Predicting radiotherapy toxicity in patients treated with radical radiotherapy using predictive assays and circadian rhythm.

A thesis submitted for the degree of
Doctor of Medicine
at the University of Leicester

By Dr Kerstie Anne Johnson

(BMedSci, BMBS, MRCP, MSc Onc, FRCR)

Clinical Oncology SPR

Department of Genetics, University of Leicester

June 2017

ABSTRACT:

Predicting radiotherapy toxicity in patients treated with radical radiotherapy using predictive assays and circadian rhythm.

Dr Kerstie Anne Johnson

Radiotherapy is a fundamental cancer treatment and plays a pivotal role in the improving outcomes for the disease. Depending on the cancer site and organ at risk rates of moderate to severe acute toxicity range between 15 to 30 percent and rates of late toxicity between 5 and 15 percent. If a patient's individual risk of radiotherapy toxicity could be predicted then their treatment could be tailored appropriately.

In this thesis two cohorts have been used to analyse predictive measures for acute and late radiotherapy toxicity: the REQUITE cohort (prospective international observational study of breast, prostate and lung cancer patients) and the LeND cohort (retrospective local study of breast cancer patients). Three main areas have been examined to establish whether they can be used to predict for radiotherapy reactions. In the prostate and lung patients associations between clinical and treatment variables and acute toxicity were reviewed. The second area was predictive assays: DNA damage and repair were assessed using the comet and γ-H2AX assays and apoptosis in lymphocytes using the RILA (radiation induced lymphocyte apoptosis assay). Finally the effect of circadian rhythm and its underlying genetics on radiotherapy toxicity were assessed.

Many of the variables were significantly associated with increased toxicity on univariate analysis. Three were significantly associated with toxicity on multivariate analysis. Acute toxicity in prostate patients was associated with intended duration of hormones ($p=0.05$) and V50 bladder ($p=0.01$)). Morning radiotherapy was associated with increased overall bivariate STAT score ($p=0.03$) in the LeND volunteers.

The results of this study indicate clinical and genetic variables and the use of predictive assays can be utilised to create more personalised radiotherapy treatments that strive for better cancer and quality of life outcomes for patients.

ACKNOWLEDGMENTS

First of all I would like to thank my amazing husband for putting up with me during this research. Especially over more recent months in my sleep deprived new parent state surrounded by nappies and everything that goes along with being a first time mum. I would like to thank my gorgeous son Finlay who kindly arrived fifteen days late giving me plenty of extra time to complete the first draft of my write up.

Next I would like to thank my supervisors Dr Chris Talbot, Professor Paul Symonds and Professor George Don Jones. Without their help and support I would not have made it this far. I would especially like to thank them for their patience particularly when training me up in the lab and not making the assumption that a medical doctor would know anything about the running of a laboratory.

I would like to thank Mr Tim Rattay for being my office buddy, statistics guru and sharing the workload of recruiting 650 patients and most importantly keeping me supplied with cups of tea during long days of data inputting.

The work and help assessing and recording REQUITE participants from Theresa Beaver, Amy Dineen and Sara Barrows has been greatly appreciated. Along with all the radiographers and clinic staff who have kindly identified patients and handed back questionnaires. Many thanks also go to the medical teams in the Leicester Royal Infirmary, Royal Derby Hospital and Nottingham City hospital particularly Dr Vasanthan, Dr Osman, Dr Kancherla, Dr Asnar-Garcia, Dr Kent, Dr Sridhar and Dr Das as well as the clinical oncology registrars for handing out patient information sheets and completing assessments for the REQUITE trial.

I would like to thank Sheila Smith, Kosalie Redman, Diana Martin, Eva Saiti, Anna Critchley, Sam Lavers, Hannah Dobbelaere, Chiara Batini and Maxime Boy for all their hard work in the lab. Without them I would not have had access to results from such large sample numbers.

Finally I would like to say a big thankyou to everyone who I have not been able to mention by name who have provided advice, friendly banter and overall made my research successful and enjoyable.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

°C	Degrees centigrade
95%CI	95% confidence intervals
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related kinase
bps	Base pairs
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CHART	Continuous Hyperfractionated Accelerated Radiotherapy
CIGMR	Centre for integrated Genomic Medical Research
CTCAE	Common terminology criteria for adverse events
CTV	Clinical target volume
DCIS	Ductal carcinoma in situ
ddH20	Double distilled water
DLCO	Carbon monoxide diffusion capacity
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSBs	Double strand breaks
EDTA	Ethylenediaminetetraacetic acid
EORTC	European Organisation for Research and Treatment of Cancer
FACS	Fluorescence-activated cell sorting (Flow Cytometry)
FBS	Fetal Bovine Solution
FITC	Fluorescein isothiocyanate
GI	Gastrointestinal
GTV	Gross Tumour volume
GU	Genitourinary
GWAS	Genome wide association study
Gy	Gray (units of dosing for radiotherapy)
H2AX	Member of the histone family
HDR	High dose rate brachytherapy
HR	Homologous recombination
IMRT	Intensity Modulated Radiotherapy
ITV	Internal Target Volume
KCO	Carbon monoxide transfer coefficient
KV	Kilovoltage
L	Litre
LeND	Leicester, Nottingham, Derby study
LENT-SOMA	Late effects of normal tissue-subjective objective management analytical
M	Molar
MMR	Mismatch repair
MRN	Mre11-Rad50-Nbs complex
MV	Megavoltage
NHEJ	Non-homologous end joining
NOC	Nocturnin
NTCP	Normal Tissue Complication Probability
OTM	Olive Tail moment
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction

PER3	Period 3 gene
PET	Positron emission tomography
PI	Propidium Iodide
PSA	Prostate Specific Antigen
PTD	Percentage Tail DNA
PTV	Planned Target Volume
RID	Radiation Induced Damage
RILA	Radiation induced lymphocyte apoptosis
RNA	Ribonucleic acid
ROR	Rate Of Repair
RPS	Reps per second
RRR	Relative rate of repair
rs	Reference SNP
RTOG	Radiation Therapy Oncology Group
SABR	Stereotactic ablative radiotherapy
S.E.	Standard Error
SNP	Single nucleotide polymorphisms
SSD	Source to surface distance
STAT	Standardised Total Average Toxicity
TANC1	Tetratricopeptide Repeat, Ankyrin Repeat And Coiled-Coil Containing 1
TCP	Tumour control probability
TNF	Tumour Necrosis Factor
µl	Microlitre
µm	Micrometer
µM	Micromolar
UV	Ultraviolet (light)
V	Volt

CHAPTER 1 : INTRODUCTION

1.1 Use of Radiotherapy

In the UK around 40% of patients who have curative cancer treatment receive radiotherapy as part of their management plan (UK 2014).

1.1.1 Prostate cancer and radiotherapy

In the UK over 47,000 men are diagnosed each year with prostate cancer, this equates to a lifetime risk of about one in eight men in the country (UK). Of these patients 85% present with localised disease (Serag et al. 2012).

The use of radiotherapy in addition to hormones in locally advanced and high risk localised prostate cancer comes from two randomised controlled trials. The first was the Scandinavian SPCG-7 trial (Widmark et al. 2009) which showed a reduction in cancer-specific ($p < 0.001$) and overall mortality ($p = 0.004$) when radiotherapy was added to androgen blockade. The second was a phase three randomised study, the PRO7 study (Amini et al. 2016, Mason et al. 2015) which has recently been reviewed at twenty years follow up and again shows a benefit for radiation to the prostate in addition to androgen therapy compared to androgen therapy alone. PR07 showed biochemical-free progression as well as all-cause mortality and cancer-specific mortality were improved by the addition of radiotherapy to hormones.

Management of organ confined prostate cancer is more controversial with options including active surveillance, radical prostatectomy, external beam radiotherapy, brachytherapy, cryotherapy and High Intensity Focused Ultrasound (HIFU). A meta-analysis (Xiong et al. 2014) and systematic review (Wolff et al. 2015) of randomised control trials comparing management options for localised prostate cancer was unable to conclude that any of the treatment options were superior. The PIVOT trial (Wilt et al. 2012) compared radical prostatectomy to observation in men under the age of 75 years with organ confined prostate cancer (T1-T2) and PSA less than 50ng/ml. It found there was no significant survival difference between the two arms. The Protec-T trial (Lane et al. 2014) published their ten year follow up data in September 2016 (Hamdy

et al. 2016). This is the first randomised trial comparing active surveillance, radiotherapy and prostatectomy in low/intermediate risk patients aged 50-69 years. There were only seventeen (out of 1643 study participants) cancer related deaths in this ten year follow period. No significant difference was observed in overall survival between the treatment arms. Disease-free progression was lower in the surgery and radiotherapy arms compared to the active surveillance group (p=0.0004).

An important factor in decision making is the risk of side effects. The Protect-T study in addition to survival looked at patient reported outcome measures for bowel, urinary and sexual function (Donovan et al.) following the three interventions. Urinary and sexual function were worst after surgery. Bowel symptoms, particularly per rectal bleeding, was more significant in the radiotherapy arm.

When considering the treatment options for localised prostate cancer there are increasing possibilities and options as discussed above (Chang et al. 2015). Even in high risk cancer not amenable to resection addition of prophylactic lymph node irradiation (Morikawa and Roach 2011) or high dose rate brachytherapy (Khor et al. 2013) to external beam radiotherapy are often considered when deciding a management plan. This can be a confusing time for patients and they agonise over making the right decision. The ability to more accurately predict what the likely side effects would be following treatment would help patients and clinicians to make these difficult decisions.

1.1.2 Breast cancer and radiotherapy

Breast cancer is the most common female cancer in the UK with 53,696 new cases of invasive disease being diagnosed in 2013 (CRUK 2013). Of these 94% will have curable disease and radiotherapy will be offered to all patients who have a wide local excision or high risk patients following mastectomy.

A meta-analysis (Early Breast Cancer Trialists' Collaborative 2005, Darby et al. 2011) looking at the benefits of radiotherapy in breast cancer patients showed that radiotherapy reduces rates of five year local recurrence following wide local excision (absolute reduction of 19%) and improves fifteen year overall survival rates (absolute

difference of 5.4%). However the same meta-analysis showed an increase in non-breast cancer associated mortality of the irradiated patients particularly from heart disease and lung cancer. The START trial (Haviland et al. 2013) reported that ten year rates of moderate or severe breast induration or breast shrinkage were between 23.0% to 26.3% in both the standard fractionated arm (2Gy per fraction) and the moderately hypofractionated arm (2.6Gy per fraction). Both these papers highlight the relevance and problems of late toxicity following radiotherapy in breast cancer patients.

1.1.3 Lung cancer and radiotherapy

43, 463 people are diagnosed with lung cancer each year (CRUK 2014) and of these only five percent are predicted to survive ten years. Lung cancer is the leading cause of cancer deaths in the UK equating to one in five cancer related deaths.

Lung cancer can be divided into non-small cell (90% of cases) and small cell (10%) subtypes. Small cell lung cancer due to its poor prognosis is largely treated with non-surgical approaches, however there is emerging literature in early stage tumours that surgery improves overall survival (Luchtenborg et al. 2014). Non small-cell lung cancer however, can if presenting at a local stage be managed with curative intent by either surgery or radiotherapy. 14.5% present as stage one and 7.3% as stage two (CRUK 2014), i.e. potentially resectable disease. As the majority of non-small cell lung cancer cases present with advanced disease then to improve mortality rates earlier detection is necessary.

Currently first line treatment for operable non-small cell lung cancer is surgery rather than radiotherapy (Baldwin et al. 2011) based on NICE guidelines updated in September 2016. Patients who are not fit for surgery should be offered radical radiotherapy with curative intent although these guidelines do not include the use of stereotactic ablative radiotherapy (SABR) as a treatment option for stage one lung cancer. A recent review of two randomised phase three trials published in the Lancet Oncology in 2015 (Chang et al. 2015) suggested that SABR had equivalent three year recurrence free survival (log rank p= 0.54) and better three year survival than

lobectomy (log rank p=0.037). Post radiotherapy toxicity grade three, four or five was also less than the equivalent post-operative morbidity (10 % vs 48%). Whilst this study only involves small numbers of patients it is becoming increasingly common for patients to be given the option of either radiotherapy or surgery for operable stage one lung cancers. Similar to prostate cancer, treatment decisions can cause anxiety to patients and their relatives. Much of this decision making process is likely to revolve around weighing up the possible toxicities that may result from either technique. A predictive assay for likelihood of radiotherapy toxicity would enable them to make a more informed decision about the management of their cancers.

1.2 How radiotherapy works

Radiotherapy is a type of ionising radiation which deposits energy in materials through a series of excitation and ionisation events. Ionisation occurs as a result of electrons being displaced from an atom or molecule to leave a charged ion. The types of radiation used in radiotherapy are predominately composed of photons (X-rays and gamma rays) and electrons. Photons are created in the head of a radiotherapy linear accelerator (linac) by firing electrons at a tungsten target. Depending on the initial energy of the striking electrons different energy photon beams are created.

The energy of photons used in radiotherapy range from 90 KV to 20MV. The energy of the photon will influence the type of interaction which occurs when it passes through matter. There are three possible interactions which can occur. The reaction most commonly seen with therapeutic radiotherapy is Compton scatter. In this a photon interacts with an unbound electron in the atom it collides with and the energy is divided between an exiting electron and photon. The resultant electron transfers energy to the material it is passing through via a process of excitations and absorptions.

1.2.1 Radiotherapy induced DNA damage

Ionisations can occur in two different ways either directly or indirectly. In direct interactions DNA or protein molecules are struck by radiation and cause either cell

death or mutations of the DNA (Bolus 2001). This can occur due to double or single strand breaks. Indirect ionisations occur when radiation interacts with water molecules to create a hydrogen molecule and one charged free radical. This free radical can then go on to cause damage to the chemical bonds in DNA (Joiner 2009).

Cells detect DNA damage and attempt to repair it using one of several DNA repair pathways. Double strand breaks (DSBs) induced by radiation are repaired by two DNA repair pathways: Homologous recombination and non-homologous end joining. The Mre11-Rad50-Nbs1 (MRN) complex detects DSBs, activates cell cycle checkpoints and regulates these two DNA repair pathways (Lamarche et al. 2010). MRN activates protein kinases (Ataxia telangiectasia mutated protein (ATM), the ATM and Rad3-related kinase (ATR), and the DNA-dependent protein kinase (DNA-PK)) which in turn phosphorylate H2AX (to γ-H2AX) at the site of DNA damage setting off a chain of events resulting in DNA repair via homologous recombination or the more error prone non homologous end joining. This is shown in figure 1.

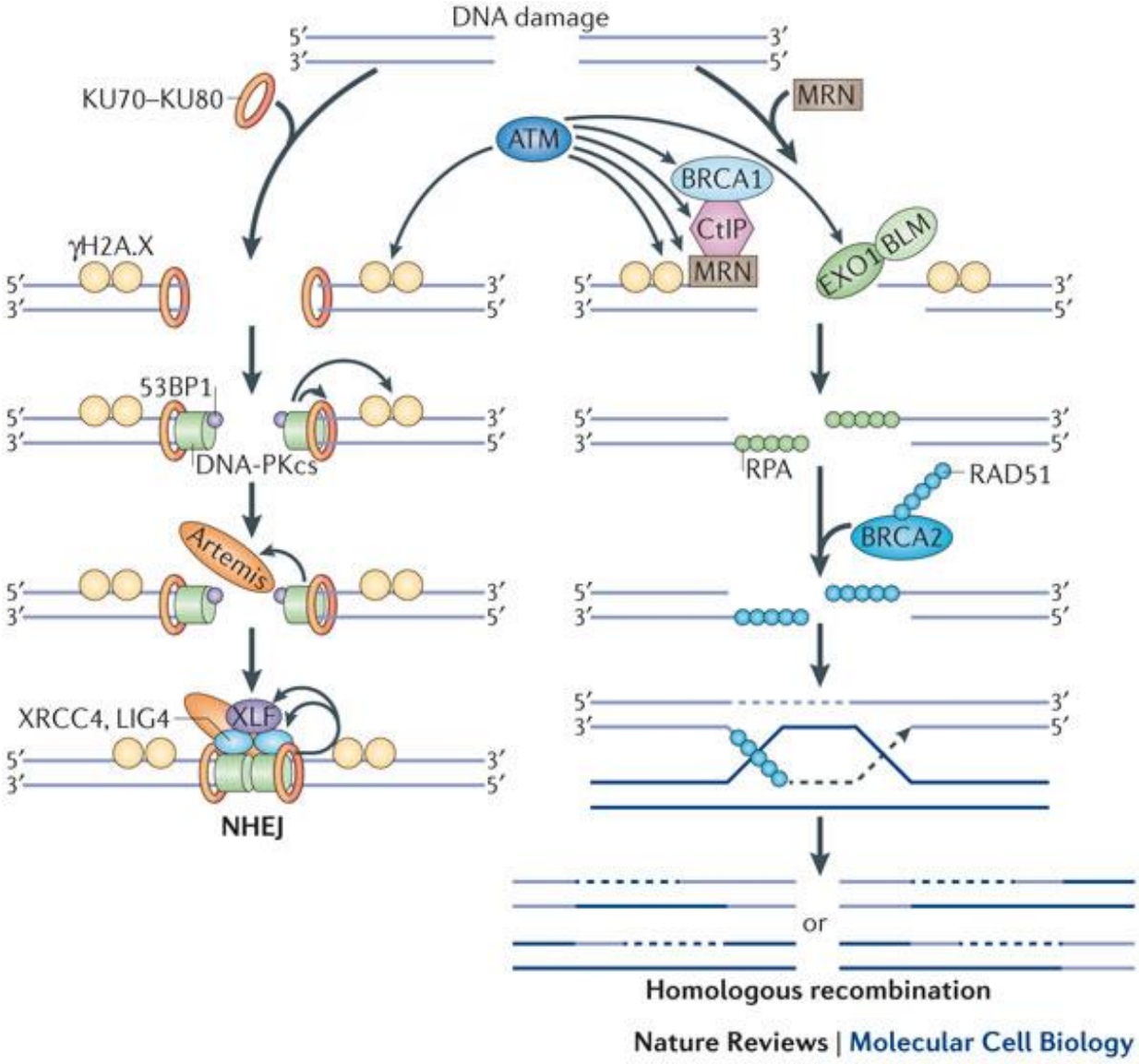

Figure 1: Diagram taken from Chowdhury 2013 Nature review (Chowdhury et al. 2013) depicting the chain of events which occurs following DNA damage and ultimately leads to either Non Homologous End Joining (NHEJ) or Homologous Recombination (HR). DNA-dependent protein kinase (DNA-PKcs); p53-binding protein 1 (53BP1); Ataxia telangiectasia mutated (ATM); phosphorylated histone H2A.X (γ-H2AX); LIG4 (DNA ligase 4); XRCC4 (X-ray repair cross-complementing protein 4); XLF (XRCC4-like factor); ATM and Rad3-related kinase (ATR); CtIP (CtBP-interacting protein), BRCA1 (breast cancer type 1 susceptibility); EXO1 (exonuclease 1); BLM (Bloom's syndrome helicase); replication protein A (RPA); RAD51 (DNA recombination/repair protein).

Cells which divide rapidly go through regular cell cycling and as such are more sensitive to DNA damage induced by radiotherapy. This includes cancer cells but also normal tissues such as hair follicles, skin and gut epithelium. The ultimate goal of radiotherapy is to damage the DNA resulting in death of the cancer. If enough cancer cells are eradicated in this way then radiotherapy can result in long term control and cure of

some tumours. Rapid division and repopulation of tumours must be overcome when giving curative regimens of radiotherapy to eradicate the cancer. This is complicated by the effect radiotherapy has on normal tissues and the resulting side effects that it may cause.

1.2.2 Radiotherapy in normal tissues

The therapeutic ratio is a balance between the probability of tumour control (TCP) and the risk of normal tissue complications (NTCP) (Beasley et al. 2005). This is demonstrated in figure 2, where dose "a" has a 60% chance of tumour control and a relatively low risk of radiotherapy related complications. Whereas dose "b" has an almost 100% chance of tumour control but a much higher risk of radiotherapy related toxicity.

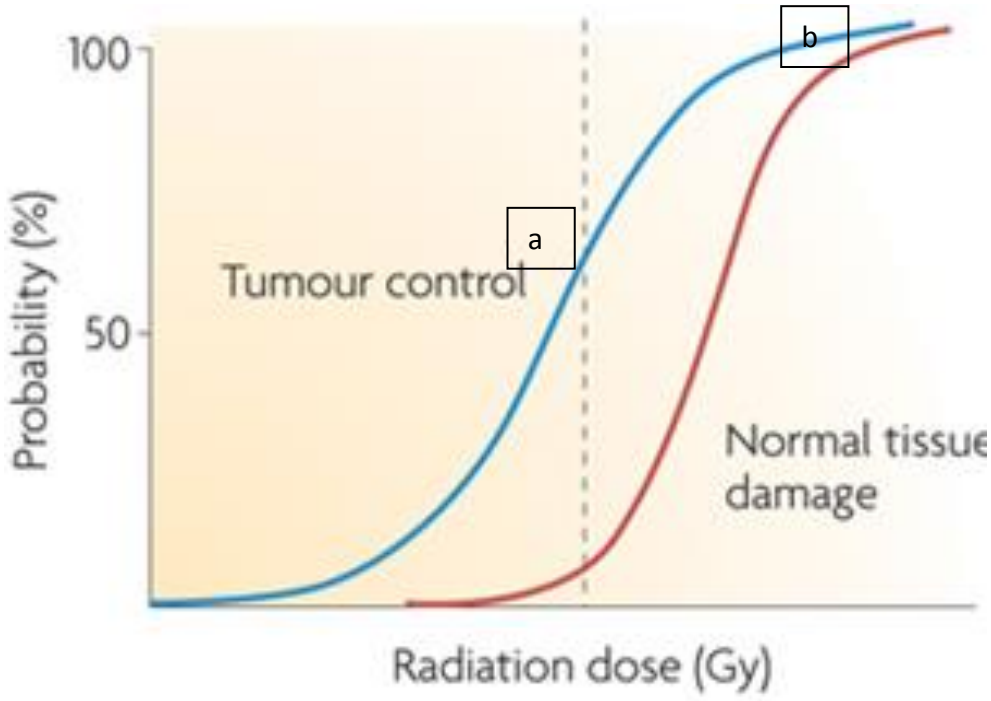

Figure 2: Tumour control probability diagram. The probability of tumour cure increases with increasing radiation dose. As a small volume of normal tissue is unavoidably included in the radiation field, the probability of severe late, normal-tissue damage also increases. Radiotherapy schedules have developed to maximise cure while minimising toxicity and the dotted line shows a theoretical dose associated with ~60% tumour control and ~5% severe late toxicity. From Barnett et al. (Barnett et al. 2009)

Normal tissue toxicity cannot be avoided altogether as the radiotherapy beam must pass through other organs and tissues to reach its target in the tumour or tumour bed. Dose limits are calculated based on the experience of radiotherapy over many decades which has given us the pivotal Emami (Emami et al. 1991) in 1991 and later in 2010 Quantec (Bentzen et al. 2010, Marks et al. 2010) papers. These set out radiotherapy dose constraints above which it is deemed more likely that patients will exeperience moderate to severe complications as a result of their treatment. For example the Quantec paper sets a constraint for irradiation of the rectum that no more than 50% of the organ should receive more than 50Gy and no more than 25% of the rectum should receive more than 65Gy. Levels exceeding these are suggested to carry a risk of unacceptable toxicity with an estimate of less than ten percent of the treatment population experiencing moderate to severe complications below this level. These dose constraints have been developed to ensure that the vast majority of the population are not left with long term problems following treatment. However the models and experience used to create these cutoff points have had to take into account the variations of patients' radiosensitivity which currently is generally an unknown entity before a patient receives radiotherapy.

Certain clinical and disease risk factors are known which can increase the risk of radiosensitivity such as diabetes and smoking. There are also some rare known genetic diseases which impair the repair and recovery from radiotherapy reactions such as Ataxia Telangectasia. Yet even without these known risk factors some patients are inherintly more sensitive to radiotherapy than others. This narrows the therapeutic ratio and is a concern for patients and physicians making decisions on cancer treatments.

1.3 Radiotherapy Toxicity

Radiotherapy related side effects can be divided into acute and late. Acute being any side effect occurring during treatment or up to three months after the completion of radiotherapy and tend to be transient. Late effects occur more than three months after radiotherapy has finished, and may develop slowly over several years leading to

irreversible changes. There are standardised scoring techniques for radiotherapy toxicity, which if used correctly, should minimise inter observer variation. LENT-soma is one scale for the scoring of late radiotherapy toxicity. It was developed in 1993 by adapting the original EORTC/RTOG scoring systems. More recently the CTCAE v4 criteria have been widely adopted (full details of the scoring system can be found on the National Health Institute website (NIH 2016)).

1.3.1 Pelvic Radiotherapy

Radiotherapy to the pelvis causes damage to normal tissues and rates of toxicity vary depending on many factors. Symptoms are divided into acute (diarrhoea, proctitis, skin erythema, dysuria, frequency, haematuria) and late (diarrhoea, per rectal bleeding, proctitis, dysuria, urinary urgency, urinary frequency and impotence). These symptoms can have a huge impact on patients' lives resulting in physical and psychological disabilities.

In 2012 Ohri et al (Ohri et al. 2012) published a review on late genitourinary and gastrointestinal radiotherapy toxicity. They quoted fifteen percent of patients experienced moderate late gastrointestinal (GI) side effects and seventeen percent moderate genitourinary (GU) toxicity from prostate radiotherapy. With regards to moderate and severe acute toxicity, rates vary between 25-35% (De Meerleer et al. 2004) depending on the volume treated. Ashman et al (Ashman et al. 2005) in 2005 compared 3D conformal radiotherapy and IMRT in prostate cancer patients and up to a third of patients experienced acute GI toxicity following whole pelvic radiotherapy. This study was in a very small number of patients but found IMRT to reduce GI toxicity but not GU toxicity. This was later followed up by similar studies which showed that due to the reduced volume of normal tissue irradiated with IMRT there is a reduction in GI toxicity (Cozzarini et al. 2007, Cahlon et al. 2008, Alongi et al. 2009).

1.3.2 Breast radiotherapy

Following breast radiotherapy acute symptoms include skin erythema and desquamation, skin ulceration, oedema and pain). Late effects include breast fibrosis,

breast atrophy/retraction, telangiectasia, pain, rib fractures, lung fibrosis, cardiac complications and lymphoedema). Rates of toxicity following breast radiation are similar to those observed following pelvic radiotherapy. A Cochrane meta-analysis (Hickey et al. 2016) looking at rates of acute and late toxicity following whole breast radiotherapy quotes acute toxicity rates between 12.4% and 60% depending on the fractionation, with significantly improved acute toxicity seen in the hypofractionated patients. The same review looked at late fibrosis and reported between 18.7% and 19.2% occurrence of grade two or more.

1.3.3 Lung Radiotherapy

Radiotherapy toxicity rates following lung cancer radiation varies depending on the organ at risk, volume irradiated and the fractionation as well as the use of systemic therapies. Typical acute symptoms include dyspnoea, cough, chest wall pain, oesophagitis and skin erythema whereas late toxicity can be rib fractures, lung fibrosis (resulting in dyspnoea and cough), oesophageal ulceration or fibrosis, cardiac complications, chest wall pain, telangiectasia, neurological deficit or damage to blood vessels. Rates of moderate to severe acute lung related toxicities can range between 10-30% (Mehta 2005, Hope et al. 2006). The most commonly observed late complication of lung radiation is lung fibrosis. Moderate to severe lung fibrosis is seen in approximately fifteen percent (Kong et al. 2006) of patients following radiotherapy to treat lung cancer. These rates are for conventional radiotherapy and are different from those observed following stereotactic radiotherapy which irradiates a lower volume of normal tissue. Dose escalation is an area of interest in lung cancer and whether this can be used safely to increase survival. Studies such as the PET boost trial are looking at boosting tumours which on imaging appear to be more radioresistant. One of the main concerns of these studies however is the possible increased toxicities which may be experienced by participating patients. They require strict reporting of toxicity and although the outcome may be improved tumour control the overall morbidity and mortality could still increase as a result of severe toxicity. This was the case in previous trials which looked at combination high dose chemotherapy and

radiotherapy in non-small cell lung cancer (Kong et al. 2014) and as a result this treatment option is used with extreme caution in only the fittest patients.

1.3.4 Pathophysiology

The pathology behind radiotherapy toxicity should be considered as two separate entities causing acute and late toxicity. Traditional thinking is that there is little correlation between those experiencing acute and late effects apart from consequential effects.

1.3.4.1 Acute Toxicity

Acute toxicity normally occurs in tissues which are rapidly dividing generally when the cells attempt mitosis. The tissues tend to respond on a time scale similar to the normal rate of loss of functional cells (Agency 2010). These responses are not usually limiting for fractionated radiotherapy because of the ability of the tissue to undergo rapid repopulation to regenerate the parenchymal cell population.

Skin erythema is believed to be related to the release of 5-hydroxytryptamine by mast cells, increasing vascular permeability. Moist desquamation and ulceration depends on the relative rates of cell loss and cell proliferation of the basal cells.

The extent of these reactions and the length of time for recovery depend on the dose received and the volume (area) of skin irradiated, because early recovery depends on the number of surviving basal cells that are needed to repopulate the tissue. Increasing the total dose and accelerated treatment time can increase the risk of acute toxicity however as long as the interval between the fractions is sufficient then acute tissues will overcome accelerated schedules.

1.3.4.2 Late Toxicity

Late toxicity occur in tissues which divide infrequently. They also occur in tissues that manifest early reactions, such as skin/subcutaneous tissue and intestine, but the pathology is different to acute effects.

Late effects are irreversible and as such limit the dose of radiotherapy regimens. It is thought that many of the late side effects result from fibroblast proliferation and vascular damage. Fibrosis is seen in multiple organs following radiotherapy including the skin, lung, heart and liver. Post radiation experiments looking at the different types of fibroblasts demonstrate increase in the number of post mitotic fibroblasts in comparison to progenitor fibroblasts resulting in increased collagen production (Rodemann and Bamberg 1995). This is thought to be in response to pro-inflammatory cytokines such as TGF-β. Irradiation of vasculature results in release of vasoactive cytokines, this in turn allows fibrin to leak into tissues and promote deposition of collagen (Stone et al. 2003). Similarly vascular damage occurs due to production of pro-inflammatory cytokines in the endothelin which attracts pro-inflammatory cells (Brush et al. 2007). This results in late fibrosis and changes in the dermal layer such as atrophy and telangiectasia (Westbury and Yarnold 2012). Figure 3 shows a schematic of the pathological process which is thought to underlie radiation fibrosis.

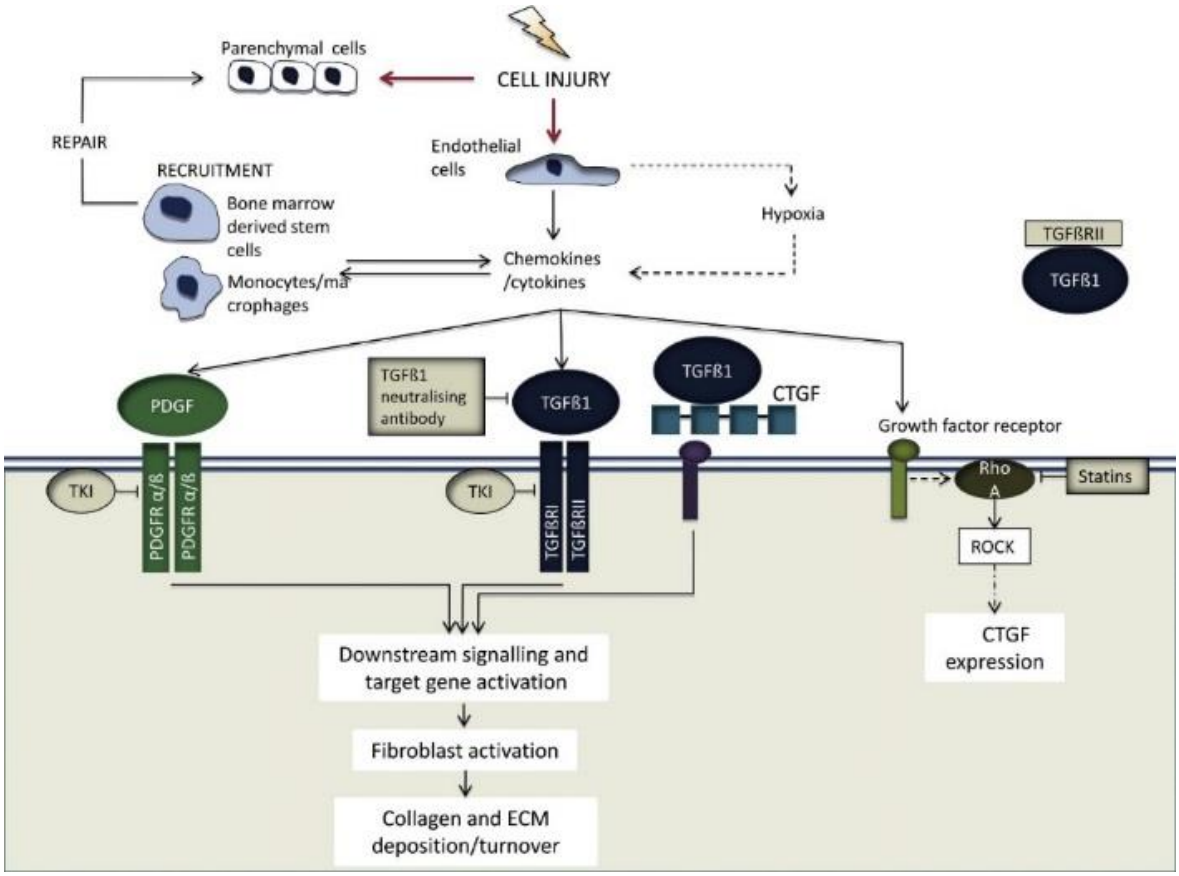

Figure 3: (Taken from Westbury and Yarnold, 2012) Simplified overview of the pathogenesis of radiation fibrosis and its therapeutic targets. Direct cellular targets of ionising radiation include cells within both parenchymal and stromal compartments. Endothelial cells are an important source of pro-fipro-fibrotic activity. CTGF has a binding site for TGF ß1 and their interaction may result in enhanced TGF ß1 signaling activity. The binding of pro-fibrotic cytokines to their receptors activates down-stream signaling pathways and target gene responses, which lead to fibroblast activation and collagen and extracellular matrix (ECM) deposition and turnover.

1.3.4.3 Dose, fractionation and treatment time

Increasing total dose will often increase chance of cure particularly in diseases traditionally difficult to provide long term survival such as lung cancer. However by increasing total dose there is also an increased risk of causing both acute and late toxicity.

Fraction size was traditionally set at 2Gy per fraction as late reacting tissues were believed to be at unacceptable risk if greater than 2Gy was used. The START and FAST forward trials have challenged this concept showing no increase in toxicity by hypofractionating a course of radiotherapy (Brunt et al. 2016). The theory behind this

being that by hypofractionating you are reducing the total two gray equivalent dose to the tissue.

Another potential way to improve survival with radiotherapy is to accelerate the total treatment time for example by giving multiple fractions per day. The idea behind this being to overcome the rate of repopulation of tumour cells. Accelerated radiotherapy has been shown to be effective in head and neck cancer (Saunders et al. 1996) and NSCLC (Saunders et al. 1999). However the tradeoff is increased risk of acute toxicity. To minimize this risk at least six hours should be left between fractions as these tissues undergo rapid cell division and regrowth and as long as the interval is greater than six hours between fractions then acute toxicity is normally recoverable (Fowler 1988).

1.3.5 Radiogenomics

Radiogenomics studies how polymorphisms in genes can affect an individual's risk of developing radiotherapy toxicity. The ultimate aim is to develop single nucleotide polymorphism-based risk models that can be used to stratify patients to more precisely tailored radiotherapy and identify potential targets for radioprotective agents that can be used in the clinic.

Early candidate gene studies showed conflicting results about whether individual genes or Single nucleotide Polymorphisms (SNPs) could influence the chances of a patient suffering radiotherapy side effects. In 2012 two replicated candidate gene associations were found. Talbot et al reported an association between genetic alterations close to the Tumour Necrosis Factor (TNF) alpha gene and late adverse reactions following breast irradiation in 2036 breast cancer patients (Talbot et al. 2012). They identified that alleles of the class III major histocompatibility complex region associate with overall radiotherapy toxicity in breast cancer patients.

Radiogenomic studies have identified associations between Ataxia Telangectasia Mutation gene (ATM) and late adverse radiotherapy reactions. Initially several smaller studies and 3 meta-analysis (Dong et al. 2015, Zhang et al. 2016, Su et al. 2014) demonstrated conflicting opinions on its relationship to adverse radiotherapy reactions. In 2016 Andreassan et al published a further meta-analysis using robust

STROGAR guidelines examining for associations between ATM and radiotherapy toxicity (Andreassen et al. 2016). They concluded that ATM rs1801516 increases acute and late radiotherapy reactions in breast and prostate cancer patients.

In 2014 Barnett et al (Barnett et al. 2014) reported the first adequately-sized Genome wide association study (GWAS) in this area. They examined more than two million different SNPs and showed a number were linked to (although not statistically significant) increased risk of late side effects in breast and prostate cancer patients. In the same year Fachal (Fachal et al. 2014) published data from another GWAS which identified TANC1 to increase overall late radiotherapy toxicity in prostate cancer patients. Their findings were validated in 2 replication cohorts and gave a significant result ($p= 4x10^{-11}$) for a locus in TANC1.

1.3.6 The REQUITE study

REQUITE is an international observational study of radiotherapy related late toxicity in breast, prostate and lung cancer patients undergoing curative treatment (West et al. 2014); www.requite.eu). The aim is to collect data on more than 5000 patients undergoing treatment and perform a GWAS to validate previously noted SNPs which increase the risk of late radiotherapy side effects. In addition a FACS-based assay of radiation-induced lymphocyte apoptosis (RILA) is being evaluated for predictive value for late radiotherapy toxicity. (Azria et al. 2015, Ozsahin et al. 2005).

1.3.7 Predictive Assays

In order to predict an individual's risk of developing radiotherapy toxicity we must look to develop a test which is reproducible and reliable. The use of predictive assays in this field is not a new entity but previous studies have contradicted as they were often performed in small populations. In the current REQUITE population in the Leicester cohort alone there were 650 patient samples which were available for testing. Coupled with other REQUITE centres gives an extremely large sample size and an advantage over previous populations.

Markers of DNA damage induced by radiation has been a popular technique examined previously. This includes single strand DNA breaks measured using the comet assay, or double strand breaks using the γ-H2AX assay, 53BPI foci (Somaiah et al. 2016) and pulsed field gel electrophoresis (Pinar et al. 2007). Other methods include radiation induced lymphocyte apoptosis (Azria et al. 2008) and the micronucleus assay. The assays chosen for this thesis are discussed in more detail below.

Previous studies have used skin fibroblasts or peripheral blood lymphocytes for the predictive assays. Whilst skin fibroblasts are potentially closer in the pathological process to radiotherapy toxicity the benefit of using lymphocytes is that they are more readily available for testing and are more reliable.

1.3.7.1 γ-H2AX assay

Radiotherapy induces DNA damage which is repaired at varying rates. One theory is that patients with slower rates of DNA repair are at increased risk of radiotherapy toxicity (van Oorschot et al. 2014).

The γ-H2AX assay detects rate of DNA double strand break repair. When radiation induces double strand breaks in DNA, repair mechanisms rebuild the damaged DNA structure. H2AX is found in normal DNA and contributes to nucleosome formation. When DNA damage occurs H2AX is phosphorylated at serine 139 to become γ-H2AX. γ-H2AX acts as a signal for downstream DNA repair mechanisms to occur (Scully and Xie 2013) and is found at every site of double strand break. γ-H2AX appears rapidly post irradiation and peaks at around 30 minutes and remains elevated for 3-4 hours (Venkateswarlu et al. 2015) then tailing off over the next 48 hours. Quantification of γ-H2AX therefore can be used as an estimate of DNA damage (Pilch 2003). Quantification is normally achieved with immunohistochemistry by counting γ-H2AX foci in each nuclei (Figure 4). However because there is always a background level of γ-H2AX present it is better to look at γ-H2AX intensity rather than foci number. Intensity is calculated using flow cytometry produced histograms and taking the ratio of mean intensity in the irradiated lymphocytes versus the unirradiated lymphocytes (Venkateswarlu et al. 2015).

