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Original Article

Genome-wide association study of treatment-related toxicity two years following radiotherapy for breast cancer

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ABSTRACT

Background and purpose: Up to a quarter of breast cancer patients treated by surgery and radiotherapy experience clinically significant toxicity. If patients at high risk of adverse effects could be identified at diagnosis, their treatment could be tailored accordingly. This study was designed to identify common single nucleotide polymorphisms (SNPs) associated with toxicity two years following whole breast radiotherapy.

Materials and Methods: A genome-wide association study (GWAS) was performed in 1,640 breast cancer patients with complete SNP, clinical, treatment and toxicity data, recruited across 18 European and US centres into the prospective REQUITE cohort study. Toxicity data (CTCAE v4.0) were collected at baseline, end of radiotherapy, and annual follow-up. A total of 7,097,340 SNPs were tested for association with the residuals of toxicity endpoints, adjusted for clinical, treatment co-variates and population substructure.

Results: Quantile-quantile plots showed more associations with toxicity above the $p < 5 \times 10^{-5}$ level than expected by chance. Eight SNPs reached genome-wide significance. Nipple retraction grade ≥ 2 was associated with the rs188287402 variant ($p = 2.80 \times 10^{-8}$), breast oedema grade ≥ 2 with rs12657177 ($p = 1.12 \times 10^{-10}$), rs75912034 ($p = 1.12 \times 10^{-10}$), rs145328458 ($p = 1.06 \times 10^{-9}$) and rs61966612 ($p = 1.23 \times 10^{-}$

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[9]), induration grade $\geq$ 2 with rs77311050 ($p = 2.54 \times 10^{-8}$) and rs34063419 ($p = 1.21 \times 10^{-8}$), and arm lymphoedema grade $\geq$ 1 with rs643644 ($p = 3.54 \times 10^{-8}$). Heritability estimates across significant endpoints ranged from 25% to 39%. Our study did not replicate previously reported SNPs associated with breast radiation toxicity at the pre-specified significance level.

Conclusions: This GWAS for long-term breast radiation toxicity provides further evidence for significant association of common SNPs with distinct toxicity endpoints.

Following surgery, radiotherapy is the second most frequent treatment for breast cancer. Radiotherapy reduces local recurrence rates with a modest improvement in long-term overall survival [1]. However, up to a quarter of patients may experience clinically significant side-effects (toxicity). Late radiation toxicity is defined as complications arising >90 days from the start of radiotherapy. These tend to develop slowly up to several years after the end of treatment [2]. They are generally irreversible and may decrease health-related quality of life. In the breast, late radiation toxicity can manifest as scar tissue (fibrosis), shrinkage (atrophy), firm subcutaneous tissue (induration), nipple changes (retraction) and subcutaneous swelling (oedema). Women may also experience reddish-purple "spider veins" on the top of the skin (telangiectasia) due to vascular injury [3]. If the axilla and/or the supra-/infraclavicular fossa are irradiated, this can lead to arm lymphoedema [4].

A multitude of factors can affect the incidence and severity of toxicity, the most important being radiotherapy dose and fractionation, patient co-morbidities, co-medications and co-treatments such as chemotherapy or tamoxifen [5]. Nevertheless, after adjusting for known risk factors, there remains extensive patient variation in radiation toxicity, suggesting that toxicity is also determined by an individual's intrinsic radiosensitivity. Heritability studies on the susceptibility to radiation toxicity are limited. However, it has been shown that cellular radiosensitivity is a heritable trait [6].

The majority of published studies in the field of radiogenomics have used a candidate gene approach, in which single nucleotide polymorphisms (SNPs) in or near genes thought to be important in the pathogenesis of radiation toxicity phenotypes were investigated [7]**.** However, it has proven difficult to replicate most of these associations and some of the more recently reported SNPs are yet to be validated [8–11]. Like other disease genetics fields, radiogenomics has shifted towards a broader, genome-wide approach to identifying common SNPs associated with adverse effects. Common variants refer to SNPs present in the population with a minor allele frequency (MAF) of at least 1%. This ensures that genetic predictors largely reflect the characteristics of the general population, rather than identifying rare genetic mutations found in radiosensitivity syndromes (such as *ATM* gene mutations) [11]. Published genome-wide association studies (GWAS) in the field of radiogenomics have confirmed that normal radiation sensitivity is determined through complex polygenic inheritance rather than single gene alterations [12–14].

The aim of this study was to investigate common SNPs associated with toxicity two years after breast radiotherapy and to validate previously published associations.

Materials and methods

Study cohort

Between 2014 and 2016, 2,071 patients who had undergone breast-conserving surgery were recruited across 18 radiation oncology centres in Europe and the US into the prospective REQUITE cohort study (https://www.requite.eu). Toxicity (CTCAE v4.0) data were collected at baseline, end of radiotherapy and annual follow-up (±1 month). Patient baseline characteristics and methodology have been described in detail elsewhere [15]. The STROGAR guidelines were followed to ensure transparency and completeness of reporting [16].

Inclusion criteria included females aged 18 and over with cancer of the breast (invasive or in situ), suitable for adjuvant external beam radiotherapy (EBRT). All breast patients receiving chemotherapy completed their course of treatment (anthracyclines +/- taxanes) at least two weeks prior to radiotherapy. Patients with metastatic disease, previous breast irradiation or bilateral breast cancer were excluded. For the full list of exclusion criteria, see [15]. All patients gave written informed consent. The study was approved by local ethics committees in participating countries (UK NRES Approval 14/NW/0035) and registered at https://www.controlled-trials.com (ISRCTN98496463).

