prior to radiotherapy.

Taken together the results indicate that separate thresholds will be needed for each cancer type. An alternative approach is to include RILA as a continuous variable in a predictive model or nomogram, like that patented by ICM under EP #18305213 and #16306097-3 [22].

4.3. Biological factors

An inverse correlation between tobacco use and RILA score was observed. There was no observable effect of patient smoking habit on lymphocyte apoptosis at 0 Gy in our data, suggesting smoking has an interactive rather than a direct effect with radiation. This observation helps explain the apparent contradiction with published laboratory studies on the effect of smoking on lymphocyte apoptosis [27–29]. The observation of reduced radiation-induced apoptosis in smokers does however concord with the hypothesis that low apoptosis is associated with increased radiation toxicity, because smoking has been identified as a risk factor for toxicity by published clinical studies in a range of cancer types [30–34].

Rheumatoid arthritis is an inflammatory condition in which many cell types are involved, including the activation of cytotoxic T lymphocytes [35]. As activated effector cells have a higher tendency to undergo apoptosis it is consistent with the finding that rheumatoid arthritis patients have a higher RILA score. Despite somewhat mixed evidence [36,37], individuals with rheumatoid arthritis are considered to have increased radiotherapy side effects, contrary to any expectation based on their higher RILA score.

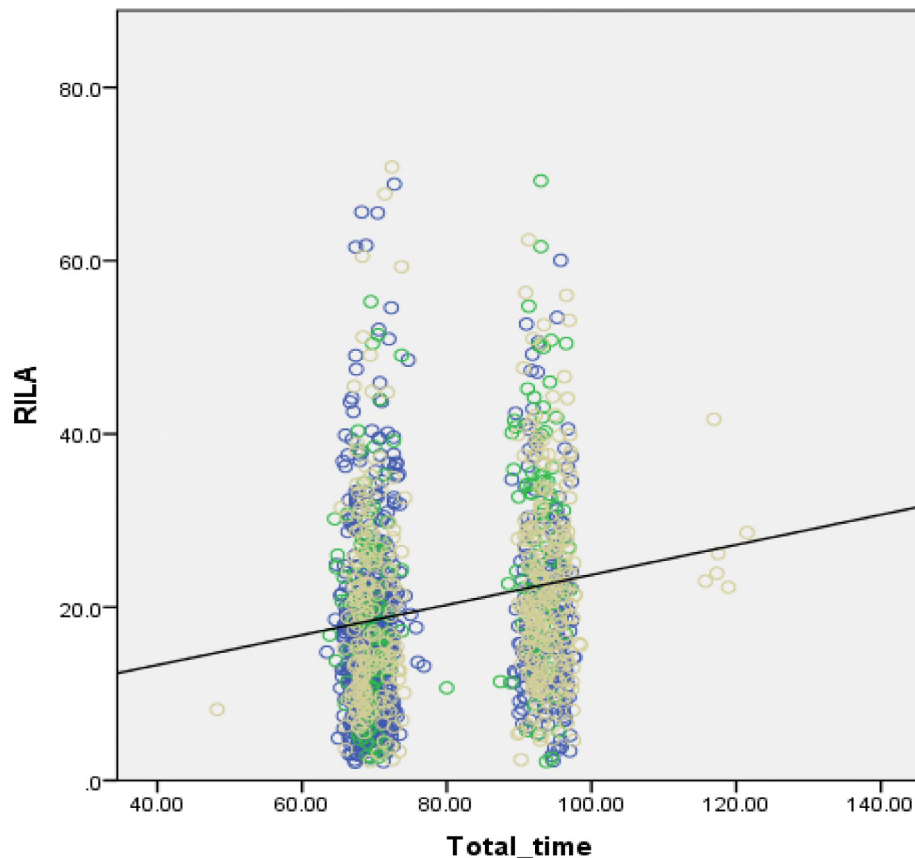


Fig. 3. RILA score against total time in hours from venepuncture to FACs lysis. Patients from all three clinical centres ($n = 1190$). Yellow circles are Leicester, green are Mannheim, blue are Montpellier. Slope = $+0.17\%/hour$, $p = <0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
RILA scores by centre and cancer type.