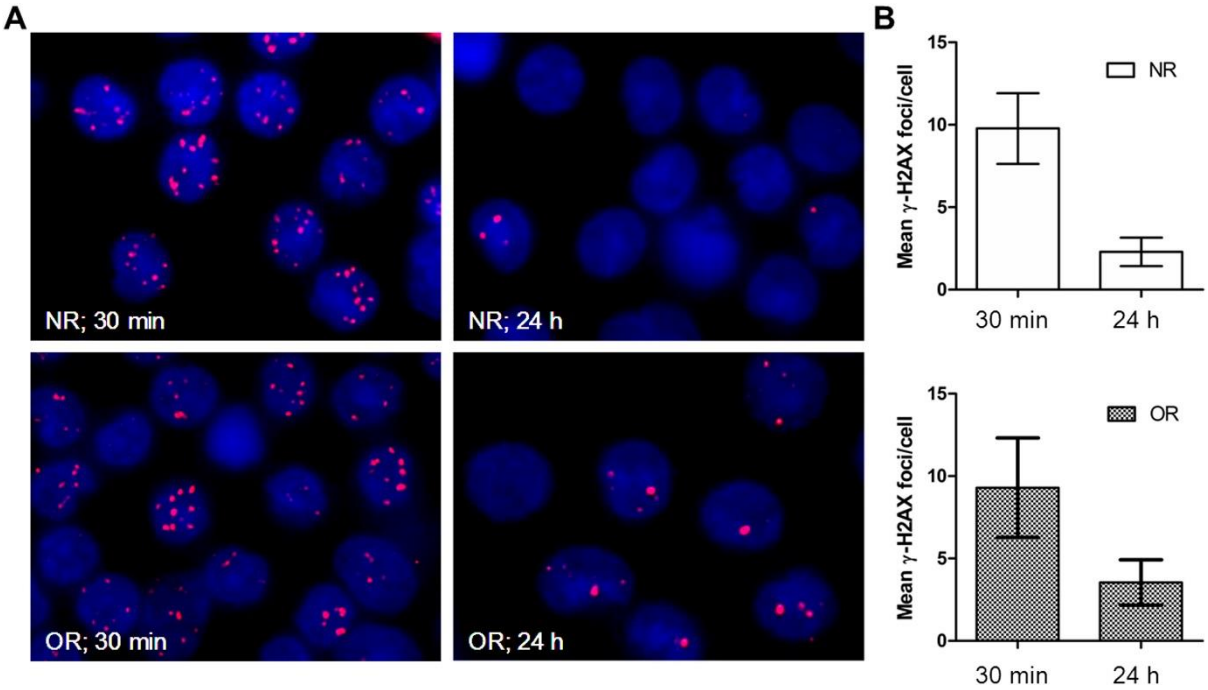

Figure 4: γ-H2AX visualised by immunofluorescence 30 minutes and 24 hours post irradiation of peripheral blood lymphocytes in patients with normal radiotherapy reactions (NR) and those with extreme reactions (OR). Taken from Oorschot et al, 2014 (van Oorschot et al. 2014)

Mumbrekar (Mumbrekar et al. 2014) looked at γ-H2AX foci in peripheral blood lymphocytes following ex-vivo irradiation and found significantly increased levels of persistent DNA damage at six hours correlated to increased rates of acute toxicity following breast radiotherapy when compared to control and normal toxicity groups. Van Oorschot (van Oorschot et al. 2014) noted a similar effect with higher rates of persistent DNA damage 24 hours post ex-vivo irradiation for prostate cancer patients having severe late toxicity compared to those with low toxicity.

Bourton (Bourton et al. 2011) used a flow cytometry based method to detect antibody to γ-H2AX. Again they concluded that patients with prolonged DNA damage up to 24 hours after a single 2Gy irradiation were at significantly increased risk of late radiotherapy toxicity. They also concluded that this method for detecting DNA damage was reliable and reproducible.

Not all studies using γ-H2AX as a predictive assay were conclusive of its predictive value. Werbrouck (Werbrouck et al. 2010) failed to show any correlation to late toxicity in gynaecological patients undergoing radical doses of radiotherapy when they used the foci method. Li (Li et al. 2013) used a flow cytometry based method and came

up with mixed results when examining the relationship of γ-H2AX to acute oral mucositis in head and neck patients receiving radiotherapy.

1.3.7.2 The comet assay

The Comet assay uses single cell gel electrophoresis to mobilise supercoiled DNA towards the anode and form characteristic comet tails. The relative proportion of DNA in the tail is representative of the amount of DNA damage present in eukaryotic cells (figure 5).

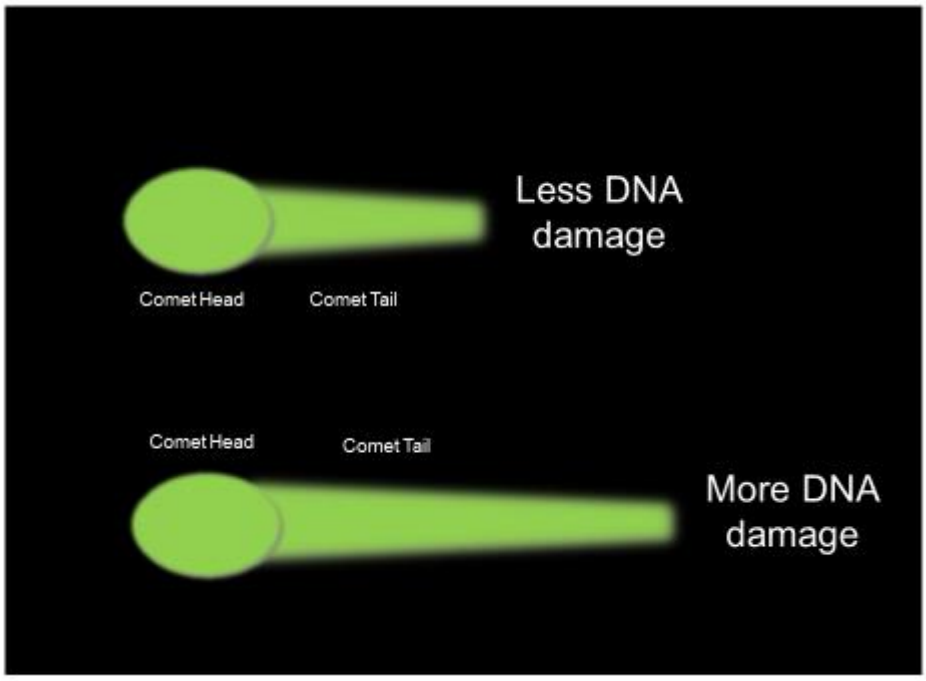

Figure 5: Schematic of comet with characteristic tail lengths depending on more or less DNA damage

The comet assay has been shown to be a reliable measure of DNA damage induced by irradiation in peripheral blood lymphocytes (Zhu et al. 2013). Use of this method has demonstrated that patients with worse adverse normal tissue reactions had greater levels of DNA damage induced by ex vivo irradiation of whole blood samples as well as reduced repair kinetics. The also showed that there was a correlation between DNA repair assessed using this method and DNA repair genes (XRCC1, XRCC3 and OGG1) (Sterpone et al. 2010). In 1999 Alapetite (Alapetite et al. 1999) used the comet assay to

demonstrate breast cancer patients suffering severe late radiotherapy toxicity had significantly higher residual DNA damage compared to control volunteers at 60 mins after irradiation of peripheral blood lymphocytes.

The comet assay can also be performed on other tissues for example tumour cells and fibroblasts. Oppitz (Oppitz et al. 1999) compared DNA damage in fibroblasts taken from Ataxia telangiectasia patients to damage seen in patients with normal radiation reactions. They also showed that those predisposed to increased normal tissue sensitivity have impaired DNA repair using the comet assay.

Other studies have however failed to show that the alkaline comet assay can be used as a reliable technique for predicting radiotherapy toxicity. Twardella (Twardella et al. 2003) performed the comet assay on 113 samples of haemagglutin stimulated lymphocytes taken from breast cancer patients receiving radiotherapy. They failed to show a correlation between DNA repair kinetics assessed with the comet assay and acute skin reactions following radiotherapy.

1.3.7.3 Radiation Induced Lymphocyte Apoptosis assay (RILA)

The Radiation induced lymphocyte apoptosis assay (RILA) has shown great promise in predicting late radiotherapy toxicity. The assay detects loss of propidium iodide (PI) staining of cellular DNA using flow cytometry as a marker of late apoptosis induced in peripheral blood lymphocytes. The first pivotal paper to report this assay in 2005 demonstrated that patients with lower levels of radiation-induced apoptosis in CD4 or CD8 lymphocytes had higher chances of moderate or severe late complications (Ozsahin et al. 2005). This has been validated in a number of later studies in different cancer types (Schnarr et al. 2009, Foro et al. 2014) and is being assessed as part of the REQUITE study in the largest cohort to date. The RILA has not been found to predict for acute toxicity (Cozzarini 2015).

In most of the papers reporting a positive association between RILA and late radiotherapy toxicity the samples are divided into three groups (low, medium and high) based on the levels of apoptosis observed. One of the issues that arises is that different centres and experiments have different cut off points for these apoptosis

levels. Whilst some degree of variation is something that can be expected for protocols being ran in labs across a variety of sites it does cast some doubt as to the reliability of the assay.

The mechanism behind the predictive value of the RILA has yet to be fully explained. Some studies have observed an inverse relationship between levels of DNA repair and apoptosis in peripheral blood lymphocytes (Ozsahin et al. 2005). Whilst more recent studies have looked at different techniques such as proteomics and propose a possible link to ROS (Lacombe et al. 2013). The REQUITE study will assess whether there is a link between genetic predictive markers and levels of apoptosis.

1.3.8 Circadian Rhythm

Circadian rhythm is any biological process which oscillates over a 24 hour time period and includes physical, mental and behavioural changes. Biological processes influenced by circadian rhythm are centrally controlled by the suprachiasmic nucleus in the anterior hypothalamus of the brain.

1.3.8.1 CLOCK Genes

Genes which govern the molecular circadian processes are called clock genes. These genes encode a series of transcription factor proteins which interact with each other through an auto-regulatory feedback loop for which one cycle takes about 24 hours.

1.3.8.1.1 BMAL1 AND CLOCK

BMAL1 is at the centre of the circadian rhythm system. It encodes a transcription factor protein. Proteins from *Bmal1* (brain and muscle ARNT-like protein 1) and *Clock* (or Npas2 in neuronal tissue) form heterodimers in the cytoplasm. The BMAL1–CLOCK heterodimer is the primary genome-wide driver for transcription of clock genes (including three *period* [*Per1*, *2*, and *3*] and two cryptochrome [*Cry1* and *2*] clock genes) and clock-controlled output genes (CCGs) via binding to E-box containing promoters. The BMAL1–CLOCK complex also drives transcription of the retinoic acid receptor related orphan receptor α (*Rorα*) and nuclear receptor subfamily 1, group D, member 1 (*NR1D1* or *Rev-erbα*) orphan nuclear receptor genes (Tamaru et al. 2015).

1.3.8.1.2 PER and CRY

The Period genes (*Per1, 2 and 3*) and Cryptochrome (*CRY 1 and 2*) encode proteins which peak during midday. During this peak period they form a complex and this then associates with BMAL1–CLOCK. This combination represses the expression of E-box clock (controlled) genes and accounts for the negative limb of the circadian feedback loop.

In addition to the feedback loop the group of Period genes and their products are associated with many circadian molecular and physiological functions including responses to light following signals from photoreceptors in the eye. In addition to circadian function Per2 knockout mice have increased sensitivity to radiation, reduced apoptosis and increased tumour formation (Fu et al. 2002).

Per3 is the least studied of the Period genes however unlike Per1 and 2 it is only found in homosapians. It is found on chromosome 1 and one of the most common variants is a variable number tandem repeat (VNTR) with either 4 or 5 copies. Its molecular function is protein and ubiquitin protein kinase binding. It has been associated with inhibition of RNA polymerase II as well as regulation of the sleep-wake cycle (Matsumura and Akashi 2017).

1.3.8.1.3 Radiotherapy and Circadian Rhythm

In 2015 Gomes et al., (Gomes et al. 2015) published data showing that irradiation of mice resulted in alterations in gene expression of the paternal offspring. Circadian rhythm genes were especially prominent in these findings suggesting that the effects of radiation may in some ways be linked to circadian rhythm.

In a 2012 report of a GWAS study Barnett (Barnett et al. 2014) showed that some genetic variants predisposed to late adverse reactions following radiotherapy. One of the strongest associations with toxicity was seen in a SNP (rs13116075) close to the CCRN4L gene (for the protein nocturnin). CCRN4L is a gene controlled by circadian rhythm and is linked to metabolism of lipids (Douris et al. 2011). Levels of this protein undergo diurnal variation with peaks at night. It has also been linked to inflammation (Stubblefield et al. 2012) which may be the possible mechanism for increasing radiotherapy toxicity. Circadian rhythm genes cause diurnal variations in proteins CRY, PER, CLOCK and BMAL1 (Sancar et al. 2014). These then in turn exert effects on other proteins which have a wide physiological effect. This includes DNA damage checkpoints and apoptosis mechanisms which govern repair in normal tissues following radiotherapy.

Variations in circadian genes can influence a person's sleep wake cycle (von Schantz 2008) and ability to repair DNA damage. This may mean that time of radiotherapy treatment could affect the chances of developing side effects and may also vary between patients depending on their own levels of expression of these genes.

Gupta et al, 2012 (Gupta et al. 2012) proposed a theory that circadian variations in endothelin 1 (a potent vasoconstrictor peptide) could affect cardiac complications and telangiectasia following radiotherapy at different times of day. They hypothesised that there would be increased risk of complications in patients treated in the morning due to higher levels of this peptide being present before noon resulting in increased vasoconstriction of coronary vasculature and thus reduced myocardial perfusion. In contrast to this theory Noh et al, 2014 (Noh et al. 2014) looked at the relationship with radiotherapy treatment time and acute skin toxicity in 395 breast cancer patients.

They reported that patients treated in the afternoon had more chance of developing acute skin toxicity (RTOG grade 2 or more) ($p = 0.0088$). Bjarnason et al, 2009 (Bjarnason et al. 2009b) looked at the relationship of treatment time to grade three or more mucositis in head and neck patients treated with radical doses of radiotherapy but failed to show a significant difference between morning and afternoon groups. However on subgroup analysis when patients were divided by gender there was a trend that females had worse toxicity in the morning but males in the afternoon.

1.4 Rationale for project

In addition to the routine management decisions that are complicated by unclear evidence, potentially being able to predict radiotherapy toxicity could have implications on how effective a cancer treatment would be. For instance in lung and prostate cancer being able to dose escalate, use hypofractionation or stereotactic radiotherapy. These low fractionation schedules have so far shown promising survival advantages over conventional radiotherapy but anecdotally have been associated with increased rates of late toxicity. If there was a reliable way of selecting those at least risk of toxicity these low fractionation schedules could be adopted. On the other hand if patients were predicted to be at increased risk of toxicity then it would be beneficial to adopt more stringent dose constraints or even utilise radioprotectors or devices such as rectal spacers to reduce the likelihood of these patients experiencing severe toxicity.

In the UK, which has a publically funded health care system, the use of these novel therapies has to be rationalised and prioritised for those most in need. For instance proton therapy is being widely used now in parts of Europe and the USA. The UK plans to open three new treatment centres to provide this modality from 2018. However demand will far exceed the ability to provide the service and clinicians and health care managers will need some way of deciding which patients will benefit most. Because protons deposit their energy over a very small range as opposed to photons which deposit energy throughout their whole track, then in theory (although this is yet to be proven in randomised controls studies) the risk to normal tissues is less. If a reliable

screening tool could be developed to predict those at highest chance of developing radiotherapy toxicity then these patients could be put forward for proton therapy.

The aim of my research is to look at the potential causes and factors involved in predicting radiotherapy sensitivity so in the future these could be utilised to stratify patients and aid in management decisions.

1.5 Study Aims

This thesis has been undertaken to establish potential predictive factors for acute and late radiotherapy toxicity in breast, prostate and lung cancer patients receieving radical radiotherapy. Funding has come from the international REQUITE study EU FP7 grant (no. 601826) and HOPE small projects grant. It has been conducted with collaberations from the University of Leicester Talbot group, and Oncology departments at the Leicester Royal Infirmary, Royal Derby Hospital and Nottingham City Hospital.

1.6 Study Objectives

I. Assess whether the comet assay can be used to predict radiotherapy toxicity.
II. Assess whether the γ-H2AX assay can be used to predict radiotherapy toxicity.
III. Assess whether the RILA can be used to predict radiotherapy toxicity.
IV. Assess if combination of these assays can improve predictive value of the individual assays.
V. Review clinical and treatment predictive varaibles in the prostate and lung cancer cohorts for acute radiotherapy toxicity.
VI. Perform genotyping for circaidan rhythm genes and examine for a correlation to acute and late radiotherapy toxicity.
VII. Assess if time of day can influence incidence of acute and late radiotherapy toxicity.

CHAPTER 2 : Clinical Data and Predictive Assays

The majority of this work was carried out on a prospective cohort of patients recruited prior to radiotherapy treatment for the REQUITE trial. For validation purposes a second cohort of patients previously recruited to the LeND study was also included in a parts of this work.

2.1 STUDY METHODOLOGY

2.1.1 REQUITE COHORT

2.1.1.1 Patient Recruitment

Patients diagnosed with breast, prostate or lung cancer were suitable for entry into this study if they were planned to receive radiotherapy alone or in conjunction with chemotherapy or surgery with the aim of cure. Inclusion criteria and exclusion criteria can be found in appendix 1. Recruitment into the REQUITE project was conducted in hub clinical centres across Europe and the USA (Manchester, Leicester, Heidelberg, Montpellier, Mount Sinai, Ghent, Maastricht, Leuven, Santiago, Milan and Barcelona). Leicester was the hub for the English East Midlands region (Leicester, Derby and Nottingham). In the East Midlands, patients were identified as appropriate in either oncology or breast surgery outpatient clinics. At time of identification patients were provided with a written information sheet about the study by a member of the research team. They were given at least 24 hours to consider entry into the study before being approached for their decision. Breast patients were recruited predominately by Mr Tim Rattay with help from myself at either the Leicester Royal Infirmary or the Glenfield Hospital. Prostate patients were recruited by myself at the Leicester Royal Infirmary alone. Lung cancer patients were recruited predominantly by myself, with some help from Professor R P Symonds at three different sites (The Leicester Royal Infirmary, The Royal Derby hospital and Nottingham City Hospital). Across all study sites, lung cancer recruitment was slower than predicted. To increase recruitment new sites were added and eligibility criteria were altered. The specific changes to criteria were patients could be recruited if they had a radiological diagnosis

of lung cancer i.e. no histology; and patients with a history of previous malignancy outside of the thorax could be recruited if this was more than 5 years previous.

In order not to duplicate work, data relating to the breast cancer patients will only be used in relation to predictive assays and circadian rhythm, Tim Rattay will report in his thesis on the clinical and genetic predictors of acute toxicity in breast cancer patients.

2.1.1.2 Baseline Visit

If patients agreed to take part they were consented prior to their fifth fraction of radiotherapy (other than lung cancer patients undergoing stereotactic radiotherapy (SABR) who were consented prior to any radiotherapy). At the same time as consent patients underwent a baseline assessment of co-morbidities and medications as well as recording any current symptoms which could later be attributed to radiotherapy toxicity (these assessment forms can be found in appendix 2).

Patients were asked to complete a baseline quality of life questionnaire including EORTC, MFI and GPAQ standardised questionnaires (these can be viewed at http://www.requite.eu).

2.1.1.3 Assessment of Radiotherapy Toxicity

Study volunteers were assessed for acute toxicity following radiotherapy (within the last five fractions of radiotherapy for breast and prostate cancer patients and at three months for lung cancer patients); as well as late toxicity at one year and two years (breast and prostate) and for lung patients six and twelve months. At these time-points participants again completed a quality of life questionnaire and were assessed by a clinician for late toxicity using the REQUITE scoring system (developed from the CTCAE V4 scoring system). Acute assessments were made myself for the prostate and lung patients and by either myself or Mr Tim Rattay for the breast patients. Late follow up was completed by the physician reviewing the patient in out-patient clinic.

In this thesis acute toxicity is taken to be the primary outcome and was calculated by deducting any baseline score from the score following radiotherapy. Any symptom with a score of two or more was taken as a significant toxicity event.

When analysis was performed on the whole cohort bivariate STAT score was used as an overall measure of acute toxicity. As described in Barnett et al, 2012 (Barnett et al. 2012) a STAT score is calculated by first calculating the Z score for every patient and all toxicity end points (Z = (score for patient – Mean of whole population)/Standard deviation for whole population)). A Z score therefore shows whether for that toxicity end-point a patient has more or less toxicity than the mean of the population. The STAT score is then calculated by taking an average of the Z scores for that patient. Toxicity was then divided to create the bivariate STAT score with the top quartile taken to be the over-responders group in terms of acute toxicity.

Due to the timeframe of this study data on late toxicity was collected on some patients but not all and therefore has not been included in the analysis.

2.1.1.4 Radiotherapy Treatment

2.1.1.4.1 Breast Patients

All breast patients received radiotherapy to the whole breast following wide local excision. Patients receiving chest wall radiotherapy (post mastectomy patients) were excluded from the study. Patients were CT planned on a breast board with arms above their head. Patients were treated with 6 or 10MV tangential field arrangement to the whole breast with a field-in-field boost in some patients to improve homogeneity. Most patients (90.8%) with invasive breast cancer were treated with hypofractionated (40Gy in 15 fractions) radiotherapy; Ductal carcinoma in situ histology (DCIS) and patients with clinical factors indicating high risk of toxicity (e.g. raised BMI, large breast size) received 2Gy per fraction (9.2% of total cohort) (50Gy total dose). Lymph node areas (axilla and supraclavicular fossa) were included in patients deemed to have high risk for recurrence or occult involvement in these areas. Very young patients (<40 years) or young patients (<50 years) with three high risk factors (grade 3, lymph nodes

involved, T3 or T4 and LVI positive), received a boost to the tumour bed. A boost field consisted of a photon or electron five centimetre square field centred on the surgical clips.

2.1.1.4.2 Prostate Patients

Patients received either radiotherapy to the prostate or prostate bed following prostatectomy. Post prostatectomy patients were referred either immediately post-surgery if there were involved excision margins or referred if there was evidence of biochemical progression with a PSA rise above 0.2.

All patients receiving radiotherapy alone received neoadjuvant luteinising releasing hormone analogues for at least three months prior to commencing radiotherapy and continued during radiotherapy. High risk patients also received adjuvant hormones to complete at least two years in total.

Prophylactic pelvic lymph node irradiation was administered to patients deemed fit enough and with more than 30% risk of occult lymph node involvement assessed using the modified Roach formula ([(2/3) x PSA + (Gleason score - 6) x 10]).

Reproducibility techniques included use of a self-administered micro enema to control for rectal filling then once this had been effective patients drank 300 millilitres of water and were treated 30 minutes later with instructions not to void their bladder until after treatment.

All patients were CT planned in the supine position with knee and neck supports. Clinicians marked up the high risk CTV (clinical target volume) of the whole prostate and seminal vesicles which was treated to a dose of 74Gy (66Gy if prostate bed). Pelvic lymph nodes were then marked up as a second lower dose (54 or 56Gy) CTV in those patients deemed at high risk for lymph node recurrence. A margin of 5 millimetres was then added to both CTV to create the PTV (planned treatment volume). The vast majority of patients were treated with standard 2Gy per fraction using RapidArc® IMRT. Image guided adjustments were made using daily cone beam CT.

Brachytherapy is not available in Leicester but if patients required high dose rate brachytherapy (HDR) they were referred to Northampton General Hospital to receive a single treatment using iridium sources prior to receiving 37.5Gy in 15 fractions external beam radiotherapy as per the above protocol.

2.1.1.4.3 Lung Patients

Patients received radiotherapy to intact tumours only, no post-operative patients were included in the study. Some patients received either neoadjuvant or concurrent chemotherapy with their radiotherapy treatments.

Patients with peripheral T1 or small T2 tumours and no lymph node involvement were treated if possible with alternate day SABR. These patients had a 4D planning CT and daily cone beam CT checks. Immobilisation for upper lobe tumours was via thermoplastic shell and for lower two thirds via vac bag. All other patients received intensity modulated radiotherapy (IMRT) and underwent a contrast enhanced CT planning scan in the supine position with knee and neck supports.

For IMRT, clinicians marked up the gross tumour volume (GTV) as the visible tumour and nodal involvement then added a 1cm margin for CTV and then a further 5mm margin for a PTV. Those undergoing SABR had a GTV marked on the static CT images then an internal target volume (ITV) from the 4D planning CT scan. A further 5mm margin was added to this to create the PTV. Image guided adjustments were made using daily cone beam CT.

Patients receiving IMRT without chemotherapy were treated with either 55Gy in 20 daily fractions or continuous hyperfractionated accelerated radiotherapy (CHART) with 56Gy in 36 fractions over 12 days. Those also receiving chemotherapy were treated in 2Gy per fraction up to 60Gy. SABR patients received either 54Gy in 3 alternate day fractions or 55Gy in 5 fractions alternate days if there were concerns about proximity to major vessels or ribs.

Data on treatment was collected retrospectively following completion of radiotherapy and included any surgery, systemic therapies (hormones, chemotherapy or targeted therapies) and radiotherapy (including brachytherapy) received.

2.1.1.5 Sample Collection and Processing

At baseline visit patients provided a venous blood sample using a vacutainer system which was divided into three tubes – 2 EDTA and 1 Lithium Heparin (breast and prostate) and 1 EDTA, 1 Lithium Heparin and 1 PAX gene tube (lung). Bloods were then transported to G10 lab in the Adrian building, University of Leicester for processing and storage.

The two EDTA bottles were placed in the -20°C freezer for at least 24 hours before transferring to the -80°C freezer for longer term storage. Shipments of the CIGMR EDTA samples were sent at various intervals to CIGMR on dry ice for DNA extraction. The second EDTA tube remained at the University of Leicester for processing in future experiments.

The Lithium heparin tube was placed in a 25°C water bath overnight before processing for live cell assays the next day. The Pax gene tube was stored at room temperature for between 24 and 72 hours in an upright position in a test tube rack before being slow frozen for 24 hours at -20°C then transferred to the -80°C freezer.

2.1.2 LeND Cohort

This retrospective cohort was used for validation purposes and were previously recruited at oncology clinics in the Leicester Royal Infirmary, Royal Derby Hospital and Nottingham City hospital. A total of 664 breast cancer patients participated in this study. All had complete macroscopic excision by either wide local excision (n = 513) or mastectomy (n = 147), two patients received radiotherapy alone and two patients had axillary surgery only. Surgery was then followed by adjuvant radiotherapy to either the breast or chest wall plus the lymph node areas if clinically indicated. Patients were treated with tangential field arrangement and the most commonly used fractionation was 2Gy per fraction (50Gy) but this varied slightly between centres. In addition to

whole breast radiotherapy, women with poor prognostic features received an additional boost to the tumour bed (9-15Gy). Patients were recruited to the LeND study at least three years post radiotherapy. All patients were tumour free at time of recruitment. Additional information on their comorbidities, radiotherapy treatment and tumours was retrospectively collected at the same time as assessment of late adverse reactions. Radiotherapy toxicity was recorded by a clinician using the Late Effects of Normal Tissue-Subjective Objective Management Analytical (LENT-SOMA) criteria (Pavy et al. 1995). Information on acute toxicity was assessed via patient recall and from medical records.

In the current study a new ethics application was applied for to conduct additional research on these patients which was granted by the REC committee (15/LO/0866) on 15/05/2015. The new ethics approval allowed a further blood sample to be obtained from the surviving LeND volunteers as well as to collect additional data from questionnaires. The additional blood would be used for DNA damage assays and lymphocyte subpopulations analysis.

The blood sample was collected in a Lithium Heparin tube on Mondays between 9 am and 11am in January and February 2016 on the HOPE unit in the Leicester Royal Infirmary. Bloods were performed by myself, Professor R.P. Symonds or Sam Levey (medical student). Bloods were then transferred to a 25°C water bath at the University of Leicester Genetics department for overnight storage before processing for live cell assays. Any remaining blood was spun down and buffy coat and plasma were stored at -80°C for future experiments.

2.1.3 PREDICTIVE ASSAYS

2.1.3.1 The Comet Assay

The comet assay was performed by myself with help from students working in G10 lab (Maxime Boy, Hannah Dobbelare, Eva Saiti and Kosalie Redman).

In three microeppendorf tubes 10µl of blood was mixed with 90µl tissue culture medium (RPMI1640-20%FBS (4:1)) and tubes labelled 0Gy, 8Gy 0 Mins and 8Gy 30 mins. The 8Gy 0 mins and 30 mins tubes were then irradiated in the Xstrahl superficial

irradiator with 190kV X-rays at 30cm FSD for eight minutes to a dose of 8Gy. Tubes were transferred to and from the irradiator on ice but irradiated off ice. The 0Gy tube and 8Gy 0 mins tube were immediately placed in a pre-cooled box in a -20°C freezer. At the same time the 8Gy 30 min tube was placed at 37°C for 30 minutes. After a 30 minute incubation the 8Gy 30 mins tube was placed in the -20°C freezer with the other samples. After at least 24 hours all three tubes were transferred to the -80°C freezer and stored until ready to process.

When ready to perform the comet assay pre-coated slides were prepared by dipping plain glass microscope slides into 1% normal melting point (NMP) agarose and left to dry overnight. The following day tubes containing blood/culture media mix were thawed at 4°C in the dark for 30 minutes and then placed on ice. 50µl blood/culture media was mixed with 150µl 0.8% low melting point (LMP) agarose (prepared at 37°C). This was done in duplicate. 80µl of blood/agarose mix was then pipetted on to a pre-coated slide and a cover slip applied. Each slide was also prepared in duplicate. i.e. four gels per patient condition. Slides were left to set on ice for approximately one hour. Once set coverslips were removed and slides placed in a Coplin jar containing lysis buffer then left at 4°C overnight.

The next day slides were washed with ice cold water twice for ten minutes then arranged in an electrophoresis tank in dim light. Slides were incubated with electrophoresis buffer for 20 minutes before electrophoresing for 20 minutes at 30V, 300mA. Following electrophoresis slides were removed from the tank and flooded with neutralisation buffer for 20 minutes then washed in double distilled water (ddH2O). Slides were left to dry overnight then rehydrated in ddH2O for 30 minutes. After hydrating each gel was flooded with propidium iodide (PI) (2.5µl/ml) and left for 20 minutes before washing for 30 minutes in ddH2O and finally leaving to dry in the incubator. Once dry slides were scored by myself, using a fluorescent microscope and Komet software. 100 comets were scored per slide and an average of the results per slide calculated from this.

Figure 6 shows an illustration of the “comet” seen by fluorescent microscope. The comet is divided into two sections the head and tail. The head portion is found at the

negative pole and contains fragmented and intact DNA. The tail is created when fragmented negatively charged DNA is attracted towards the positive electrode. Different measures are used to quantify the amount of DNA damage. In this experiment Percentage Tail DNA (DNA in tail/Total DNA in comet); Tail Length and Olive tail moment ((amount of DNA in tail) x distance between head and tail regions) were used.

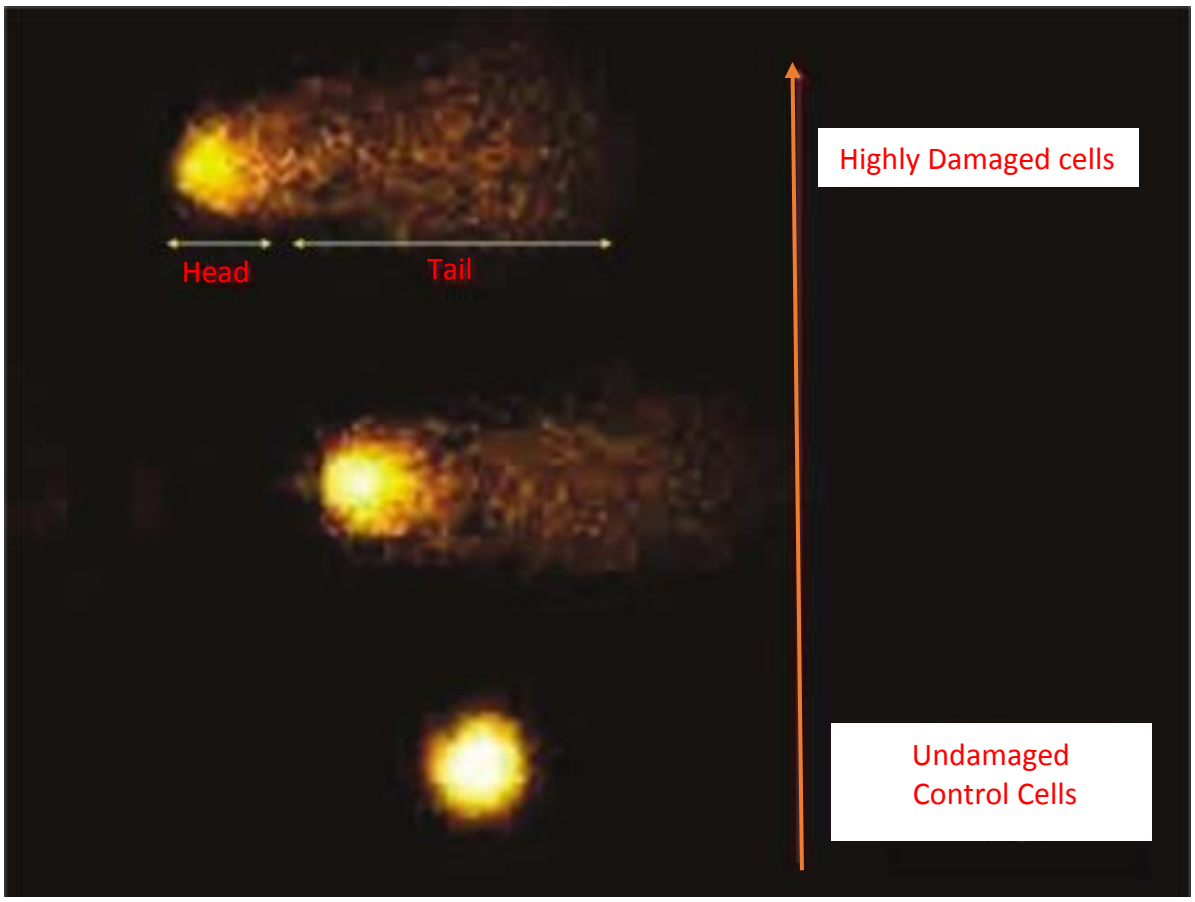

Figure 6: Image of different levels of DNA damage assessed following administration of hydrogen peroxidase using the comet assay (Benhusein et al. 2010)

From these results radiation induced damage (8Gy 0 minutes – 0Gy), rate of repair (8Gy 0 mins – 8Gy 30 mins) and relative rate of repair (8Gy 0 minutes/8Gy 30 minutes) were calculated.

2.1.3.2 Radiation Induced Lymphocyte Apoptosis Assay (RILA)

2.1.3.2.1 Methodology

The RILA assay was already part of the REQUITE trial prior to this project and has been investigated as a predictive assay for late radiotherapy toxicity by some other researchers. The main reason for inclusion in this study was to compare to other DNA damage assays and clinical variables to explain a possible mechanism of action and establish if combinations of assays could improve their predictive value. For this reason, this thesis will only report limited information on how the RILA relates to toxicity and baseline demographics in the whole cohort.

The RILA was performed from April 2014 to October 2015 by Sheila Smith, from October 2015 to March 2016 by Chiara Batini and from April 2016 to May 2016 by Kosalie Redman. Due to the technician and FACS machine availability only samples collected on a Monday or a Tuesday were processed for RILA.

The following procedure unless otherwise stated was carried out in a tissue culture hood. 200µl of fresh blood was added to 2ml of tissue culture serum (FBS) (RPMI1640-20%FBS) in a T25 tissue culture flask. This was prepared in duplicate for an unirradiated sample and an irradiated sample. The flasks were then left at 37°C, 5% CO_2 overnight and the following day the irradiated flasks were placed at 30cm SSD in the Xstrahl superficial irradiator and exposed to 8Gy (190 kv) for eight minutes. To ensure identical environments both the control (unirradiated sample) and the test (8Gy) samples were transferred to and from the radiation bunker. Following irradiation the blood/culture mix was transferred back to the incubator for 48 hours to culture. Following this the contents of each flask were transferred to centrifuge tubes and centrifuged at 300 rpm for five minutes. The supernatant was aspirated and 200µl of phosphate buffered solution (PBS) was added then 10µl of anti-CD8 antibody (CD8-FITC) was added and mixed before placing in a dark incubator at 25°C for 20 minutes. 4ml of lysis buffer was then added to the tubes, mixed and then incubated for 20 minutes. After incubation the tubes were centrifuged at 300 rpm for 5 minutes. The supernatant was then once again aspirated and the lysis stage repeated. Following spinning and aspiration of the supernatant 200µl of PBS was added then 5µl of

propidium iodide (PI) (0.4mg/ml) and 5µl of RNAase (10mg/ml). This was mixed and then transferred on ice to the flow cytometer for analysis.

2.1.3.2.2 FACS analysis of RILA

The contents of each centrifuge tubes was transferred to a filtered falcon tube and run through the FACS machine until 10,000 events were recorded in the lymphocyte gate. The RILA score was calculated by deducting the levels of apoptosis in the CD8 positive lymphocytes in the control (0Gy) sample from the test sample (8Gy). An average was then calculated between the duplicate tubes.

2.1.3.2.3 Gating for RILA

The gating strategy had previously been determined to establish the correct position for the gates. Slight variations in PI preparation between samples required minimal adjustments of the apoptotic cell gates to ensure only apoptotic cells were being counted. In figure 7 plot A represents all lymphocytic cells (estimated using forward/side scatter). Plot B shows how all lymphocytes divide into CD8 positive and negative. Plot C is made up of only CD8 positive lymphocytes and divided by PI staining. PI binds to DNA in the permeabilised cells, cells in the final stages of apoptosis loose DNA and thus have reduced PI staining.

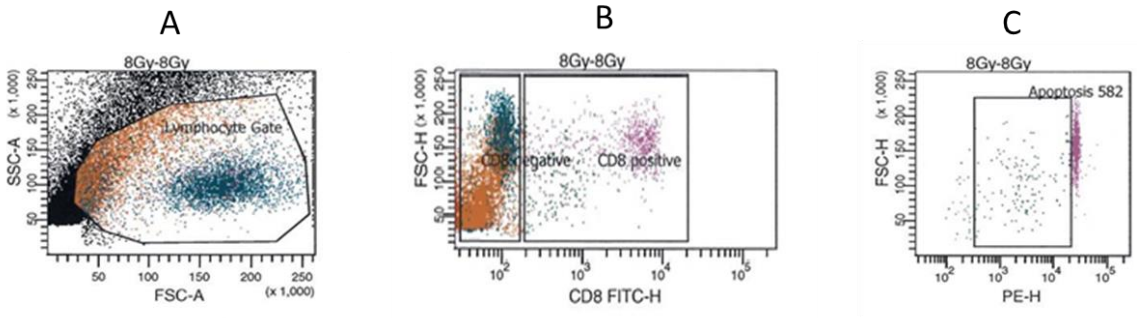

Figure 7: Gating used for RILA. A: All lymphocytes; B: All lymphocytes split into CD8 positive and negative; C: CD8 positive lymphocytes stained with PI. Those with PI staining are apoptotic

2.1.3.3 γ- H2AX assay

2.1.3.3.1 Methodology

The γ – H2AX assay was set up and performed by myself. The protocol was established to be carried out alongside the RILA protocol. Initially it was hoped that the antibody for γ – H2AX (Anti-Human/Mouse phospho-H2AX (S139) eFluor® 660 from ebioscience) would be added to the RILA samples. In set-up testing there was noted to be overlapping between the FITC stain (for CD8 cells) and γ – H2AX antibody staining so it was decided to run the 2 assays separately as further compensation needed to be done which would affect the gating and hence results on the samples already collected for RILA. During set up testing the γ –H2AX assay results were analysed on the FACS machine at 3 hours, 24 hours and 48 hours post irradiation (see figure 8). Maximal staining was seen at 3 hours with reduction over the following 45 hours. To simplify the protocol and keep in line with RILA testing it was therefore decided to only run the 3 and 48 hour assays. Only samples collected on a Monday or a Tuesday were processed for the assay due to FACS machine availability.

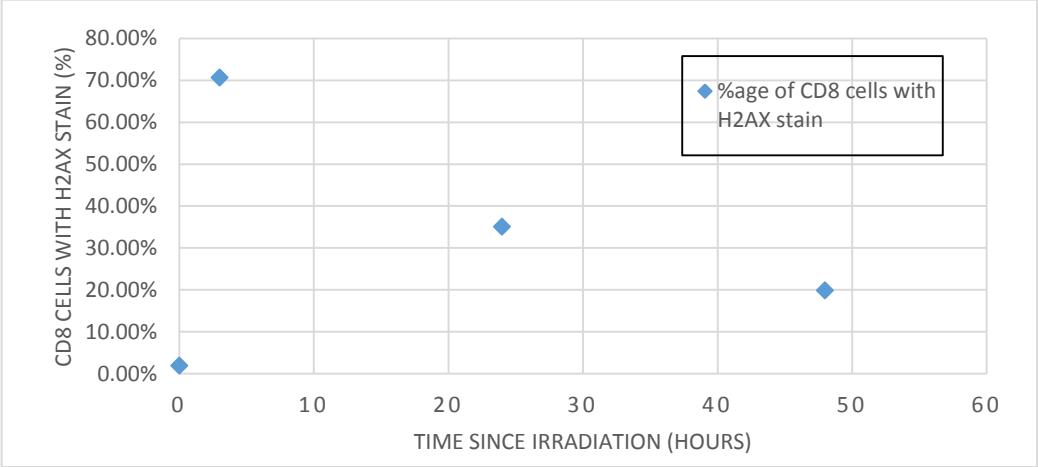

Figure 8: Scatter plot of percentage CD8 positive lymphocytes staining with γ-H2AX antibody at different time points after irradiation (0 hours, 3 hours, 24 hours and 48 hours). The 0 hours sample represents the control (unirradiated sample)

The following procedure unless otherwise stated was carried out in a tissue culture hood. 200µl of fresh blood was added to 2ml of tissue culture medium (RPMI1640-20%FBS) in a T25 tissue culture flask. For each patient sample an unirradiated sample (control), and two irradiated flasks were prepared. The flasks were then left at 37°C,

5% CO_2 overnight and the following day one of the irradiated flasks were placed at 30cm SSD in the Xtrahl superficial irradiator and exposed to 8Gy (190 KV) for eight minutes. To ensure identical environments both the control (unirradiated sample) and the test (8Gy) samples were transferred to and from the radiation bunker on ice. Following irradiation the blood/culture mix was transferred back to the incubator for 48 hours to culture. On day four the second test sample was irradiated using the same irradiation protocol.