Patients were treated according to local protocol; 47.9% underwent intensity-modulated radiotherapy (IMRT), with a lower proportion in France and no IMRT at Italian or US centres. The majority patients received a tumour-bed boost (71.7%), ranging from less than 20% at the French, Italian and Spanish centres to over 80% at the Belgian centres, given either simultaneously (n = 212) or sequentially (n = 964) (see Table 1). Patients with invasive breast cancer in Belgium and the UK were treated using the START-B hypofractionated regimen [17].

Genotyping, imputation and quality control

All patients were genotyped in one batch using Illumina OncoArrays with $\sim$ 600,000 SNPs including a GWAS backbone and a similar number of cancer-specific SNPs, of which 2,000 were selected from previous radiogenomics studies. Datasets were imputed prior to analysis according to OncoArray Network methods [18]. The reference dataset included the 1000 Genomes Project (GP) Phase 3 (Haplotype release date October 2014) for chromosomes 1 to 22. The 1000 GP Phase 1 dataset (Haplotype ChrX release date Aug 2012) was used for chromosome X, since the phased data for Chr X from the 1000 GP Phase 3 was not available. Whole genome data were imputed in a two-stage procedure using SHAPEIT (shapeit.v2.r790.Ubuntu_12.04.4.static) to derive phased genotypes, and IMPUTEv2 (impute_v2.3.2_x86_64_static) to perform imputation of the phased data.

Standard quality control procedures were performed. Initially, 9 samples and 7,132 variants with a call rate of < 0.8 and 13 samples and 4,874 variants with call rate < 0.95 were excluded. A further 1,610 variants with MAF < 1% and call rate < 0.98 were removed. Unexpected sample duplicates were also removed (n = 5), as were those with recorded gender/genomic gender discrepancy (n = 7). Principal component analysis (PCA) was performed by EigenStrat software ensuring only European ancestry populations were included, and those with less than 80% European ancestry were excluded (n = 167). Samples which were heterozygosity outliers were also removed ($p < 10^{-6}$, n = 18). Finally, 2,746 variants were excluded due to deviation from Hardy Weinberg Equilibrium (HWE) ($p < 10^{-7}$), and a further 33,752 variants were removed from the analysis whose frequency did not match the 1,000 GP European

Table 1
Distribution of patient and treatment variables. Means are given for quantitative variables while the percentage of patients positive for the variable is given for categorical variables e.g. percentage of patients who smoke. *BED = Biologically Effective Dose. This is a widely used method of comparing different radiotherapy fractionation schedules and assigns a numerical score based on the linear quadratic model. The alpha value is the number of logs of cell kill per Gy (gray) from the linear portion of the cell survival curve and the beta is the number of logs of cell per Gy squared from the quadratic component. BED is the product of the number of fractions (n), dose per fraction (d), including any boost doses, and a factor determined by the dose and α/β ratio (3 Gy for late effects):..$BED = nd(1 + \frac{d}{\alpha/\beta})$.

Characteristic	REQUITE Breast Cohort (N = 1,640)	
Age (years, Mean, SD)	58.5 (10.8)	
BMI (kg/m2, Mean, SD)	26.3 (5.3)	
Smoking (ever smoked)	42.4%	
Alcohol drinker	54.6%	
Cardiovascular disease	7.1%	
Hypertension	28.9%	
Rheumatoid arthritis	3.1%	
Diabetes	5.3%	
Breast size (cup size ordinal + band size ordinal) (Mean, SD)	9.8 (2.6)	
*BED (Gy)	79.0 (4.8)	
Breast dose (Gy) (median range)	50 (28.5–56.0)	
Breast fractions (median, range)	25 (5–31)	
Hypo-fractionation (vs conventional fractionation)	46.3%	
Radiation Boost	71.7%	
Chemotherapy	Adjuvant	22.7%
	Neo-adjuvant	9.4%
Co-medications	ACE inhibitor	7.3%
	Anti-diabetic agent	4.3%
	Analgesic use	9.0%
	Anti-depressant	12.1%
	Statin	14.2%
	Tamoxifen	40.4%
	Aromatase Inhibitor	50.9%
Radiation to the axilla	11.9%	
Surgery to the axilla	Axillary node dissection	7.6%
	Sentinel lymph node biopsy (SLNB)	74.5%
	Axillary node dissection followed by SLNB	10.7%
	No axillary surgery	7.3%

population frequency, based on the formula (p1 − p0)^2/((p1 + p0) *(2-p1-p0)), where p1 represents the allele frequency in OncoArray and p0 in the reference population. Any variant with a value > 0.007 was excluded. The final imputed genomic dataset included 1,932 samples and 21,465,139 variants.

Assessment of late radiotherapy toxicity

Toxicity (CTCAE v4.0) was assessed prospectively by the treating physician at baseline, end of radiotherapy, and annual follow-up (+/- 1 month). The CTCAE grading system was used to characterise the severity: Grade (G)1 (mild), G2 (moderate), and G3 (severe or medically significant but not immediately life-threatening). Supplementary Table 1 lists the CTCAE v4.0 endpoints that were studied. Two-year toxicity endpoints were dichotomized to derive binary phenotypes (e.g. nipple retraction ≥ G1, ≥G2; atrophy ≥ G1, ≥G2 etc.). The phenotypes induration ≥ G2 and telangiectasia ≥ G2 included patients with any G2 or G3 toxicity in or outside the tumour bed. Patients with ≥ G2 toxicity at baseline were excluded from cases i.e. they were classed as controls. Patients with G1 toxicity at baseline which did not change at 2-year follow-up, were excluded from cases. For any patients with ≥ G2 toxicity at follow-up, baseline scores > G0 were included as co-variate in the regression model.

Statistical analysis

Multivariable generalised linear models were generated for each dichotomized toxicity endpoint. Clinical/treatment variables including co-treatments, patient co-morbidities, body mass index (BMI) and age as well as radiotherapy dose, regimen and technique were used to estimate the residuals for each model. The model residuals quantify the toxicity not explained by patient- and treatment-related factors. Therefore, patients with residuals of zero have toxicity entirely accounted for by the clinical and treatment factors included in the model. On the contrary, patients with negative or positive residuals have less or greater toxicity, respectively, than explained by the factors in the model.