Centre	Cancer type	Number	Mean RILA (%)	Lower tertile cut-point (%)	Upper tertile cut-point (%)
UK	Combined	414	21.4	14.8	24.7
	Breast	190	19.4	13.6	22.6
	Lung	25	17.8	12.1	22.6
	Prostate	198	23.9	16.8	26.8
Germany	Combined	194	23.1	16.4	26.7
	Breast	142	22.3	15.4	26.1
	Prostate	52	25.3	18.2	28.9
France	Combined	678	18.6	12.3	20.9
	Breast	392	18.0	11.4	19.8
	Lung	27	12.5	8.7	14.7
	Prostate	221	19.7	14.2	22.8

It is possible that these factors are part of the, as yet unexplained, mechanism underlying the correlation between RILA and radiation toxicity. As such, whether to adjust RILA for these factors needs to consider the effect on predictive value of the test measured, for example as measured by multi-parameter nomograms or Receiver Operating Characteristic (ROC) curve analysis. The REQUITE late toxicity analysis should be well placed to consider this question.

4.4. Acute toxicity

Previous studies found no evidence that RILA predicts acute radiotherapy toxicity, and the present study confirmed that for all clinical endpoints with the exception of acute breast pain. The reason for the latter is unknown, although as the frequency of pain

is higher than the other end-points there is a greater statistical power. It may also reflect a genuine distinction between acute toxicity endpoints: principal components analysis demonstrates that pain does not cluster with any other endpoint, suggesting it has a separate biological basis. Pain may be a marker of inflammation or deep tissue damage caused by radiotherapy, separate from the other acute breast end-points that reflect skin damage.

4.5. Limitations and strengths of the assay

A limitation of the current RILA protocol to routine implementation is the necessity for culturing the cells for one day prior to ex-vivo irradiation and for two days after irradiation. The current study presents preliminary evidence that using shorter incubation times causes reduced RILA levels but which are correlated with the

three-day protocol. Further work is required to evaluate shorter protocols with no pre-irradiation incubation and a shortened post-irradiation incubation.

The strengths of RILA are multiple and facilitate its use in daily clinical practice: one simple blood sample (without any tissue biopsy); obtaining the results in four days, and generation of a significant continuous variable capable of being incorporated in a nomogram.

There is now sufficient evidence to underpin increased use of the RILA assay in daily practice as soon as possible before breast irradiation. For widespread clinical use, additional work is required to show implementation has patient benefit and is cost effective.

4.6. Mechanism

Identifying the mechanism by which RILA predicts late toxicity might be useful for the design of ameliorative interventions. Currently it is unknown whether RILA discriminates between sequential and generic late effects, but the lack of predictive power for acute reactions might suggest it is the latter [38]. The finding in this study that acute breast pain exceptionally is predicted by RILA, may provide a more nuanced dissection of pathological pathways. When toxicity data are available on the REQUITE patients it may be possible to discriminate between types of late reactions.

4.7. Recommendations

The SOP produced for the study presented here should be used by others carrying out the RILA assay. Any use of the protocol in multi-centre studies requires standardisation of the reagents and culture conditions. It is recommended that future inter-laboratory comparisons of radiosensitivity tests accumulate sufficient pilot data on comparable cancer patients. The pilot data can be used either to define centre and cancer-site specific tertile cut-offs or construct nomograms, prior to testing associations with clinical end-points.

5. Conclusion

This report presents data from the collection phase of a multi-national study to validate the RILA assay as a predictive test for adverse reactions to radiotherapy, part of the larger REQUITE study. Despite efforts to use a common protocol and reagents, there was variation in the results generated in different centres. Adjusting for some variables identified allowed results to be combined across centres. Evidence was found for different levels of radiation-induced lymphocyte apoptosis between cancer types, which may be of biological interest but would need to be accounted for in studies involving multiple malignancies. Lastly several experimental and lifestyle factors were found to affect RILA score and will need to be included in the future analysis of predictive value of the assay for acute and late toxicity.

Declaration of Competing Interest

Dr. Azria has a patent on RILA assay testing for breast and prostate cancer issued to NovaGray. The other authors have nothing to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ctro.2019.06.001>.

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