The contents of each flask were then transferred to centrifuge tubes and centrifuged at 300g for five minutes. The supernant was aspirated and 200µl of PBS was added then 10µl of CD8 antibody (CD8-FITC) was added and mixed before placing in a dark incubator at 25°C for 20 mins. 4ml of lysis buffer (Lysing Buffer (Becton Dickinson ref: 349202) diluted 1:10 in water) was then added to the tubes, mixed and then incubated for 20 minutes. After incubation the tubes were centrifuged at 300g for five minutes. The supernant was then once again aspirated and the lysis stage repeated. Following repeat spinning and aspiration of the supernant 200µl of PBS was added then 5µl of γ-H2AX antibody. The remaining solution was mixed then left for 60 minutes in a dark incubator at 25°C. Finally 5µl of PI (0.4mg/ml) and 5µl of RNAase (10mg/ml) was added and mixed before transferring on ice to the flow cytometer for analysis.

2.1.3.3.2 Selection of lasers

Single cells are drawn in a linear fashion past the lasers of the flow cytometer. Antibodies conjugated to fluorophores attach to the cell surface antigens, the fluorophores fluoresce and emit a specific wavelength of light when excited by lower wavelengths light. Using this information the flow cytometer can then process information on the amount of fluorescence in cells present in a sample per a set number of events.

Table 1 gives the lasers which were selected for detecting the antibodies/stains for the γ -H2AX assay.

Table 1: Lasers and fluorochromes used for γ -H2AX assay

Antigen/Target	Flurochrome	Laser
CD8	FITC	530nm Blue
γ -H2AX	Efluor660	670nm Red
DNA	PI	582nm Yellow/Green

2.1.3.3.3 Compensation

Compensation corrects for overlapping of emission spectrum between fluorophores in the same panel. The degree of overlap is automatically calculated by the FACS computer programme and taken into account when the FACS is run. Compensation is run first using beads which bind the antibody then applied to blood samples.

2.1.3.3.4 Gating

The gating strategy for γ - H2AX was set up with help from Jenny Higgins (FACS operator) and Sheila Smith by adapting the gates from the RILA assay. The first step involved a gate for all lymphocytes and as per the RILA a small gate was set up with the highest concentration of cells. However because this was a new assay we also set up a larger gate to capture any other possible cells which may stain with H2AX antibody. A further difference from the RILA gating was that we examined both strong staining cells and those with weak staining as a separate gate. Figure 9 shows the different gates. Plot A represents all lymphocytic cells small and large gates (estimated using forward/side scatter). Plot B shows how all lymphocytes divide into CD8 positive and negative. Plot C is made up of only CD8 positive lymphocytes and divided by γ -H2AX staining (strong and weak). Plot D is made up of all lymphocytes and divided by γ - H2AX staining (strong and weak).

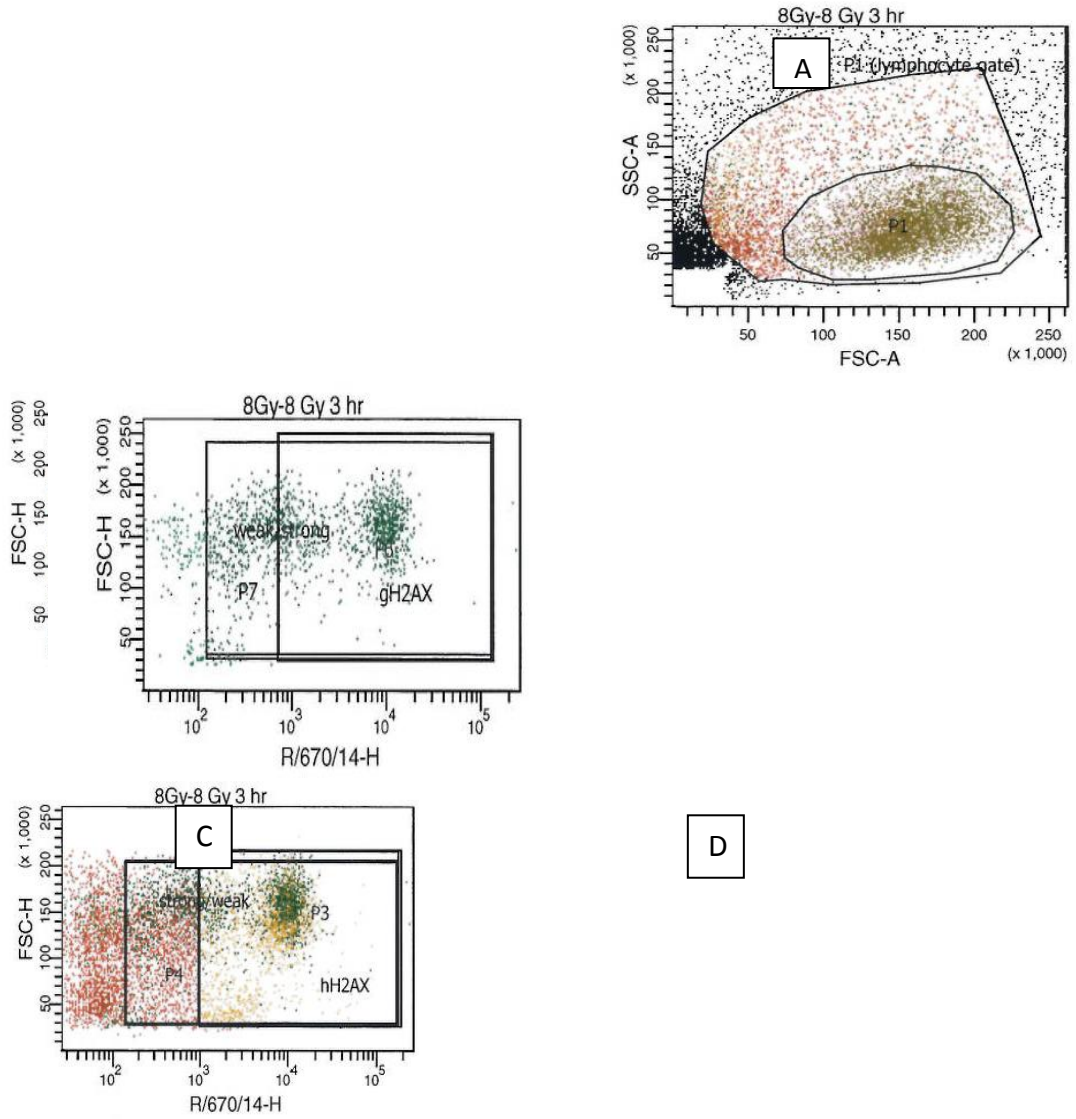

Figure 9: Gating used for γ -H2AX assay. A: All lymphocytes; B: All lymphocytes split into CD8 positive and negative; C: CD8 positive lymphocytes stained with γ -H2AX; D: All lymphocytes stained with γ -H2AX

2.1.3.3.5 FACS analysis

The contents of the centrifuge tubes were transferred to filtered falcon tubes and run through until 10,000 events were recorded in the lymphocyte gate. γ -H2AX staining was counted in all lymphocytes as well as CD8 positive lymphocytes.

2.1.4 STATISTICAL ANALYSIS

Statistical analysis was carried out by myself using SPSS v24. Univariate analysis for categorical variables was carried out using the chi squared test for equivalence. Groups

with expected values less than five were analysed using a fisher's exact test. Univariate analysis of continuous variables if normally distributed was performed using a T test or a Mann Whitney U for non-parametric variables. Linear regression was used for multivariate analysis. Variables included for the multivariate analysis model were smoking status, diabetes, age, gender, cancer type, BMI, depression and radiotherapy dose. For breast cohorts use of hormones, BED (biologically effective dose), boost to radiotherapy dose, use of chemotherapy and breast cup size were added to the model. In the prostate model prophylactic pelvic nodal radiotherapy, intended duration of hormone therapy, V30 rectum, V50 bladder, V50 large bowel and PSA were added to the model. In the lung cohort baseline lung function, PTV (cm^3), V30 heart, mean heart dose, V5 lung, V20 lung and cancer T stage were also included in the model. Numbers were corrected to 2 decimal places and P values less than 0.05 were taken to be significant.

2.2 RESULTS

2.2.1 Patient Demographics

642 patients were recruited for this study, 250 prostate cancer patients, 350 breast cancer patients and 42 lung cancer patients. 627 were recruited in Leicester, ten of the lung cancer patients were recruited in Nottingham and five in Derby. As previously stated to avoid duplication of work the breast patients will only be analysed in the context of the predictive assays.

Table 2 summarises the baseline demographics for the overall cohort.

Table 2: Demographics for REQUITE cohort recruited in Leicester

Variable	n=	Median	Range
Age (years)	642	66.0	38.0 - 89.0
BMI	642	27.3	13.1 - 71.0
Gender		NA	NA
Female	375		
Male	267		
Smoking status		NA	NA
Non smokers	295		
Ex-smokers	263		
Current smokers	82		
Missing	2		
Alcohol		NA	NA
Never drank	135		
Previous alcohol	70		
Current alcohol	434		
Missing	3		
Menopausal status		NA	NA
Pre-menopausal	42		
Post-menopausal	270		
Peri-menopausal	54		
Missing	9		

2.2.2 Site Specific Clinical Variables

2.2.2.1 Prostate Cohort

2.2.2.1.1 Baseline Demographics

The median age of prostate patients was 69 years and the median BMI was 27.4. 112 (44.8%) were non-smokers and 138 (55.2%) were either current smokers or ex-smokers. Table 3 shows the prevalence of comorbidities in the prostate cohort.

Table 3: Comorbidities present in prostate cohort

Comorbidity	Number with	Number without
Diabetes	36 (14.4%)	214 (85.6%)
Ischaemic Heart Disease	53 (21.2%)	197 (78.8%)
Rheumatoid Arthritis	3 (1.2%)	247 (98.8%)
Hypertension	112 (44.8%)	138 (55.2%)
Haemorrhoids	39 (15.6%)	211 (84.4%)
Inflammatory bowel disease or diverticular disease	10 (4.0%)	239 (95.6%) 1 unknown (0.4%)
Family History of radiotherapy toxicity	3 (1.2%)	245 (98.0%) (2 (0.8%) unknown)

2.2.2.1.2 Tumour Specifics

The most common MRI T stage was T3a but ranged from not visible to T4 on MRI scanning. The most frequent overall Gleason score was 7 (range 5-9) and the median PSA score was 13 ng/ml (range 0.1 to 214). Of note on the REQUITE data collection forms Gleason score was only recorded as total score not “x+y=z” as it is more commonly staged.

2.2.2.1.3 Treatment Details

At time of analysis treatment information was available on 236 of the 250 prostate cancer patients recruited. The remaining fourteen patients had a delayed start of radiotherapy so had not completed treatment.

Sixteen patients received radiotherapy post prostatectomy and five of these patients also underwent lymphadenectomy.

All patients receiving radiotherapy as primary treatment and one post-operative patient received hormone therapy. Median intended length of intended hormone therapy was 36 months.

92.8% of patients with available treatment data received 74Gy total dose to the PTV. One patient received 37.5Gy external beam radiotherapy following a single 15Gy high dose rate brachytherapy treatment. Of the sixteen post-operative patients fourteen received 66Gy (in 2Gy per fraction) to the PTV and the remaining two had hypofractionated treatment with 52.5Gy in 20 fractions (2.63Gy/fraction). 44.7% of patients also received prophylactic pelvic nodal therapy with a dose range between 50Gy to 60Gy.

2.2.2.1.4 Dosimetry

Table 4 summarises the dosimetry for the radiotherapy treatment received by the prostate cohort.

Table 4: Summary table of dosimetry for prostate cohort (REQUITE) external beam radiotherapy treatment.

	Median	Range	Standard deviation		Median	Range	Standard deviation
PTV	113 cm3	49 – 376 cm3	53.6	CTV	50 cm3	20 – 135 cm3	22.5
Median %age volume of organ receiving "x" Gray (range)							
ORGAN	*V30*	*V40*	*V50*	*V60*	*V65*	*V70*	*V75*
Rectum	80% (22-100%)	60% (0-95%)	40% (0-72%)	27.5% (0-60%)	21.0% (0-37%)	11% (0-21%)	0% (0-3%)
Bladder	x	x	42.5% (0-100%)	28% (0-97%)	x	x	0% (0-3%)
Large Bowel	x	x	12.0% (0-50%)	x	x	x	x
Femoral head	x	x	Left 0% (0-1%) Right 0% (0-1%)	x	x	x	x

x = not recorded

2.2.2.1.5 Toxicity

2.2.2.1.5.1 Baseline Symptoms

Table 5 shows the prevalence of baseline symptoms when assessed prior to radiotherapy using the REQUITE scoring criteria.

Table 5: REQUITE prostate patients baseline symptoms assessed prior to commencing radiotherapy

ORGAN	Symptom	Grade of toxicity (n= (%))		
		0	*1*	*2*
Gastrointestinal	*Proctitis*	242 (96.8%)	6 (2.4%)	2 (0.8%)
	Diarrhoea	242 (96.8%)	8 (3.2%)	
	Flatus	189 (75.6%)	60 (24.0%)	1 (0.4%)
	Rectal Bleeding	234 (93.6%)	16 (6.4%)	
Genitourinary Tract	*Haematuria*	246 (98.4%)	4 (1.6%)	
	Incontinence	224 (89.6%)	21 (8.4%)	5 (2.0%)
	Frequency	127 (50.8%)	111 (44.4%)	12 (4.8%)
	Urgency	182 (72.8%)	59 (23.6%)	9 (3.6%)
	Retention	193 (77.2%)	45 (18.0%)	12 (4.8%)
	Erectile dysfunction	13 (5.3%)	233 (94.3%)	1 (0.4%)

2.2.2.1.5.2 Overall Toxicity

Acute toxicity was calculated by deducting any baseline symptom score from score at the end of radiotherapy assessment. A significant acute event was classed as grade 2 or more. Because nearly all patients were on neoadjuvant LHRH analogues prior to baseline assessment, scoring of erectile dysfunction was not meaningful in terms of an acute radiotherapy toxicity. 222 patients had completed radiotherapy and were eligible for acute toxicity scoring at time of analysis. Table 6 summarises the results.

Table 6: Prostate volunteers (REQUITE) acute symptoms assessed in the last 5 fractions of radiotherapy, any baseline symptom score was deducted from the acute score.

ORGAN	SYMPTOM	GRADE OF TOXICITY (N= (%))	
		Low (0-1)	*Moderate/Severe (≥2)*
Gastrointestinal	*Proctitis*	210 (94.6%)	12 (4.8%)
	Diarrhoea	201 (90.5%)	21 (8.4%)
	Flatus	218 (98.2%)	4 (1.8%)
	Rectal Bleeding	219 (98.6%)	3 (1.4%)
Genitourinary Tract	*Haematuria*	221 (99.5%)	1 (0.5%)
	Incontinence	217 (97.7%)	5 (2.3%)
	Frequency	189 (85.1%)	33 (14.9%)
	Urgency	189 (85.1%)	33 (14.9%)
	Retention	187 (84.2%)	35 (15.8%)
	Erectile dysfunction	219 (100%)	3 declined to answer

110 patients had been assessed one year after completion of radiotherapy (plus or minus four weeks) at time of analysis. As per acute toxicity baseline score was deducted from assessed score at this time point. Table 7 shows the distribution of late toxicity at one year in this 110 patients. In view of the low overall number of significant events at this one year time point further analysis was not appropriate.

Table 7: Prostate Volunteers (REQUITE) symptoms assessed 1 year after completion of radiotherapy (plus or minus 4 weeks), any baseline symptom score was deducted from the 1 year score.

ORGAN	Symptom	Grade of toxicity (n= (%))	
		Low (0-1)	*Moderate/Severe (≥2)*
Gastrointestinal	*Proctitis*	106 (96.4%)	4 (3.6%)
	Diarrhoea	109 (99.1%)	1 (0.9%)
	Flatus	110 (100%)	0 (0%)
	Rectal Bleeding	107 (97.3%)	3 (2.7%)
	Perforation	109 (99.1%)	1 (0.9%)
Genitourinary Tract	*Haematuria*	110 (100%)	0 (0%)
	Incontinence	109 (99.1%)	1 (0.9%)
	Frequency	106 (96.4%)	4 (3.6%)
	Urgency	106 (96.4%)	4 (3.6%)
	Retention	106 (96.4%)	4 (3.6%)
	Erectile dysfunction	106 (96.4%)	1 (0.9%) 3 declined to answer

2.2.2.1.5.3 Clinical Variables and Toxicity

Univariate analysis for categorical variables was analysed using a chi squared test for equal distributions. The following categorical variables were analysed for acute toxicity: smoking status, alcohol consumption, comorbidities (diabetes, rheumatoid arthritis, hypertension, ischaemic heart disease, inflammatory bowel disease or diverticular disease, haemorrhoids, history of hip replacement, previous abdominal surgery and depression), medications (ace inhibitors, beta blockers, statins, oral antidiabetic medication and antidepressants), family history of radiotherapy toxicity, tumour factors (MRI T stage, lymph node involvement on imaging), treatment factors (TURP pre-radiotherapy, post-operative radiotherapy, lymphadenectomy, hormone therapy, prophylactic pelvic nodal radiotherapy). Table 8 shows the significant univariate categorical variables for gastrointestinal and genitourinary symptoms as well as overall toxicity assessed by bivariate STAT score.

Table 8: Significant results on univariate analysis of categorical variables for acute GI and GU toxicity in REQUITE prostate volunteers

ORGAN	Symptom	Variable
Gastrointestinal Tract	Proctitis	Ace Inhibitor (p=0.05)
	Diarrhoea	Pelvic nodal radiotherapy ($p=3.0x10^{-4}$)
	PR bleeding	Haemorrhoids (p=0.03)
Genitourinary	Incontinence	Depression ($p=4.0x10^{-3}$)
		Antidiabetic Meds (p=0.03)
	Frequency	Pelvic nodal radiotherapy (p=0.05)
	Urgency	Pelvic nodal radiotherapy (p=0.02)
	Retention	Hip replacement (p=0.03)
Bivariate STAT score		Diabetes (p=0.02)
		Pelvic Radiotherapy ($P=4.0x10^{-3}$)

Univariate analysis for continuous variables was performed using Mann Whitney U testing for difference in means between low and high toxicity groups. The variables included were age; weight; height; PSA pre-biopsy; external beam radiotherapy dose; dose per fraction; PTV (cm^3); CTV (cm^3); dosimetry (percentage volume of organ receiving "x" dose) values for rectum, bladder and large bowel). A summary of these results is shown below in table 9.

Table 9: Significant results on univariate analysis of continuous variables for acute GI and GU toxicity in REQUITE prostate cohort

ORGAN	Symptom	Variable
Gastrointestinal Tract	Proctitis	N/A
	Diarrhoea	PSA (p=0.05)
		V30 rectum (p=0.01)
		V40 rectum ($p=4.0x10^{-3}$)
	PR bleeding	N/A
Genitourinary	Incontinence	Bladder v50 (p=0.04)
	Frequency	Rectum v30 (p=0.02)
		Rectum v40 (p=0.02)
	Urgency	Large Bowel v50 (p=0.02)
	Retention	Large Bowel v50 (p=0.03)
Bivariate STAT score		Length of hormone therapy (p=0.01)
		PTV (P=0.05)
		V50 large bowel ($p=6.0x10^{-3}$)

Multivariate analysis was performed using linear regression (see table 10). Intended duration of hormones (p=0.05) and V50 bladder (p=0.01) remained significant on multivariate analysis. All other significant observations seen on univariate analysis were no longer statistically significant.

Table 10: Multivariate analysis for REQUITE prostate cohort for effects on acute diarrhoea.

Variable	Sig.	Exp (B)	95% C.I. for Exp (B) Lower	Upper
Age	0.34	0.97	0.91	1.03
Smoker	0.92	1.03	0.62	1.71
Diabetes	0.82	1.18	0.29	4.78
Depression	0.39	2.11	0.39	11.55
RT Dose	1.00	6.41	0.00	
Pelvic Radiotherapy	0.11	5.15	0.69	38.41
V30 rectum	0.50	1.00	0.99	1.01
Intended duration of hormones	0.05	1.08	1.00	1.17
PSA	0.82	1.00	0.99	1.01
BMI	0.73	0.98	0.89	1.09
V50 Bladder	0.01	1.05	1.01	1.09
V50 Large Bowel	0.32	1.00	1.00	1.00
PTV	0.20	0.99	0.97	1.01

2.2.2.1.5.4 Integral Dose Analysis

Mean integral dose (Gy/L) was calculated by multiplying the total body volume (litres) by the mean dose to the body (Gy). This was obtained by reviewing the radiotherapy plans and dosimetry. Data was available for 130 of the 250 prostate patients at time of analysis. Figure 10 shows the distribution of the mean integral dose in the prostate cohort. There are two peaks in the histogram, the first peak represents the patients who received radiotherapy to the prostate only, and the second peak is made up of the patients who also underwent prophylactic pelvic nodal radiotherapy as well.

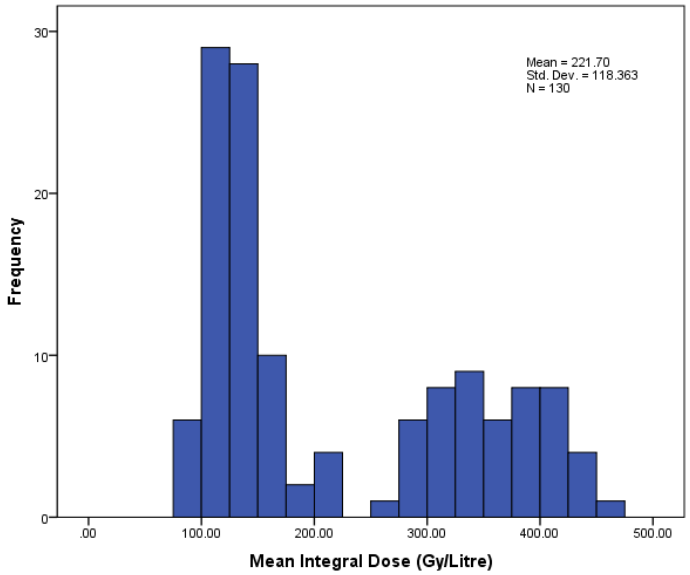

Figure 10: Histogram of the distribution of mean integral dose in the prostate cohort

To establish if there was any connection to acute radiotherapy toxicity a Mann Whitney U test for significant difference between the means was performed. As previously an overall measure of acute toxicity was calculated with a STAT score then the cohort divided by bivariate STAT score. This analysis demonstrated that patients with a higher mean integral dose had increased acute radiotherapy toxicity ($p = 0.04$) shown in figure 11.

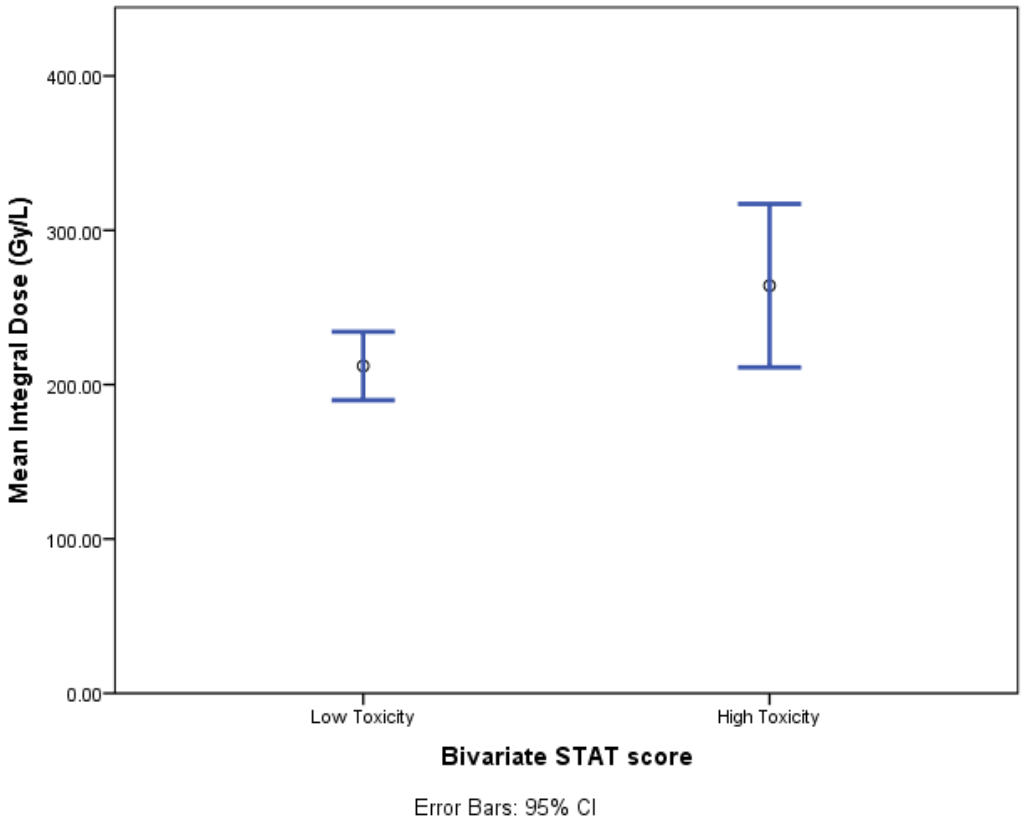

Figure 11: Bar chart of the different levels of mean integral dose split by acute radiotherapy toxicity (bivariate STAT score)

Putting mean integral dose into the same model as the above clinical variables it was no longer significant (p=0.18) likely due to the positive correlation to pelvic nodal radiotherapy dose (p=0.04). There was no significant association with the individual toxicity measures.

2.2.2.1.5.5 Exercise and Toxicity

The activity levels of the prostate patients was calculated from the GPAQ questionnaire using the standardised scoring technique from the World Health association (WHO 2016) for calculating average number of minutes per day of any activity (work, travel or recreational). Questionnaire responses were available in 152 of the prostate cohort with median time being 83.6 minutes (range 0 to 964.3).

Daily baseline activity level was compared to overall acute toxicity (bivariate STAT score) using a Mann Whitney U test and there was no significant effect observed

(p=0.30). Univariate analysis of daily activity levels assessed at the end of radiotherapy however revealed that patients who managed to maintain a higher level of activity had lower levels of acute toxicity (p=0.02). When the cohort was divided into three equal groups by activity level (low, moderate and high) further analysis was performed to review any associations with acute radiotherapy toxicity. On this grouped analysis a chi squared test revealed that acute diarrhoea was seen less in those with higher levels of baseline activity (p=0.02). These associations were not however replicated on multivariate analysis.

2.2.2.2 Lung Cohort

In total 42 lung patients were recruited to this study from Leicester, Nottingham and Derby.

2.2.2.2.1 Baseline Demographics

The median age of lung patients was 76 years and the median BMI was 23.6. There were no non-smokers and 42 (100%) were either current smokers or ex-smokers. The demographic details of the lung patients are summarised in table 11.

Table 11: Demographics of REQUITE lung cancer patients

Comorbidity	Number with	Number without
Diabetes	8 (19%)	34 (81%)
Ischaemic Heart Disease	14 (33.3%)	28 (66.7%)
Rheumatoid Arthritis	1 (2.4%)	41 (97.6%)
Hypertension	21 (50%)	21 (50%)
COPD	15 (35.7%)	27 (64.3%)
Family History of Radiotherapy toxicity	1 (2.4%)	41 (97.6%)

2.2.2.2.2 Baseline Lung function

If available baseline lung function (FEV1 (Litres and % predicted); DLCO (predicted carbon monoxide diffusion capacity) and KCO (predicted carbon monoxide transfer

coefficient) were collected from the notes. Lung function testing was not repeated unless there was a clinical indication at subsequent visits. Median FEV1 was 1.5L (range 0.8 – 3.6) and 65% (range 4.6-128.0) predicted. Median DLCO (diffusion lung capacity for carbon monoxide) was 66.0% predicted (range 54-96) and median KCO (transfer coefficient) was 85.5% predicted (range 55-102).

2.2.2.2.3 Tumour Details

The most common radiological T stage was T1a (23.8%) but ranged from T1a to T4. 55.9% had no lymph nodes involved, 14.7% were N1 and 29.4% were N2. The most common histological subtype was adenocarcinoma (40%), then squamous (36.7%), then non-small cell of non-specific type (10%). The remainder (13.3%) of tumours with available data had no histology and diagnosis was made radiologically based on a positive PET-CT scan.

2.2.2.2.4 Treatment Details

At time of analysis treatment information was available on 35 of the 42 lung cancer patients recruited. The remaining seven patients were recruited at sites other than Leicester and there was a delay in data entry due to staff shortages. Only one patient had received previous lung surgery; seven patients also received chemotherapy (four induction and three concurrent).

2.2.2.2.4.1 Radiotherapy

A mixture of dose/fractionation schedules were used which are shown in table 12. The most common was 55Gy in 20 fractions. 2Gy per fraction was used in any patients also undergoing chemotherapy. Other than CHART radiotherapy all treatments were delivered by RapidArc® or other types of IMRT. CHART was planned using 3D conformal techniques.

Table 12: Dose and fractionation radiotherapy schedule for lung cancer cohort

Dose/Fractionation	Number	Percentage
54Gy/3# (SABR)	2	5.9%
55Gy/5# (SABR)	8	23.5%
55Gy/20# (IMRT/RapidArc®)	11	32.4%
60Gy/30# (IMRT/RapidArc®)	5	14.7%
54Gy/36# (CHART)	8	19.0%

2.2.2.2.4.2 Dosimetry

Table 13 summarises the dosimetry for the radiotherapy treatment received by the lung cohort. Not all patients had a GTV marked up, for instance patients who had received previous chemotherapy or who had lymph node involvement were often marked up with CTV only. Patients receiving SABR had a GTV marked up then an ITV based on all the available imaging (4D CTV, static CT, contrast CT and MIP). In the REQUITE trial return forms there was no option for marking up an ITV so this was marked as a CTV also.

Table 13: Dosimetric results for radiotherapy treatment in lung cancer cohort

	Median	Range		Median	Range
GTV	17.5cm3	1.8 - 160 cm3	*CTV*	33.1 cm3	4.1 – 315.3 cm3
PTV	186.7 cm3	10.0 – 813.6 cm3			
ORGAN	*Median %age volume of organ receiving "x" Gy (range)*			*Average Mean dose to organ (Gy) (Range)*	*Average Max dose to 1cc of organ (Gy) (Range)*
Lung	*V5:* 39.2% (14.8-91.9)	*V20:* 11.6% (1.8-30.5)		9.2Gy (3.0-20.7)	
Oesophagus	*V35:* 0% (0-52.6)	*V50:* 0% (0-32.1)	*V60:* 0% (0-10.1)	9.6Gy (1.2-29.6)	20.1Gy (4.7-62.5)
Heart	*V5*: 9.6% (0-100)	*V30:* 0% (0-9.4)	*V40:* 0% (0-9.0)	4.1Gy (0.1-16.9)	24.4Gy (0.9-64.3)

2.2.2.2.5 Toxicity Data

2.2.2.2.5.1 Baseline Toxicity

Table 14 shows the prevalence of baseline symptoms when assessed using the REQUITE scoring criteria.

Table 14: Baseline symptoms assessed prior to commencing radiotherapy in REQUITE lung cancer cohort

Symptom	Grade of toxicity (n= (%))		
	0	*1*	*2*
Cough	17 (42.5%)	20 (50.0%)	3 (7.5%)
Dyspnoea	15 (37.5%)	17 (42.5%)	8 (20.0%)
Haemoptysis	31 (77.5%)	9 (22.5%)	x
Chest Wall Pain	28 (70.0%)	11 (27.5%)	1 (2.5%)
Oesophagitis	36 (90.0%)	4 (10.0%)	x
Dysphagia	36 (90.0%)	4 (10.0%)	x

2.2.2.2.5.2 Overall Toxicity

Acute toxicity was calculated by deducting any baseline symptom score from score at the end of radiotherapy assessment. A significant acute event was classed as grade two or more. In the lung patients due to the differences in fractionation schedule acute toxicity was collected at three months post commencement of radiotherapy. 34 patients were eligible for acute toxicity scoring at time of analysis. Table 15 summarises the results.

Table 15: REQUITE lung cohort acute symptoms assessed 3 months following completion of radiotherapy, any baseline symptom score was deducted from the acute score. Grade 2 or more was classed as a significant toxicity.

SYMPTOM	Grade of toxicity (n= (%))	
	Low/No toxicity (0-1)	*Moderate/Severe toxicity (≥2)*
Cough	34 (100%)	0
Dyspnoea	31 (91.2%)	3 (8.8%)
Haemoptysis	33 (97.1%)	1 (2.9%)
Chest Wall Pain	33 (97.1%)	1 (2.9%)
Oesophagitis	33 (97.1%)	1 (2.9%)
Dysphagia	34 (100%)	0
Pneumonitis	31 (91.2%)	3 (8.8%)

2.2.2.2.5.3 Clinical Variables and Toxicity

In view of the small number of significant toxicity events univariate analysis for categorical variables was performed using a Fisher's exact test for equal distributions. The following categorical variables were analysed for acute toxicity: gender; smoking status; alcohol consumption; menopausal status; comorbidities (diabetes, rheumatoid arthritis, hypertension, ischaemic heart disease, COPD and depression), medications (ace inhibitors, statins, oral analgesics and antidepressants), family history of radiotherapy toxicity, tumour factors (T stage, lymph node involvement on imaging, histology, tumour site), treatment factors (chemotherapy, post-operative radiotherapy, radiotherapy technique). No statistically significant results were obtained during this analysis presumably due to the low numbers of events/patients.

Univariate analysis for continuous variables was performed using Mann Whitney U testing for difference in means between low and high toxicity groups. The variables included were age; BMI; baseline lung function; external beam radiotherapy dose; dose per fraction; GTV (cm^3); PTV (cm^3); CTV (cm^3); dosimetry parameters. The only significant result was V30 to the heart (0.03) and mean heart dose (p=0.03) as shown in figure 12.

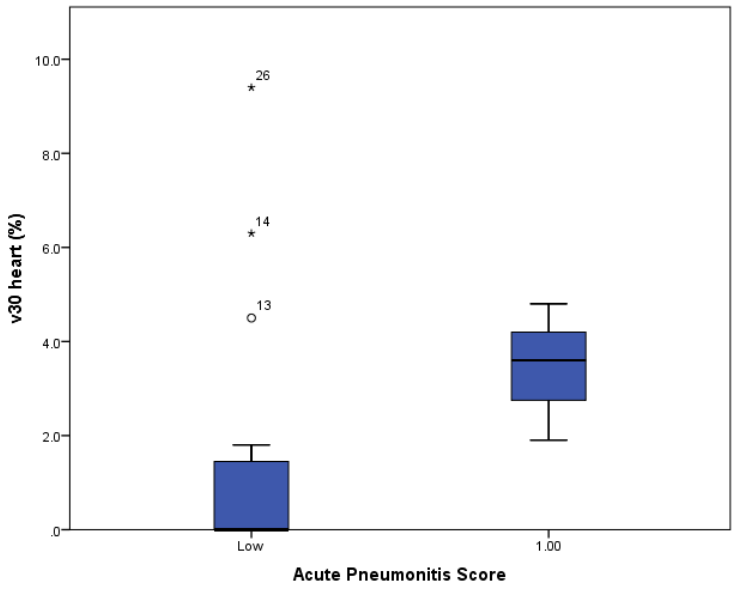

Figure 12: Boxplot for v30 heart values split by acute pneumonitis score. Grade 2 or more is a significant event.

Multivariate analysis was performed using bimodal regression, none of the variables remained significantly associated with acute toxicity.

2.2.2.3 Breast Cohort

The breast cohort data was collected predominantly by Mr Tim Rattay but is included for completeness in this section.

2.2.2.3.1 Baseline Demographics

The median age of the breast patients was 60.0 years and the median BMI was 27.2. 183 (52.6%) were non-smokers and 165 (47.4%) were either current or ex-smokers. 42 (12.1%) were premenopausal, 250 (72.3%) were post-menopausal and 54 (15.4%) were peri-menopausal. Table 16 shows the prevalence of comorbidities in the breast cohort.

Table 16: Comorbidities in breast cohort (REQUITE)

Comorbidity	Number with	Number without
Diabetes	29 (8.3%)	319 (91.7%)
Ischaemic Heart Disease	26 (7.5%)	322 (92.5%)
Rheumatoid Arthritis	8 (2.3%)	340 (97.7%)
Hypertension	106 (30.5%)	242 (69.5%)
Family History of radiotherapy toxicity	8 (2.3%)	340 (97.7%)

2.2.2.3.2 Tumour Specifics

At time of analysis data on tumour details was only available for 282 patients. The most common pathological T stage was T1c but ranged from Tis to T3. 199 (56.7%) patients were lymph node negative, 21 (5.7%) had unknown lymph node status (due to no axillary surgery or prior neo-adjuvant chemotherapy), and 42 (12.5%) were lymph node positive. There were 69 (25.1%) grade 1, 141 (51.3%) grade 2 and 65 (23.6%) grade 3 tumours. 24 (8.5%) were HER2 positive, 226 (80.1%) HER2 negative and 32 (11.3%) had unknown HER2 status. 219 (77.7%) were ER (oestrogen receptor) positive, 33 (11.7%) ER negative and 30 (10.6%) had unknown ER status.

2.2.2.3.3 Treatment Details

At time of analysis treatment information was available on 284 of the 350 breast cancer patients recruited.

28 (9.8%) patients received chemotherapy (22 adjuvant and 6 neo-adjuvant). 152 (53.7%) received endocrine therapy and 3 (1.1%) received Trastuzumab.

250 (88.3%) patients with available treatment data received 40Gy total dose to the whole breast field. 26 (9.2%) received 50Gy to the breast. 20 patients (5.7%) underwent a boost to the tumour bed in addition to whole breast radiotherapy and 15 (5.6%) had axillary lymph nodes treated.

2.2.2.3.4 Dosimetry

Table 17 summarises the dosimetry for the radiotherapy treatment received by the breast cohort.

Table 17: Dosimetry for Breast (REQUITE) cohort

	Median	Range	Standard deviation
Max Skin Dose	42.5Gy	2.8-53.7Gy	4.0
Mean Lung Dose	3.6Gy	1.1-7.3Gy	1.2
Mean Heart Dose	0.7	0.2-2.7	0.5

2.2.2.3.5 Toxicity

2.2.2.3.5.1 Baseline Symptoms

Table 18 shows the prevalence of baseline symptoms when assessed prior to radiotherapy using the REQUITE scoring criteria.

Table 18: REQUITE breast patients baseline symptoms assessed prior to commencing radiotherapy

Symptom	Grade of toxicity (n= (%))		
	0	*1*	*2*
Breast Oedema	321 (91.7%)	29 (8.3%)	0
Skin Induration	264 (75.4%)	85 (24.3%)	1 (0.3%)
Breast Erythema	336 (96.0%)	12 (3.4%)	2 (0.6%)
Breast Pain	206 (59.0%)	143 (41.0%)	0

2.2.2.3.5.2 Overall Toxicity

Acute toxicity was calculated by deducting any baseline symptom score from score at the end of radiotherapy assessment. A significant acute event was classed as grade two or more. 335 patients had completed radiotherapy and were eligible for acute toxicity scoring at time of analysis. For some of the acute criteria there was missing data hence less than 335 patients. Table 19 summarises the results.

Table 19: Breast volunteers (REQUITE) acute symptoms assessed in the last 5 fractions of radiotherapy, any baseline symptom score was deducted from the acute score.

SYMPTOM	GRADE OF TOXICITY (N= (%))	
	Low (0-1)	*Moderate/Severe (≥2)*
Breast Oedema	319 (100%)	0
Skin Induration	319 (100%)	0
Breast Atrophy	313 (98.4%)	5 (1.6%)
Breast Erythema	279 (83.3%)	56 (16.7%)
Breast Pain	322 (100%)	0

2.2.3 PREDICTIVE ASSAY RESULTS

2.2.3.1 Comet Assay

2.2.3.1.1 REQUITE cohort

2.2.3.1.1.1 Baseline Demographics

The comet assay was performed on 148 of the REQUITE patient samples and results were obtained for 136 of these. The twelve patient samples which failed were due to technical issues resulting in loss of gel.

Table 20 shows the baseline demographics for the patients the comet assay was performed on. On univariate analysis none of the baseline demographics or comorbidities significantly related to comet assay results.

Table 20: Baseline demographics for REQUITE patients comet assay performed on

Variable	N= (%)
Tumour site	Breast: 70 (47.3%) Prostate: 67 (45.3%) Lung: 11 (7.4%)
Gender	Male: 72 (48.6%) Female: 76 (51.4%)
Age	Median 67 years (range 40-89)
Smoking status	Non Smoker: 74 (50%) Current or Ex-Smoker: 74 (50%)

2.2.3.1.1.2 Comet Assay Results

Results for percentage tail DNA and Olive tail moment were obtained for 0Gy, 8Gy 0 minutes post irradiation and 8Gy 30 minutes post irradiation samples. From these results radiation induced damage (8Gy 0 minutes – 0Gy), rate of repair (8Gy 0 mins – 8Gy 30 mins) and relative rate of repair (8Gy 0 minutes/8Gy 30 minutes) were calculated. Table 21 summarises these results.