Analysis was conducted in R, using the *glm* function to generate the logistic regression models. A stepwise regression approach, using the Akaike Information Criterion method (AIC) was used to avoid overfitting and to identify parsimonious multivariable models for each toxicity outcome. Regression models are shown in Supplementary Table 2.

Association analysis

A p-value < 5×10^{-8} was considered statistically genome-wide significant. All Genetic Association analyses used PLINK version 2 [20]. LocusZoom was used to display local linkage disequilibrium (LD), recombination patterns and the positions of genes in the region [21]. We included a total of 7,097,340 SNPs with MAF ≥ 0.05 and imputation score ≥ 0.3 in the analysis, which were tested for association with the residuals of toxicity endpoints at 2 years (Fig. 1, panel B). Variants with MAF ≥ 0.01 but < 0.05 were excluded. To control for European population substructure the top 15 principal components were included.

Power calculation

Power was estimated using the Genetic Association Study (GAS) Power Calculator [19]. For the toxicity endpoint with the greatest number of cases (Atrophy ≥ G1; 470 cases vs. 1,107 controls), the study had 99.9% power to detect a genotype relative risk (RR) of 1.5 ($\alpha = 5 \times 10^{-5}$, allele frequency = 0.3, toxicity prevalence = 29.8%). For different values of RR, power was 96.0% for RR = 1.4

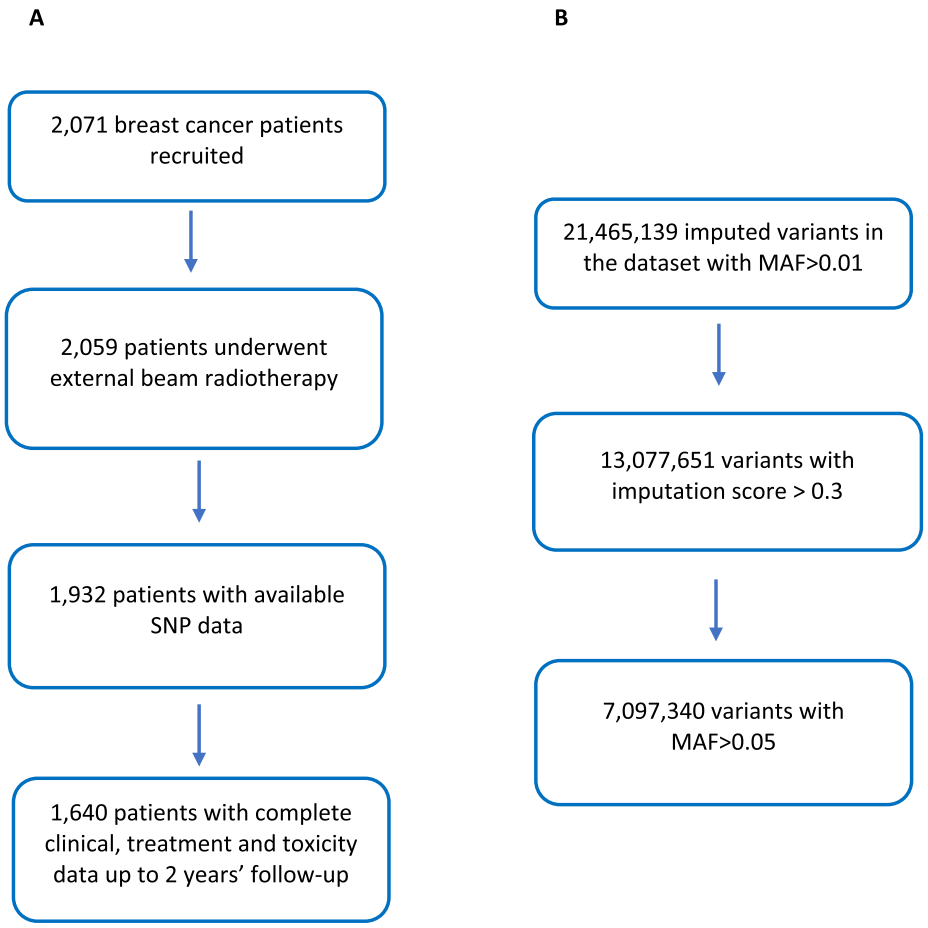

Fig. 1. Flow diagram showing the selection of patients (A) and genetic SNP data for GWAS (B) based on the inclusion or exclusion criteria (outlined in the methods, MAF = minor allele frequency).

and 67.2% for RR = 1.3. Assuming the same allele and phenotype frequencies, at the $\alpha = 5 \times 10^{-8}$ significance threshold, the study had 94.5% power to detect a RR of 1.5.

Heritability analysis

GCTA software was used to estimate heritability, which is defined as the proportion of phenotypic variation in a population attributed to the genetic variance between individuals [22]. Genetic relationship matrices (GRM) were computed using the SNPs on the autosome. A total of 6,389,759 valid SNPs were used. GRMs were input into a Restricted Estimated Maximum Likelihood (REML) analysis to estimate the proportion of phenotypic variance explained by the SNPs. All analyses were adjusted for the top 15 principal component analyses to account for possible confounding by population substructure.

Candidate gene analysis

Ninety candidate SNPs previously reported in the literature were tested in association with radiation toxicity in our GWAS results as a replication analysis (Supplementary Table 3-4). A Bonferroni correction for the number of SNPs tested was applied to correct for multiple testing ($p = 0.05/90 = 5.6 \times 10^{-4}$).

Polygenic risk score analysis

Polygenic risk scores (PRS) were developed using summary GWAS data for each toxicity endpoint. Only those SNPs with a GWAS association P value below the threshold ($P < 1 \times 10^{-5}$) were included in the calculation of the PRS, while all other SNPs were excluded.

To avoid using multiple SNPs in a risk locus, linkage disequilibrium clumping was performed, such that weakly correlated SNPs as well as SNPs most associated with the phenotype under study were preferentially retained. SNPs that had r2 higher than 0.1 with the index SNPs were removed while SNPs within 250 k of the index SNP were considered for clumping. PLINK V1.9 was used for clumping and for the construction of effect-size weighted PRS. The PRS was then used to stratify the cohort into two groups (upper and lower quartiles) and analysed against their respective toxicity traits using logistic regression models.

Results

There were 1,640 breast cancer patients in the REQUITE cohort with complete SNP, clinical, treatment and toxicity data two years after treatment (Fig. 1, panel A). Table 1 describes the patients' clinical and treatment characteristics. Mean age at treatment was 58.5 years. The median breast dose was 50 Gy (range 28.5–56.0) given in a median of 25 fractions (5–31). Boost was received by 71.1% of patients. Axillary node dissection was performed in 18.3% of patients and 11.9% received axillary irradiation.

Table 2 summarises the distribution by grade for each toxicity endpoint. Toxicity increased from baseline to 2-year follow-up across all toxicity endpoints. G3 toxicity was seldom reported. Correlation between the toxicity phenotype model residuals was gen-

erally weak except in cases with the same endpoint but different cut-off by grade (Supplementary Fig. 1).

Fig. 2A-E shows the Manhattan plots for individual toxicity endpoints where GWAS significant SNPs were identified including the telangiectasia endpoint where a SNP reached borderline significance, along with their corresponding QQ-plots. The GWAS results that did not reach statistical significance can be found in Supplementary Fig. 2A-F. Each dot on the Manhattan plot represents a SNP along the genome and the corresponding p-value for association with the phenotype residual (i.e. after adjusting for clinical and treatment variables). The QQ plots showed no evidence of genomic inflation (all $\lambda < 1.1$) and there were more associations with toxicity above the $p < 5 \times 10^{-4}$ level than expected by chance. The QQ plots for atrophy $\geq$ G2, induration inside tumour bed $\geq$ G1, induration outside tumour bed $\geq$ G1, nipple retraction $\geq$ G1 and oedema $\geq$ G1 demonstrate deflation over the smallest p-values (Supplementary Fig. 2B-F).

For arm lymphoedema $\geq$ G1, one SNP reached genome-wide significance (rs643644, $p = 3.54 \times 10^{-8}$, beta = 0.34, 95% CI: 0.22, 0.45). There were 33 SNPs with $p < 1 \times 10^{-6}$ (Fig. 2A). In association with breast atrophy $\geq$ G1, no SNP reached genome-wide significance although the rs7336605 SNP was borderline at $p = 8.06 \times 10^{-7}$ (Supplementary Fig. 2A). There were 15 SNPs with $p < 1 \times 10^{-6}$. For atrophy $\geq$ G2, the rs7227232 SNP was close to the genome-wide threshold ($p = 1.37 \times 10^{-7}$) (Supplementary Fig. 2B). No SNPs analysed for induration inside tumour bed $\geq$ G1 and induration outside tumour bed $\geq$ G1 reached genome-wide significance. However, several chromosomal regions contained groups of SNPs aligning almost vertically with p-values $< 10^{-5}$ (Supplementary Fig. 2C-D). For induration $\geq$ G2, two SNPs reached genome-wide significance (rs77311050, $p = 2.54 \times 10^{-8}$ and rs34063419, $p = 1.21 \times 10^{-8}$) (Fig. 2B). There were seven SNPs with $p < 1 \times 10^{-6}$. For nipple retraction $\geq$ G2, the rs188287402 variant was significant ($p = 2.80 \times 10^{-8}$). There were 89 SNPs below the p-value threshold of 1×10^{-6} (Fig. 2C). For nipple retraction $\geq$ G1 or oedema $\geq$ G1, there were no significant genome-wide SNPs (Supplementary Fig. 2E-F). However, the rs12657177 ($p = 1.12 \times 10^{-10}$) and the rs61966613 variants ($p = 1.06 \times 10^{-9}$) were significant for oedema $\geq$ G2 (Fig. 2D). For telangiectasia $\geq$ G1 (inside or outside the tumour bed), the rs12443861 variant (6.17×10^{-8}) reached close to genome-wide significance (Fig. 2E). There were 13 further SNPs below $p < 1 \times 10^{-6}$.

Table 3 summarises the eight genome-wide significant hits and two close to signifcant hits and their corresponding 2-year toxicity endpoint. Fig. 3A-E shows the LocusZoom plots for phenotypes with genome-wide significant hits. These plots demonstrate the extent of the association signal and the position relative to nearby genes, local linkage disequilibrium (LD) and recombination rates. The plots for arm lymphoedema $\geq$ G1 (Fig. 3A) show that the top SNP (rs643644) is in the intronic region of the Paired Box 7 (*PAX7*) gene on chromosome 1. The lead SNP on chromosome 7 (7:105482642_GT/GTT, $p = 5.78 \times 10^{-8}$) has several SNPs in LD spanning the Ataxin 7-Like 1 (*ATXN7L1*) and Cadherin-Related Family Member 3 (*CDHR3*) genes. For induration $\geq$ G2, Locuszoom plots of the two significant loci on chromosome 1 (rs77311050) and 4 (4: 48,467,985 G/GA) show a single causative SNP each (Fig. 3B). For nipple retraction $\geq$ G2, a group of SNPs in the *ANOS1* gene (all $p < 10^{-5}$) were in LD with the peak SNP rs188287402 (Fig. 3C). The plots for oedema $\geq$ G2 (Fig. 3D) show the rs75912034 variant on chromosome 5 represented by the red triangle, is in LD with the index SNP rs12657177. On chromosome 13, the rs61966613 variant (beta = 0.206) represented by the purple diamond located in the intron of Glypican 5 (*GPC5*) gene is in high LD with the rs61966612 SNP (beta = 0.205). The top hit for telangiectasia $\geq$ G1, the rs12443861 variant on chromosome 16 ($p = 6.17 \times 10^{-8}$), is in LD with a cluster of SNPs spannnig the Ankyrin Repeat and Sterile Alpha Motif Domain Containing 4B *(ANKS4B)* and Crystallin Mu (*CRYM)* genes (Fig. 3E).