Table 21: Comet assay results summary in REQUITE cohort

	Valid	Missing	Median	Std. Deviation	Minimum	Maximum
0Gy (PTD)	136	12	9.5	7.9	0	37.9
0Gy (OTM)	117	31	2.2	2.2	0.4	12.9
8Gy 0mins (PTD)	121	27	45.8	13.4	12.3	71.3
8Gy 0mins (OTM)	121	27	11.3	5.2	2.5	27.6
8Gy 30 mins (PTD)	75	73	29.5	10.1	8.4	57.2
8Gy 30 mins (OTM)	75	73	6.9	3.7	2	21.9
RID (PTD)	120	28	37.2	17.6	0.2	68.9
RID (OTM)	120	28	8.8	6.2	1	26.5
ROR (PTD)	108	40	24.2	12	-0.4	41.3
ROR (OTM)	108	40	6.2	4.2	0	19.3
RRR (PTD)	113	35	1.8	1	0.8	5.7
RRR (OTM)	113	35	2.2	1.1	0.8	6.4

PTD = percentage Tail DNA; OTM = Olive Tail Moment; RID = Radiation induced damage; ROR = Rate of Repair; RRR = Relative rate of repair

2.2.3.1.1.3 Comet assay results and Toxicity

Univariate analysis for the effect of comet assay parameters on toxicity was performed using a Mann Whitney U test for the whole cohort. None of the parameters showed any correlation to toxicity (table 22).

Table 22: Univariate analysis of comet assay parameters in whole REQUITE cohort

(Percentage Tail	p value	(Olive Tail Moment)	p value

DNA)			
0Gy	0.34	*0Gy*	0.64
8Gy 0mins	0.99	*8Gy 0mins*	0.82
8Gy 30 mins	0.44	*8Gy 30 mins*	0.85
RID	0.71	*RID*	0.88
ROR	0.76	*ROR*	0.52
RRR	0.19	*RRR*	0.05

RID = Radiation induced damage; ROR = Rate of Repair; RRR = Relative rate of repair

The cohort was split by tumour site and there were no significant results in the prostate or lung cohorts but in the breast cohort relative rate of repair for percentage tail DNA (PTD) (p=0.004) and Olive tail moment (OTM) (p=0.008) were both statistically significant (figure 13). Multivariate analysis using binary logistic regression however did not show the same significance (p=0.54 (PTD), p=0.27 (OTM)).

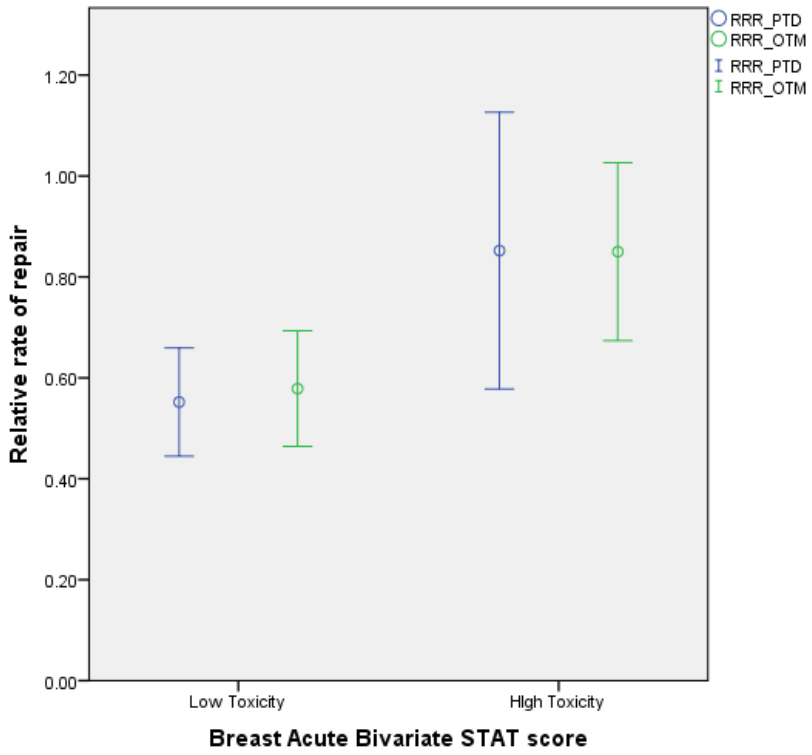

Figure 13: Comet assay Relative rate of repair in the REQUITE breast patients. RRR = Relative rate of repair; PTD = Percentage Tail DNA; OTM = Olive Tail moment

2.2.3.1.2 LeND cohort

2.2.3.1.2.1 Baseline Demographics and Treatment Data

In order to try and replicate the findings in the REQUITE cohort the comet assay was performed on 82 of the LeND cohort patients. Results were obtained for 80 of these, two samples failed due to technical issues resulting in loss of gel.

Table 23 shows the baseline demographics for the patients the comet assay was performed on.

Table 23: Demographics and treatment related data for patients in LeND cohort who provided a sample for the comet assay

Variable	N= (%)
Age at radiotherapy	Mean: 56.5 years
	Range:34 – 87 yrs.
Smoking Status	Non Smoker: 64/82 (78%)
	Current/Ex-Smoker: 15/82 (18.3%)
	Unknown – 3/82 (3.7%)
Hypertension	Yes – 19/82 (23.2%)
	No – 61/82 (74.4%)
	Unknown – 2/82 (2.4%)
Diabetes Mellitus	Yes – 2/82 (2.4%)
	No – 78/82 (95.1%)
	Unknown – 2/82 (2.4%)
TREATMENT FACTORS	
Radiotherapy Dose	40Gy/15# - 8/82 (9.8%)
	45Gy/20# - 23/82 (28%)
	50Gy/25# - 48/82 (58.5%)
	Unknown – 3/82 (3.7%)
Boost	Yes – 14/82 (17.1%)
	No – 65/82 (79.3%)
	Unknown – 3/82 (3.7%)
Hormone Therapy	Yes – 68/82 (82.9%)
	No – 11/82 (13.4%)
	Unknown – 3/82 (3.7%)
Chemotherapy	Yes – 68/82 (82.9%)
	No – 11/82 (13.4%)
	Unknown – 3/82 (3.7%)

2.2.3.1.2.2 Comet Assay Results

The parameters were collected as described above for the REQUITE cohort. Table 24 summarises these results.

Table 24: Comet assay results summary in LeND cohort

	Valid	Missing	Median	Std. Deviation	Minimum	Maximum
RID (PTD)	77	3	32.46	6.22	6.31	52.35
RID (OTM)	77	3	8.25	2.30	1.55	15.22
ROR (PTD)	80	0	26.10	8.69	0	45.53
ROR (OTM)	77	3	7.06	2.37	0.09	13.58
RRR (PTD)	77	3	279.76	72.37	114.34	497.69
RRR (OTM)	77	3	305.55	87.41	100.91	503.56

PTD = Percentage Tail DNA; OTM = Olive Tail Moment. RID = Radiation induced damage; ROR = Rate of Repair; RRR = Relative rate of repair

None of the baseline characteristics from table 23 were linked to comet assay results.

2.2.3.1.2.3 Comet Assay and Toxicity

Univariate analysis for the effect of comet assay parameters on toxicity was performed using a Mann Whitney U test for the whole cohort. For Olive tail moment both rate of repair (p=0.03) and relative rate of repair were significantly associated with increased late toxicity (p=0.049) (table 25, figure 14). Multivariate analysis however failed to demonstrate a significant relationship (p=0.78 (ROR), p=0.29 (RRR)).

	Olive Tail Moment			Percentage Tail DNA		
	Bivariate STAT score	*Mean Values*	*P Value*	*Bivariate STAT score*	*Mean Values*	*P Value*
Radiation Induced Damage	0	8.3	0.07	0	32.8	0.05*
	1	9.4		1	36.2	
Rate Of Repair	0	6.8	0.03	0	25.3	0.11
	1	8.2		1	29.1	
Relative Rate of Repair	0	300.1	0.05	0	279.3	0.19
	1	348.3		1	306.1	

Table 25: Univariate analysis of comet assay parameters in LeND cohort.

* *p =0.051 so not significant*

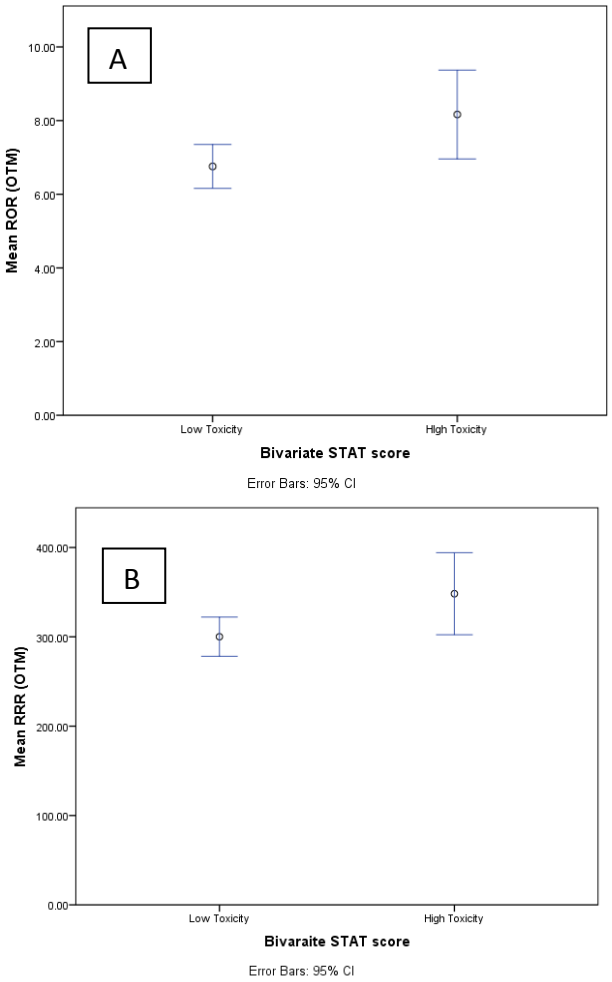

Figure 14: Olive tail moment mean results for comet assay rate of repair and relative rate of repair in the LeND cohort. A= ROR (Rate of repair); B = RRR (Relative rate of repair); OTM = Olive Tail moment.

2.2.3.1.3 LeND vs REQUITE cohort

2.2.3.1.3.1 Univariate Analysis

A Mann Whitney U test was performed to compare the comet results between the two cohorts. Table 26 summarises the mean results and compares the two cohorts. Both residual DNA damage 30 minutes post irradiation ($p=2.61x10^{-13}$ (PTD) /$p=2.18x10^{-10}$ (OTM)) and the relative rate of repair ($p=1.11x10^{-7}$ (PTD); $p=4.10x10^{-5}$ (OTM)) measured with percentage tail DNA and Olive tail moment were statistically different between the 2 cohorts. Faster relative rate of repair in the LeND cohort due to lower residual DNA damage at 30 minutes was observed as shown in figure 15.

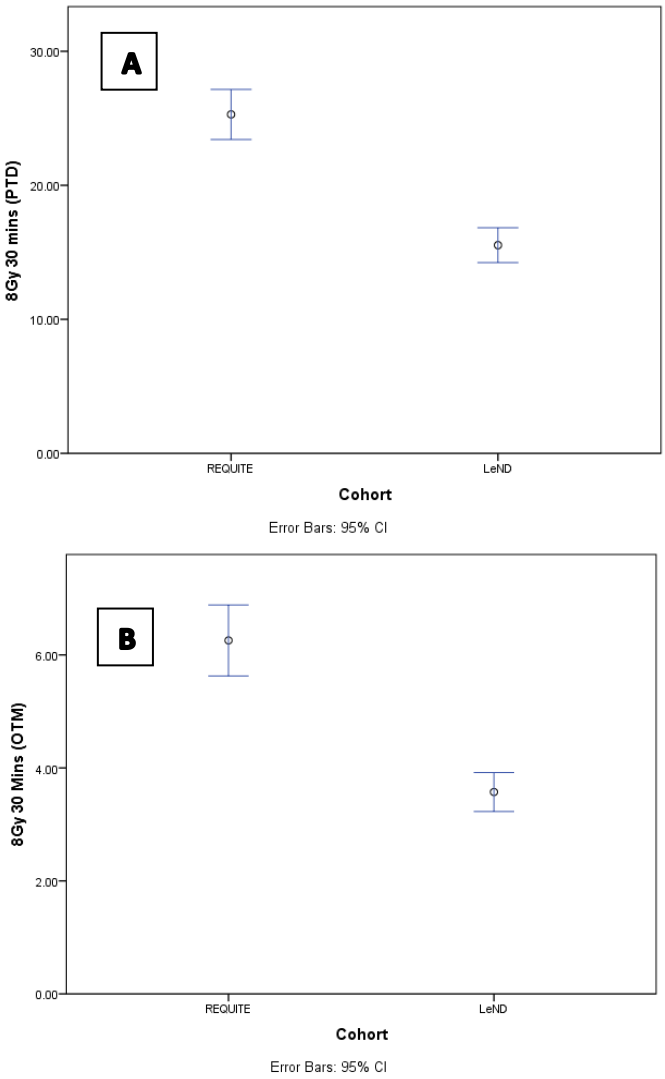

Figure 15: Difference in DNA damage induced by 8Gy left after 30 mins repair in REQUITE and LeND cohorts using comet assay. A = PTD (Percentage Tail DNA); B = OTM (Olive Tail Moment)

Table 26: Differences in mean comet variables by cohort.

Comet variable	Cohort	N	Mean	Std. Deviation	P value (2 tailed significance)
0Gy	REQUITE	118	10.29 [a] /2.74 [b]	7.87 [a] /2.25 [b]	0.86 [a] /0.44 [b]
	LeND	78	9.45 [a] /2.15 [b]	3.65 [a] /0.85 [b]	
8Gy 0mins	REQUITE	121	44.89 [a] /12.51 [b]	13.43 [a] /5.24 [b]	0.24 [a] /0.07 [b]
	LeND	78	42.10 [a] /10.73 [b]	2.54 [a] /5.83 [b]	
8Gy 30mins	REQUITE	117	25.29 [a] /6.26 [b]	10.22 [a] /3.46 [b]	$2.61x10^{-13}$ [a] /$2.18x10^{-10}$ [b]
	LeND	77	15.53 [a] /3.57 [b]	6.22 [a] /2.30 [b]	
RID	REQUITE	120	34.3[a]/10.1[b]	17.6 [a] /6.16 [b]	0.49 [a] /0.59[b]
	LeND	80	33.47 [a] /8.53 [b]	23.4 [a] /6.22 [b]	
ROR	REQUITE	108	22.2 [a] /6.85 [b]	12.0 [a] /4.22 [b]	0.10[a] / 0.25[b]
	LeND	80	26.10 [a] 7.05 [b]	8.69 [a] /2.37[b]	
RRR	REQUITE	113	2.11 [a] /2.41 [b]	0.99 [a] /1.11 [b]	$1.11x10^{-7}$ [a] / $4.10x10^{-5}$ [b]
	LeND	77	7.74 [a] /2.98 [b]	2.37 [a] /0.89 [b]	

a = Percentage tail DNA, b = Olive tail moment. RID = Radiation induced damage; ROR = Rate of Repair; RRR = Relative rate of repair

2.2.3.1.3.2 Multivariate Analysis

On univariate analysis in this combined cohort dataset diabetes was significantly related to radiation induced damage (p=0.008) as was age (p=0.02). Age also was significantly correlated to rate of repair. (p=0.02). Relative rate of repair had no recorded significant variables other than cohort. Smoking status was significantly correlated to PTD after 30 minutes repair (p=0.04). The effect of cohort was put into the multivariate analysis model and PTD 8Gy 30 mins remained significantly different in the cohorts ($p=6.16x10^{-13}$), no other comet parameters were significantly different between the two cohorts on multivariate analysis.

2.2.3.2 γ-H2AX assay

The γ-H2AX assay was performed on 30 REQUITE patient samples (10 breast, 17 prostate and 3 lung). After an assessment of the results it was decided to stop at this point to concentrate on other aspects of the project. Baseline demographics for the 30 patients with γ-H2AX assay results available are shown in table 27.

Table 27: Baseline demographics for patients with H2AX assay results

Variable	N =
Age	Median: 64.6 years (range 47-84)
Tumour	Breast - 10; Lung - 3; Prostate - 17
Gender	Female - 11; Male - 19
Smoking Status	Never - 15; Current or ex - 15
Diabetes	No - 25; Yes - 5
Hypertension	No - 21; Yes - 9
IHD	No - 24; Yes - 6
Depression	No - 28; Yes - 2
Ace Inhibitor	No - 25; Yes - 5
Statin	No - 21; Yes - 9
Antidiabetics	No - 26; Yes - 4
FH of radiotherapy toxicity	No - 30

2.2.3.2.1 Spread of Data

Figure 16 shows the mean results for the different parameters measured for the γ-H2AX assay. As expected the lowest levels of DNA damage were observed in the 0Gy (control sample). The highest levels of DNA damage were observed 3 hours following irradiation falling close to basal levels 48 hours post irradiation.

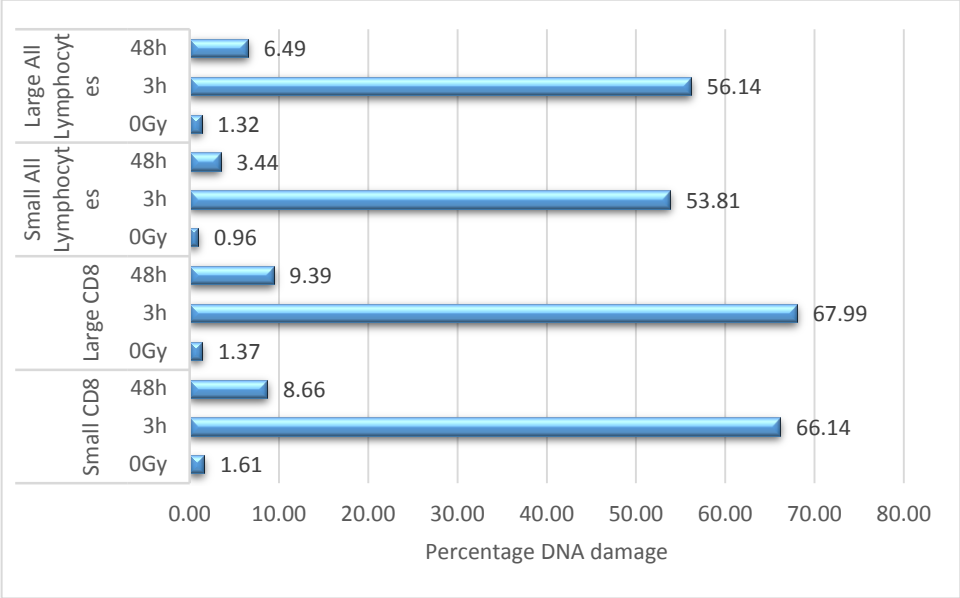

Figure 16: Bar chart of mean results for γ -H2AX parameters in all 4 gating strategies: CD8 positive lymphocytes, small and large gates; all lymphocytes, small and large gates

Table 28 shows the calculated parameters for the CD8 positive lymphocytes and all lymphocytes using the small gating.

Table 28: Summary of results for γ-H2AX assay (small gates only).

γ -H2AX assay result	Lymphocyte Population	Mean	Std. Deviation	Minimum	Maximum
Absolute damage 3h	All	53.81	11.85	16.71	72.12
(8Gy 3h - 0Gy)	CD8	66.14	11.71	34.13	78.78
Absolute damage 48h	All	3.44	2.19	0.20	8.54
(8Gy 48h - 0Gy)	CD8	8.66	6.20	0	26.35
% Residual damage 3h	All	0.019	0.02	0	0.11
(0Gy/8Gy 3h)	CD8	0.02	0.04	0	0.25
% Residual damage 48h	All	0.24	0.20	0	0.73
(0Gy/8Gy 48h)	CD8	0.18	0.21	0	1.16
ROR	All	50.56	11.73	13.03	63.94
(8Gy 3h - 8Gy 48h)	CD8	56.93	11.16	31.42	71.56
RRR	All	0.07	0.05	0	0.22
(Absolute DNA damage 48h/ Absolute DNA damage 3h)	CD8	0.13	0.09	0	0.39

RRR = relative rate of repair; ROR = rate of repair

γ -H2AX results were compared to baseline demographics using a Mann Whitney U test for categorical variables (gender, tumour type, smoking status, diabetes) and a spearman's rank correlation for continuous variables (age, BMI). No significant effect was observed in either the categorical or the continuous variables on univariate analysis of the γ -H2AX results.

2.2.3.2.2 γ- H2AX and acute radiotherapy toxicity

Acute radiotherapy toxicity was assessed as previously using bivariate STAT score. The γ -H2AX assay results were not normally distributed so univariate analysis was performed using a Mann Whitney U test to assess if there was any difference in mean γ -H2AX assay results split by acute bivariate STAT score. These results are summarised in table 29, none of the variables were significantly different between the low toxicity group and the high toxicity group.

Table 29 : γ -H2AX univariate analysis for differences in mean results split by bivariate STAT score.

Test Statistics	Mann-Whitney U	Z	P value (2-tailed sig)
RRR All	34	-1.90	0.05
RRR CD8	41.5	-1.50	0.15
ROR All	65	-0.09	0.93
ROR CD8	63	-0.20	0.84
% Residual DNA 48h All	51.5	-1.03	0.31
% Residual DNA 48h CD8	64	-0.33	0.74
% Residual DNA 3h All	63.5	-0.38	0.70
% Residual DNA 3h CD8	49	-1.22	0.22
Absolute DNA damage 48H All	42.5	-1.52	0.13
Absolute DNA damage 48h CD8	41	-1.61	0.11
Absolute DNA damage All3h	66	-0.22	0.83
Absolute DNA damage CD8 3h	56	-0.78	0.44

There were too few patients to perform any meaningful analysis for acute toxicity for the separate tumour types with the γ -H2AX assay results.

2.2.3.3 RILA

The RILA assay was performed on 419 of the patients recruited at Leicester. The RILA score was calculated as described above and median score was 20.10. In previous publications reporting the predictive value of RILA the score has been divided into three equal groups. In this cohort low score is less than 14.8; mid score = 14.8-24.6; high score is greater than 24.6.

2.2.3.3.1 RILA and clinical variables

Of the patients the RILA was performed on 212 were female and 207 were male. 198 were prostate cancer patients, 200 were breast cancer patients and 21 lung cancer patients. An independent T test for equivalence of mean RILA between the genders showed that females had a significantly lower mean RILA score than males ($p= 3.0 \times 10^{-5}$). Using 1 way ANOVA testing to compare the results by tumour site breast cancer patients had a significantly lower mean RILA ($p=0.001$) than prostate patients. There was no difference in mean score between the lung patients and either the breast or the prostate cohorts. These findings are summarised in table 30.

Table 30: RILA mean values by cancer type and gender. ANOVA test results.

	Gender		Cancer		
	Female	*Male*	*Prostate*	*Breast*	*Lung*
N	212	207	198	200	21
Mean	19.09	23.93	23.87	19.4	18.84
s.d	11.2	12.24	12.33	11.32	10.44
s.e	0.77	0.85	0.88	0.8	2.28
Mean Difference	-4.84		4.47 (breast) 5.03 (lung)	4.47 (prostate) 0.56 (lung)	5.03 (prostate) 0.56 (breast)
Lower 95% CI	-7.09		1.69 (breast) -1.32 (lung)	-7.24 (prostate) -5.79 (lung)	11.39 (prost) -6.91 (breast)
Upper 95% CI	-2.59		7.24 (breast) 11.39 (lung)	-1.69 (prostate) 6.91 (lung)	1.32 (prostate) 5.79 (breast)
Significance (2 tailed p)	3x10-5		$1x10^{-3}$ (breast) 0.15 (lung)	$1x10^{-3}$ (prostate) 0.98 (lung)	0.15 (prostate) 0.98 (breast)

S.E. = Standard error, S.D = standard deviation, CI = Confidence interval

2.2.3.3.2 Baseline comorbidities and RILA

Categorical variables (comorbidities: diabetes, smoking status, heart disease, hypertension, depression; medications: antidiabetics, ACE inhibitors, statins, antidepressants) were compared to mean RILA score with an independent T test and continuous variables (age and BMI) were compared using spearman's rank. Only depression (p=0.01) and age (p=0.006) were significantly associated with RILA as shown in figures 17 and 18. Patients with underlying depression have a lower mean RILA score (22.04 vs 17.46) and with increasing age mean RILA score also increases.

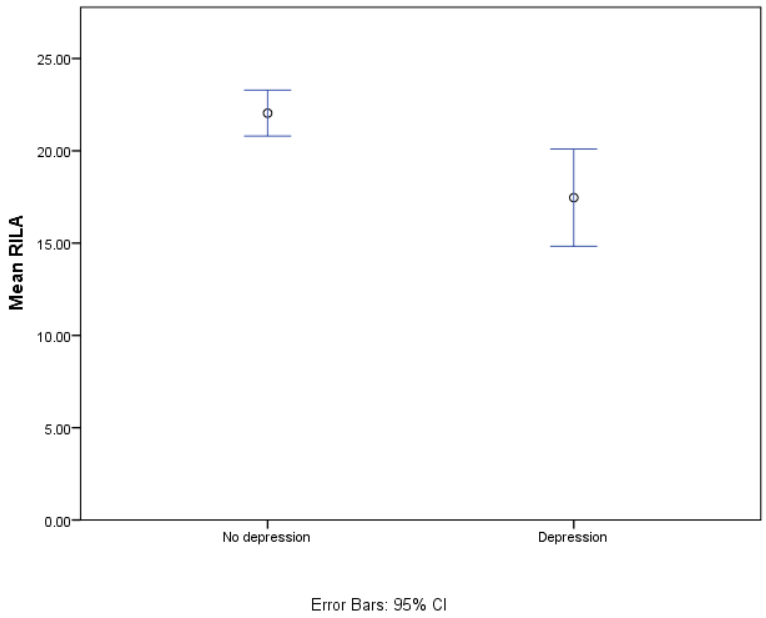

Figure 17: Mean RILA score in patients with and without history of depression

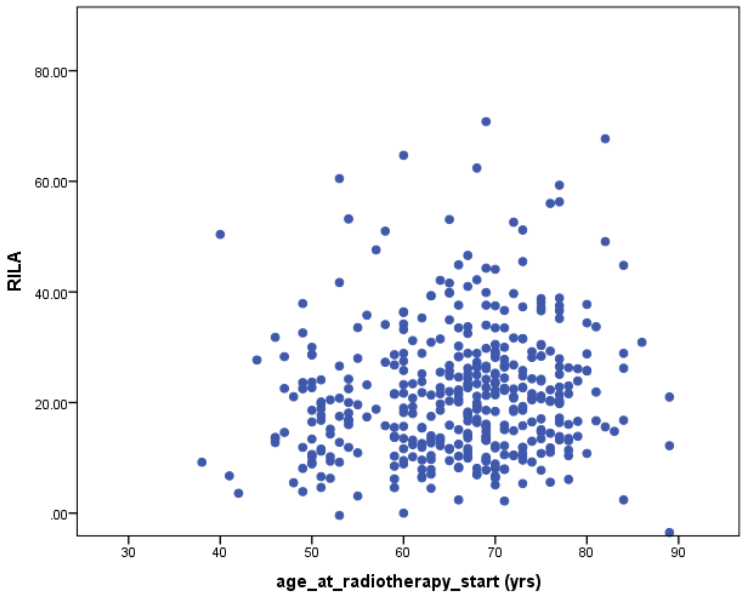

Figure 18: Correlation between RILA score and age

Multivariate analysis using linear regression was performed to analyse if any of the baseline variables had a significant effect on RILA score. Smoking status (p=0.05) and gender (p=0.02) were significantly associated with RILA score (table 31). Patients who

have a positive smoking history having a lower mean RILA score. And females have a lower RILA score than males.

Table 31: Multivariate analysis for baseline demographics and comorbidities effect on mean RILA score

	Exp (B)	P value	95% C.I. of Exp (B) Lower Upper	
Gender	-7.21	0.02	-13.20	-1.21
Tumour	-0.40	0.88	-5.48	4.68
Age	0.04	0.76	-0.20	0.27
smoker	-2.38	0.05	-4.85	0.09
diabetes	-1.74	0.61	-8.38	4.91
depression	-3.19	0.35	-9.90	3.52

2.2.3.3.3 RILA and Toxicity

Patients were split as previously described using bivariate STAT score to assess for overall acute radiotherapy toxicity. An independent T test failed to demonstrate any significant difference in mean RILA score between the two groups (p = 0.47). When RILA score was divided into three groups as described in previous publications and acute toxicity was compared using a chi squared test for equivalence no significant difference was found between the three groups (p=0.79), figure 19.

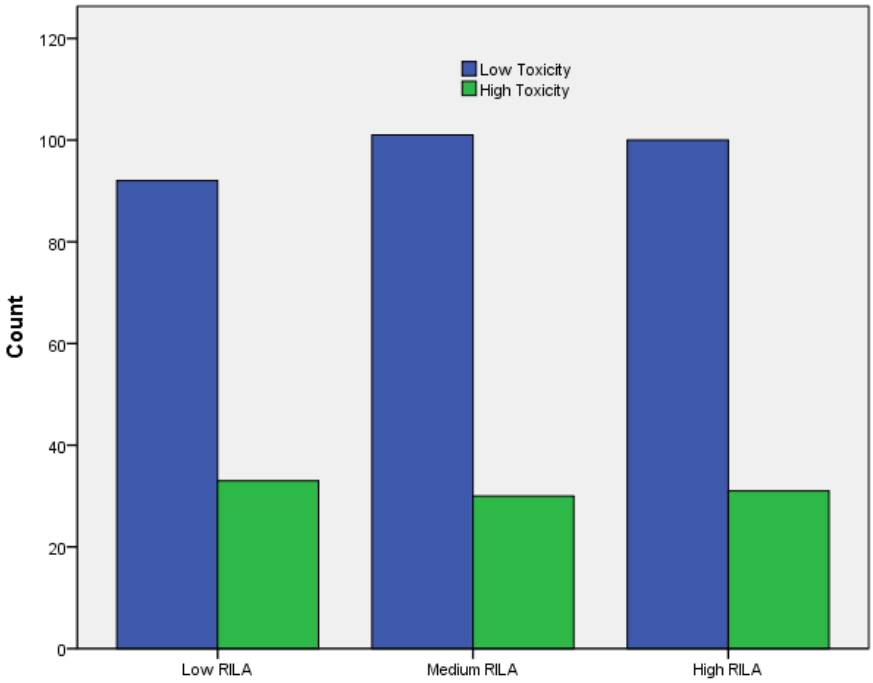

Figure 19: Acute toxicity split by 3 mean RILA score groups.

Subgroup analysis was also performed on the basis of gender and cancer type but there remained no significant predictive value of RILA for acute toxicity.

2.2.3.4 Cross Assay Analysis

2.2.3.4.1 RILA and Comet assay

There were 91 patient samples on which the RILA and comet assay were both performed. A Spearman's rank test was performed to assess if there was any significant correlation (table 32). There was a significant correlation between RILA and rate of repair (Percentage tail difference, $p = 0.01$) and relative rate of repair (percentage tail difference ($p= 3.0 \times 10^{-3}$) and Olive tail moment $p = 8.0 \times 10^{-3}$). Patients with a faster rate of repair and relative rate of repair have a lower RILA score (figure 20).

Table 32: Correlation between RILA and Comet assay results.

Comet Parameter		RID (8Gy 0mins-0Gy)		ROR (8Gy 0 mins - 8Gy 30 mins)		RRR (8Gy 0mins/8Gy 30 mins)	
		PTD	*OTM*	*PTD*	*OTM*	*PTD*	*OTM*
RILA	*Correlation Coefficient*	-0.03	0.12	-0.40	-0.27	-0.43	-0.39
	Sig. (2-tailed)	0.85	0.40	0.01	0.08	$3X10^{-3}$	$8X10^{-3}$

RID = Radiation induced damage; ROR = Rate of repair; RRR = relative rate of repair; PTD = Percentage tail difference; OTM = olive tail moment

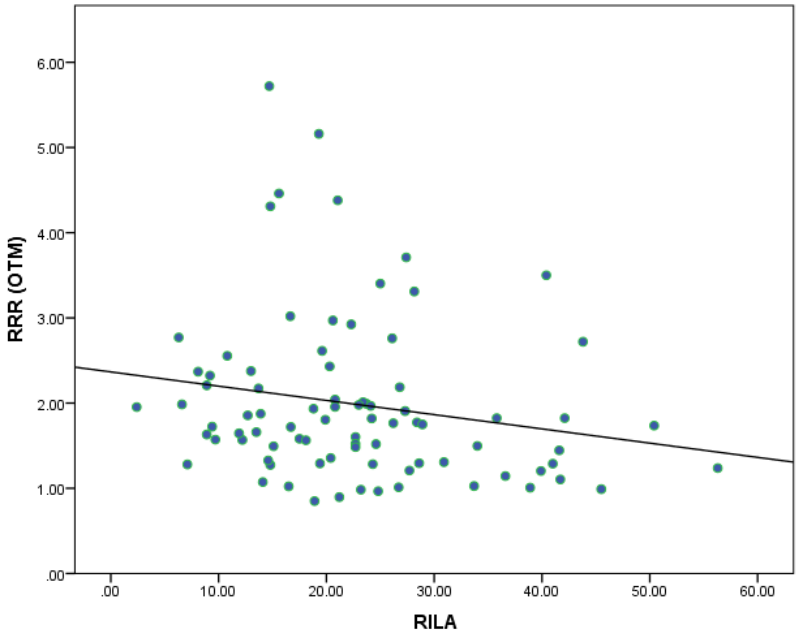

Figure 20: Scatter diagram showing the correlation between RILA score and Relative Rate of repair measured using the comet assay. RRR = Relative rate of repair; OTM = Olive Tail Moment

2.2.3.4.2 γ-H2AX and comet assay

Fifteen patient samples had both the comet and γ -H2AX assays performed on them. Spearman's rank was performed and there was no significant correlation between any of the outcome measures between the two assays (table 33).

Table 33: Correlation between γ -H2AX and Comet assay results

		γ -H2AX assay result					
Comet result		*RID CD8*	*RID All Lymph*	*ROR CD8*	*ROR All Lymph*	*RRR CD8*	*RRR All Lymph*
RID PTD	*Correlation Coefficient*	-0.05	-0.46	-0.02	-0.44	-0.23	-0.15
	Sig. (2-tailed)	0.86	0.08	0.94	0.10	0.42	0.60
RID OTM	*Correlation Coefficient*	-0.15	-0.50	-0.17	-0.48	-0.15	-0.11
	Sig. (2-tailed)	0.58	0.06	0.55	0.07	0.59	0.69
ROR PTD	*Correlation Coefficient*	0.10	-0.31	0.03	-0.29	0.01	0.00
	Sig. (2-tailed)	0.75	0.30	0.91	0.33	0.97	1.00
ROR OTM	*Correlation Coefficient*	0.22	-0.17	0.12	-0.15	0.09	0.06
	Sig. (2-tailed)	0.48	0.58	0.71	0.63	0.78	0.84
RRR PTD	*Correlation Coefficient*	0.19	-0.26	0.10	-0.24	0.11	0.04
	Sig. (2-tailed)	0.53	0.38	0.75	0.43	0.72	0.90
RRR OTM	*Correlation Coefficient*	0.19	-0.26	0.10	-0.25	0.07	0.04
	Sig. (2-tailed)	0.53	0.39	0.75	0.42	0.83	0.90

RID = Radiation induced damage; ROR = Rate of repair; RRR = relative rate of repair; PTD = Percentage tail difference; OTM = Olive tail moment

2.2.3.4.3 γ-H2AX and RILA

28 patient samples had both the RILA and γ -H2AX assays performed on them. Spearman's rank was performed and there was no significant correlation between any of the outcome measures between the two assays (table 34).

Table 34: Correlation between γ -H2AX and RILA assay results. RID = Radiation induced damage; ROR = Rate of repair; RRR = relative rate of repair

		γ -H2AX assay results					
		RID CD8	*RID All Lymph*	*ROR CD8*	*ROR All Lymph*	*RRR CD8*	*RRR All Lymph*
RILA	*Correlation Coefficient*	-0.21	0.01	-0.25	-0.02	0.06	0.01
	P value	0.29	0.98	0.20	0.90	0.76	0.96

2.3 DISCUSSION

2.3.1 Prostate Cohort

Prostate cancer patients treated with radiotherapy in Leicester tend to have locally advanced, inoperable disease (T3a on MRI being the most common T stage). Reflective of this is the high proportion therefore that receive prophylactic pelvic lymph node radiotherapy (44.7%). Because of the larger treatment volume involved in pelvic lymph node radiotherapy the standard treatment is conventional 2Gy per fraction with only two patients in this cohort receiving hypofractionated courses and prostate nodal radiotherapy. In addition very few patients receive post-operative radiotherapy (only sixteen patients in total). This makes analysis more reliable when looking for other predictors of toxicity as the treatment was largely uniform.

As expected the dosimetric data collected shows a large spread but all median values were within the parameters set by QUANTEC. The fact that there are extremes which exceed these guidelines allows for a meaningful analysis of whether dosimetry predicts for toxicity. The large standard deviation for the PTV is likely as a result of 44.7% of patients receiving prophylactic pelvic nodal radiotherapy in addition to the pelvic radiotherapy the whole cohort received.

In this study there were lower than expected levels of acute GI (maximal 8.4%) and GU (maximal 15.8%) toxicity. Previously studied prostate cohorts using similar radiotherapy techniques observed rates around 30% grade two or more acute toxicity (Arcangeli et al. 2011). In the current study patients were assessed at baseline using the REQUITE trial scoring criteria. There was a high proportion who had at least grade one symptoms at initial assessment e.g. grade one urinary frequency was present in 44.4% of the patients. To correct for this any baseline toxicity score was deducted from the acute radiotherapy toxicity score and may thus underestimate the percentage of patients with acute events. For some of the toxicity end points e.g. frequency and urgency the maximal score was two, it may have been better for urinary symptoms to assess for a change from baseline like with diarrhoea. Another notable factor was retention grade two included any kind of medical intervention from drug therapy to catheterisation and therefore may not have picked up the extreme reactions. Erectile function was not possible to assess in the acute term in the present cohort as all

patients (except fifteen of the post-operative patients) were on LHRH analogues. One year toxicity rates were also very low with a maximal of 3.6% of the patients having a GU or GI toxicity at this time point. Therefore no meaningful analysis of late toxicity could be drawn at this time.

On univariate analysis the strongest clinical predictor of acute radiotherapy toxicity (individual GI and GU endpoints and combined bivariate STAT score) was pelvic nodal radiotherapy. This was not however significant on multivariate analysis although intended duration of hormone therapy was. It is possible that longer use of hormones could be a surrogate marker of pelvic nodal radiotherapy as patients with a longer intended duration are more likely to undergo prophylactic nodal radiotherapy. These findings are consistent with previously reported studies where pelvic nodal irradiation increases acute GI toxicity (Deville et al. 2010) however has not been proven to correlate to increased late toxicity or GU toxicity.

On univariate analysis mean integral dose was calculated as a possible predictive variable for acute toxicity. Mean integral dose is essentially a measure of the average radiation dose to the body. A recent presentation (Nuradh Joseph 2016) by members of the Radiogenomics Consortium suggests that patients with a higher pelvic mean integral irradiation dose have worse fatigue and functional outcomes. On univariate analysis in the current study increased mean dose correlated with a worse acute bivariate STAT score ($p=0.04$). This was not however replicated on multivariate analysis. There is a high degree of correlation between integral dose and pelvic nodal radiotherapy as expected and in our cohort the two should be considered surrogate markers of each other.

Data on the activity levels of patients was collected pre and post radiotherapy with the GPAQ WHO questionnaire. Patients who managed to maintain higher levels of activity to the end of radiotherapy had lower levels of acute radiotherapy toxicity on univariate analysis. This was not replicated on multivariate analysis however there were only questionnaire responses from 117 patients and with low levels of acute toxicity it is possible in a larger cohort the findings may be significant. This is in keeping with previous evidence (Mishra et al. 2012, Kapur et al. 2010) that suggests patients

experience less cancer treatment related toxicity if they are able to maintain a moderate degree of activity during treatment. One possible avenue of further work would be to see if toxicities improved quicker in those with higher activity levels which would be possible by looking at the one year and two year toxicity data in comparison to acute toxicity. The explanation may be that patients who maintain some level of activity are less likely to become low in mood during their treatment so under report toxicities in comparison to those with a more sedentary lifestyle. Or it may be that the well documented effect of aerobic exercise (Brolinson and Elliott 2007) has on the immune system is in some way protective of the observed toxicities in the organs at risk. Another possible explanation could be that patients experiencing moderate or severe toxicity may find it harder to exercise.

2.3.2 Lung Cohort

Recruitment for the lung cohort was lower than expected for Leicester and the whole REQUITE trial consortium. Despite lung cancer being the second most common cancer in the UK the vast majority of patients still present with advanced disease which is not amenable to radical treatment. In addition to this many of the known causes for lung cancer are also linked to other health problems for instance smoking causes COPD; heart disease and peripheral vascular disease. These comorbidities in addition to the cancer can mean that even patients who are clinically suitable for radical treatment are not fit enough to cope with the potential morbidity from the diagnosis and treatment options. A third factor locally is that current NICE guidelines suggest that all patients with resectable disease should be offered surgery as a first option and only radiotherapy if the patients declines surgery or is not fit for an operation. There is increasing evidence that if patients are suitable for SABR then this may be equivalent in terms of survival and carries a better side effect profile than lobectomy (Chang et al. 2015). However without robust randomised clinical trial data it is difficult to change protocol.

In total 42 lung cancer patients were recruited to the current study with heterogeneous stage and treatment interventions. On multivariate analysis cancer T

stage and dose per fraction was entered to control for the differences in cancer presentation and treatment. The most common cancer stage was T1a reflecting the increasing use of SABR as a treatment option for lung cancer. All patients recruited had a smoking history being either current or ex-smokers. As expected therefore many (35.7%) had underlying chronic obstructive pulmonary disease and on baseline assessment 20% had already grade two dyspnoea and 42% grade one. Dyspnoea score is the primary end point for the REQUITE trial but because of the high proportion of patients with baseline symptoms in this thesis other end points particularly pneumonitis which would not have been present at baseline have been examined.

A further factor that impacted the data available is the different dose and fractionation schedules meant the acute assessment for lung patients was performed three months after the start of radiotherapy. At this time point it is standard to get a follow up CT scan to assess response. Any patients who progressed in this time frame were withdrawn from the study, resulting in only 35 patients that it was possible to analyse acute toxicity on. In this small group only mean dose to the heart and v30 heart had a significant association with pneumonitis on univariate analysis (p=0.03). Irradiation of the heart although not a well-documented risk factor for pneumonitis has previously been reported in a study (Huang et al. 2011) of 209 patients to be significantly associated with radiation pneumonitis. A possible explanation is that mean dose to the heart is a surrogate marker of the overall volume of lung irradiated, however in our analysis the V5 and V20 to the lungs were not significantly associated with pneumonitis so this would need to be investigated in a larger cohort such as the complete international REQUITE study population.

2.3.3 Breast cohort

There were two breast cohorts available for analysis in this thesis. The REQUITE cohort with 350 prospectively recruited patients assessed for acute toxicity only and the LeND cohort recruited years after their radiotherapy treatment assessed for both acute and late toxicity.

For the REQUITE cohort there were robust and reliable assessment tools in place for toxicity scoring including review of patients at baseline (up to the fifth fraction of radiotherapy) and at the end of radiotherapy (within the last three fractions of radiotherapy). 16.7% of the assessed population experienced a grade two or higher level of acute toxicity. This is somewhat lower than previously reported levels which are often closer to 30%. One possible explanation is that the lower levels are because of improved radiotherapy techniques and that the previous reports were based on old data and treatments. Another explanation is that peak acute toxicity for hypofractionated radiotherapy is not apparent until one to two weeks after the radiotherapy has completed (Brunt et al. 2016) so the protocol used may have missed some significant events thus under reporting acute toxicity. A further proposed theory is that acute skin reactions are less sensitive to fraction size than late reacting normal tissues. When total two gray equivalent dose is calculated using the EQD2 equation for hypofractionated regimens it is lower and may reduce their severity and duration of toxic effects, despite the shorter overall treatment time.

The LeND cohort were assessed for late toxicity many years after their radiotherapy had taken place. Data on treatments, acute toxicity and comorbidities was collected retrospectively from patient recall and available medical notes. Using a mature cohort has the benefit of ready availability of data for assessment of predictive measures of toxicity, however this method of collection of acute toxicity data is highly unreliable and any results should be interpreted with care. The fact radiotherapy had taken place much earlier than the REQUITE cohort treatments it was more common practice then to use standard two gray per fraction radiotherapy with very few patients receiving hypofractionated courses. As discussed above this has radiobiological implications for incidence of observed acute toxicities and may impact the ability of univariate analysis to compare the two cohorts.

A final consideration when examining the differences between acute and late effects observed in these two breast populations is the effect of biological effective dose (BED). BED is calculated using the following formula

$$BED = nd\left(1+\frac{d}{\alpha/\beta}\right)$$

For early effects, α/β is large (10), for late effects, α/β is small (2-3). For the REQUITE cohort which was used as acute toxicity population when BED was included in multivariate analysis an α/β ratio of 10 was used. For the LeND cohort an α/β ratio of 3 was used as this was assessing predominately late toxicity. Multivariate analysis was performed using dose and radiobiological factors in the model for any variables found to be significant on univariate analysis. The differences in α/β ratios may have resulted in some inaccuracies for multivariate analysis for acute toxicity in the LeND cohort.

Taking the above points into consideration the best way to interpret the breast data would be to use the two cohorts to report acute toxicity in REQUITE and late toxicity in the LeND cohort. Due to the inaccuracies with the acute data in LeND and the different underlying pathological and radiobiological processes it is more difficult to use to the two populations to validate findings. In the future once the REQUITE cohort matures then the late toxicity predictors observed in the LeND cohort may be assessed for validation purposes.

2.3.4 DNA damage assays

2.3.4.1 Comet Assay

Of the 136 REQUITE patient samples with results there was a mixture of tumour types. When the whole cohort was analysed together there was no association with the DNA damage observed during the comet assay and acute radiotherapy toxicity. However on univariate analysis in the breast cohort (n=70) it was observed that patients with higher values of relative rate of repair had more acute radiotherapy toxicity (p=0.004 percentage tail DNA; p = 0.008 Olive tail moment).

The comet assay was also performed on samples obtained from the LeND cohort for replication and to determine relationship to late toxicity. The acute toxicity data from the LeND cohort was collected by patient recall or from review of medical notes. These samples were taken at least eight years post radiotherapy and when the initial study

was set up the primary outcome was predictors of late radiotherapy toxicity. 80 samples were successfully analysed using the comet assay and it was observed on univariate analysis that patients with a higher relative rate of repair value had worse late toxicity (p=0.049). In addition the rate of repair was also faster in the patients with a higher bivariate STAT score (p=0.03).

The comet assay results from the REQUITE cohort were validated in the LeND cohort indicating it could be used as a possible predictive assay for detecting both acute (REQUITE) and late (LeND) radiotherapy toxicity. Although neither cohort was significant on multivariate analysis it may be that the sample size was too small with too few events to detect. Samples are stored on the rest of the REQUITE cohort so it would be possible to do future experiments and increase the sample size.

The exact pathophysiology for radiotherapy toxicity is not yet known, but there has been significant work examining post radiation levels of lymphocyte apoptosis measured using the RILA assay. Patients with a lower level of lymphocyte apoptosis 48 hours after ex vivo irradiation have increased risk of radiotherapy toxicity (Ozsahin et al. 2005). In this study it has been proposed that there could be an inverse relationship between DNA damage and levels of apoptosis. In the REQUITE cohort we see that this is the case and there is a correlation with patients who have a lower RILA score having a higher relative rate of repair value (p=0.003 PTD, p=0.008 OTM) as well as faster rate of repair (p=0.01 PTD). One possible explanation for these observations is that patients with a faster repair of DNA damage are better able to cope with radiation-induced damage therefore leading to less apoptosis.

Another possible mechanism is the interaction with the immune system. As depicted in figure 21 parts of the immune system promote inflammation in response to external stresses such as radiotherapy. Following inflammation there is impaired healing and poor vascularisation leading to fibrosis (Straub et al. 2015).

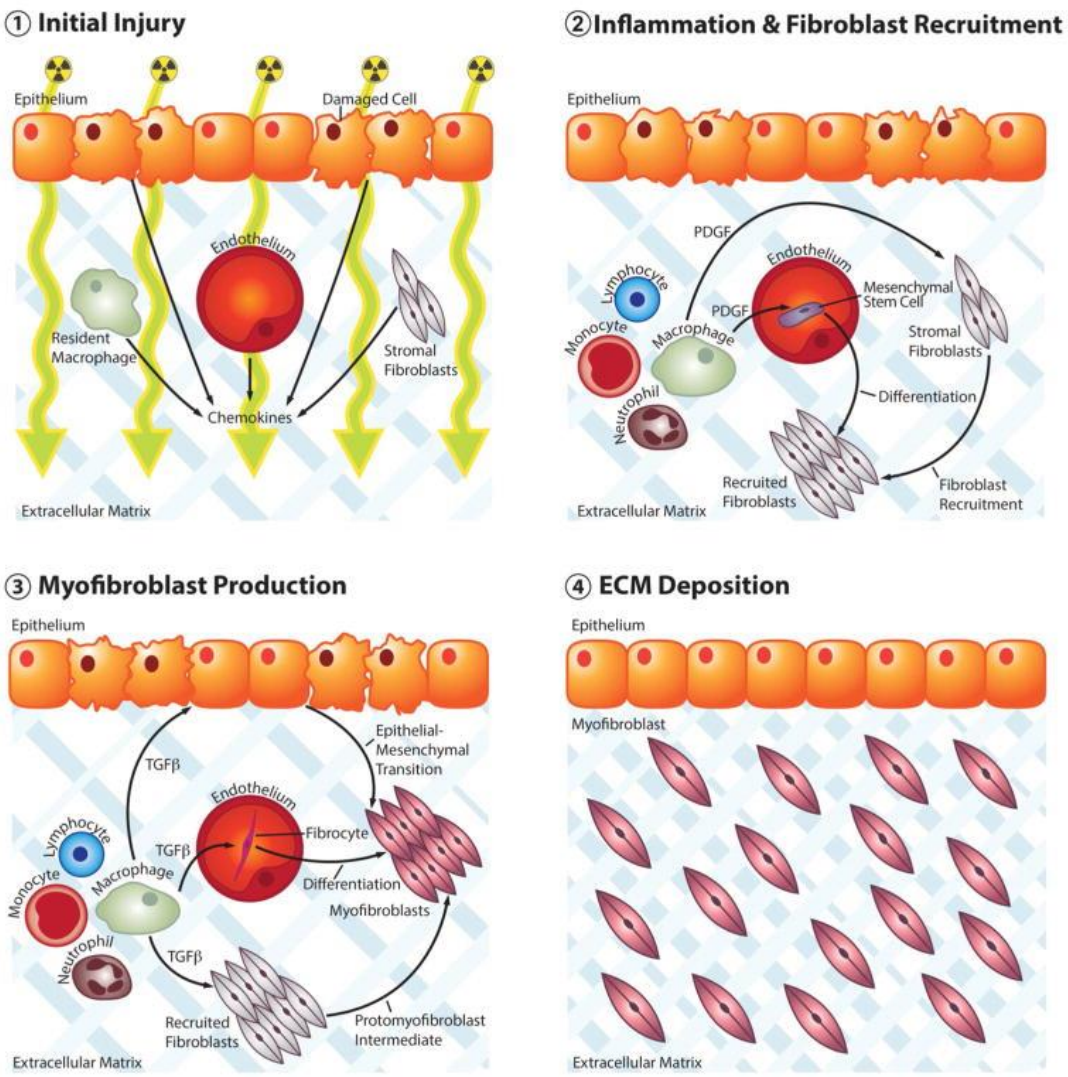

Figure 21: Pathogenic process involved in development of fibrosis and the interaction of parts of the immune system (Straub et al. 2015)

TGF-β is a leading cytokine implicated in this process (Anscher 2010, Rube et al. 2000). It is a pro-inflammatory cytokine and also inhibits Th1 and 2 which in turn leads to depletion in CD4 and CD8 lymphocytes (Banchereau et al. 2012). Low levels of CD8 apoptosis detected via the RILA therefore may be a marker of higher levels of TGF- β mediated inflammation and fibrosis.

IL-6 is another cytokine important in the inflammatory process. One of its known functions is inhibition of T cell apoptosis. It has been shown to promote DNA repair and reduces apoptotic death in lung cancer stem cells following radiation therapy (Chen et al. 2015). It has also been shown that early rises in IL-6 following radiation of

lung tissue are associated with higher levels of toxicity (Rube et al. 2000). It may be that the comet and RILA assays are therefore acting a surrogate marker for parts of the immune system which are inducing toxicity effects. Patients with faster rate of repair may repair DNA damage in genes responsible for secretion of pro-inflammatory anti-apoptotic cytokines like IL-6 and TGF-β.

In the current study the prospective REQUITE cohort had higher levels of residual DNA damage after 30 minutes when compared to the retrospective LeND cohort resulting also in significantly slower relative rate of repair in the REQUITE cohort. One possible explanation is inter-observer variability. The comet assay was performed and scored in the two cohorts by independent technicians and it would therefore be reasonable to assume that slight variations in technique could account for the current observations. However the baseline DNA damage and immediate DNA damage observed between the two cohorts do not vary significantly. A second possible explanation could be that the volunteers for the LeND cohort have previously undergone radiotherapy to the breast or chest wall unlike the REQUITE cohort in which the samples were all taken at the start or pre-radiotherapy. It is possible that radiotherapy has caused long term changes in DNA repair genes which could account for the differences. A further possible explanation is that previous radiotherapy kills cells with ineffective DNA repair mechanisms and there is clonal survival of those with more efficient repair mechanisms. The final theory is that the REQUITE participants immune system is in a different state due to the presence or recent presence of tumour cells, whereas the LeND volunteers are now (mostly) tumour free.

The comet assay results suggest there could be a possible link with DNA repair and acute and late toxicity however there were a number of faults with our methodology which mean that the results should be interpreted with caution. The comet assay was conducted by a number of different members of the research team and there was a lot of inter observer variation. There are automated systems available for both the scoring and running of samples which could reduce this confounding factor. A second issue is that although DNA repair was controlled for immediately following radiation by placing samples on ice, in the Xstrahl irradiator samples were irradiated off ice and as such could already start to repair for the 8 minutes the irradiator samples were in the

bunker. In future experiments this would need modifying to ensure that the 0 minutes and 0Gy samples remain on ice at all times.

2.3.4.2 γ-H2AX assay

In the current study we failed to detect any correlation between the γ -H2AX assay results and toxicity nor baseline comorbidities and demographics. Only 30 samples were available for analysis using the γ -H2AX assay and these were a mixture of breast, prostate and lung cancer patients. As such any meaningful analysis for toxicity would be unlikely. There was also no correlation with the results of the other two assays.

However in terms of developing a reproducible method and pilot study for detecting DNA double strand breaks it has been successful. Showing an appropriate rise and fall at three hours and 48 hours post irradiation respectively. One interesting observation that can be drawn is that some patient samples had a much higher percentage of residual DNA damage at 48 hours post irradiation than others which returned to baseline levels. A recent study (van Oorschot et al. 2016) using the γ -H2AX assay showed that patients with impaired homologous recombination had higher levels of DNA double strand breaks at 24 hours post irradiation compared with those with intact homologous recombination pathways. In the REQUITE study data will be available on the genomics of the patients the γ -H2AX assay was performed on. It would therefore be possible to look at SNPs in the DNA double strand break repair genes and how they correlate to the results obtained. Previous studies (Mumbrekar et al. 2014, van Oorschot et al. 2014) which have observed a correlation with γ -H2AX and radiotherapy toxicity have counted γ -H2AX foci with microscopes rather than using fluorescence methods. Counting foci gives quantitative results to analyse rather than fluorescence which only determines if γ -H2AX is present or not in a cell. A possible improvement to the current method would be to use the mean fluorescence from the FACS to determine a more quantitative result. This may allow a more meaningful analysis of a smaller sample size. Also as with the comet assay samples should remain on ice during irradiation to control for DNA repair inside the bunker.

2.3.4.3 RILA

The RILA assay was carried out on just over two thirds of the Leicester REQUITE cohort (66.8%). There was no correlation with acute toxicity in the cohort when assessed as a whole or on sub group analysis by tumour type and gender. The assay has never previously been shown to predict for acute radiotherapy only late toxicity so our findings would be in keeping with previous evidence. As previously discussed one possible theory is that the RILA is a surrogate assay for parts of the immune system including cytokines such as IL-6 and TNF-β which are ultimately involved in the pathology of late radiotherapy toxicity. Acute toxicity is more likely to relate to an inflammatory process and therefore correlated with different immune mechanisms and cytokines.

The RILA assay was carried on the patients included in this thesis using the predetermined method set out in the REQUITE protocol. As such no amendments were possible to the agreed protocol. Whilst I accept this method had been developed after years of preliminary testing there are a number of changes I would have liked to consider. The irradiation of samples off ice as already discussed may contribute to some variation in findings. Whilst the RILA is not measuring DNA repair it still could be that apoptosis levels may undergo a similar repair process off ice. A second area of concern is that blood samples were allowed to be collected both pre and up to 5 days into radiotherapy. This could clearly impact on any results seen. A sample of 10 unmatched cases was used to determine that the RILA results did not vary significantly up to 5 fractions in however this is a very small sample and as such may not be adequately powered to confirm this. It would have been a much more robust test if all blood samples for the RILA were collected before patients had received any radiotherapy.

In the current study there was an association on univariate and multivariate analysis with mean RILA score and gender with females had a significantly lower mean RILA score ($p= 0.02$ on multivariate analysis) and tumour type (breast cancer patients had a significantly ($p=0.001$) lower than prostate patients mean RILA score). One possible explanation for this is that the prostate patients had their blood samples taken at their planning CT visit and as such had not received any radiotherapy yet. Due to logistics

however this was not possible for the breast patients who instead had their samples collected once they had started radiotherapy. As discussed above it is quite reasonable to assume that apoptosis levels may vary once a patient has received any radiotherapy treatment and could account for the observed difference between the sexes.

There was also a univariate association between depression (p=0.01) and age (p=0.006). Patients with underlying depression have a lower mean RILA score (22.04 vs 17.46) and with increasing age mean RILA score also increases. The effect of age on radiation induced lymphocyte apoptosis has previously been noted (Radojcic and Crompton 2001) and is in keeping with our current findings. It has also been well documented with regards to the interaction between the immune system and depressive symptoms with suggestions that patients with a history of depression have a less effective immune systems (Herbert and Cohen 1993). The only study to date looking at markers of lymphocyte apoptosis in fact suggested that there was a relationship but in the opposite direction with patients who suffer with depression having a higher level of lymphocyte apoptosis. However their study only included a small number of patients (n=46) and used morphological features assessed via light microscopy to determine levels of apoptosis. The RILA is a more reliable technique for detecting lymphocyte apoptosis. The only other variable on multivariate analysis which was significantly associated with RILA score was smoking status (p=0.05). Those with a positive smoking history having a significantly lower mean RILA score. One study looking at the effect of smoking on lymphocyte apoptosis in COPD patients demonstrated that there were differences in levels of apoptosis assessed by flow cytometry between non-smokers, healthy smokers and smokers with COPD (Chen et al. 2016). As with our data they showed that cigarette smoke exposure inhibited apoptosis of T cells. They also noted that the number of regulatory T cells was increased in smokers. A future avenue of further work would therefore be to look at whether this is the mechanism by which smoking raises toxicity, and test if smokers with a high RILA score still have raised toxicity when compared to smokers with a low RILA score.

CHAPTER 3 : CIRCADIAN RHYTHM

3.1 METHOD

3.1.1 PATIENT SELECTION

3.1.1.1 LeND cohort (retrospective)

As previously described in chapter 2.

3.1.1.2 REQUITE cohort (prospective)

As previously described in chapter 2.

An ethics amendment was made allowing the Leicester breast and prostate cancer patients to be sent the same Munich chronotype questionnaire as the LeND cohort.

In order to do a combined analysis of acute toxicity in the breast and prostate cancer patients a STAT score was calculated as a measure of overall acute toxicity. This has been described in previous sections. Toxicity was then divided to create the bivariate STAT score with the top quartile taken to be the over-responders group in terms of acute toxicity.

3.1.2 Data Collection

3.1.2.1 Time of radiotherapy treatment

I reviewed the radiotherapy records for the patients treated at the Leicester Royal Infirmary to obtain time of treatment for every fraction. Patients receiving more than 66% of their radiotherapy before noon were classed as morning treatment; patients who received more than 66% after noon were classified as afternoon treatment; and those falling outside these criteria as a mixed group.

Season was classified using the astronomical calendar which is determined by the orbit of the Earth around the sun relative to the amount of light during a day (Met_Office 2014). The summer solstice (June 20th/21st) is the longest day in the Northern hemisphere and the Winter solstice (December 21st) is the shortest day. Spring was defined as months March 21st to June 20th; summer as June 21st to September 22nd;

autumn as September 23rd to December 20th; winter as December 21st to March 20th. For the purposes of this analysis seasons were grouped around the solstices with the darkest half of the year being 20th September to 20th of March and the lightest half of the year being 21st of March to the 19th of September.

3.1.3 Genotyping

3.1.3.1 DNA extraction

3.1.3.1.1 LeND cohort

For volunteers with study numbers between 150 to 633 DNA had previously been isolated from whole blood at the University of Leicester and stored at -80°C at stock concentration. A number of samples also had been diluted to 10ng/µl and loaded onto 96 well verti plates and frozen at -80^{0}C. Volunteers 1 to 149 had no stored DNA available for the current study.

3.1.3.1.2 REQUITE cohort

DNA was isolated by CIGMR from frozen EDTA blood. Blood volumes less than 1ml were deemed inadequate for quality control purposes by CIGMR. DNA extraction was performed using magnetic bead robotic extraction technique at their biobank in Manchester and then couriered on dry ice to the University of Leicester where it was stored at -80°C until ready to use. 100µl of 20ng/µl DNA was sent by CIGMR for each sample unless DNA quality was poor and then the closest to this concentration was provided instead.

3.1.3.2 DNA preparation

1µl of stock DNA was pipetted into a well on a 96 well plate. This was repeated for each volunteer's extracted DNA sample.

3.1.3.3 PER3 PCR

3.1.3.3.1 Selection and preparation of primers

Primers in the forward and reverse direction were designed using Primer3. The primers were then verified using UCSC In silico PCR online software to ensure they only amplified DNA in the region of interest. Lyophilised PER3 oligonucleotides were then purchased from Sigma as below:

PER3-F: 5'-CAAAATTTTATGACACTACCAGAATGGCTGAC

PER3-R: 5'- AACCTTGTACTTCCACATCAGTGCCTGG

Stock PER3 primers (100 µM) were made by adding 467 µl of autoclaved deionised water to the dried forward PER3 oligonucleotide and 673 µl of autoclaved deionised water to the reverse oligonucleotide. For the PCR reaction stock solutions were further diluted to 10 µM.

3.1.3.3.2 DNA amplification

DNA was amplified by Polymerase Chain reaction using a Veriti 96 well thermal cycler. 10 µl PCR solution was made by adding to the contents of table 35 to 1ul DNA in each well of a 96 well PCR plate. To establish the best annealing temperature a gradient was set up with 6 identical DNA samples run at different temperatures and the temperature with the best results (63°C) from this was selected (52- 68 °C).

Table 35: Contents of each well to make 10µl for PCR reaction

Reagent	*Volume*	*Concentration*
DNA	1 µl	10ng/µl (LeND)/ 20ng/µl (REQUITE)
dNTPs	1 µl	2mM
PER-3F	1 µl	10µM
PER-3R	1 µl	10µM
Buffer (Kappa B)	1 µl	X10
Taq	0.1 µl	5U/ µl
Water	4.9 µl	
Total	10 µl	

Following an initial four minute denaturing step at 95°C 30 cycles of: 30 seconds denaturing at 95°C, 30 seconds annealing at 63°C and 30 seconds elongation at 72°C took place. Followed by a final extension phase of two minutes at 72°C then cooling for 30 seconds at 4°C.

3.1.3.3.3 Gel Electrophoresis

2% agarose was made by adding two grams of agarose to 100ml of Tris Borate EDTA (TBE). To make the agarose plate the agarose mix was heated until melting point then 1µl of 10mg/ml Ethidium bromide added for every 100 ml of agarose mix. After being allowed to cool slightly the mixture was poured into a gel plate, a comb placed near the top and just below half way down, then the gel was left to set.

Whilst the agarose was setting 2µl of blue dye was added to each of the wells containing the amplified DNA. Once set the comb was removed from the gel plate and the samples loaded into the gel. In the centre well on the gel plate a DNA ladder was placed for reference. The gel was then placed in an electrophoresis tank set at 5 V/cm and ran for 90 minutes. Following this the results were viewed using a UV transilluminator and camera.

3.1.3.3.4 Genotyping of PER3 VNTR

In each column of the gel the number and position of the bands were counted. Figure 22 shows a gel for PER3 VNTR.

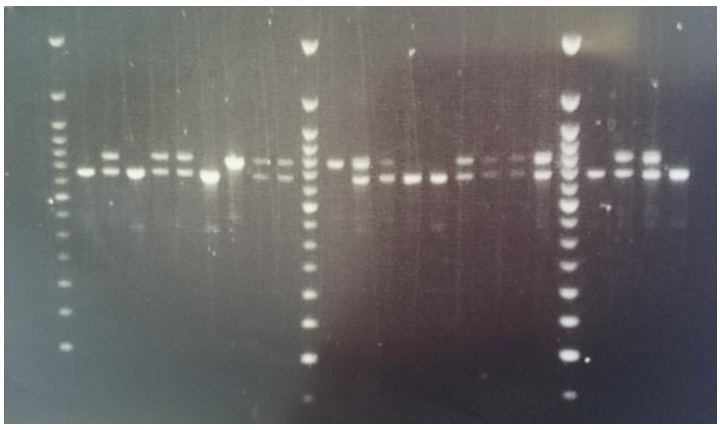

Figure 22: PER3 VNTR PCR example plate

A single band at 639bp represents a 4/4 genotype, a double band heterozygote at 639 bp and 685 bp represents a 4/5heterozygote and a single homozygote band at 685 bp represents a 5/5 genotype. The genotyping was verified by a second scorer (Dr Chris Talbot) and any gels with discrepancy were repeated.

3.1.3.4 NOC rs13116075 PCR (LeND cohort)

This lab work was undertaken by Anna Critchley a third year Genetics undergraduate student.

3.1.3.4.1 Selection and preparation of primers

Primers designed and prepared as above using the following sequence:

NOC-F	GTCCCCATCTTGACCTCTTG
NOC-R	GGATGTCCTTAACCTTGGCA

3.1.3.4.2 DNA amplification

DNA was amplified as above for PER3. 61°C was selected as the annealing temperature.

3.1.3.4.3 Restriction Enzyme Step

The amplified DNA was then left to run overnight at 37°C in the Veriti 96 well thermal cycler with restriction enzyme solution (table 36).

Table 36: Content of each well for restriction enzyme step of PCR for NOC rs13116075

Reagent	Volume
Amplified DNA	5 µl
Xba I 10U/ µl	1 µl
Cut Smart Buffer	1.5 µl
Deionised Autoclaved Water	12.5 µl
Total	20 µl

The correct restriction enzyme was selected to ensure that it cut the genomic sample at the correct site and fragment size for the NOC SNP of interest (NOC rs13116075).

3.1.3.4.4 Gel Electrophoresis

Electrophoresis performed as above with 3% agarose, 90 mins at 5V/cm.

3.1.3.4.5 Genotyping of NOC rs13116075

In each column of the gel the number and position of the bands was counted. Figure 23 shows a gel for NOC SNP rs13116075.

Figure 23: Photograph of a gel electrophoresis plate for NOC rs 13116075 taken from Anna Critchley's work.

A single band at the 161bp represents GG homozygote, a triple band at 46 bp, 115 bp and 161 b is AG heterozygote and a double band at 46 bp and 115 bp is AA homozygote. The genotyping was verified by a second scorer (Dr Chris Talbot) and any gels with discrepancy were repeated.

3.1.3.5 NOC rs13116075 and CLOCK rs1801260 genotyping (REQUITE cohort)

96 well plates containing 1µl of 20ng/µl DNA were transferred to the Genomics Core facility at the University of Leicester and quantitative PCR was performed using

Taqman assays. Primers had previously been obtained for SNPs CLOCK rs1801260 and NOC rs13116075. Figure 24 shows the plots for each SNP.

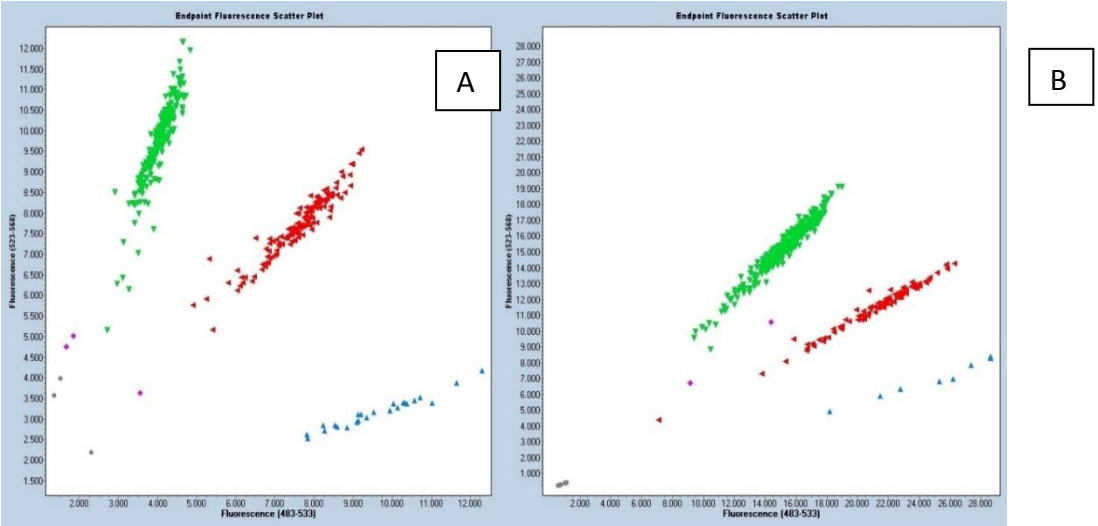

Figure 24: Taqman plots for CLOCK rs1801260 (A): Green dots are CLOCK AA homozygotes, red dots CLOCK AG heterozygotes and blue dots CLOCK GG homozygotes. Graph B shows NOC rs13116075: Green dots are NOC AA homozygotes, red NOC AG heterozygotes and blue NOC GG homozygotes.

3.1.4 Chronotype questionnaire

The Munich Chronotype questionnaire (see appendix 3) was developed by researchers at the Ludwig-Maximilians University (LMU) Munchen. It assesses a person's chronotype by asking questions about sleep – wake patterns on work days and rest days. Factors which can influence chronotype include age, sex and exposure to daylight. This data was collected on our cohorts to allow correction for these confounders during analysis. Study participants who worked shift patterns (outside of the hours 07:00 – 20:00) were excluded from the analysis.

3.1.4.1 LeND cohort

A new ethics application was approved allowing collection of further information from the surviving original LeND cohort (15/LO/0866). Once this was approved NHS records were reviewed for the Leicester LeND volunteers to obtain up-to-date addresses and survival status on the original participants. Surviving volunteers were then posted a patient information sheet and details of a patient education event. Those wishing to attend the event were informed they could complete a copy of the Munich

Chronotype questionnaire on the evening and return either then or post back at a later date. Those not able to attend but wishing to partake in the new study were asked to contact the research team who posted a copy of the questionnaire and consent form with a return envelope. A second postal invite was sent three months later to increase numbers. Information from the questionnaires was input into the online algorithm system provided by the Ludwig-Maximilians University (LMU) Munchen to generate study participants' chronotype.

3.1.4.2 REQUITE cohort

A new ethics application (REC number 14/NW/0035) was approved for a protocol substantial amendment allowing collection of further information from REQUITE study participants. Once ethics was approved survival status and study continuation status was confirmed then breast (n=350) and prostate (n=250) patients were sent the Munich Chronotype questionnaire in the post. If they agreed to take part in this sub-study they were asked to sign a separate consent form and return with the questionnaire in a pre-paid envelope. Information from the questionnaires was then processed as per the LeND cohort.

3.1.5 Predictive assays

At time of consent REQUITE volunteers provided a 30 ml peripheral blood sample. 10mls of this was taken in a Lithium Heparin tube which was subsequently used for DNA damage assays and lymphocyte assays (as described in an earlier section). Blood sampling was performed by a qualified member of the research team using a vacutainer system and aseptic technique. Time of blood sampling ranged between 08:00 and 18:00.

The LeND follow up study patients (n=106) were asked to attend and provide a fresh 10ml peripheral blood sample. Blood sampling was performed as per the REQUITE cohort. Time of blood sampling was controlled and study volunteers were asked to attend between 09:00 and 10:30 on a Monday to the HOPE clinical trials unit.

3.1.5.1 Comet Assay

The comet assay was performed with help from students working in G10 lab (Maxime Boy, Hannah Dobbelare, Eva Saiti and Kosalie Redman) using the previously described protocol.

3.1.5.2 RILA

The RILA was performed on the REQUITE and LeND cohort using the protocol described previously.

3.1.5.3 Lymphocyte Subpopulations

Blood from the LeND cohort was examined for lymphocyte subpopulations. This work was carried out by medical student Sam Leavers under the supervision of Dr Chris Talbot using a protocol adapted from the RILA assay. In brief flow cytometry was used to detect antibodies for lymphocyte subpopulations in lysed unirradiated blood. Table 37 shows the antibodies which were selected and the lymphocyte subpopulation they were intended to detect.

Table 37: Antibodies added to detect lymphocyte subpopulations

Antibody	Lymphocyte Subpopulation
CD3	T cells
CD4	T helper cells
CD8	Cytotoxic T cells
CD45RA	Memory cells
CCR7	Effector cells
CD28	T cell survival/activation
CD27	Long term T cell immunity

Appropriate lymphocyte gating was selected to detect these antibodies. An approximate lymphocyte population was first selected using forward and side scatter

then CD3 positive cells selected from this. From the CD3 positive cells CD4 and CD8 subpopulations were identified. Both of these subgroups were then examined for the presence and absence of CD45RA, CCR7, CD28 and CD27.

3.1.6 Statistical Analysis

IBM SPSS version 24.0 was used for statistical analysis. P values <0.05 were taken to be significant. All samples were tested for normality then the appropriate statistical tests performed. Difference in group proportions was tested using chi squared, difference in mean for continuous variables was assessed using Kruskal- Wallis or Mann Whitney U. Spearman's rank was used to test for correlation to continuous variables and liner regression was performed for multivariate analysis using the model descried in chapter 2.

3.2 RESULTS

3.2.1 Time of radiotherapy treatment

3.2.1.1 LeND cohort

Of the 664 patients included in the original study time of radiotherapy treatment was available on 536 patients. Of the remaining 128 patients 77 were recruited at sites other than Leicester and 51 received radiotherapy before digital records began (pre-1998). Patients were then divided into three treatment groups as described previously. 185 received treatment in the morning, 170 in the afternoon and 181 a mix of morning and afternoon treatments.

Based on these treatment times a chi squared test for equal distribution of radiotherapy toxicity between the groups was performed. For acute toxicity presence of moderate or severe reactions at any time during and immediately after radiotherapy was taken as a significant event. No significant difference was observed between the three treatment groups (p = 0.33) or if the mixed group is removed from the analysis (p = 0.19). Table 38 shows the distributions of the three groups.

Table 38: LeND cohort acute toxicity split by treatment times

Radiotherapy Treatment Time	Low acute Toxicity	Moderate/severe acute Toxicity	Total
Morning	168 (90.2%)	18 (9.8%)	184
Afternoon	144 (85.7%)	24 (14.3%)	168
Mixed	155 (85.6%)	26 (14.4%)	181
Total	465	68	533

On binary logistic regression analysis presence of moderate or severe acute toxicity was not significantly affected by time of treatment (p=0.11).

For late toxicity groups were split based on treatment time and presence or absence of late toxicity determined by bivariate STAT score. Table 39 shows the split of the groups. On univariate analysis using a chi squared test there was a significant

difference with a p value of 0.03. Removal of the mixed group reduces significance (p = 0.08).

Table 39: LeND cohort late toxicity split by treatment times

Treatment group	Bivariate STAT score (75% lowest)	Bivariate STAT score 25% highest	Total
Morning	131 (31.6%)	54 (44.3%)	185
Afternoon	134 (32.4%)	36 (29.5%)	170
Mixed	149 (36.0%)	32 (26.2%)	181
Total	414	122	536

Known variables for late toxicity were reviewed for relationship to radiotherapy treatment time to determine if a confounder could be causing the observed effect. Significantly more patients (p=0.04) received a boost in the morning compared to the afternoon group. To account for this boost was multiplied by radiotherapy treatment time and this was input into the model. Multivariate analysis was performed using binary logistic regression and radiotherapy treatment time remained significant (p=0.01). Table 40 summarises this information.

Table 40: Multivariate analysis (LeND cohort) for effect on late toxicity (Bivariate STAT score)

Variable	Sig.	Exp (B)	95% C.I. for Exp (B)	
			Lower	Upper
Age	0.79	1.00	0.97	1.03
Season	0.55	1.17	0.45	1.54
RT Dose	0.02	0.05	0.00	0.60
Boost	0.58	2.32	0.12	45.52
BED	0.02	944.95	3.10	288157.55
Hormones	0.32	1.57	0.64	3.81
Chemo	0.81	1.09	0.54	2.21
Diabetes	0.27	0.50	0.15	1.72
Smoker	0.51	0.86	0.54	1.35
Cup Size	$1.25x10^{-7}$	1.30	1.18	1.44
Radiotherapy Treatment time	0.01	0.58	0.38	0.89
Boost x Time	0.005	12.19	2.14	69.27

3.2.1.2 REQUITE cohort

343 breast patients and 216 prostate cancer patients were included in the analysis. Treatment times were divided as previously into morning; afternoon and mixed treatment time groups. Figure 25 shows the distribution of treatment times by cancer site.

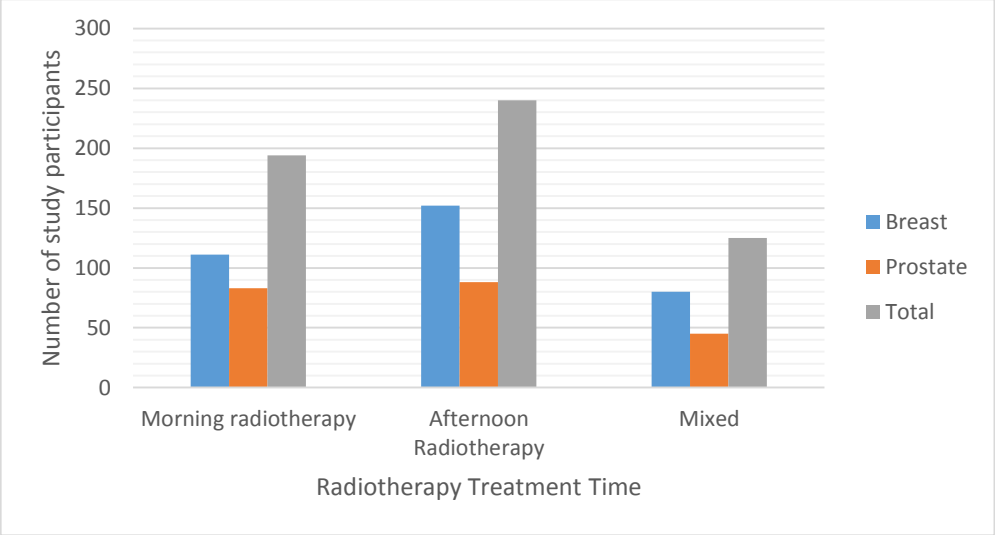

Figure 25: Proportion of study volunteers split by radiotherapy treatment time (REQUITE cohort)

Based on these treatment times a chi squared test for equal distribution of radiotherapy toxicity was performed. No significant difference was observed between the three treatment time groups (p = 0.98) for acute toxicity nor if the mixed group is removed from the analysis (p = 0.87). Table 41 shows the distributions of the three groups.

Table 41: Acute toxicity distribution split by treatment time (REQUITE cohort)

Bivariate STAT score	Morning radiotherapy	Afternoon radiotherapy	Mixed treatment times	TOTAL
Low toxicity	138 (74.6%)	167 (73.9%)	85 (73.3%)	390
High Toxicity	47 (25.4%)	59 (26.1%)	31 (26.7%)	137
TOTAL	185	226	116	527

On subgroup analysis of separate tumour sites. Breast erythema was scored within the last three days of radiotherapy, a score of grade two or above was taken as an indicator of moderate to severe acute toxicity. Table 42 shows the distribution of breast erythema by radiotherapy treatment time.

Table 42: Acute breast erythema distribution split by treatment time (REQUITE cohort)

Breast Erythema Score	Morning Radiotherapy (more than two thirds pre 12:00)	Afternoon radiotherapy (more than two thirds after 12:00)	Mixed treatment times	Total
Grade 0 or 1	81 (76.4%)	130 (89.0%)	64 (81.0%)	275
Grade 2 or above	25 (23.6%)	16 (11.0%)	15 (19.0%)	56
TOTAL	106	146	79	331

Based on these treatment times a chi squared test for equal distribution of radiotherapy toxicity was performed. A significant difference was observed between the three treatment time groups ($p = 0.03$) with increased toxicity observed in patients treated in the morning. If the mixed group is removed from analysis and afternoon and morning groups are compared alone there is again a significant difference ($p = 7x10^{-3}$).

As with the LeND cohort significantly more patients (p=0.002) received a boost in the morning treatment group (n=12) versus the afternoon group (n=3) so as previously radiotherapy treatment time was multiplied by boost and input into the multivariate analysis model.

On multivariate analysis using binary logistic regression treatment time was no longer significant (p=0.22) (see table 43).

Table 43: Multivariate analysis REQUITE breast cohort with addition to radiotherapy time, season and boost x RT time to model.

Variable	Sig.	Exp (B)	95% C.I. for Exp (B)	
			Lower	Upper
BMI	0.01	1.06	1.01	1.12
Smoker	0.11	1.49	0.92	2.41
Diabetes	0.52	1.53	0.41	5.68
Age	0.96	1.00	0.96	1.04
Bra cup size	$3x10^{-4}$	1.71	1.28	2.30
Depression	0.01	0.05	0.00	0.48
Chemotherapy use	1.00	0.00	0.00	
Adjuvant endocrine therapy	0.19	0.54	0.21	1.37
Boost to tumour bed	0.80	1.41	0.09	21.13
Boost x RT time	0.25	4.98	0.33	75.01
Season	0.58	1.28	0.53	3.05
Radiotherapy Treatment time	0.22	0.72	0.42	1.22
BED	$6x10^{-5}$	1.44	1.23	1.68

For prostate patients grade two or more diarrhoea within the last week of radiotherapy was taken as a marker of acute toxicity. Table 44 shows the distribution of toxicity by treatment time.

Table 44: Acute diarrhoea distribution split by treatment time (REQUITE prostate cohort)

Acute diarrhoea score	Morning Radiotherapy (more than two thirds pre 12:00)	Afternoon radiotherapy (more than two thirds after 12:00)	Mixed treatment times	Total
Grade 0 or 1	77 (96.2%)	74 (85.1%)	41 (91.1%)	192
Grade 2 or above	3 (3.8%)	13 (14.9%)	4 (8.9%)	20
TOTAL	80	87	45	212

Based on these treatment times a chi squared test for equal distribution of radiotherapy toxicity was performed. A significant difference was observed between the three treatment time groups (p = 0.05) with increased toxicity observed in patients

treated in the afternoon. If the mixed group is removed and afternoon and morning groups are compared again there is a significant difference (p = 0.01). On multivariate analysis using binary logistic regression treatment time was no longer significant (p=0.15).