Polygenic risk scores (PRS) for each toxicity endpoint were developed using SNPs with $p < 1 \times 10^{-5}$ to estimate the odds ratio. As expected, these were normally distributed (not shown). Having a score in the highest PRS quartile was associated with each toxicity endpoint with the exception of arm lymphoedema $\geq$ G1 (p = 0.684), nipple retraction $\geq$ G2 and oedema $\geq$ G2 (p = 0.986) (Table 4).

SNP heritability was calculated as the percentage of phenotypic variance explained by all SNPs tested in the study (Table 5). The highest heritability was estimated for induration $\geq$ G2 (39%) with only atrophy $\geq$ G1 and telangiectasia $\geq$ G1 also being statistically significant at 25% and 20%, respectively.

Previous candidate SNPs (n = 90) reported as significantly associated with long-term breast radiation toxicity are listed in Supplementary Table 3. At the pre-selected significance level, none of the previously reported SNPs were replicated in our analysis (Supplementary Table 4). A total of 45 SNPs achieved p-values < 0.05 across different toxicity phenotypes with 10 SNPs 'replicated' at the 0.05 level across two or more endpoints. The lowest p values identified were for the rs12562052 variant (beta = 0.09, p = 0.002) near the *FCRL5* gene and telangiectasia $\geq$ G1, and for rs754692 situated between *PEPD* and *CHST8* (beta = 0.07, p = 0.003) and oedema $\geq$ G2.

Discussion

This study provides further evidence for the association of common SNPs with distinct breast radiation toxicity endpoints. We have identified new genomic regions associated with toxicity at 2-year follow-up for arm lymphoedema $\geq$ G1, nipple retrac-

Table 2
Distribution of patients by CTCAEv4.0 grade of toxicity (baseline and 2 years following radiotherapy).

Grade	Arm Lymphoedema		Atrophy		Telangiectasia outside tumour bed		Telangiectasia inside tumour bed	
	Baseline	2 years	Baseline	2 years	Baseline	2 years	Baseline	2 years
0	1603	1579	1160	857	1620	1568	1624	1563
1	33	52	396	564	15	68	12	73
2	2	9	66	191	2	5	2	5
3	-	-	11	23	-	-	-	–
	Induration inside tumour bed		Induration outside tumour bed			Nipple retraction		Oedema
	Baseline	2 years	Baseline	2 years	Baseline	2 years	Baseline	2 years
0	986	986	1524	1405	1449	1395	1449	1425
1	572	546	95	210	156	206	177	187
2	70	103	15	23	14	18	13	29
3	9	7	2	3	-	-	-	-

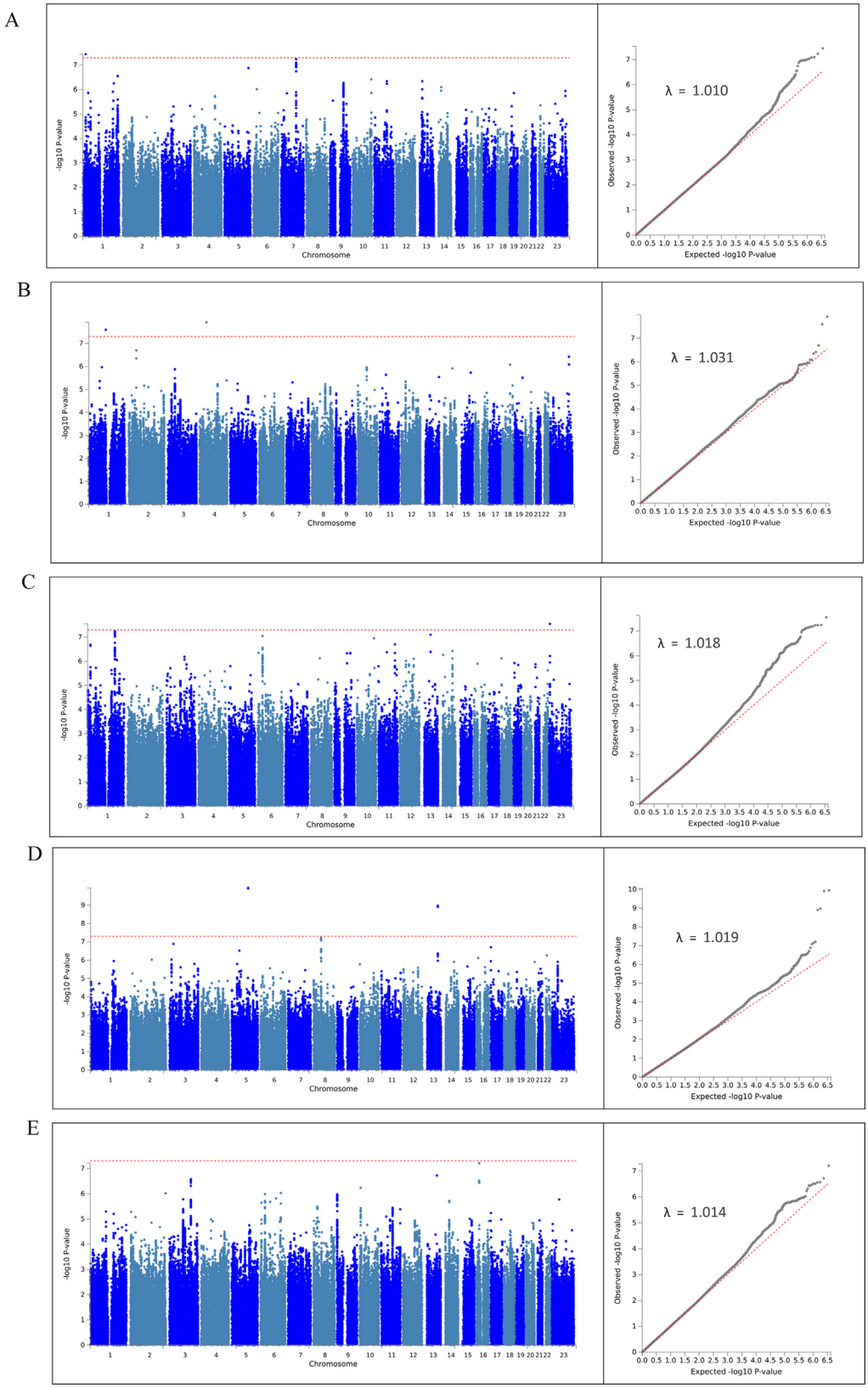

Fig. 2. A-E. Manhattan plots for late toxicity endpoints with statistically significant GWAS associations. Each point represents a single nucleotide polymorphism (SNP), with numbers on the x-axis denoting the location in the genome. The red dashed line indicates the threshold for genome-wide significance: $p < 5 \times 10^{-8}$. The Y-axis shows − log10 p-values for the association of each of the tested SNPs with the phenotypes. Quantile-quantile (QQ) plots represent the deviation of the observed p-values from the null hypothesis. The red diagonal indicates the expected and the grey line shows observed p-values. The QQ plots display deviation from the null distribution above P values of 4 (-log10) suggesting that common SNPs are associated with risk of 2-year radiation toxicity. The genomic inflation factor (λ) is shown on the QQ-plots.

Table 3
Genome-wide significant hits and corresponding toxicity phenotype. Two variants which reached closed to genome-wide significance in association with arm lymphoedema G1 and telangiectasia G1 are also included.

Phenotype	Chromosome: position: ref/alt	Alt frequency	Peak SNP	Nearest gene(s)	Odds Ratio (Confidence Interval)	P value
Arm Lymphoedema ≥ G1	1: 19,050,896 A/G	0.94	rs643644	*PAX7*	1.40 (1.24, 1.57)	3.54×10^{-8}
	7: 105,482,642 GT/GTT	0.17	rs11345494	*ATXN7L1*	1.13 (1.08, 1.18)	5.78×10^{-8}
Induration ≥ G2	1: 116,746,614C/T	0.06	rs77311050	*LINC01779*	1.34 (1.21, 1.48)	2.54×10^{-8}
	4: 48,467,985 G/GA	0.95	rs34063419	*SLC10A4*	1.71 (1.42, 2.05)	1.21×10^{-8}
Nipple Retraction ≥ G2	X: 8537499 T/C	0.07	rs188287402	*ANOSI*	1.14 (1.09, 1.19)	2.80×10^{-8}
Oedema ≥ G2	5: 113,214,053C/T	0.08	rs12657177	*AC093240.1*	1.24 (1.16, 1.33)	1.12×10^{-10}
	5: 113,229,284C/G	0.08	rs75912034		1.24 (1.16, 1.33)	1.21×10^{-10}
	13: 92938752 T/C	0.06	rs145328458	*GPC5*	1.23 (1.15, 1.31)	1.06×10^{-9}
	13: 92938236 T/A	0.06	rs61966612		1.23 (1.15, 1.31)	1.23×10^{-9}
Telangiectasia ≥ G1	16: 21,259,515 A/T	0.24	rs12443861	*ANKS4B, CRYM*	1.20 (1.12, 1.28)	6.17×10^{-8}