3.2.2 Season

3.2.2.1 Effects in the LeND cohort

Table 45 summarises the division of toxicity by season.

Table 45: LeND cohort acute and late toxicity split by season

		Moderate/Severe Acute toxicity		Late Toxicity (Bivariate STAT score)	
Season	Time of Radiotherapy	No	Yes	75% lowest	25% highest
Dark	*Morning*	74 (93.7%)	5 (6.3%)	56 (70%)	24 (30%)
	Afternoon	55 (78.6%)	15 (21.4%)	57 (79.2%)	15 (20.8%)
	Mixed	82 (89.1%)	10 (10.9%)	72 (78.6%)	20 (21.4%)
Light	*Morning*	91 (89.2%)	11 (10.8%)	73 (71.6%)	29 (28.4%)
	Afternoon	86 (90.5%)	9 (9.5%)	74 (77.9%)	21 (22.1%)
	Mixed	71 (83.5%)	14 (16.5%)	74 (87.1%)	11 (12.9%)
Total		459	64	406	120

When examined as a whole cohort acute toxicity did not vary by season (acute: p= 0.92; late: p=0.91). However, by splitting the group into time they received radiotherapy patients treated in the afternoon during the darker months had significantly greater acute toxicity than those treated in the morning during the darker months (p = 0.007); this effect was not present during the lighter months (p=0.76). Late toxicity did not vary by season alone or when taking treatment time into account (dark: morning vs afternoon p=0.20; light: morning vs afternoon p=0.31).

3.2.2.2 Seasonal effects in REQUITE cohort

Table 46 summarises the division of acute toxicity (bivariate STAT score) by season in combined breast and prostate patients.

Table 46: REQUITE cohort acute toxicity split by season

Season	Acute Toxicity (Bivariate STAT score) n=		TOTAL
	75% lowest	*25% highest*	
Dark	203 (72.8%)	76 (27.2%)	279
Light	186 (74.4%)	64 (25.6%)	250
TOTAL	276	56	529

A chi squared test for significant difference between the groups did not demonstrate radiotherapy toxicity varied by season (p= 0.67). There was no significant effect observed by time of treatment in either the lighter or darker months. The distribution of these groups is shown in table 47.

Table 47: REQUITE cohort acute toxicity divided by season and radiotherapy treatment time.

Season	Radiotherapy treatment time	Acute Combined Toxicity (Bivariate STAT score) n=		TOTAL	P value
		75% lowest	*25% highest*		
Light	*Morning*	65 (74.7%)	22 (25.3%)	87	$0.47^{a}/0.78^{b}$
	Afternoon	75 (72.1%)	29 (27.9%)	104	
	Mixed	43 (81.1%)	10 (18.9%)	53	
Dark	*Morning*	70 (73.7%)	25 (16.3%)	95	$0.39^{a}/0.88^{b}$
	Afternoon	90 (75.0%)	30 (25.0%)	120	
	Mixed	40 (65.6%)	21 (34.4%)	61	
TOTAL		383	137	520	

a Includes mixed treatment time group in analysis; b excludes mixed treatment time group from analysis

On subgroup analysis split by tumour site table 48 summarises the division of acute breast toxicity by season.

Table 48: REQUITE breast cohort acute erythema divided by season

Season	Acute Erythema n=		TOTAL
	Grade 0/1	*Grade 2 or above*	
Dark	142 (81.6%)	32 (18.4%)	174
Light	134 (84.8%)	24 (15.2%)	158
TOTAL	276	56	332

A chi squared test for significant difference between the groups did not demonstrate radiotherapy toxicity varied by season (p= 0.44). During the darker months treatment in the morning was associated with significantly greater toxicity than those treated in the afternoon ($p = 0.03^{a}/9x10^{-3\ b}$); this effect was not observed during the lighter months ($p = 0.18^{a}/0.25^{b}$). The distribution of these groups is shown in table 49.

Table 49: REQUITE breast cohort acute erythema divided by season and radiotherapy treatment time

Season	Radiotherapy treatment time	Acute Erythema		TOTAL	P value
		Grade 0/1	*Grade 2 or above*		
Light	*Mixed*	26 (76.5%)	8 (23.5%)	34	
	Morning	44 (83.0%)	9 (17.0%)	53	$0.18^{a}/0.25^{b}$
	Afternoon	63 (90.0%)	7 (10.0%)	70	
Dark	*Mixed*	37 (84.1%)	7 (15.9%)	44	
	Morning	36 (69.2%)	16 (30.8%)	52	$0.03^{a}/9x10^{-3\ b}$
	Afternoon	66 (88.0%)	9 (22.0%)	75	
TOTAL		272	56	328	

a Includes mixed treatment time group in analysis; b excludes mixed treatment time group from analysis

Table 50 summarises the division of acute prostate toxicity by season.

Table 50: Acute prostate toxicity on REQUITE cohort split by season

Season	Acute Diarrhoea		TOTAL
	Grade 0/1	*Grade 2 or above*	
Dark	101 (91.8%)	10 (8.1%)	111
Light	96 (89.7%)	11 (10.3%)	107
TOTAL	197	21	218

In the prostate cohort a chi squared test for significant difference between the groups did not demonstrate radiotherapy toxicity varied by season (p= 0.75). The effect of afternoon treatment causing increased rates of acute diarrhoea was only observed in the lighter months when the mixed group was excluded (p= 0.10 [a] /0.04 [b]), in the darker months time of day had no effect on toxicity (p= 0.39 [a] /0.17 [b]). This is shown is table 51.

Table 51: REQUITE prostate cohort acute diarrhoea divided by season and radiotherapy treatment time

Season	Radiotherapy treatment time	Acute Diarrhoea		TOTAL	p value
		Grade 0/1	*Grade 2 or above*		
Light	*Morning*	35 (97.2%)	1 (2.8%)	36	
	Afternoon	33 (82.5%)	7 (17.5%)	40	0.10[a]/0.04[b]
	Mixed	22 (91.6%)	2 (8.4%)	24	
Dark	*Morning*	42 (95.5%)	2 (4.5%)	44	
	Afternoon	41 (89.1%)	6 (10.9%)	47	0.39[a]/0.17[b]
	Mixed	18 (90.0%)	2 (10.0%)	20	
TOTAL		191	20	211	

a Includes mixed treatment time group in analysis; b excludes mixed treatment time group from analysis

3.2.3 Circadian rhythm genes and toxicity

3.2.3.1 PER 3

3.2.3.1.1 LeND cohort

Of the 664 LeND patients DNA was available for genotyping on 508 patients. Of these 508 patients genotyping results obtained via PCR for PER3 VNTR was successful in 476 patients, in the remaining 32 samples there was inadequate DNA to produce reliable

results. Genotyping results were tested and found to be in Hardy Weinberg Equilibrium. Table 52 shows the distribution of radiotherapy toxicity by PER 3 genotype.

Table 52: Acute and late toxicity split by PER3 genotyping results in LeND cohort

		Acute Toxicity n=		p value	Late Toxicity (Bivariate STAT score) n=		p value
		No/Low	*Moderate/ Severe*		*75% lowest*	*25% highest*	
PER3 VNTR	*4/4*	188 (83.6%)	37 (16.4%)	p=0.43	170 (75.6%)	55 (24.4%)	p=0.89
	4/5	168 (88.0%)	23 (12.0%)		146 (76.4%)	45 (13.6%)	
	5/5	52 (86.7%)	8 (13.3%)		44 (73.3%)	16 (16.7%)	
TOTAL		408	68		360	116	

There was no significant relationship between PER3 VNTR and acute or late toxicity (Bivariate STAT score).

Taking time of radiotherapy treatment into consideration shows a significant effect of PER3 VNTR (table 53) on late toxicity with 4/4 PER3 VNTR being associated with increased toxicity if treated in the mornings ($p=6.0x10^{-3}$). No significant relationship was observed between acute toxicity and PER3 VNTR by treatment time.

Table 53: Acute and late toxicity split by PER3 genotyping and radiotherapy treatment time in LeND cohort

		Radiotherapy treatment time	Acute Toxicity n=		p value	Late Toxicity (Bivariate STAT score) n=		p value
			No/ Low	*Moderate/ Severe*		*75% lowest*	*25% highest*	
PER3 VNTR	*4/4*	*Morning*	52 (82.5%)	11 (17.5%)	0.70	43 (68.3%)	20 (31.7%)	$6x10^{-3}$
		Afternoon	53 (86.9%)	9 (13.1%)		49 (79.0%)	13 (21.0%)	
		Mixed	47 (80.0%)	12 (20.0%)		54 (91.5%)	5 (8.5%)	
	4/5	*Morning*	45 (93.7%)	3 (6.3%)	0.26	37 (77.1%)	11 (22.9%)	0.49
		Afternoon	50 (83.3%)	10 (16.7%)		51 (85.0%)	9 (15.0%)	
		Mixed	51 (87.9%)	7 (12.1%)		45 (77.6%)	13 (22.4%)	
	5/5	*Morning*	13 (86.7%)	2 (13.3%)	0.89	11 (73.3%)	4 (26.7%)	0.75
		Afternoon	16 (88.9%)	2 (11.1%)		12 (66.7%)	6 (33.3%)	
		Mixed	15 (83.3%)	3 (16.7%)		14 (77.8%)	4 (22.2%)	
TOTAL			342	59		316	85	

3.2.3.1.2 REQUITE cohort

Of the 600 breast and prostate patients recruited in Leicester DNA was available for genotyping on 539 patients (323 breast and 216 prostate). Of these samples genotyping results obtained via PCR for PER3 VNTR was successful in 525 patients, for the remaining thirteen samples PCR failed to produce reliable results on repeat analysis. Genotyping results were tested and found to be in Hardy Weinberg Equilibrium. Table 54 shows the distribution of radiotherapy toxicity by PER 3 VNTR.

Table 54: Acute toxicity split by PER3 genotyping results in REQUITE cohort

		Acute Toxicity (Bivariate STAT score) n=		p value
		75% lowest	*25% highest*	
PER3 VNTR	*4/4*	169 (78.2%)	47 (21.8%)	p=0.14
	4/5	150 (71.1%)	61 (28.9%)	
	5/5	36 (67.9%)	17 (32.1%)	
TOTAL		355	125	

There was no significant relationship with PER3 VNTR and acute toxicity (Bivariate STAT score). Taking time of radiotherapy treatment into consideration showed no significant effect of PER3 (table 55) on acute toxicity with any of the genotypes.

Table 55: Acute toxicity split by PER3 genotyping and radiotherapy treatment time in REQUITE cohort

Breast AND Prostate Patients (REQUITE)		Radiotherapy treatment time	Acute (Bivariate STAT score) n=		p value
			75% lowest	*25% highest*	
PER3 VNTR	*4/4*	*Morning*	53 (76.8%)	16 (23.3%)	0.26
		Afternoon	71 (74.7%)	24 (25.3%)	
		Mixed	44 (86.3%)	7 (13.7%)	
	4/5	*Morning*	58 (77.3%)	17 (22.7%)	0.06
		Afternoon	67 (72.8%)	25 (27.2%)	
		Mixed	24 (57.1%)	18 (42.9%)	
	5/5	*Morning*	16 (69.6%)	7 (30.4%)	0.85
		Afternoon	12 (70.6%)	5 (29.4%)	
		Mixed	8 (61.5%)	5 (38.5%)	
		TOTAL	353	124	

On subgroup analysis for breast patients acute toxicity score was only available on 294 of the 331 patients with PER3 VNTR genotyping results. PER3 VNTR alone did not

significantly correlate with acute toxicity using acute erythema as a surrogate marker for acute toxicity. Table 56 shows the distribution of toxicity by genotype.

Table 56: Acute breast erythema split by PER3 genotyping results in REQUITE breast cohort

Breast Patients only (REQUITE)		Acute Erythema n=		p value
		Grade 0/1	*Grade 2 or more*	
PER3 VNTR	*4/4*	112 (84.8%)	20 (15.2%)	0.79
	4/5	107 (81.7%)	24 (18.3%)	
	5/5	26 (83.9%)	5 (16.1%)	
	TOTAL	245	49	

Taking time of radiotherapy treatment into consideration showed no significant effect of PER3 VNTR (table 57) on acute toxicity with any of the genotypes, however if the mixed treatment time group is excluded from analysis patients with 4/5 PER3 VNTR have increased acute toxicity in the mornings (p=0.02)

Table 57: Acute breast erythema split by PER3 genotyping and radiotherapy treatment time in REQUITE breast cohort

Breast Patients only (REQUITE)		Radiotherapy treatment time	Acute Erythema n=		p value
			Grade 0/1	*Grade 2 or more*	
PER3 VNTR	*4/4*	*Morning*	31 (81.6%)	7 (18.4%)	0.71[a]/0.71[b]
		Afternoon	49 (84.5%)	9 (15.5%)	
		Mixed	31 (88.6%)	4 (11.4%)	
	4/5	*Morning*	32 (72.7%)	12 (27.3%)	0.07[a]/0.02[b]
		Afternoon	53 (89.8%)	6 (10.2%)	
		Mixed	20 (76.9%)	6 (23.1%)	
	5/5	*Morning*	9 (81.8%)	2 (18.2%)	0.19 [a]/0.16 [b]
		Afternoon	10 (100%)	0	
		Mixed	7 (70.0%)	3 (30.0%)	
		TOTAL	242	49	

a chi squared test for significance including all treatment times, b chi squared test for significance excluding mixed treatment times group.

On subgroup analysis of the prostate patients, acute toxicity score was available on 200 of the 216 patients with genotyping data available. Diarrhoea assessed within the last 5 fractions of radiotherapy was taken as a surrogate marker for overall acute toxicity. PER3 VNTR alone did not significantly correlate with acute diarrhoea. Table 58 shows the distribution of toxicity by genotype.

Table 58: Acute diarrhoea split by PER3 genotyping results in REQUITE prostate cohort

Prostate Patients only (REQUITE)		Acute Diarrhoea (n=)		p value
		Grade 0/1	*Grade 2 or more*	
PER3 VNTR	*4/4*	80 (89.9%)	9 (10.1%)	0.43
	4/5	83 (93.3%)	6 (6.7%)	
	5/5	18 (81.8%)	4 (18.2%)	
	TOTAL	181	19	

Taking time of radiotherapy treatment into consideration showed no significant effect of PER3 VNTR (table 59) on acute toxicity with any of the genotypes.

Table 59: Acute diarrhoea split by PER3 genotyping and radiotherapy treatment time in REQUITE prostate cohort

Prostate Patients only (REQUITE)		Radiotherapy treatment time	Acute diarrhoea n=		p value
			Grade 0/1	*Grade 2 or more*	
PER3 VNTR	*4/4*	*Morning*	31 (91.2%)	1 (8.8%)	$0.22^{a}/0.08^{b}$
		Afternoon	32 (84.2%)	6 (15.8%)	
		Mixed	17 (89.5%)	2 (10.5%)	
	4/5	*Morning*	30 (96.8%)	1 (3.2%)	$0.42^{a}/0.23^{b}$
		Afternoon	33 (89.2%)	4 (10.8%)	
		Mixed	20 (95.2%)	1 (4.8%)	
	5/5	*Morning*	11 (91.7%)	1 (8.3%)	$0.12^{a}/0.08^{b}$
		Afternoon	4 (57.1%)	3 (43.9%)	
		Mixed	3 (100%)	0	
		TOTAL	181	19	

a chi squared test for significance including all treatment times, b chi squared test for significance excluding mixed treatment times.

3.2.3.2 NOC rs13116075

3.2.3.2.1 LeND cohort

Of the 664 patients DNA was available for genotyping on 508 patients. Of these 508 patients genotyping results obtained via PCR for NOC rs13116075 was successful in 466 patients, in the remaining 42 samples there was inadequate DNA to produce reliable results. Genotyping results were tested and found to be in Hardy Weinberg Equilibrium. Table 60 shows the distribution of radiotherapy toxicity by NOC rs13116075 genotype.

Table 60: Acute and late toxicity split by NOC rs13116075 genotyping results in LeND cohort

		Acute Toxicity n=		p value	Late Toxicity (Bivariate STAT score) n=		p value
		No/Low	*Moderate/ Severe*		*75% lowest*	*25% highest*	
NOC rs13116075	*AA*	278 (87.7%)	39 (22.3%)	0.15	243 (76.7%)	74 (23.3%)	0.37
	AG	114 (87.0%)	27 (13.0%)		101 (71.6%)	40 (28.4%)	
	GG	7 (87.5%)	1 (12.5%)		5 (62.5%)	3 (37.5%)	
TOTAL		399	67		349	117	

There was no significant relationship with NOC rs13116075 and acute or late toxicity (Bivariate STAT score).

Taking time of radiotherapy treatment into consideration shows a significant effect of NOC rs13116075 (table 61) on late toxicity. Patients with AA NOC rs13116075 genotype have increased toxicity if treated in the mornings ($p=5.0 \times 10^{-3}$) but no significant relationship was observed with acute toxicity and NOC rs13116075 by treatment time.

Table 61: Acute and late toxicity split by NOC rs13116075 genotyping and radiotherapy treatment time in LeND cohort

	Treatment	Acute	p	Late (Bivariate STAT	p

		time	n=		value	score) n=		value
			No/ Low	*Mod/ Severe*		*75% lowest*	*25% highest*	
	AA	*Morning*	79 (87.8%)	11 (12.2%)		61 (76.7%)	29 (23.3%)	
		Afternoon	82 (87.2%)	12 (12.8%)	0.98	80 (85.1%)	14 (14.9%)	$5x10^{-3}$
		Mixed	75 (88.2%)	10 (11.8%)		72 (84.7%)	13 (15.3%)	
	A G	*Morning*	25 (86.2%)	4 (13.8%)		22 (75.9%)	7 (24.1%)	
NOC rs13116075		*Afternoon*	36 (80.0%)	9 (20.0%)	0.64	34 (75.6%)	11 (24.4%)	0.74
		Mixed	34 (77.3%)	10 (22.7%)		36 (81.8%)	8 (18.2%)	
	G G	*Morning*	2 (66.7%)	1 (33.3%)		3 (100%)	0	
		Afternoon	3 (100%)	0	0.27	1 (33.3%)	2 (66.7%)	0.08
		Mixed	0	0		0	0	
TOTAL			336	57		316	85	

3.2.3.2.2 REQUTE cohort

Of the 600 breast and prostate REQUITE patients DNA was available for genotyping on 539 patients (323 breast and 216 prostate). Of these samples Taqman genotyping for NOC rs13116075 was successful in 538 patients, one sample failed to produce reliable results on repeat analysis. Genotyping results were tested and found to be in Hardy Weinberg Equilibrium. Table 62 shows the distribution of radiotherapy toxicity by NOC rs13116075 genotype.

Table 62: Acute toxicity split by NOC rs13116075 genotyping results in the REQUITE cohort

Breast AND Prostate patients (REQUITE)	Acute Toxicity (Bivariate STAT score) n=	p value

		75% lowest	*25% highest*	
NOC rs13116075	*AA*	285 (76.2%)	89 (23.8%)	0.16
	AG	79 (66.4%)	40 (33.6%)	
	GG	9 (81.8%)	2 (18.2%)	
TOTAL		373	131	

There was no significant relationship with NOC rs13116075 and acute toxicity (Bivariate STAT score).

Taking time of radiotherapy treatment into consideration patients with AG NOC rs13116075 treated with a mix of treatment times had more acute toxicity than the other treatment groups (p=0.05). Table 63 shows the distribution of acute toxicity by genotype and treatment time.

Table 63: Acute toxicity split by NOC rs13116075 genotyping and radiotherapy treatment time in REQUITE cohort

Breast AND Prostate Patients (REQUITE)		Radiotherapy treatment time	Acute (Bivariate STAT score) n=		p value
			75% lowest	*25% highest*	
NOC rs13116075	*AA*	*Morning*	102 (78.5%)	28 (21.5%)	0.41
		Afternoon	115 (72.8%)	43 (27.2%)	
		Mixed	65 (79.3%)	17 (20.7%)	
	AG	*Morning*	28 (65.1%)	15 (34.9%)	0.05
		Afternoon	39 (76.5%)	12 (23.5%)	
		Mixed	12 (48.0%)	13 (52.0%)	
	GG	*Morning*	2 (100%)	0	0.75
		Afternoon	4 (80.0%)	1 (20.0%)	
		Mixed	3 (75.0%)	1 (25.0%)	
		TOTAL	370	130	

On subgroup analysis acute toxicity score was available for 308 of the 323 breast patients with genotyping data available. Acute erythema was used as a surrogate marker for acute toxicity. NOC rs13116075 AG genotype was significantly related to

increased toxicity (p=0.04). The other genotypes were not related to toxicity. Table 64 shows the distribution of toxicity by genotype.

Table 64: Acute breast erythema split by NOC rs13116075 genotyping results in the REQUITE breast cohort

Breast Patients only (REQUITE)		Acute Erythema n=		p value
		Grade 0/1	Grade 2 or more	
NOC rs13116075	*AA*	195 (86.7%)	30 (13.3%)	**0.04**
	AG	55 (75.3%)	18 (24.7%)	
	GG	7 (70.0%)	3 (30.0%)	
	TOTAL	257	51	

Taking time of radiotherapy treatment into consideration showed no significant effect of NOC rs13116075 (table 65) on acute toxicity with any of the genotypes, however if the mixed treatment time group is excluded from analysis patients with AG NOC rs 13116075 have increased acute toxicity in the mornings (p=0.04).

Table 65: Acute breast erythema split by NOC rs13116075 genotyping and radiotherapy treatment time in REQUITE breast cohort.

Breast Patients only (REQUITE)		Radiotherapy treatment time	Acute Erythema n=		p value
			Grade 0/1	*Grade 2 or more*	
NOC rs13116075	*AA*	*Morning*	59 (80.8%)	14 (19.2%)	0.21^{a}/0.18^{b}
		Afternoon	83 (88.3%)	11 (11.7%)	
		Mixed	49 (90.7%)	5 (9.3%)	
	AG	*Morning*	15 (65.2%)	8 (34.8%)	0.06^{a}/**0.04**^{b}
		Afternoon	30 (88.2%)	4 (11.8%)	
		Mixed	10 (62.5%)	6 (37.5%)	
	GG	*Morning*	1 (100%)	0	N/A
		Afternoon	5 (100%)	0	
		Mixed	1 (100%)	0	
		TOTAL	253	51	

a chi squared test for significance including all treatment times, b chi squared test for significance excluding mixed treatment times

On subgroup analysis of the prostate patients, acute toxicity score was available on 211 of the 215 patients with NOC rs13116075 genotyping data available. Diarrhoea assessed within the last 5 fractions of radiotherapy was taken as a surrogate marker

for overall acute toxicity. NOC rs13116075 alone did not significantly correlate with acute diarrhoea. Table 66 shows the distribution of toxicity by genotype.

Table 66: Acute diarrhoea split by NOC rs13116075 genotyping results in the REQUITE prostate cohort

Prostate Patients (REQUITE)		Acute diarrhoea n=		p value
		Grade 0/1	*Grade 2 or more*	
NOC rs13116075	*AA*	149 (90.9%)	15 (9.1%)	0.96
	AG	41 (89.1%)	5 (10.9%)	
	GG	1 (100%)	0	
	TOTAL	191	20	

Patients with AA NOC rs13116075 genotype treated in the afternoon had significantly increased acute toxicity (p= 0.02[a]/0.005[b]). Toxicity divided by treatment time and genotype is shown in table 67.

Table 67: Acute diarrhoea split by NOC rs13116075 genotyping and radiotherapy treatment time in REQUITE prostate cohort.

Prostate Patients only (REQUITE)	Radiotherapy treatment time	Acute diarrhoea n=		p value
		Grade 0/1	*Grade 2 or more*	

NOC rs13116075	*AA*	*Morning*	59 (98.3%)	1 (1.7%)	$0.02^{a}/5.0x10^{-3}$ b
		Afternoon	58 (84.1%)	11 (15.9%)	
		Mixed	32 (91.4%)	3 (8.6%)	
	AG	*Morning*	16 (88.9%)	2 (11.1%)	$1.00^{a}/1.00^{b}$
		Afternoon	16 (88.9%)	2 (11.1%)	
		Mixed	9 (90.0%)	1 (10.0%)	
	GG	*Morning*	1 (100%)	0	N/A
		Afternoon	0	0	
		Mixed	0	0	
		TOTAL	191	20	

a chi squared test for significance including all treatment times, b chi squared test for significance excluding mixed treatment times

3.2.3.3 CLOCK rs1801260 (REQUITE cohort only)

Of the 600 breast and prostate patients DNA was available for genotyping on 539 patients (323 breast and 216 prostate). Of these samples Taqman genotyping for CLOCK rs1801260 was successful in 537 patients, 2 samples failed to produce reliable results on repeat analysis. Genotyping results were tested and found to be in Hardy Weinberg Equilibrium. Table 68 shows the distribution of radiotherapy toxicity by CLOCK rs1801260 genotype.

Table 68: Acute toxicity split by CLOCK rs1801260 genotyping results in the REQUITE cohort

Breast AND Prostate patients (REQUITE)		Acute Toxicity (Bivariate STAT score) n=		p value
		75% lowest	*25% highest*	
CLOCK rs1801260	*AA*	243 (74.3%)	84 (25.7%)	0.45
	AG	118 (74.7%)	40 (25.3%)	
	GG	11 (61.1%)	7 (38.9%)	
TOTAL		372	131	

There was no significant relationship with CLOCK rs1801260 and acute toxicity (Bivariate STAT score).

Taking time of radiotherapy treatment into consideration CLOCK rs1801260 had no effect on acute toxicity rates. Table 69 shows the distribution of acute toxicity by genotype and treatment time.

Table 69: Acute toxicity split by CLOCK rs1801260 genotyping and radiotherapy treatment time in REQUITE cohort

Breast AND Prostate Patients (REQUITE)		Radiotherapy treatment time	Acute (Bivariate STAT score) n=		p value
			75% lowest	*25% highest*	
CLOCK rs1801260	*AA*	*Morning*	86 (75.4%)	28 (24.8%)	0.66
		Afternoon	102 (71.8%)	40 (28.2%)	
		Mixed	54 (77.1%)	16 (22.9%)	
	AG	*Morning*	44 (74.6%)	15 (25.4%)	0.33
		Afternoon	51 (79.7%)	13 (20.3%)	
		Mixed	21 (65.2%)	11 (34.8%)	
	GG	*Morning*	2 (100%)	0	0.43
		Afternoon	5 (62.5%)	3 (37.5%)	
		Mixed	4 (50.0%)	4 (50.0%)	
		TOTAL	369	130	

On subgroup analysis of breast patients, acute toxicity score was available on 307 of the 323 breast patients with genotyping data available. Acute erythema was used as a surrogate marker for acute toxicity. CLOCK rs1801260 did not significantly relate to increased toxicity. Table 70 shows the distribution of toxicity by genotype.

Table 70: Acute breast erythema split by CLOCK rs1801260 genotyping results in the REQUITE breast cohort

Breast Patients only (REQUITE)		Acute Erythema n=		p value
		Grade 0/1	*Grade 2 or more*	
NOC rs13116075	*AA*	146 (84.4%)	27 (15.6%)	0.56
	AG	93 (80.9%)	22 (19.1%)	
	GG	17 (89.5%)	2 (10.5%)	
	TOTAL	256	51	

Taking time of radiotherapy treatment into consideration showed no significant effect of CLOCK rs1801260 (table 71) on acute toxicity with any of the genotypes. Similarly if

the mixed treatment time group is excluded from analysis patients there is no significant effect on toxicity.

Table 71: Acute breast erythema split by CLOCK rs1801260 genotyping and radiotherapy treatment time in REQUITE breast cohort.

Breast Patients only (REQUITE)		Radiotherapy treatment time	Acute Erythema n=		p value
			Grade 0/1	*Grade 2 or more*	
CLOCK rs1801260	*AA*	*Morning*	44 (78.6%)	12 (21.4%)	0.20[a]/0.08[b]
		Afternoon	69 (89.6%)	8 (10.4%)	
		Mixed	32 (82.1%)	7 (17.9%)	
	AG	*Morning*	30 (75.0%)	10 (25.0%)	0.30[a]/0.14[b]
		Afternoon	41 (87.2%)	6 (12.8%)	
		Mixed	19 (76.0%)	6 (24.0%)	
	GG	*Morning*	1 (100%)	0	0.94/0.73
		Afternoon	8 (88.9%)	1 (11.1%)	
		Mixed	8 (88.9%)	1 (11.1%)	
		TOTAL	252	51	

a chi squared test for significance including all treatment times, b chi squared test for significance excluding mixed treatment times

On subgroup analysis of the prostate patients, acute toxicity score was available on 211 of the 215 patients with CLOCK rs1801260 genotyping data available. Diarrhoea assessed within the last 5 fractions of radiotherapy was taken as a surrogate marker for overall acute toxicity. CLOCK rs1801260 alone did not significantly correlate with acute diarrhoea. Table 72 shows the distribution of toxicity by genotype.

Table 72: Acute diarrhoea split by CLOCK rs1801260 genotyping results in the REQUITE prostate cohort

Prostate Patients only (REQUITE)		Acute Diarrhoea n=		p value
		Grade 0/1	*Grade 2 or more*	
CLOCK rs1801260	*AA*	106 (90.6%)	11 (9.4%)	0.99
	AG	65 (90.3%)	7 (9.7%)	
	GG	20 (90.9%)	2 (9.1%)	
	TOTAL	191	20	

Time of radiotherapy treatment had no effect on toxicity in the different genotypes, however if the mixed time group were excluded from the analysis then patients with

AA genotype treated in the afternoon had significantly increased acute toxicity (p= 0.03). Table 73 shows acute toxicity by CLOCK rs1801260 genotypes and radiotherapy treatment time.

Table 73: Acute diarrhoea split by CLOCK rs1801260 genotyping and radiotherapy treatment time in REQUITE prostate cohort.

Prostate Patients only (REQUITE)		Radiotherapy treatment time	Acute diarrhoea n=		p value
			Grade 0/1	Grade 2 or more	
CLOCK rs1801260	*AA*	*Morning*	43 (97.7%)	1 (2.3%)	0.10[a]/0.03[b]
		Afternoon	39 (84.8%)	7 (15.2%)	
		Mixed	24 (88.9%)	3 (11.1%)	
	AG	*Morning*	24 (92.3%)	2 (7.7%)	0.24[a]/0.36[b]
		Afternoon	27 (84.4%)	5 (15.6%)	
		Mixed	14 (100%)	0	
	GG	*Morning*	10 (100%)	0	0.20[a] /0.28 [b]
		Afternoon	8 (88.9%)	1 (11.1%)	
		Mixed	2 (66.7%)	1 (33.3%)	
		TOTAL	191	20	

a chi squared test for significance including all treatment times, b chi squared test for significance excluding mixed treatment times

3.2.3.4 Combined circadian rhythm genotypes

3.2.3.4.1 LeND cohort

Of the 664 patients DNA was available for genotyping on 508 patients. Of these 508 patients genotyping results obtained via PCR for both NOC rs13116075 and PER3 VNTR was successful in 441 patients, in the remaining 67 samples there was inadequate DNA to produce reliable results. Table 74 shows the distribution of radiotherapy toxicity by NOC rs13116075 and PER3 VNTR genotype.

Table 74: Acute and late toxicity distribution when split by combined genotyping (PER3 VNTR and NOC rs13116075) results for LeND cohort

LeND cohort	Acute Toxicity n=	p value	Late Toxicity (Bivariate STAT score) n=	p value

		NOC rs13116075	*No/Low*	*Moderate/ Severe*		*75% lowest*	*25% highest*	
PER3 VNTR	*44*	*AA*	122 (83.0%)	25 (17.0%)		113 (76.9%)	34 (23.1%)	
		AG	48 (81.3%)	11 (18.7%)	0.70	42 (71.2%)	17 (29.8%)	0.18
		GG	3 (100%)	0		1 (33.3%)	2 (66.7%)	
TOTAL			173	36		156	53	
PER3 VNTR	*45*	*AA*	111 (92.5%)	9 (7.5%)		93 (77.5%)	27 (22.5%)	
		AG	41 (78.8%)	11 (21.2%)	0.03	37 (71.2%)	15 (28.8%)	0.35
		GG	3 (75.0%)	1 (25.0%)		4 (100%)	0	
TOTAL			155	21		134	42	
PER3 VNTR	*55*	*AA*	32 (86.5%)	5 (13.5%)		26 (70.3%)	11 (29.7%)	
		AG	16 (84.2%)	3 (15.8%)	0.82	16 (84.2%)	3 (15.8%)	0.25
		GG	0	0		0	0	
TOTAL			48	8		42	14	
Overall TOTAL			376	65		332	109	

Patients with 45, AG genotype had increased risk of acute toxicity (p=0.03) otherwise there was no significant relationship with NOC rs13116075 and PER3 VNTR and acute or late toxicity (Bivariate STAT score).

Taking time of radiotherapy treatment into consideration shows a significant effect of NOC rs13116075 and PER3 VNTR (table 75) on late toxicity. Patients with AA NOC rs13116075 genotype and 4/4 PER3 VNTR have increased late radiotherapy toxicity if treated in the mornings ($p = 0.45x10^{-4}$). No significant relationship was observed with acute toxicity, genotype and treatment time.

Table 75: Acute and late toxicity distribution when split by radiotherapy treatment time and combined genotyping (PER3 VNTR and NOC rs13116075) results for LeND cohort.

LeND cohort	NOC rs131	Radiotherapy	Acute Toxicity n=	p value	Late Toxicity (Bivariate STAT	p value

		16075	treatment time				score) n=		
				No/Low	*Mod/ Severe*		*75% Lowest*	*25% Highest*	
			Morning	34 (81.0%)	8 (19.0%)	0.94 [a] /0.74 [b]	26 (61.9%)	16 (38.1%)	$4.54x10^{-4}$ [a] /0.02 [b]
		AA	*Afternoon*	36 (83.7%)	7 (16.3%)		36 (83.7%)	7 (16.3%)	
			Mixed	29 (82.9%)	6 (17.1%)		34 (97.1%)	1 (2.9%)	
			TOTAL	99	21		96	24	
PER 3 VNT R	*4/4*		*Morning*	11 (78.6%)	3 (21.4%)	0.73 [a] /0.56 [b]	11 (78.6%)	3 (21.4%)	0.86 [a] /0.74 [b]
		AG	*Afternoon*	13 (86.7%)	2 (13.3%)		11 (73.3%)	4 (26.7%)	
			Mixed	16 (76.2%)	5 (23.8%)		17 (81.0%)	4 (19.0%)	
			TOTAL	40	10		39	11	
			Morning	1 (100%)	0	N/A	1 (100%)	0	0.08 [a] /0.08 [b]
		GG	*Afternoon*	2 (100%)	0		0	2 (100%)	
			Mixed	0	0		0	0	
			TOTAL	3	0		1	2	
			Morning	32 (94.1%)	2 (5.9%)	0.55 [a] /0.41 [b]	25 (73.5%)	9 (26.5%)	0.25 [a] /0.11 [b]
		AA	*Afternoon*	31 (88.6%)	4 (11.4%)		31 (88.6%)	4 (11.4%)	
			Mixed	36 (94.7%)	2 (5.3%)		29 (76.3%)	9 (23.4%)	
			TOTAL	99	8		85	22	
PER 3 VNT R	*4/5*		*Morning*	9 (100%)	0	0.20 [a] /0.08 [b]	8 (88.9%)	1 (11.1%)	0.75 [a] /0.46 [b]
		AG	*Afternoon*	16 (72.7%)	6 (27.3%)		17 (77.3%)	5 (22.7%)	
			Mixed	10 (71.4%)	4 (28.6%)		11 (78.6%)	3 (21.4%)	
			TOTAL	35	10		36	9	
			Morning	1 (50.0%)	1 (50.0%)	0.39 [a] / 0.39 [b]	2 (100%)	0	N/A
		GG	*Afternoon*	1 (100%)	0		1 (100%)	0	
			Mixed	0	0		0	0	
			TOTAL	2	1		3	0	
			Morning	10 (90.9%)	1 (9.1%)	0.72 [a] / 0.94 [b]	8 (72.7%)	3 (27.3%)	0.99 [a] /0.89 [b]
		AA	*Afternoon*	9 (90.0%)	1 (10.1%)		7 (70.0%)	3 (30.0%)	
PER 3 VNT R	*5/5*		*Mixed*	8 (80.0%)	2 (20.0%)		7 (70.0%)	3 (30.0%)	
			TOTAL	27	4		22	9	
			Morning	2 (66.7%)	1 (33.3%)	0.74 [a] / 0.49 [b]	2 (66.7%)	1 (33.3%)	0.28 [a] /0.88 [b]
		AG	*Afternoon*	6 (85.7%)	1 (14.3%)		5 (71.4%)	2 (28.6%)	
			Mixed	6 (85.7%)	1 (14.3%)		7 (100%)	0	

	TOTAL	14	3		14	3	
	Morning	0	0	N/A	0	0	N/A
	Afternoon	0	0		0	0	
GG	*Mixed*	0	0		0	0	
	TOTAL	0	0		0	0	
	Overall TOTAL	319	57	376	296	80	376

a chi squared test for significance including all treatment times, b chi squared test for significance excluding mixed treatment times.

3.2.3.4.2 REQUITE cohort

Of the 600 patients DNA was available for genotyping on 539 patients. Of these patients genotyping results for NOC rs13116075, PER3 VNTR and CLOCK rs1801260 was successful in 476 patients. Table 76 shows the distribution of radiotherapy toxicity by NOC rs13116075 and PER3 VNTR genotype.

Table 76: Acute toxicity distribution when split by combined genotyping (PER3 VNTR and NOC rs13116075) results for REQUITE cohort

REQUITE cohort		Acute Toxicity n=		p value
	NOC rs13116075	*No/Low*	*Moderate/*	

				Severe	
PER3 VNTR	*44*	*AA*	127 (79.4%)	33 (20.6%)	0.29
		AG	37 (72.4%)	14 (27.4%)	
		GG	5 (100%)	0	
	TOTAL		169	47	
PER3 VNTR	*45*	*AA*	121 (73.8%)	43 (26.2%)	0.23
		AG	26 (60.5%)	17 (39.5%)	
		GG	3 (75.0%)	1 (25.0%)	
	TOTAL		150	61	
PER3 VNTR	*55*	*AA*	24 (77.4%)	9 (22.6%)	0.25
		AG	11 (61.1%)	7 (38.9%)	
		GG	0	1 (100%)	
	TOTAL		35	17	
Overall TOTAL			354	125	

There was no significant relationship between NOC rs13116075 and PER 3 genotype and acute toxicity (Bivariate STAT score).

Taking time of radiotherapy treatment into consideration shows no significant effect of NOC rs13116075 and PER3 VNTR (table 77) on acute toxicity.

Table 77: Acute toxicity distribution when split by radiotherapy treatment time and combined genotyping (PER3 VNTR and NOC rs13116075) results for REQUITE cohort.

Per3 VNTR	NOC rs13116075	RT treatment time	Acute Toxicity n=		p value
			No/Low	*Mod/ Severe*	
4/4	*AA*	*Morning*	39 (78.0%)	11 (22.0%)	0.23[a] /0.67[b]
		Afternoon	50 (74.6%)	17 (25.4%)	

		Mixed	37 (88.1%)	5 (11.9%)	
		TOTAL	126	33	
	AG	*Morning*	13 (72.2%)	5 (27.8%)	0.99[a] /0.95[b]
		Afternoon	19 (73.1%)	7 (26.9%)	
		Mixed	5 (71.4%)	2 (28.6%)	
		TOTAL	37	14	
	GG	*Morning*	1 (100%)	0	N/A
		Afternoon	2 (100%)	0	
		Mixed	2 (100%)	0	
		TOTAL	5	0	
	AA	*Morning*	49 (81.7%)	11 (18.3%)	0.18[a] /0.19[b]
		Afternoon	51 (71.8%)	20 (28.2%)	
4/5		*Mixed*	20 (64.5%)	11 (35.5%)	
		TOTAL	120	42	
	AG	*Morning*	8 (57.1%)	6 (42.9%)	0.08[a] /0.21[b]
		Afternoon	14 (77.8%)	4 (22.2%)	
		Mixed	4 (36.4%)	7 (63.6%)	
		TOTAL	26	17	
	GG	*Morning*	1 (100%)	0	N/A
		Afternoon	2 (66.7%)	1 (33.3%)	
		Mixed	0	0	
		TOTAL	3	1	
5/5	*AA*	*Morning*	9 (69.2%)	5 (30.8%)	0.24[a]/0.79[b]
		Afternoon	9 (69.2%)	4 (30.8%)	
		Mixed	6 (100%)	0	
		TOTAL	24	9	
	AG	*Morning*	6 (75.0%)	2 (25.0%)	0.23[a]/1.0[b]
		Afternoon	3 (75.0%)	1 (25.0%)	
		Mixed	2 (33.3%)	4 (66.7%)	
		TOTAL	11	7	
	GG	*Morning*	0	0	N/A
		Afternoon	0	0	
		Mixed	0	1 (100%)	
		TOTAL	0	1	
		Overall TOTAL	352	124	476

a chi squared test for significance including all treatment times, b chi squared test for significance excluding mixed treatment times

In the REQUITE cohort genotyping was also available for CLOCK rs1801260. Addition of this information into the combined analysis of PER3 VNTR and NOC rs13116075 had no effect on toxicity either with or without treatment times. Due to the numerous layers most cells had an expected count less than 5.

On subgroup analysis of the breast patients combination of PER3 VNTR, NOC rs13116075 and CLOCK rs1801260 and treatment time had no effect on toxicity due to the small numbers in each group.