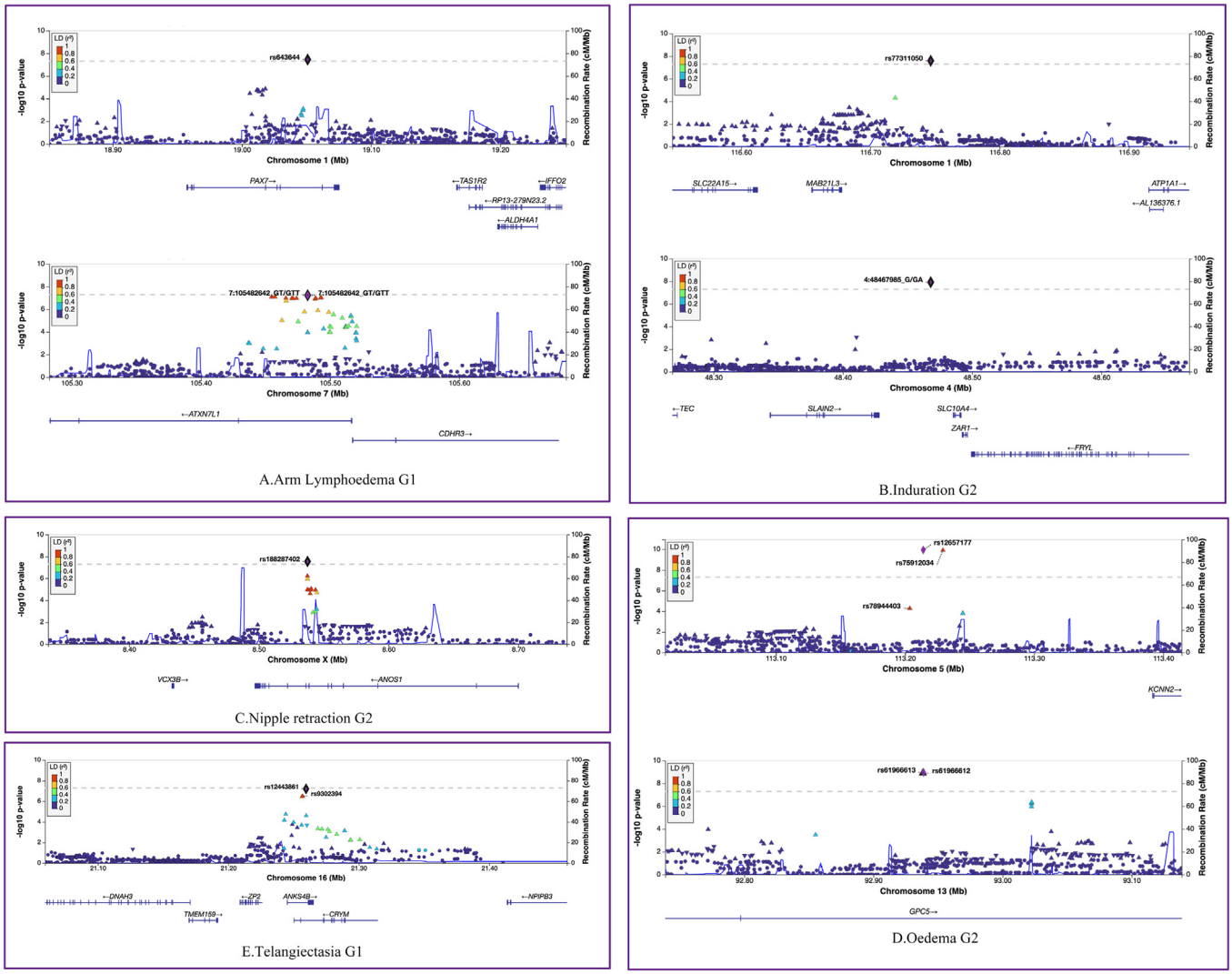

Fig. 3. A-E: Locuszoom plots of the top reported SNPs. Purple diamond represents the SNP of interest. Points representing nearby SNPs are color-coded according to linkage disequilibrium r2 value as indicated in the legends. The variants in orange or red colour are in high Linkage Disequilibrium with the specified SNP. The X-axis shows the genomic coordinates of the relevant chromosome. The Y1 axis shows − log10 P-values for each of the SNPs in the genome. The Y2 axis shows the combined recombination rate which is estimated from the international HapMap project.

tion ≥ G2, induration ≥ G2, and oedema ≥ G2. None of the significant SNPs in this study have previously been reported in association with clinical radiation toxicity or breast cancer susceptibility. Although these would require further investigation, there are biologically plausible mechanisms by which these SNPs could exert a clinical effect.

Table 4
Polygenic risk score (PRS) results developed for each dichotomised phenotype using all SNPs from the GWAS with $p < 1 \times 10^{-5}$. For each PRS, corresponding odds ratios, their standard errors, and p-values are shown. N/S = non-significant.

	Odds ratio	Std. Error	P value	SNPs in PRS
Arm Lymphoedema ≥ G1	0.89	0.27	0.684	39
Breast Atrophy ≥ G1	4.39	0.13	2.0×10^{-16}	12
Breast Atrophy ≥ G2	4.46	0.19	7.0×10^{-15}	16
Induration outside tumour bed ≥ G1	1.74	0.19	2.0×10^{-16}	16
Induration inside tumour bed ≥ G1	4.26	0.13	2.0×10^{-16}	12
Induration inside tumour bed ≥ G2	3.72	0.59	2.4×10^{-10}	26
Nipple Retraction ≥ G1	6.39	0.23	4.8×10^{-16}	10
Nipple Retraction ≥ G2	N/S	N/S	0.986	76
Oedema ≥ G1	4.13	0.19	2.8×10^{-13}	13
Oedema ≥ G2	N/S	N/S	0.986	57
Telangiectasia ≥ G1	7.55	0.27	1.3×10^{-13}	25

Table 5
Heritability analysis for each radiotherapy associated toxicity endpoint. V(G)/Vp represents the proportion of genotypic to phenotypic variation.

Toxicity phenotype	V(G)/Vp or H^2	SE	p-value
Arm lymphoedema ≥ G1	0.02	0.15	0.46
Atrophy ≥ G1	0.25	0.15	3.5×10^{-2}
Atrophy ≥ G2	0.07	0.14	0.29
Nipple Retraction ≥ G1	<0.01	0.15	0.50
Nipple Retraction ≥ G2	<0.01	0.15	0.50
Oedema ≥ G1	0.01	0.15	0.46
Oedema G ≥ 2	0.14	0.15	0.16
Induration inside tumour bed ≥ G1	0.04	0.16	0.41
Induration outside tumour bed ≥ G1	0.16	0.15	0.13
Induration inside tumour bed ≥ G2	0.39	0.15	2.6×10^{-3}
Telangiectasia ≥ G1	0.20	0.15	7.4×10^{-2}

The top reported SNP, rs11345494, for arm lymphoedema ≥ G1 is located near the protein coding Cadherin related family member 3 *(CDHR3)* gene. This gene is thought to be functionally related to cadherins which have a role in cell-adhesion and cell-to-cell signalling [23]. Since disturbance of intracellular adhesion is a prerequisite for invasion and metastasis of tumour cells, cadherins are considered prime candidates for tumour suppressor genes. However, little is known about the link between *CDHR3* and cancer in the literature. The other variant associated with arm lymphoedema, rs643644, is found in the paired box *(PAX7)* gene. The *PAX7* gene is a transcription factor that plays a role in myogenesis during embryonic development, however, the specific function of this gene is unknown. The top reported SNP for telangiectasia, rs12443861 is found in the protein coding Ankyrin Repeat And Sterile Alpha Motif Domain Containing 4B *(ANKS4B)* gene and the Crystallin Mu *(CRYM)* gene. *ANKS4B* Is essential for intermicrovillar adhesion complex formation [24].

The top genomic risk loci in this study varied across the phenotypes, which suggests there are differences in the mechanisms underlying the pathogenesis of different radiation toxicity endpoints. This finding is supported by previous radiogenomics studies [25]. There is evidence to suggest that the underlying SNP profiles are tissue-specific and there may not be a single SNP profile that is representative of all radiotherapy side-effects [12]. The weak correlation between the different toxicity endpoints, as shown in this study (Supplementary Fig. 1), corroborates similar findings in previous studies including a lack of correlation in radiotherapy response between two different tissues [26]. In the present study, the peaks in the Manhattan plots differed whether the cut-off for cases was G1 or G2 for the same phenotype. This may be explained by the relatively smaller number of cases for the G2 phenotypes compared to G1 leading to more imbalanced data, e.g. oedema ≥ G2, which only had 29 cases; or it may represent a true difference in the underlying SNP profile for clinically significant vs. the relatively more frequent milder cases.

Most of the 2-year radiation toxicity phenotypes studied here had heritability estimates below 20%, the highest being induration ≥ G2 at 39%. These relatively moderate estimates are likely due to a combination of insufficient power and polygenicity [27]. For example, the heritability estimation for Atrophy ≥ G1 is 25%, which is significant. However, for Atrophy ≥ G2, the heritability drops to 7% due to the reduced number of cases compared to controls, resulting in a loss of statistical significance. Some of the QQ plots also showed deflation from the diagonal at very small p-values below 10^{-5}. This is again likely due to limited statistical power. The sample size of the present analysis, while large for a radiogenomics study, is modest in comparison to those used in other fields such as cancer risk. The discovery of risk SNPs through GWAS often depends on very large sample sizes of genotyped data, especially if the aim is to capture a large fraction of the SNP heritability. As larger GWAS are conducted, additional variants with small effect sizes are likely to be discovered. Heritability estimates may also be low due to unknown sources of variation in the environment. These include differences in clinicians' scoring of the phenotype, or management of acute toxicity by clinicians which could impact the incidence of late toxicity, as well as other differences between the treatment centres.

The lack of validation of SNPs previously reported in association with long-term breast radiation toxicity may also be explained by a lack of statistical power, both in the present and in the original studies. For example, a previous GWAS conducted using the RAPPER cohort with 778 patients at 2-year follow-up identified the following SNPs potentially associated with telangiectasia: rs16837908, rs11854033 and rs169585362 [12]. However, none of these SNPs were statistically significant in the REQUITE breast cohort: rs11854033 (beta = 0.042, p value = 0.12) and rs169585362 (beta = 0.053, p value = 0.066), while rs16837908 was not genotyped in the REQUITE dataset. The use of different toxicity scales between the studies may pose an additional source of heterogeneity. The START, LENT-SOMA and CTCAE v3 scales were used for clinical assessment in earlier studies whereas CTCAE v4 was used to grade toxicity in REQUITE.

Future work

The Manhattan plots in this study confirm that radiation toxicity is a complex polygenic trait and there are many variants that contribute to each phenotype. Continued patient recruitment into new studies and pooling of existing studies in a meta-GWAS approach is essential so that methodological challenges may be addressed. This will continue to eliminate any false positives and increase statistical power to detect definite variants which individually are likely to have small effect sizes. Studies should also

increase recruitment from genetic ancestry groups other than White Europeans.

One approach is to summarise the effects of multiple individual SNPs, which are typically small, into a single polygenic risk score (PRS), in order to obtain a better estimate of the risk attributed to common genetic variants [10]. Our future work will include a meta-GWAS and validation of the PRS developed in the present study in an independent dataset. If validated, the PRS could then be incorporated into predictive risk algorithms, with or without clinical and treatment risk factors. These algorithms can then be evaluated in clinical trials or incorporated into radiotherapy planning software, to optimise treatment delivery for patients at elevated risk of toxicity. Furthermore, detailed bioinformatic and experimental analysis should elucidate the causative genes and biological pathways involved in the development of radiation toxicity. Our future work will also focus on longer-term toxicity beyond two years and the long-term outcome data from current radiogenomics cohorts is eagerly anticipated to be able to examine cumulative toxicity over time.

Conclusions

The present GWAS provides further evidence for significant association of common SNPs with distinct breast radiation toxicity endpoints two years following radiotherapy. It extends the field of radiogenomics and adds a piece to the increasing evidence that patients are genetically predisposed to the development of toxicity following radiotherapy. Further collaborative efforts combining patient cohorts with high-quality data collection are vital to enable us to identify genetic variants with lower penetrance and validate the SNPs discovered in current studies. This will allow us to develop predictive risk models and PRS to guide treatment decision-making and augment the precision oncology approach for breast cancer patients.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CJT, TRat and HKJ conceived the study design. HKJ wrote the first draft of the paper. HKJ, CDV, LF an CL analysed the data. HKJ, LF, CJT, and TRat contributed to the interpretation of the data. CMW was the lead investigator and CJT is the deputy lead investigator of the REQUITE study. MA-B, MA, DA, AB, CB, RB, RRC, MPF-J, SG, ML, CM, VR, TRan, TRat, DDR, MCdS, ES, RPS, BT-V, LV, and CW recruited patients into the study. HS is the breast cancer patient advocate on the REQUITE study. AJW curated the database for the REQUITE study. PS and RME were study managers. All authors commented on and approved the final manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radonc.2023.109806.

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