On subgroup analysis of the prostate patients combination of PER3 VNTR, NOC rs13116075 and CLOCK rs1801260 had no effect on toxicity due to the small numbers in each group.

3.2.4 Chronotype and toxicity

3.2.4.1 LeND cohort

119 study participants of the previously recruited LeND cohort completed a Munich Chronotype questionnaire. Patients were divided according to wake and sleep time was normally distributed (see figure 26).

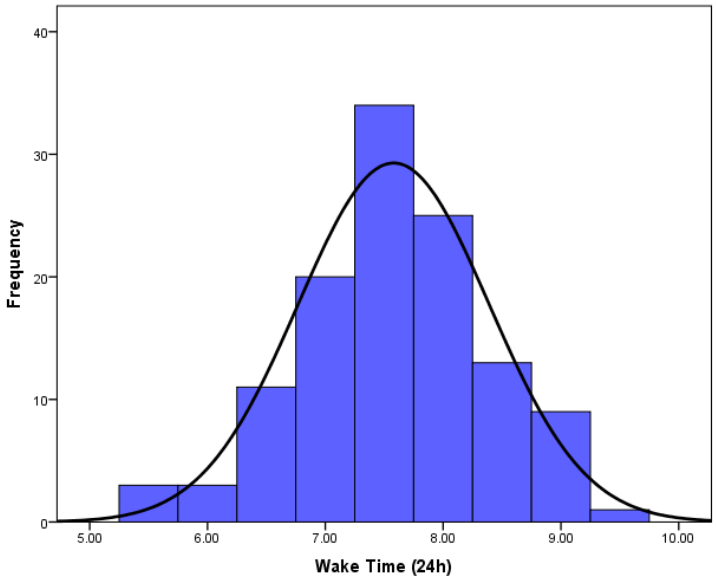

Figure 26: LeND cohort chronotype distribution (time of waking in 24h clock).

They were then further subdivided into normal (wake between 07:30 and 08:00), early (wake pre 07:30) and late (wake post 08:00) chronotypes based on these results. A chi squared test for equivalence did not demonstrate any difference in acute or late toxicity (p=0.41 and p=0.49 respectively) in patients by chronotype. However when patients were grouped into time of radiotherapy treatment a chi squared test for equivalence showed that patients with a late chronotype had a reduced risk of late radiotherapy toxicity if treated in the afternoon (p=0.02). There was no effect on acute toxicity. Table 78 summarises these findings.

Table 78: spread of toxicity in radiotherapy treatment time groups divided by chronotype. NB Acute score only available for 94 patients, late toxicity score available in 96 patients.

There is no correlation (p=0.58) between chronotype and time of radiotherapy treatment.

Treatment time	Chronotype	Acute Toxicity n=		p value	Late Toxicity (Biv STAT score)		p value
		No/ low score	*Mod/ Severe score*		*Lower 3 quartiles*	*Upper quartile*	
Morning (more than two thirds before 12:00)	*Early (Pre 07:30)*	10 (83.3%)	2 (16.7)		9 (75.0%)	3 (25.0%)	
	Normal (07:30 – 08:00)	6 (66.7%)	3 (33.3%)	0.17	7 (70.0%)	3 (30.0%)	0.93
	Late (post 08:00)	9 (100%)	0		7 (77.8%)	2 (22.2%)	
Afternoon (more than two thirds after 12:00)	*Early (Pre 07:30)*	8 (100%)	0		6 (66.7%)	3 (33.3%)	
	Normal (07:30 – 08:00)	7 (87.5%)	1 (12.5%)	0.61	3 (37.5%)	5 (62.5%)	**0.02**
	Late (post 08:00)	9 (90.0%)	1 (10.0%)		10 (100%)	0	
Mixed treatment times	*Early (Pre 07:30)*	8 (88.9%)	1 (11.1%)		8 (88.9%)	1 (11.1%)	
	Normal (07:30 – 08:00)	9 (81.8%)	2 (18.2%)	0.78	8 (72.7%)	3 (27.3%)	0.67
	Late (post 08:00)	14 (77.8%)	4 (22.2%)		14 (77.8%)	4 (22.2%)	

3.2.4.2 REQUITE cohort

268 REQUITE participants completed a Munich Chronotype questionnaire (breast and prostate only). Patients were divided according to wake and sleep time and distribution was not normally distributed due to the presence of 2 outliers with extreme late type (see figure 27).

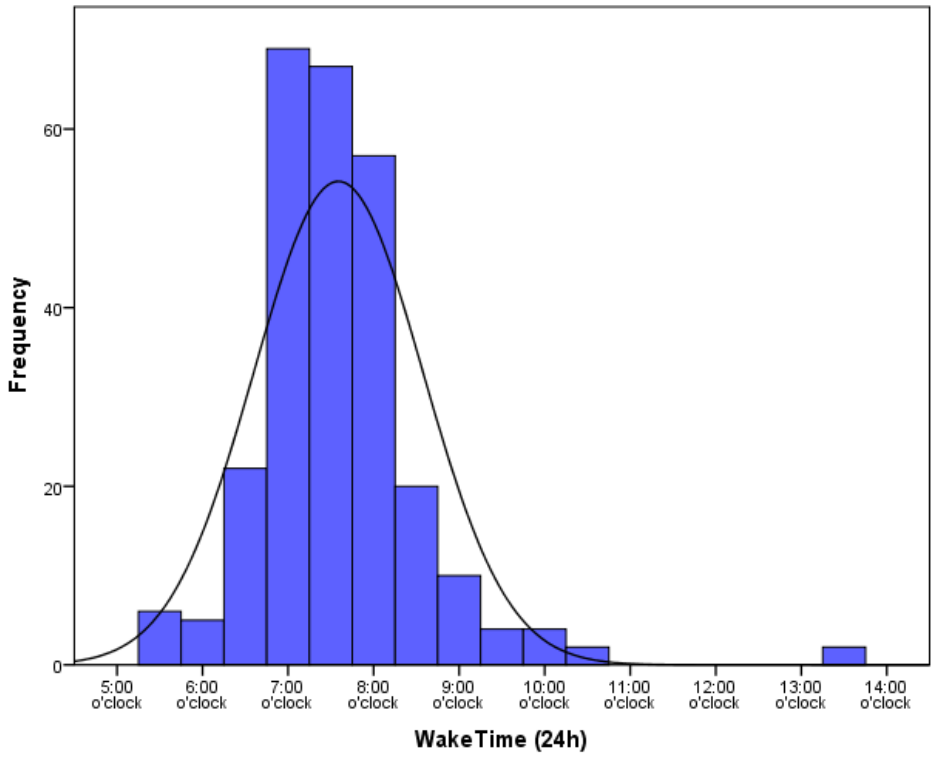

Figure 27: REQUITE cohort chronotype distribution (time of waking in 24h clock).

They were then further subdivided into 3 groups based on wake time: normal (wake between 07:00 and 08:00), early (wake pre 07:00) and late (wake post 08:00).

A chi squared test for equivalence revealed that patients with a normal wake time had an increased risk of acute radiotherapy toxicity (p=0.03). However combining time of radiotherapy treatment and chronotype had no significant effect on radiotherapy toxicity. On subgroup analysis in the prostate cohort the normal chronotype had a significantly higher rate of acute radiotherapy toxicity (p=0.03) and if they were treated in the afternoon again they had significantly higher rates of toxicity (p=0.01). There were no significant findings in the REQUITE breast cohort.

3.2.5 Genotype and Chronotype

A Kruskall Wallis test for equal distribution showed no significant relationship between time of waking and PER 3 VNTR (p= 0.85) and NOC rs 13116075 (p= 0.48) in the LeND cohort. In the REQUITE cohort again there was no association between genotype and chronotype (PER 3 VNTR p=0.57; NOC rs 13116075 p=0.88; CLOCK rs 1801260 p=0.79).

3.2.6 Circadian rhythm and comet assay

3.2.6.1 DNA repair and time of bleeding

In the REQUITE patients data on the time of blood sampling was collected and correlated to the outputs from the comet assay. Time of bleeding was available on 146 of the 148 patients the comet assay was performed on and ranged between 09:10 and 17:00 (figure 28). Spearman's rank was performed to test for correlation between time of bleeding and DNA repair.

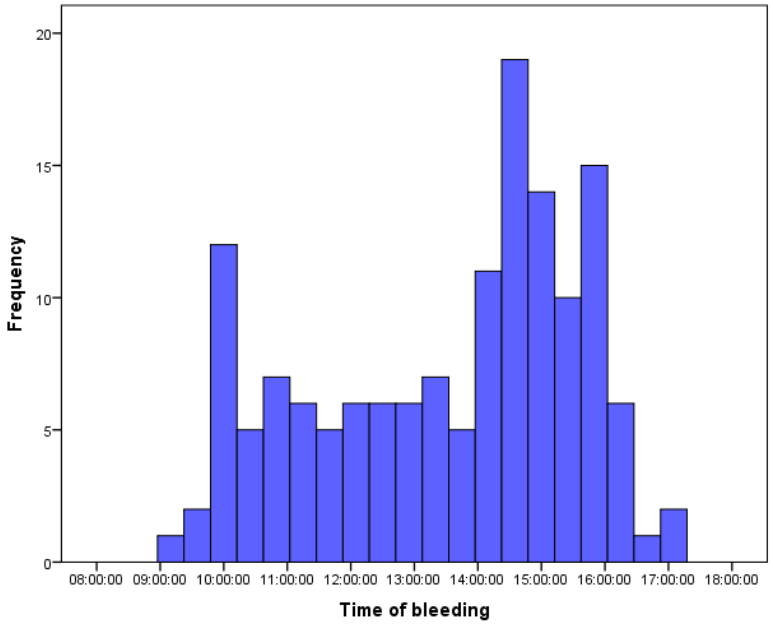

Figure 28: Histogram of the distribution of times REQUITE study participants were bled (24h)

There was no significant correlation between time of bleeding and the comet assay observed (Table 79). Nor on subgroup analysis by gender was any significant correlation observed.

Table 79: Correlation between comet assay and time of bleeding in REQUITE patients comet assay performed on (Spearman's rank).

Comet assay output	Correlation coefficient (p value)	Comet assay output	Correlation coefficient (p value)
0Gy (PTD)	0.06 (0.49)	*8Gy 30 mins (TL)*	0.03 (0.78)

0Gy (OTM)	0.06 (0.56)	*RID (PTD)*	-0.03 (0.77)
0Gy (TL)	-0.12 (0.22)	*RID (OTM)*	-0.08 (0.42)
8Gy 0 mins (PTD)	0.02 (0.87)	*ROR (PTD)*	-0.04 (0.71)
8Gy 0 mins (OTM)	-0.06 (0.52)	*ROR (OTM)*	-0.07 (0.46)
8Gy 0 mins (TL)	0.03 (0.78)	*RRR (PTD)*	-0.02 (0.81)
8Gy 30 mins (PTD)	0.13 (0.28)	*RRR (OTM)*	-0.03 (0.78)
8Gy 30 mins (OTM)	0.10 (0.40)		

PTD = Percentage tail DNA; OTM = Olive Tail Moment; TL = Tail Length; rid = Radiation Induced Damage; ROR = Rate of Repair; RRR = Relative rate of repair

The REQUITE patients were also divided into 2 groups split at the median time (14:05) of bleeding and there was again no significant difference in DNA repair. There was no significant result on subgroup analysis split by gender.

The comet assay was performed by different operators during the course of this study. When results were stratified by operator a significant difference in DNA repair was observed on sub-group analysis for the operator who performed most assays (Maxime Boy, n = 43). Patients bled later in the day had a lower median relative rate of repair than those bled earlier (p=0.03). If the patients are split by gender then this relationship only remained significant in the male patients (p=0.03).

In the LeND cohort patients were all bled at the same time of day (between 10:00 and 11:00) so this analysis was not performed in the retrospective LeND cohort.

3.2.6.2 DNA repair and circadian rhythm genes

Results from the comet assay were tested for an association with the circadian rhythm genes (PER3 VNTR, NOC rs13116075 and CLOCK rs1801260). The comet assay outputs were not normally distributed so differences in the mean were assessed using the Kruskal –Wallis test. The only significant difference was Olive tail moment 8Gy 0 minutes and 8Gy 30 minutes results and the percentage tail DNA 8 Gy 30 minutes in the REQUITE samples. This showed that patients with the most common NOC rs13116075 AA (n=63) had lower levels of DNA damage induced by radiation (see figure 29).

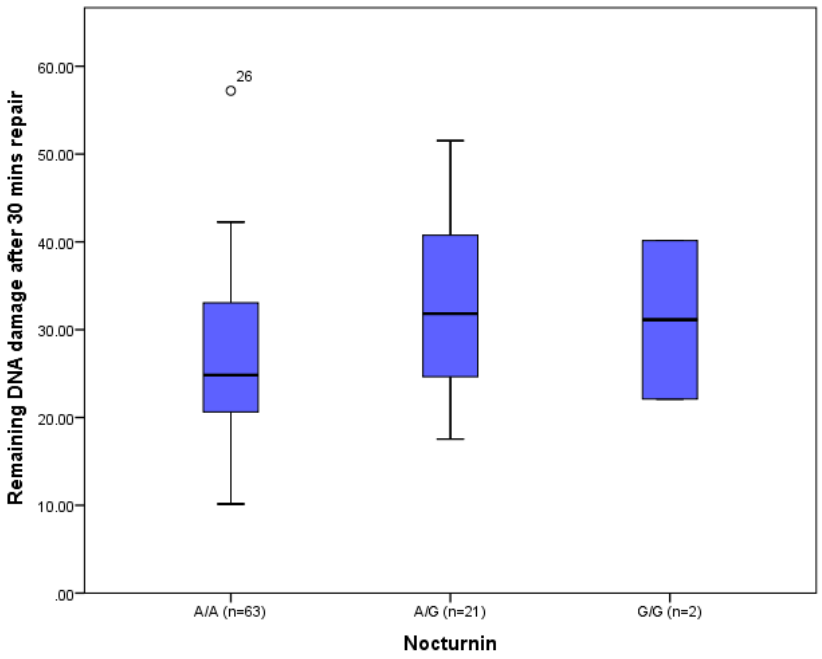

Figure 29: Boxplot showing difference in Olive tail moment results 30 minutes after 8Gy irradiation in the REQUITE cohort by NOC rs13116075 genotype.

This was not replicated in the LeND cohort and none of the other genotypes showed any significant variation in comet results as shown in table 80. In the LeND cohort no genotyping data was available for CLOCK rs1801260.

Table 80: Kruskal-Wallis test for significant difference in mean comet results by circadian rhythm genotype

Comet assay result	LeND			REQUITE		
	PER3 VNTR	*NOC rs13116075*	*CLOCK rs1801260*	*PER3 VNTR*	*NOC rs13116075*	*CLOCK rs1801260*
0Gy (PTD)	0.07	0.60	X	0.51	0.98	0.85
0Gy (OTM)	0.16	0.73	X	0.13	0.55	0.71
0Gy (TL)	0.18	0.80	X	0.14	0.47	0.32

8Gy 0 mins (PTD)	0.56	0.77	X	0.34	0.28	0.33
8Gy 0 mins (OTM)	0.96	0.28	X	0.38	0.03	0.63
8Gy 0 mins (TL)	0.96	0.48	X	0.27	0.21	0.25
8Gy 30 mins (PTD)	0.30	0.27	X	0.76	0.02	0.85
8Gy 30 mins (OTM)	0.46	0.41	X	0.66	0.03	0.91
8Gy 30 mins (TL)	0.87	0.81	X	0.48	0.14	0.81
RID (PTD)	0.94	0.81	X	0.26	0.30	0.78
RID (OTM)	0.99	0.41	X	0.27	0.15	0.91
ROR (PTD)	0.77	0.94	X	0.22	0.87	0.38
ROR (OTM)	0.97	0.55	X	0.26	0.58	0.81
RRR (PTD)	0.54	0.75	X	0.26	0.58	0.06
RRR (OTM)	0.75	0.99	X	0.17	0.22	0.28

PTD = Percentage tail DNA; OTM = Olive Tail Moment; TL = Tail Length; rid = Radiation Induced Damage; ROR = Rate of Repair; RRR = Relative rate of repair

On subgroup analysis of the female patients only, those with AA NOC genotype had lower levels of DNA damage observed at 0 mins (p=0.05) and 30 mins (p=0.03) post irradiation (Spearman's rank analysis). Female patients in the REQUITE cohort with 44 PER3 VNTR were also noted to have higher levels of DNA damage at 0 mins (p=0.04) and overall radiation induced damage (p=0.05).

3.2.7 RILA and circadian rhythm

No significant difference was observed in RILA dependent on time of bleeding when a Mann Whitney U test was performed when the cohort was split by median time of bleeding or on correlation testing by spearman's rank. Sub-group analysis again failed to demonstrate any relationship between apoptosis levels and time of bleeding. A Kruskal-Wall test for significant difference in mean RILA result was performed for the 3 circadian rhythm genes tested. Again only NOC rs13116075 demonstrated a significant difference ($p=3.0x10^{-3}$) in the REQUITE cohort (see figure 30). This was not replicated in the retrospective LeND cohort. Applying a Bonferroni correction for six tests gives p=0.018.

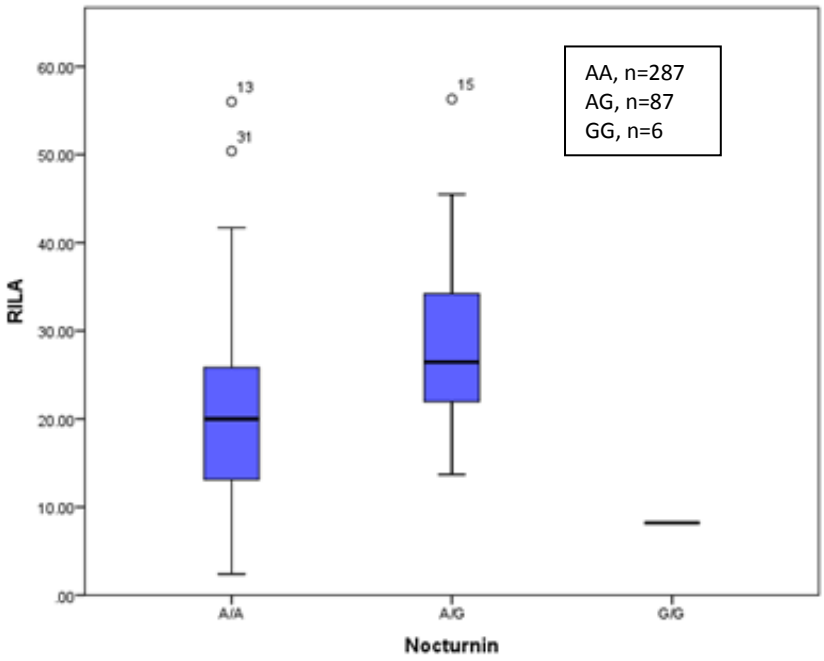

Figure 30: Boxplot showing difference in mean RILA results in the REQUITE cohort by NOC rs13116075 genotype.

Table 81 shows the RILA results for both cohorts.

Table 81: p values from Kruskal-Wallis test for significant difference in mean RILA results by circadian rhythm genotype

	LeND			REQUITE		
	PER3 VNTR	*NOC rs13116075*	*CLOCK rs1801260*	*PER3 VNTR*	*NOC rs13116075*	*CLOCK rs1801260*
P value	0.56	0.52	x	0.13	$3X10^{-3}$	0.67

3.2.8 Lymphocyte subpopulations and circadian genes

In the LeND cohort 80 patients were rebled for lymphocyte subpopulation assays. Of these results were available on 51 study participants. A Kruskal-Wallis test for a significant difference in mean lymphocyte subpopulations by circadian genotype was performed for overall lymphocytes, cytotoxic (CD8) and helper (CD4) T cells. Volunteers with AA NOC rs13116075 (n=29) had significantly lower CD4 lymphocyte

levels (p=0.02) and overall lymphocyte count (p=0.02) than AG (n=7) and GG (n=1) genotypes (see figure 31). There was no significant difference in mean CD8 T cells (p=0.11) by NOC rs13116075 genotype, nor was there any difference in any of the lymphocyte subpopulations by PER3 VNTR (figure 32). These differences are not significant after Bonferroni correction for six tests.

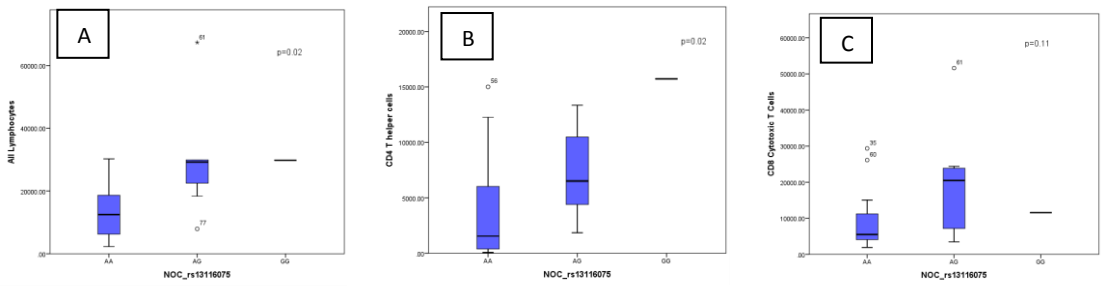

Figure 31: Boxplots showing difference in mean overall lymphocytes (A), CD4 T helper cells (B) and CD8 Cytotoxic T cells (C) in the LeND cohort by NOC rs13116075genotype

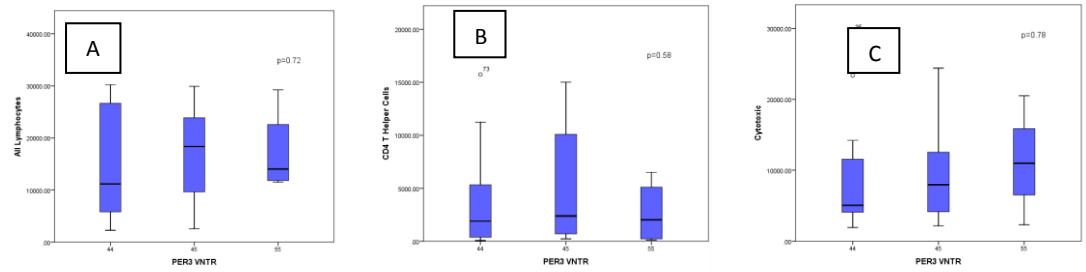

Figure 32: Boxplots showing difference in mean overall lymphocytes (A), CD4 T helper cells (B) and CD8 cytotoxic T cells (C) in the LeND cohort by PER3 VNTR genotype.

3.2.9 Circadian results summary

3.2.9.1 Relationship to toxicity

Table 82: Summary table for circadian chapter results

	ACUTE toxicity				LATE toxicity
Variable	LeND	REQUITE			LeND
		Overall	Breast	Prostate	
*Time of RT**	N/S	N/S	Morning RT	Afternoon p	Morning RT

			p=0.03^a/p=0.007 ^b; N/S†)	=0.05^a/p=0.01 ^b	p=0.03^a; p=0.08 ^b, p= 0.01†)
Season	N/S	N/S	N/S	N/S	N/S
Season + RT time*	Afternoon RT* in darker month p = 0.007	N/S	Morning RT* in darker months: p= 0.03[a]; p=9x10^{-3}[b]	Afternoon RT* in lighter months p =0.1[a]; 0.04[b]	N/S
PER3 VNTR	N/S	N/S	N/S	N/S	N/S
PER3 VNTR + RT time*	N/S	N/S	Morning RT and 45 genotype p =0.07[a]; p=0.02[b]	N/S	Morning RT* and 4/4 genotype: p=6x10^{-3}^
NOC rs13116075	N/S	N/S	AG genotype: p= 0.04^	N/S	N/S
NOC rs13116075 + RT time*	N/S	N/S	Morning RT* and AG genotype p =0.06[a]; p=0.04[b]	Afternoon RT* and AA p=0.02[a]; 5.0x10^{-3}[b]	Morning RT and AA genotype: p= 5x10^{-3}
CLOCK rs1801260	N/A	N/S	N/S	N/S	N/A
CLOCK rs1801260 + RT time*	N/A	N/S	N/S	Afternoon RT* and AA genotype p= 0.10a ;0.03b	N/A
PER3 VNTR + NOC rs13116075	45 and AG genotypes p= 0.03	N/S	N/S	N/S	N/S
PER3 VNTR + NOC rs13116075 + RT time*	N/S	N/S	N/S	N/S	Morning RT* and 44, AA genotype 4.54x10-4 [a] ;0.02[b]
PER3 VNTR + NOC rs13116075 + CLOCK rs1801260	N/A	N/S	N/S	N/S	N/A
PER3 VNTR + NOC rs13116075 + CLOCK rs1801260 + RT time*	N/A	N/S	N/S	N/S	N/A
Chronotype	N/S	Normal wake time increased toxicity (p=0.03)	NS	Normal wake time increased toxicity (p=0.03)	N/S
Chronotype + RT Time*	N/S	NS	NS	Normal wake time increased toxicity with pm RT (p=0.01)	Late radiotherapy protective in early type p = 0.02^
Chronotype +	N/S	NS	NS	NS	N/S

Genotype	

*N/S = not significant; *RT=radiotherapy; ^ = univariate; [a] = includes mixed treatment time group, [b] = excludes mixed treatment times in analysis; † = multivariate analysis; N/A = not applicable*

3.2.9.2 Possible Mechanism of action

Table 83: Summary table of possible mechanistic circadian effects in both LeND and REQUITE cohorts

	LEND	REQUITE		
Variable		Overall	Female	Male
DNA repair and time of bleeding	N/A	N/S (Subgroup analysis slower rate of DNA repair (RRR) in	N/S	N/S (Subgroup analysis slower rate of DNA repair (RRR) in

		those bled later in the day p =0.03)		those bled later in the day p =0.03)
DNA repair and Circadian Genotypes	N/S	NOC rs13116075 AA genotype associated with lower levels of DNA damage at 0 mins and 30 mins	NOC rs13116075 AA genotype associated with lower levels of DNA damage at 0 mins and 30 mins and 44 PER3 VNTR higher DNA damage at 0 mins and radiation induced damage	N/S
RILA and circadian genotypes	N/S	NOC rs13116075 genotypes differ in RILA values ($p=3X10^{-3}$)	N/S	N/S
Lymphocyte subpopulations	AA NOC genotype associated with lower levels of overall lymphocytes (p=0.02) and CD4 lymphocytes (p=0.02)	N/A	N/A	N/A

N/S = not significant; N/A = not applicable

3.3 DISCUSSION

From the results of these investigations we have been able to ascertain some important connections between radiotherapy toxicity and circadian rhythm. Tables 82 and 83 summarise the overall results.

3.3.1 Time of radiotherapy treatment

In both the LeND (retrospective breast cohort) and the REQUITE (prospective mixed cohort) time of radiotherapy treatment had an impact on radiotherapy toxicity. On univariate analysis morning radiotherapy is associated with increased late radiotherapy reaction assessed by bivariate STAT score in the LeND breast patients ($p=0.03$); this is maintained on multivariate analysis ($p=0.01$). More patients did receive a boost during the morning treatment for unknown reasons but possibly due to boost treatments taking longer and there being more staff in the mornings. The effect of boost treatment was accounted for in the multivariate model. When looking for the same effect in the REQUITE cohort overall there is no appreciable difference in toxicity by time of radiotherapy treatment, however if the cohort is split into males and females then subgroup analysis becomes significant. Again we observed that in the female breast cancer patients morning radiotherapy was associated with increased toxicity (univariate analysis $p=0.03$ and if the mixed treatment time group is removed $p<0.01$; although not significant on multivariate analysis). In the REQUITE cohort the observed toxicity is acute as opposed to late in the LeND cohort so as already discussed there are different pathologies behind acute and late effects. Another possible explanation for not seeing the effect on multivariate analysis in the REQUITE cohort is that the breast patients were assessed during their last 3 fractions of radiotherapy which may have been too early to observe acute toxicity particularly with hypofractionated courses as the majority of patients received. This was a set protocol for acute toxicity assessment laid out by the REQUITE trial, however it would have been preferable to assess them one to two weeks after radiotherapy completed.

In the male patients the opposite effect was observed with increased toxicity seen in the patients who received the majority of their radiotherapy in the afternoon (univariate analysis: $p =0.05$ all prostate patients and if the mixed treatment time group was removed from analysis $p=0.01$). Hsu et al (Hsu et al. 2016) observed the same findings in their retrospective study of prostate cancer patients receiving high dose external beam radiotherapy with higher incidence of acute GI and GU toxicity in patients treated later in the day. Bjarnason et al (Bjarnason et al. 2009a) studied toxicity following head and neck radiotherapy and showed a trend towards a

difference between the sexes with only the male patients experiencing increased grade three mucositis events with afternoon radiotherapy. The difference in circadian toxicity patterns between the genders is more established with chemotherapy administration. For example Francis Levi demonstrated in his chronotolerance experiments (Levi et al. 2007) in colorectal and lung cancer chemotherapies that the incidence of grade three and four GI toxicities was significantly greater in females and the patterns of toxicity varied between the genders dependent on the time of administration.

It is possible that the difference observed in these observations is not gender specific but in fact target organ specific. Radiotherapy works best in cells that are rapidly dividing and as such spend more time in G2/M phase of the cell cycle. Bjarnason et al. (Bjarnason and Jordan 2002) reviewed the differences between GI mucosa and skin in terms of the cells cycle and concluded that rectal mucosal DNA was in S phase earlier in the day than skin DNA. Certainly in terms of acute toxicity development of side effects depends on the ability of normal tissues to repair sub-lethal damage and behaves much like tumours in this respect. So assuming that it takes roughly six to eight hours for S phase to complete (Cameron and Greulich 1963) and the cell to enter its more vulnerable G2/M phase then rectal mucosa would be more likely to be effected with afternoon radiotherapy as observed with the REQUITE cohort. In contrast if skin epithelium is in S phase later in the evening then by morning it would be more sensitive to treatment as once again observed in the REQUITE acute cohort.

3.3.2 Seasonal Variation

Season alone had no significant effect on acute or late radiotherapy toxicity in either cohort including subgroup analysis split by gender and tumour site. However when the cohorts were split based on the time of day they received radiotherapy then there were significant differences in toxicity between the darker and lighter months. In both the LeND ($p = 0.007$) and REQUITE ($p=0.03$) breast cohorts the effects of time of radiotherapy treatment on acute breast toxicity was only observed in the darker months (although in opposite directions with afternoon radiotherapy being associated

with more acute toxicity in the LeND cohort and morning radiotherapy in the REQUITE cohort). In the prostate patients afternoon radiotherapy was only associated with acute diarrhoea in the lighter months (p=0.04 if mixed treatment group excluded from analysis).

One possible explanation for the variations by season would be differences in levels of melatonin. Melatonin levels are lower in lighter months than darker months (Shirazi et al. 2007). The anti-oxidant effects of melatonin have been studied (Reiter et al. 2003) as a potential radioprotective agent administered alongside radiotherapy treatment to reduce toxicity mainly in animal models (Zetner et al. 2016). Recently a phase two study showed than melatonin emollients reduce radiation dermatitis in breast cancer patients receiving whole breast radiotherapy (Ben-David et al. 2016). This would fit the observations seen in the LeND cohort however the REQUITE prostate and breast cohorts this explanation would not be valid. For the prostate patients there would in fact be less protective melatonin around in the lighter months so you would not expect to see a relationship to season and for the breast cohort the afternoon patients should have less melatonin available than the morning patients. The REQUITE cohorts when analysed by subgroup are smaller than the LeND cohort (n= 536 (LeND); n=328 (REQUITE breast); n=218 (REQUITE prostate)) so one possible avenue would be to increase the sample size and reanalyse.

The fact the results are contradictory to each other and to the melatonin theory means that it is likely that any positive findings are related to chance especially taking into consideration that the most plausible explanation is only observed in the unreliable LeND acute toxicity dataset. More work in a larger cohort is required to fully investigate the relationship between acute and late toxicity to season.

3.3.3 Circadian Genes

The three SNPs selected are associated with circadian rhythm genes. PER3 VNTR for Period 3 gene; NOC rs13116075 for nocturnin; CLOCK rs1801260 for the Circadian Locomotor Output Cycles Kaput gene. When analysed alone PER3 and CLOCK were not associated with an increased risk of toxicity, NOC rs13116075 AG genotype on

univariate analysis was associated with increased acute toxicity in the REQUITE breast cohort but this was not observed in the LeND cohort nor present after multivariate analysis. When time of radiotherapy treatment is accounted for along with circadian genotype then more positive associations were observed. The strongest observation was with NOC rs13116075 AA genotype. In the REQUITE prostate cohort NOC rs13116075 AA genotype was associated with increased acute toxicity when patients are treated in the afternoons ($p<0.01$) and in the LeND breast cohort increased late toxicity when treated in the morning ($p<0.01$). When the genotypes of PER3 VNTR and NOC rs 13116075 were combined then the strongest observation was observed. Breast patients with 4/4 PER3 VNTR and AA NOC rs 13116075 in the LeND cohort experienced increased late toxicity assessed by bivariate STAT score if they received morning radiotherapy ($p<0.0001$). This was not validated in the REQUITE cohort however the REQUITE cohort only has data on acute radiotherapy toxicity and there are fewer breast patients with lower levels of toxicity compared with the LeND cohort. As already discussed there are several possible theories why an opposite effect is noted in males and females. It is not clear whether there is a link between patients who experience acute toxicity and late toxicity, traditional thinking is that due to the different physiological pathways involved there is no link and as such one would not necessarily expect the same genotypes to be associated with increased toxicity in the two cohorts.

Another possible explanation for the differences in the REQUITE and LeND cohorts is there are more post-menopausal women in REQUITE cohort (median age 60 years vs 57.1 year in LeND cohort) and therefore differences in adrenal cortical axis. A previous study demonstrated that pre-menopausal women had a different cortisol stress response to men at certain times of day but the same observation was not true in post-menopausal women. (Van Cauter et al. 1996) This could be a possible avenue of further work.

Circadian genes are linked with rhythmical variations in gastrointestinal function controlled by variations in hormones, motility and cell proliferation (Konturek et al. 2011). Melatonin is a well-known hormone under circadian control and is excreted predominately at night. Administration of melatonin has been shown to reduce the

toxicity following radiotherapy in numerous cancer sites. (Wang et al. 2012). Variations is expression of the different genotypes in the studied circadian genes could therefore influence hormonal secretion such as melatonin and have an impact on radiotherapy related side effects. It could also be the effect of circadian rhythm on DNA damage and repair. Skin biopsies taken following exposure to UV light show increased levels of proteins relating to DNA damage when taken in the morning compared to the afternoon patients (Guan et al. 2016).

3.3.4 Chronotype

A person's preference to morning or evening time has been quantified using a scoring questionnaire called the Munich chronotype questionnaire (Roenneberg et al. 2003). Various factors influence the resulting outcome including differences in circadian rhythm genes (Jones et al. 2016). In our study we looked at whether chronotype had any links to circadian genes and whether it could be used as a surrogate marker to predict for toxicity. In the LeND cohort there was no link with circadian genes or toxicity. However when time of day was factored in patients with a later chronotype had significantly less late toxicity if they received radiotherapy treatment in the afternoon ($p=0.02$). Chronotype had no effect on the time of day patients chose to have radiotherapy so this was not a protective factor. However in the REQUITE breast cohort no relationship between chronotype, time of radiotherapy and toxicity was observed. As noted previously though at time of analysis for this thesis the REQUITE breast cohort looked only at acute toxicity so once the late toxicity data is available then we will be able to examine for this relationship to chronotype again.

To date there have been no studies directly looking at the effect of chronotype on radiotherapy toxicity however previous investigations have looked at its relationship to stress response (Roeser et al. 2012). Volunteers with morning type had increased cardiovascular parameters when performing stressful tasks in the afternoon and vice versa for those with a later chronotype (Dunn and Taylor 2014). One possible explanation is that the chronotype differences could be reflective of circadian fluctuations in the function of the suprachiasmic nucleus. This small region of the brain

found in the hypothalamus controls circadian rhythms in a wide range of bodily functions and is influenced by fluctuations in expression of circadian genes throughout a 24 hour day (van Esseveldt et al. 2000, Hida et al. 2014). If chronotype is therefore reflective of suprachiasmic function then one or more of the processes it governs may be having an influence on radiotherapy toxicity.

3.3.5 Possible Mechanism

3.3.5.1 DNA damage and repair

We explored three possible mechanisms to explain the relationship between circadian rhythm and radiotherapy toxicity. The first being relationship to DNA damage and repair. DNA damage was assessed using the alkaline comet assay. We failed to demonstrate any difference in DNA damage and repair depending on the time of day patients were bled. However there are a few possible explanations for this. The samples were not paired samples as we only had ethics approval for one bleed per patient. Analysis was therefore performed on the whole cohort comparing the earlier bled patients to the later bled patients via a spearman's rank test. Other patient dependent factors may thus play a role in determining DNA damage repair pathways. A flaw in the method for the assay could also account for the lack of significant results: although time of bleeding was collected the samples were irradiated at different times of day and thus frozen suspending DNA repair at varying times of day. This data was not collected so could not be analysed for effect.

Another possible factor was the inter-observer differences as a result of the comet assay being performed by different operators. When stratified for operator there was a significant difference in the relative rate of repair (RRR) with patients bled in the morning having a higher RRR and thus a faster rate of repair. This analysis was performed in a mixed cohort of breast, prostate and lung cancer patients. When split by gender it was only true for the male patients ($p=0.03$). As already noted in our study male patients treated in the afternoon had an increased risk of acute diarrhoea. These observations suggest that slower DNA repair in small bowel mucosal cells could lead to more persistent DNA damage and thus development of symptoms. We also showed

that there was a possible link with DNA damage and repair mechanisms and circadian rhythm genes in the REQUITE cohort. In the REQUITE overall and breast cohorts patients with AA NOC rs13116075 had greater levels of DNA damage observed at 0 minutes and after 30 minutes of repair. In the breast patients those with 4/4 PER3 VNTR had higher levels of radiation induced damage. These findings were not validated in the LeND cohort so most likely are chance observations. However the comet assay was performed in bloods obtained from the LeND cohort years after they had received radiotherapy to the breast. In the REQUITE cohort the patients were all radiotherapy naïve. It is therefore possible that previous radiation or presence of a tumour may alter part of the DNA repair pathway and result in the differences observed. Further work would need to be done in this area to explore the possible relationship between circadian rhythm genes and DNA repair. For example in patients with known circadian genotype it would be interesting to look at their rate of repair at different times of day. Initially in healthy volunteers and then in patients and link to toxicity.

3.3.5.2 Apoptosis

Radiation induced apoptosis in various tissue types has been observed to vary dependent on the time of day the organ of interest is irradiated. For example murine intestinal crypt cells undergo varying levels of apoptosis when irradiated at different times of the day, (Ijiri and Potten 1990). In this study they proposed that the differences were due to circadian variation in the cell cycle. In our study no significant results were obtained for levels of CD8 positive lymphocyte apoptosis 48 hours after irradiation assessed by the RILA. This is not surprising as this is a 72 hour protocol and time between bleeding and irradiation varied between subjects.

We observed a significant association with the circadian rhythm genotype and RILA score ($p<0.01$) in the REQUITE cohort but this again was not validated in the LeND cohort. As previously discussed the REQUITE cohort has only data collected for acute effects at time of analysis and the LeND cohort data is predominately late effects. The RILA assay was performed on many more subjects in the REQUITE cohort (n=423) as

opposed to the LeND cohort where RILA results were only available for 56 patients, hence making it extremely unlikely that any significant associations would be observed with genotype in the LeND cohort. The second problem was that much like the comet assay the RILA was performed in the LeND cohort at least eight years after these patients had received radiotherapy and the REQUITE cohort had never received any previous irradiation. It is unknown what effect this may have on the observed RILA results and the lack of validation.

3.3.5.3 The immune system

The final possible mechanism we explored was the relationship of circadian rhythm and the immune system. There is already well documented evidence of the link between circadian rhythm and the immune system. T and B lymphocytes vary by time of day with lowest levels seen in the morning corresponding with a rise in cortisol (Labrecque and Cermakian 2015). It is therefore plausible that the circadian system through its influence on the immune system could alter the toxicity profile of patients undergoing radiotherapy.

We observed a significant correlation between NOC rs 13116075 genotype and levels of CD4 positive and overall T cell lymphocytes counts. Patients with AA genotype had the lowest levels ($p=0.02$). This is consistent with previous findings that melatonin which is strongly correlated to the circadian system is involved in T helper cell pathways and its administration favours priming and release of cytokines (Miller et al. 2006). Melatonin is also associated with release of interleukin-6 (IL-6) from T cells (Maestroni 2001). IL-6 is involved in inflammatory processes in the human body (Scheller et al. 2011) and has been shown to correlate with increased levels of radiation induced pneumonitis following radiotherapy to the lung (Chen et al. 2001). We did not perform lymphocyte subpopulation analysis in the REQUITE cohort but in the future it may be possible to extract the data from the information obtained during the RILA assay to see if we can validate our findings. Another area of future work would be to measure melatonin levels in subjects and correlate this to both radiotherapy toxicity and lymphocyte levels.

CHAPTER 4 : General Discussion

Cancer survivorship is becoming an increasingly popular topic and area of interest. With improving outcomes increasing numbers of patients are living with and beyond cancer (Macmillan 2017). In this thesis rates of moderate or severe acute radiotherapy toxicity was maximally 15.8% (urinary retention) and one year toxicity only 3.6% for GI and GU prostate symptoms. In view of the low number of events any research in to toxicity needs to therefore recruit high numbers of patients. Over the course of this project we have recruited a substantial number of study participants for the REQUITE trial (642 in total). We were able to meet our recruitment target ahead of schedule and recruited an additional 50 breast and 50 prostate patients. Lung recruitment was lower than expected so analysis on this subgroup is limited and was largely excluded from any cancer site specific analysis. We also set up a follow up study in the surviving LeND patients to look at the predictive measures in a mature dataset. 119 patients (of the 664 initial volunteers) agreed to take part in further data collection and 82 attended for a repeat blood sample. In addition to this the REQUITE trial has now completed recruitment and at last time of reporting the overall total stood at 4314 patients. Any clinical and treatment variables which we wish to look at in a larger cohort could be put to the trial steering committee to analyse for validation purposes.

In this thesis I have discussed the objectives set out in the introduction chapter and how they each relate to radiotherapy toxicity. Objectives I to IV relate to the predictive assays. Robust reproducible protocols were set up for all three. Findings from the comet assay were reproduced in both the REQUITE and LeND cohort however toxicity events were low and the comet was only performed on small numbers of patient samples so further work needs to be done. Students working in the Talbot lab at the University of Leicester have already started working on the remaining stored REQUITE samples which should bring the total closer to 400. A possible relationship between the RILA and comet assay indicates that patients with a faster rate of repair have a lower RILA score. In previous studies low RILA score has been associated with increased late radiotherapy toxicity (Azria et al. 2015). Once the REQUITE cohort matures late toxicity data will be available to establish if these patients with low RILA/fast rate of repair have increased toxicity and whether the two assays can be

used together to increase the predictive value. Another possible avenue for further work would be to look at whether the assays could be used to predict for response to radiotherapy treatment or cancer progression.

The remaining two objectives addressed the link between circadian rhythm and radiotherapy toxicity. In both the REQUITE and the LeND cohort a lot of data was generated with some exciting findings. However many of these findings did not validate between the two datasets. Once the REQUITE cohort has late toxicity data collected then further comparisons can be made to the findings in the LeND cohort. A grant application has been applied for with Breast Cancer Now for a circadian follow up study to increase sample size and look more for a possible mechanism. A second grant application to HOPE has been successfully awarded to investigate the circadian rhythm effects further. With regards to the prostate findings discussions have begun with members of the PROTECT-T study team to apply for access to their cohort of 545 patients who underwent radical radiotherapy and for which there is late toxicity data available.

Radiogenomics continues to be an increasing field of research and the REQUITE study is the largest observational study to date in this research area. Following completion of recruitment data analysis and modelling is currently underway and an application for further EU funding has been applied for (Tailored project (EU 2020 grant proposal)) to test the validity of the predictive outcomes (SNPs and RILA).

In the future cancer therapies are moving towards a much more personalised approach. Findings from this study and similar ones go hand in hand with those looking at the best cancer targeting therapy to create individual patient pathways.

CHAPTER 5 : CONCLUSION

5.1 Positive Findings

5.1.1 Clinical

- On univariate analysis acute GI toxicity was associated with pelvic nodal radiotherapy ($p=3.0x10^{-4}$), haemorrhoids (p=0.03), PSA (p=0.05), V30 rectum (p=0.01), V40 rectum ($p=4.0x10^{-3}$). Intended duration of hormones (p=0.05) and V50 bladder (p=0.01) were significantly associated with acute diarrhoea on multivariate analysis
- On univariate analysis acute GU toxicity was associated with depression ($p=4.0x10^{-3}$), antidiabetic meds (p=0.03), pelvic nodal radiotherapy (p=0.02) and hip replacement (p=0.03).
- On univariate analysis overall acute prostate toxicity (Bivariate STAT score) was associated with length of hormone therapy (p=0.01), PTV (P=0.05), V50 large bowel ($p=6.0x10^{-3}$), diabetes (p=0.02) and prophylactic pelvic lymph node radiotherapy ($P=4.0x10^{-3}$).
- Prostate patients with a higher mean integral dose (Gy/L) had increased acute radiotherapy toxicity (p = 0.04) on univariate analysis.
- Univariate analysis revealed that prostate patients who managed to maintain higher levels of mean activity had lower levels of acute toxicity (p=0.02).
- Univariate analysis (p=0.03) revealed that mean dose to the heart and v30 heart was significantly associated with pneumonitis in the lung cohort.

5.1.2 Predictive assays

- In the REQUITE breast cohort univariate analysis revealed faster relative rate of repair was significantly associated with increased acute toxicity (percentage tail DNA (p=0.004) and Olive tail moment (p=0.008)).
- In the LeND breast cohort univariate analysis revealed faster rate of repair (p=0.03) and relative rate of repair (p=0.049) was significantly associated with increased acute toxicity.
- Females had a significantly lower mean RILA score than males (p= 0.02 on multivariate analysis).

- Breast cancer patients had a significantly lower mean RILA (p=0.001) than prostate patients.
- On univariate analysis patients with underlying depression have a lower mean RILA score (22.04 vs 17.46; p=0.01) and with increasing age mean RILA score also increases (P=0.006).
- On multivariate analysis patients who have a positive smoking history having a lower mean RILA score (p=0.05).
- Using the comet assay patients with a faster rate of repair (PTD: p = 0.01) and relative rate of repair (PTD: p= 3.0 x 10^{-3}; OTM: p = 8.0 x10^{-3}) have a higher RILA score.

5.1.3 Circadian rhythm

- In the LeND cohort on univariate analysis patients who received radiotherapy treatment in the morning had significantly increased (p=0.03) risk of late radiotherapy toxicity (bivariate STAT score). This was maintained on multivariate analysis (p=0.01).
- In the REQUITE breast cohort morning radiotherapy was associated with increased acute toxicity (p = 0.03; p = 7.0 x10^{-3} (with mixed treatment group removed from analysis)).
- In the REQUITE prostate cohort afternoon radiotherapy was associated (p = 0.05; p=0.01 (mixed group removed)) with increased acute toxicity.
- In the LeND cohort patients treated in the afternoon during the darker months had significantly greater acute toxicity than those treated in the morning during the darker months (p = 0.007).
- In the REQUITE breast cohort morning radiotherapy during the darker months was associated with significantly greater acute toxicity than those treated in the afternoon (p = 0.03; 9.0x10^{-3} (mixed group removed)).
- In the LeND cohort patients with 4/4 PER3 VNTR receiving morning radiotherapy had increased late toxicity (p=6.0x10^{-3}).
- LeND patients with AA NOC rs13116075 genotype have increased late toxicity if treated in the mornings (p=5.0 x10^{-3}).

- In the REQUITE cohort patients with NOC rs13116075 AA (n=63) had lower levels of DNA damage induced by radiation assessed using the comet assay (p=0.03).
- REQUITE prostate patients with AA NOC rs13116075 genotype treated in the afternoon had significantly increased acute toxicity (p= 0.02[a]/0.005[b] (mixed group removed from analysis)).
- In the LeND cohort patients with 4/5 (PER3) and AG (Noc rs13116075) genotypes had increased risk of acute toxicity (p=0.03).
- Patients with AA NOC rs13116075 genotype and 4/4 PER3 VNTR have increased late radiotherapy toxicity if treated in the mornings ($p = 4.5x10^{-4}$).
- LeND patients with a late chronotype had a reduced risk of late radiotherapy toxicity if treated in the afternoon (p=0.02).
- REQUITE patients with a normal wake time had an increased risk of acute radiotherapy toxicity (p=0.03).
- REQUITE prostate patients with normal chronotype had a significantly higher rate of acute radiotherapy toxicity (p=0.03) and if they were treated in the afternoon they had significantly higher rates of toxicity than the morning group (p=0.01).
- In REQUITE samples received later in the day a lower relative rate of repair was observed than those bled earlier (p=0.03). If the patients are split by gender then this relationship only remained significant in the male patients (p=0.03).
- In the REQUITE cohort patients with NOC rs13116075 AA (n=63) had lower levels of DNA damage induced by radiation (p=0.03).
- Female REQUITE patients with AA NOC genotype had lower levels of DNA damage observed at 0 mins (p=0.05) and 30 mins (p=0.03) post irradiation.
- Female REQUITE patients with 4/4 PER3 VNTR have higher levels of DNA damage at 0 mins (p=0.04) and overall radiation induced damage (p=0.05).
- Mean RILA score was significantly different between the NOC rs13116075 genotypes ($p=3.0x10^{-3}$) in the REQUITE cohort.
- LeND volunteers with AA NOC rs13116075 had significantly lower CD4 lymphocyte levels (p=0.02) and overall lymphocyte count (p=0.02).

5.2 Unique Research

Table 84 summarises the areas in which this thesis has developed novel research.

Table 84: Unique research findings

Novel Research Area	Findings in current thesis
Link between lymphocyte apoptosis and DNA damage following radiation	In the REQUITE cohort patient samples with a lower RILA score having a higher relative rate of repair value (p=0.003 PTD, p = 0.008 OTM) as well as faster rate of repair (p=0.01 PTD).
Pre and post RT differences in DNA damage and repair post radiotherapy.	Pre radiotherapy (REQUITE) comet samples had higher residual DNA damage at 30 minutes post irradiation (multivariate $p=6.16x10^{-13}$) and slower relative rate of repair ($p=1.11x10^{-7}$) than the post radiotherapy (LeND) samples.
Link between RILA and depression	Patients with a history of depression have lower levels of radiation induced lymphocyte apoptosis (p=0.01).
Link between smoking status and RILA	Patients with a positive smoking status have lower RILA score (p=0.05 on multivariate analysis).
Validated link between radiotherapy treatment time and toxicity (breast patients).	Morning radiotherapy associated with increased radiotherapy toxicity in REQUITE (p=0.03) and LeND cohorts (p=0.03).
The effect of season on acute radiotherapy toxicity	Effects of time of radiotherapy on acute toxicity are only observed in the darker months (p=0.007 LeND; p=0.03 REQUITE)
Use of circadian rhythm genes in predicting radiotherapy toxicity depending on treatment time	Breast patients (LeND cohort) with 4/4 PER3 VNTR and AA NOC rs 13116075 experienced increased late toxicity assessed by bivariate STAT score if they

	received morning radiotherapy ($p<0.0001$).
Differences in DNA damage and repair between circadian rhythm genotypes	In the REQUITE overall and breast cohorts patients with AA NOC rs 13116075 had greater levels of DNA damage observed at 0 minutes and after 30 minutes of repair. In the breast patients those with 4/4 PER3 VNTR had higher levels of radiation induced damage.
Difference in RILA score by NOC rs13116075 genotype	We observed a significant association with the circadian rhythm genotype and RILA score ($p<0.01$) in the REQUITE cohort NOC rs13116075 genotypes differ in RILA values ($p=3X10^{-3}$)
Correlation between lymphocyte subpopulations and NOC rs 13116075	We observed a significant correlation between NOC rs 13116075 genotype and levels of CD4 positive and overall T cell lymphocytes counts. Patients with AA genotype had the lowest levels ($p=0.02$).

APPENDICES

A.1 Inclusion/Exclusion Criteria

Inclusion Criteria

- Confirmed diagnosis of the specified tumour types, for lung cancer confirmation either by histology or based on radiological findings
- Patients suitable for adjuvant radiotherapy* for cancer of the breast (invasive or *in situ*) including breast patients receiving neo-adjuvant chemotherapy
- Patients suitable for radical radiotherapy or brachytherapy for prostate cancer; including post-prostatectomy patients
- Patients suitable for radical radiotherapy, sequential or concurrent chemoradiotherapy or stereotactic body radiation therapy for lung cancer
- No other malignancy in the last 5 years prior to treatment for the specified tumour types except basal cell or squamous cell carcinoma of the skin
- No evidence of distant metastases
- Patients able to provide a venous blood sample
- Breast patients consent to have photos taken of both breasts
- Willingness and ability to comply with scheduled visits, treatment plans and available for follow up within country of origin
- Greater than 18 years of age; no upper age limit
- The capacity to understand the patient information sheet and the ability to provide written informed consent

*Breast patients receiving chemotherapy should have completed their course of chemotherapy (anthracyclines) at least two weeks prior to radiotherapy commencing.

Exclusion Criteria

- Patients with metastatic disease
- Prior irradiation at the same site
- Planned use of protons
- High Intensity Focal Ultrasound (HIFU)
- Breast patients receiving concomitant chemo-radiation

- Male breast cancer patients
- Mastectomy patients
- Bilateral breast cancer
- Mental disability or patient otherwise unable to give informed consent and/or complete patient questionnaires
- Limited life expectancy due to co-morbidity
- Pregnant patients
- Partial breast irradiation
- Patients with breast implants if not removed during surgery
- Patients with known HIV infection/infectious hepatitis

A.2 Baseline assessment forms (symptoms and comorbidities/medications)

Prostate

P2a

PROSTATE PATIENT FACTORS ***(to be completed pre-*** **– BASELINE** ***radiotherapy)***

Study Number RQ☐☐☐☐☐-☐

Patient Initials ☐☐☐☐

Date of Birth (dd/mm/yyyy) ☐☐/☐☐/☐☐☐☐

Date Completed (dd/mm/yyyy) ☐☐/☐☐/☐☐☐☐

Name + Signature of Person completing the CRF _____

Patient Information

Height (cm) ☐☐☐

Weight (kg) ☐☐☐

Age at start of radiotherapy (yrs) ☐☐

Smoker ☐
0=No
1=Ex before cancer diagnosis
2=Ex since cancer diagnosis
3=Current
7=Do not wish to answer

If ever smoker
Duration of smoking (yrs) ☐☐
No. of tobacco products (e.g. cigarettes) a day ☐☐☐
Tobacco product _____

If ex smoker before cancer diagnosis: Time since quitting smoking (yrs) ☐☐

Approximate number of alcoholic drinks a week ☐☐☐ 777=Do not wish to answer

Diabetes	☐ 0=No 1=Yes	If yes, duration (yrs)	☐☐
Rheumatoid Arthritis	☐ 0=No 1=Yes	If yes, duration (yrs)	☐☐
Systemic Lupus Erythematosus	☐ 0=No 1=Yes	If yes, duration (yrs)	☐☐
Other collagen vascular disease	☐ 0=No 1=Yes	If yes, duration (yrs)	☐☐
Hypertension	☐ 0=No 1=Yes	If yes, duration (yrs)	☐☐
History of heart disease	☐ 0=No 1=Yes	If yes, duration (yrs)	☐☐
Any inflammatory bowel or diverticular disease	☐ 0=No 1=Crohn's disease 2=Colitis ulcerosa 3=Diverticulosis 4=Other	If yes, duration (yrs)	☐☐

Haemorrhoids	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
			If yes, physician confirmed?	☐	0=No 1=Yes 9=Not known
Depression	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
On ACE inhibitor?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
On a beta blocker?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
On other anti-hypertensive drug?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
On statin?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
On other lipid-lowering drugs?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
On anti-diabetic drug?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
On phosphodiesterase type 5 (PDE5) inhibitor like cialis?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
On sildenafil?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
On 5 alpha-reductase inhibitor?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
On alpha blocker?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
On anti-muscarinic drug?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
On amiodarone?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐	
Hip replacement?	☐	0=No 1=Unilateral 2=Bilateral	Previous abdominal surgery	☐	0=No 1=Appendectomy 2=Cholecystectomy 3=Rectum-sigma resec 4=Nephrectomy 5=Other
Bladder TUR	☐	0=No 1=Yes			

Other co-morbidity_____

Family history of prostate cancer in first degree relative	☐	0=No 1=Yes	Family history of radiotherapy toxicity	☐	0=No 1=Yes 9=Not known

to be continued on next page

Ethnicity	1=White (European or American European) 2=White and Black Caribbean Mixed 3=White and Black African Mixed 4=White and Asian Mixed 5=Hispanic American 6=Turkish 7=Indian 8=Pakistani 9=Bangladeshi 10=Chinese 11=Japanese 12=Other Asian 13=Black Caribbean 14=Black African 15=Northern African 16=African American 17=Jewish Ashkenazi 18=Jewish Sephardi 19=Any Other Ethnic Background; please specify _____ 20=Patient refused to give

REQUITE
Validating Predictive Models and Biomarkers of Radiotherapy Toxicity to Reduce Side-Effects and Improve Quality-of-Life in Cancer Survivors

P4

HEALTH PROFESSIONAL PROSTATE TOXICITY DATA

To be completed by the Doctor or Research Nurse ONLY

Study number RQ☐☐☐☐☐-☐

Patient initials ☐☐☐☐

Date of birth (dd/mm/yyyy) ☐☐/☐☐/☐☐☐☐

Date completed (dd/mm/yyyy) ☐☐/☐☐/☐☐☐☐

Name + Signature of Person completing the CRF _____

Time point

- ☐ Pre-radiotherapy (RT)
- ☐ End of RT
- ☐ 6 weeks after end of RT*
- ☐ 1 year after RT start
- ☐ 2 years after RT start
- ☐ 3 years after RT start*
- ☐ 4 years after RT start*

*if available

Rectum/ Bowel If information: **Death** = 5; **Not Known** fill boxes with 9's, if **Not Applicable** fill boxes with 8's

Proctitis ☐
- 0 = None
- 1 = Rectal discomfort, intervention not indicated
- 2 = Symptoms (e.g. rectal discomfort, passing blood or mucus); medical intervention indicated; limiting instrumental ADL
- 3 = Severe symptoms; faecal urgency or stool incontinence; limiting self care ADL
- 4 = Life-threatening e.g. perforation; urgent intervention indicated

Perforation ☐
- 0 = None
- 2 = Symptomatic; medical intervention indicated
- 3 = Severe symptoms; elective operative intervention indicated
- 4 = Life-threatening; urgent intervention indicated

Bowel Obstruction ☐
- 0 = None
- 1 = Asymptomatic; clinical or diagnostic observations only; intervention not indicated
- 2 = Symptomatic; altered GI function e.g. vomiting, diarrhoea; IV fluids <24 h
- 3 = Hospitalization indicated; elective operative intervention; disabling
- 4 = Life-threatening; urgent intervention indicated, e.g. total colectomy

Bowel fistula ☐
- 0 = None
- 1 = Asymptomatic; clinical or diagnostic observations only; intervention not indicated
- 2 = Symptomatic; altered GI function
- 3 = Severely altered GI function; TPN or hospitalization indicated; elective operative intervention indicated
- 4 = Life-threatening consequences; urgent intervention indicated

Bowel stenosis ☐
- 0 = None
- 1 = Asymptomatic; clinical or diagnostic observations only; intervention not indicated
- 2 = Symptomatic; altered GI function
- 3 = Severely altered GI function; tube feeding or hospitalization indicated; elective operative intervention indicated
- 4 = Life-threatening consequences; urgent operative intervention indicated

Bowel Ulceration ☐
- 0 = None
- 1 = Asymptomatic/ radiographic/ endscopic findings only; intervention not indicated
- 2 = Symptomatic; altered GI function
- 3 = Symptomatic and severely altered GI function; TPN indicate, deep ulcer; elective operative or endoscopic intervention indicated; disabling
- 4 = Life threatening consequences; urgent operative intervention indicated

Diarrhoea ☐
- 0 = None
- 1 = Increase of <4 stools per day over baseline; mild increase in ostomy output compare to baseline
- 2 = Increase of 4-6 stools per day over baseline; moderate increase in ostomy output compared to Baseline
- 3 = Increase of >/=7 stools per day over baseline; incontinence; hospitalization indicated; severe increase in ostomy output compared to baseline; limiting self care ADL
- 4 = Life threatening consequences; urgent intervention indicated

Flatus ☐
- 0 = None
- 1 = Mild symptoms; intervention not indicated
- 2 = Moderate; persistent; psychosocial sequelae

Rectal bleeding ☐
- 0 = No bleeding
- 1 = Mild; no intervention
- 2 = Moderate symptoms; medical intervention/minor cauterization indicated
- 3 = Transfusion, interventional radiology, endoscopic or operative intervention indicated
- 4 = Life threatening/ major or urgent intervention indicated

Management of Sphincter Control ☐
- 0 = No problem
- 1 = Occasional use of incontinence pads
- 2 = Daily use of incontinence pads
- 3 = Severe symptoms, elective operative intervention indicated

Bladder/Urethra

If information: **Death** = 5**; Not Known** fill boxes with 9's**,** if **Not Applicable** fill boxes with 8's

Haematuria ☐
- 0 = None
- 1 = Asymptomatic; clinical or diagnostic observations only; no intervention indicated
- 2 = Symptomatic; urinary catheter or bladder irrigation indicated; limiting instrumental ADL
- 3 = Gross hematuria; transfusion, IV medication or hospitalization indicated; elective endoscopic, radiologic or operative intervention indicated; limiting self care ADL
- 4 = Life threatening/ urgent radiologic or operative intervention indicated

Urinary tract obstruction ☐
- 0 = None
- 1 = Asymptomatic; clinical or diagnostic observations only
- 2 = Symptomatic but no hydronephrosis, sepsis or renal dysfunction; urethral dilation, urinary or suprapubic catheter indicated
- 3 = Symptomatic and altered organ function (e.g. hydronephrosis, or renal dysfunction); elective radiologic, endoscopic or operative intervention indicated
- 4 = Life-threatening consequences; urgent intervention indicated

Urinary Incontinence ☐
- 0 = None
- 1 = Occasional (e.g. with coughing or sneezing), pads not indicated
- 2 = Spontaneous; pads indicated; limiting instrumental ADL
- 3 = Intervention indicated (e.g., clamp, collagen injections); operative intervention indicated; limiting self care ADL

Urinary frequency ☐
- 0 = None
- 1 = Present
- 2 = Limiting instrumental ADL; medical management indicated

Urinary urgency ☐
- 0 = None
- 1 = Present
- 2 = Limiting instrumental ADL; medical management indicated

Urinary retention ☐
- 0 = None
- 1 = Urinary, suprapubic or intermittent catheter placement not indicated; able to void with some residual
- 2 = Placement of urinary, suprapubic or intermittent catheter placement indicated; medication Indicated
- 3 = Elective operative or radiologic intervention indicated; substantial loss of affect kidney function or mass
- 4 = Life-threatening; organ failure; urgent operative intervention indicated

Fistula ☐
- 0 = None
- 2 = Non-invasive intervention indicated; urinary or suprapubic catheter placement indicated
- 3 = Limiting self care ADL; elective radiologic, endoscopic or operative intervention indicated; permanent urinary diversion indicated
- 4 = Life-threatening; urgent radiologic or operative intervention indicated

Ureter/Kidney If information: **Death** = 5; **Not Known** fill boxes with 9's, if **Not Applicable** fill boxes with 8's

Obstruction ☐
- 0 = No obstruction
- 1 = Asymptomatic, endoscopic or radiographic findings only
- 2 = Symptomatic, no hydronephrosis, sepsis or renal dysfunction; no intervention
- 3 = Symptomatic, altered organ function e.g. hydronephrosis or renal dysfunction, operative intervention indicated
- 4 = Life-threatening consequences, organ failure or operative intervention

Creatinine / acute kidney injury ☐
- 0 = Normal
- 1 = Creatinine 1 – 1.5 above baseline
- 2 = Creatinine >1.5 – 3 times above baseline
- 3 = >3 times above baseline
- 4 = > 6 x upper limit of nomal

Glomerular Filtration Rate/ chronic kidney disease ☐
- 0 = Normal
- 1 = eGFR (estimated Glomerular Filtration Rate) or CrCl (creatinine clearance) <LLN – 60 ml/min
- 2 = eGFR or CrCl 59 - 30 ml/min
- 3 = eGFR or CrCl 29 - 15 ml/min
- 4 = eGFR or CrCl <15 ml/min; dialysis or renal transplant indicated

Sexual Dysfunction *Also for patients with hormonal therapy*

Libido ☐
- 0 = Normal
- 1 = Decrease in interest, not affecting relationship
- 2 = Decrease in interest, adversely affecting relationship
- 9 = Did not wish to answer

Erectile Dysfunction ☐
- 0 = None
- 1 = Decrease in erectile function (i.e. frequency/ rigidity of erections) but intervention not indicated (e.g. medication or use of mechanical device, penile pump)
- 2 = Decrease in erectile function (i.e. frequency/ rigidity of erections) and erectile aids are indicated
- 3 = Decrease in erectile function (i.e. frequency/ rigidity of erections) but erectile aids not helpful and penile prosthesis indicated

Ejaculation Disorder ☐
- 0 = None
- 1 = Diminished ejaculation
- 2 = Anejaculation or retrograde ejaculation

Orgasmic Dysfunction ☐
- 0 = None
- 1 = Decrease in orgasmic response not adversely affecting relationship
- 2 = Decrease in orgasmic response and adversely affecting relationship

Other

Radiation Dermatitis ☐
- 0 = None
- 1 = Faint erythema or dry desquamation
- 2 = Moderate to brisk erythema; patchy moist desquamation, mostly confined to skin folds and creases; moderate edema
- 3 = Moist desquamation in areas other than skin folds and creases; bleeding induced by minor trauma or abrasion
- 4 = Life-threatening consequences; skin necrosis or ulceration of full thickness dermis; spontaneous bleeding from involved site; skin graft indicated

Pneumonitis **(must be discussed with a physician)** ☐
- 0 = None
- 1 = Asymptomatic; clinical or diagnostic observations only; intervention not indicated
- 2 = Symptomatic; medical intervention indicated; limiting instrumental ADL
- 3 = Severe symptoms; limiting self care ADL; oxygen indicated
- 4 = Life-threatening respiratory compromise; urgent intervention indicated (e.g., tracheotomy or intubation)

If information: **Death** = 5; **Not Known** fill boxes with 9's, if **Not Applicable** fill boxes with 8's

Lung

(

L2a

LUNG PATIENT FACTORS – BASELINE
(to be completed pre-radiotherapy)

Study Number RQ -

Patient Initials

Date of Birth (dd/mm/yyyy) / /

Date Completed (dd/mm/yyyy) / /

Name + Signature of Person completing the CRF _____

Patient Information

Gender 1=Male 2=Female

Height (cm)

Weight at cancer diagnosis (kg)

Age at start of radiotherapy (yrs)

Smoker 0=Never 1=Ex before cancer diagnosis 2=Ex since cancer diagnosis 3=Current 7=Do not wish to answer

If ever smoker
Duration of smoking (yrs)
No. of tobacco products a day
Tobacco product _____

If ex smoker before cancer diagnosis: Time since quitting smoking (yrs)

Alcohol intake 0=Never 1=Previously consumed alcohol, but stopped BEFORE cancer diagnosis 2=Previously consumed alcohol, but stopped AT cancer diagnosis 3=Current 7=Do not wish to answer

Previous alcohol consumption: Approximate number of alcoholic drinks a week 777=Do not wish to answer 888=Not applicable

Current alcohol consumption: Approximate number of alcoholic drinks a week 777=Do not wish to answer 888=Not applicable

If female:

Menopausal status at time of cancer diagnosis 1=Pre 2=Post 3=Peri

If postmenopausal, age of menopause (yrs)

If postmenopausal, use of menopausal hormone replacement therapy? 0=No 1=Yes

Diabetes	0=No 1=Yes	If yes, duration (yrs)
Rheumatoid Arthritis	0=No 1=Yes	If yes, duration (yrs)
Systemic Lupus Erythematosus	0=No 1=Yes	If yes, duration (yrs)
Other collagen vascular disease	0=No 1=Yes	If yes, duration (yrs)
Hypertension	0=No 1=Yes	If yes, duration (yrs)
History of heart disease	0=No 1=Yes	If yes, duration (yrs)
Depression	0=No 1=Yes	If yes, duration (yrs)
Tuberculosis of the lung	0=No 1=Yes	If yes, duration (yrs)
COPD (GOLD)	0=No 1=Yes	If yes, duration (yrs)

Medication at cancer diagnosis

On statin?	0=No 1=Yes	If yes, duration (yrs)
On other lipid-lowering drugs?	0=No 1=Yes	If yes, duration (yrs)
On ACE inhibitor?	0=No 1=Yes	If yes, duration (yrs)
On other anti-hypertensive drug?	0=No 1=Yes	If yes, duration (yrs)
On amiodarone?	0=No 1=Yes	If yes, duration (yrs)
On anti-diabetic drug?	0=No 1=Yes	If yes, duration (yrs)
On oral steroids?	0=No 1=Yes	If yes, duration (yrs)
On analgesics?	0=No 1=Yes	If yes, duration (yrs)
On anti-depressant?	0=No 1=Yes	If yes, duration (yrs)
On immunosuppressant?	0=No 1=Yes	If yes, duration (yrs)

Type of immunosuppressant _____

Family history of lung cancer in first degree relative	0=No 1=Yes	Family history of radiotherapy toxicity

0=No
1=Yes
9=Not known

Other co-morbidity _____

Ethnicity

1=White (European or American European)
2=White and Black Caribbean Mixed
3=White and Black African Mixed
4=White and Asian Mixed
5=Hispanic American
6=Turkish
7=Indian
8=Pakistani
9=Bangladeshi
10=Chinese
11=Japanese
12=Other Asian
13=Black Caribbean
14=Black African
15=Northern African
16=African American
17=Jewish Ashkenazi
18=Jewish Sephardi
19=Any Other Ethnic Background; please specify _____
77=Patient refused to give answer

Highest educational/professional qualification received

1=Primary school
2=Secondary school (Please selection an option_____)
3=Professional school (e.g. technical. Please specify type_____)
4=University (or equivalent)
5=Others, please specify _____
7=Do not wish to answer

Options for "Secondary school":
a. UK: GSCE / O level
b. UK: A level
c. US: High school
d. B: Algemeen Secundair Onderwijs
e. GER: Hauptschule
f. GER: Realschule/Mittlere Reife
g. GER: Gymnasium/Abitur
h. CH: Realschule
i. CH: Sekundarschule
j. CH: Gymnasium / Matura
k. F: college
l. F: lycée/baccalaureate
m. I: scuola secondaria di primo grado
n. I: scuola secondaria di secondo grado
o. NL: voortgezet onderwijs
p. SP: Educación Secundaria Obligatoria/Bachillerato
q. Other, please specify _____

Net household income (average) per month

1=<1.000 €
2=1.000-<2.000€
3=2.000-<3.000€
4=3.000-<4.000€
5=4.000-<5.000€
6=5.000-<6.000€
7=6.000-<7.000€
8=7.000-<8.000€
9=8.000€ and higher
77= Do not wish to answer

Number of household members

L4

HEALTH PROFESSIONAL LUNG TOXICITY DATA

To be completed by the Doctor or Research Nurse ONLY

Study Number RQ☐☐☐☐☐-☐

Patient Initials ☐☐☐☐

Date of Birth (dd/mm/yyyy) ☐☐/☐☐/☐☐☐☐

Date Completed (dd/mm/yyyy) ☐☐/☐☐/☐☐☐☐

Name + Signature of Person completing the CRF _____

Time Point

☐ Pre-radiotherapy (RT) ☐ 1 year after RT start
☐ 3 months after RT start ☐ 2 years after RT start
☐ 6 months after RT start ☐ 3 years after RT start*

* if available

If information is **Not Known** fill boxes with 9's, if **Not Applicable** with 8's

Lung function test

FEV1 (% predicted) ☐☐☐•☐

FEV1 (litres) ☐☐☐•☐

DLCO (% predicted) ☐☐☐•☐

KCO **or** DLCO/VA (% predicted) ☐☐☐•☐

Steroid use since last visit ☐
0 = None
1 = Oral
2 = Intravenous

If yes, please specify:
Drug name _____
Start dose (mg/day) _____

Duration of steroid use (days) ☐☐☐

Cough ☐
0 = None
1 = Mild symptoms; non-prescription intervention indicated
2 = Moderate symptoms; medical intervention indicated; limiting instrumental activities of daily living (ADL)
3 = Severe symptoms; limiting self care ADL

Dyspnoea ☐
0 = None
1 = Shortness of breath with moderate exertion
2 = Shortness of breath with minimal exertion; limiting instrumental ADL
3 = Shortness of breath at rest; limiting self care ADL

Study Number: RQ☐☐☐☐☐-☐

If information is **Not Known** fill boxes with 9's, if **Not Applicable** with 8's

Broncho-pulmonary haemorrhage ☐
- 0 = None
- 1 = Mild symptoms; intervention not indicated
- 2 = Moderate symptoms; medical intervention indicated
- 3 = Transfusion, radiologic, endoscopic, or operative intervention indicated (e.g. hemostasis of bleeding site)
- 4 = Life-threatening respiratory or hemodynamic compromise; intubation or urgent intervention Indicated

Chest wall pain ☐
- 0 = None
- 1 = Mild pain
- 2 = Moderate pain; limiting instrumental activities of daily living (ADL)
- 3 = Severe pain; limiting self care ADL

Pneumonitis **(must be discussed with a physician)** ☐
- 0 = None
- 1 = Asymptomatic; clinical or diagnostic observations only; intervention not indicated
- 2 = Symptomatic; medical intervention indicated; limiting instrumental ADL
- 3 = Severe symptoms; limiting self care ADL; oxygen indicated
- 4 = Life-threatening respiratory compromise; urgent intervention indicated (e.g., tracheotomy or intubation)

Pulmonary fibrosis ☐
- 0 = None
- 1 = Mild hypoxemia; radiologic pulmonary fibrosis <25% of lung volume
- 2 = Moderate hypoxemia; evidence of pulmonary hypertension; radiographic pulmonary fibrosis 25-50%
- 3 = Severe hypoxemia; evidence of right-sided heart failure; radiographic pulmonary fibrosis >50-75%
- 4 = Life-threatening consequences (e.g., hemodynamic/pulmonary complications); intubation with ventilatory support indicated; radiographic pulmonary fibrosis >75% with severe honeycombing

Bronchial fistula ☐
- 0 = None
- 1 = Asymptomatic; clinical or diagnostic observations only; intervention not indicated
- 2 = Symptomatic; tube thoracostomy or medical management indicated; limiting instrumental ADL
- 3 = Severe symptoms; limiting self care ADL; endoscopic or operative intervention indicated (e.g., stent or primary closure)
- 4 = Life-threatening consequences; urgent operative intervention with thoracoplasty, chronic open drainage or multiple thoracotomies indicated

Bronchial stricture ☐
- 0 = None
- 1 = Asymptomatic; clinical or diagnostic observations only; intervention not indicated
- 2 = Symptomatic (e.g., rhonchi or wheezing) but without respiratory distress; medical intervention indicated (e.g., steroids, bronchodilators)
- 3 = Shortness of breath with stridor; endoscopic intervention indicated (e.g., laser, stent placement)
- 4 = Life-threatening respiratory or hemodynamic compromise; intubation or urgent intervention Indicated

Trachial fistula ☐
- 0 = None
- 1 = Asymptomatic; clinical or diagnostic observations only; intervention not indicated
- 2 = Symptomatic; tube thoracostomy or medical management indicated; limiting instrumental ADL
- 3 = Severe symptoms; limiting self care ADL; endoscopic or operative intervention indicated (e.g., stent or primary closure)
- 4 = Life-threatening consequences; urgent operative intervention with thoracoplasty, chronic open drainage or multiple thoracotomies indicated

Myocardial infarction ☐
- 0 = None
- 2 = Asymptomatic and cardiac enzymes minimally abnormal and no evidence of ischemic ECG changes
- 3 = Severe symptoms; cardiac enzymes abnormal; hemodynamically stable; ECG changes consistent with infarction
- 4 = Life-threatening consequences; hemodynamically unstable

Myocarditis ☐
- 0 = None
- 1 = Asymptomatic with laboratory (e.g., BNP [B-Natriuretic Peptide]) or cardiac imaging abnormalities
- 2 = Symptoms with mild to moderate activity or exertion
- 3 = Severe with symptoms at rest or with minimal activity or exertion; intervention indicated
- 4 = Life-threatening consequences; urgent intervention indicated (e.g., continuous IV therapy or mechanical hemodynamic support)

Pericardial effusion ☐
- 0 = None
- 2 = Asymptomatic effusion size small to moderate
- 3 = Effusion with physiologic consequences
- 4 = Life-threatening consequences; urgent intervention indicated

Pericarditis ☐
- 0 = None
- 1 = Asymptomatic, ECG or physical findings (e.g., rub) consistent with pericarditis
- 2 = Symptomatic pericarditis (e.g., chest pain)
- 3 = Pericarditis with physiologic consequences (e.g., pericardial constriction)
- 4 = Life-threatening consequences; urgent intervention indicated

Radiation dermatitis ☐
- 0 = None
- 1 = Faint erythema or dry desquamation
- 2 = Moderate to brisk erythema; patchy moist desquamation, mostly confined to skin folds and creases; moderate edema
- 3 = Moist desquamation in areas other than skin folds and creases; bleeding induced by minor trauma or abrasion
- 4 = Life-threatening consequences; skin necrosis or ulceration of full thickness dermis; spontaneous bleeding from involved site; skin graft indicated

Comments_____

If information is **Not Known** fill boxes with 9's, if **Not Applicable** with 8's

Trachial stenosis

0 = None
1 = Asymptomatic; clinical or diagnostic observations only; intervention not indicated
2 = Symptomatic (e.g., noisy airway breathing) but without respiratory distress; medical intervention indicated (e.g., steroids)
3 = Stridor or respiratory distress limiting self care activities of daily living (ADL); endoscopic intervention indicated (e.g. stent, laser)
4 = Life-threatening airway compromise; urgent intervention indicated (e.g., tracheotomy or intubation)

Brachial plexopathy

0 = None
1 = Asymptomatic; clinical or diagnostic observations only; intervention not indicated
2 = Moderate symptoms; limiting instrumental ADL
3 = Severe symptoms; limiting self care ADL

Myelitis

0 = None
1 = Asymptomatic; mild signs (e.g., Babinski's reflex or Lhermitte's sign)
2 = Moderate weakness or sensory loss; limiting instrumental ADL
3 = Severe weakness or sensory loss; limiting self care ADL
4 = Life-threatening consequences; urgent intervention indicated

Esophagitis

0 = None
1 = Asymptomatic; clinical or diagnostic observations only; intervention not indicated
2 = Symptomatic; altered GI function
3 = Severely altered GI function; tube feeding; hospitalization indicated; elective operative intervention indicated
4 = Life-threatening consequences; urgent operative intervention indicated

Dysphagia

0 = None
1 = Symptomatic, able to eat regular diet
2 = Symptomatic and altered eating/swallowing
3 = Severely altered eating/swallowing; tube feeding or total parenteral nutrition (TPN) or hospitalization indicated
4 = Life-threatening consequences; urgent operative intervention indicated

Esophageal fistula

0 = None
1 = Asymptomatic; clinical or diagnostic observations only; intervention not indicated
2 = Symptomatic; altered GI function
3 = Severely altered GI function; tube feeding, total parenteral nutrition (TPN) or hospitalization indicated; elective operative intervention indicated
4 = Life-threatening consequences; urgent intervention indicated

Breast

B2a

BREAST PATIENT FACTORS – BASELINE
(to be completed pre-radiotherapy)

Study Number RQ☐☐☐☐☐-☐

Patient Initials ☐☐☐☐

Date of Birth (dd/mm/yyyy) ☐☐/☐☐/☐☐☐☐

Date Completed (dd/mm/yyyy) ☐☐/☐☐/☐☐☐☐

Name + Signature of Person completing the CRF _____

Height (cm) ☐☐☐

Weight at cancer diagnosis (kg) ☐☐☐

Age at start of radiotherapy (yrs) ☐☐

Bra cup size ☐
1=AA
2=A
3=B
4=C
5=D
6=E/DD
7=F (E Italy)
8=G (F Italy
9=H (FF Italy)
10=J (G Italy)
11> J (G Italy)

Band size ☐
1=28 (UK)
2=30 (UK)
3=32 (UK) 70 (EU) 85 (Fr) 1 (It)
4=34 (UK) 75 (EU) 90 (Fr) 2 (It)
5=36 (UK) 80 (EU) 95 (Fr) 3 (It)
6=38 (UK) 85 (EU) 100 (Fr) 4 (It)
7=40 (UK) 90 (EU) 105 (Fr) 5 (It)
8=42 (UK) 95 (EU) 110 (Fr) 6 (It)
9=44 (UK) 100 (EU) 115 (Fr) 7 (It)
10>above

Smoker ☐
0=Never
1=Ex before cancer diagnosis
2=Ex since cancer diagnosis
3=Current
7=Do not wish to answer

If ever smoker
Duration of smoking (yrs) ☐☐
No. of tobacco products a day ☐☐☐
Tobacco product _____

If ex smoker before cancer diagnosis: Time since quitting smoking (yrs) ☐☐

Alcohol intake ☐
0=Never
1=Previously consumed alcohol, but stopped BEFORE cancer diagnosis
2=Previously consumed alcohol, but stopped AT cancer diagnosis
3=Current
7=Do not wish to answer

Previous alcohol consumption: Approximate number of alcoholic drinks a week ☐☐☐
777=Do not wish to answer
888=Not applicable

Current alcohol consumption: Approximate number of alcoholic drinks a week ☐☐☐
777=Do not wish to answer
888=Not applicable

Menopausal status at time of cancer diagnosis	☐	1=Pre 2=Post 3=Peri	If postmenopausal, age of menopause (yrs)	☐☐
			If postmenopausal, use of menopausal hormone replacement therapy?	☐ 0=No 1=Yes
Diabetes	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
History of heart disease	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
Rheumatoid Arthritis	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
Systemic Lupus Erythematosus	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
Other collagen vascular disease	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
Hypertension	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
Depression	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
Medication at cancer diagnosis				
On anti-diabetic drug?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
On ACE inhibitor?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
On other anti-hypertensive drug?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
On statin?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
On other lipid-lowering drugs?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
On amiodarone?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
On analgesics?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
On anti-depressant?	☐	0=No 1=Yes	If yes, duration (yrs)	☐☐
Family history of breast cancer in first degree relative	☐	0=No 1=Yes	Family history of radiotherapy toxicity	☐ 0=No 1=Yes 9=Not known

Other co-morbidity _____

Previous Malignancies? ☐ 0=No 1=Yes

Which type?

ICD-10 / ICD-O-3 coding: ☐☐☐.☐ ☐☐☐☐/☐

Date of diagnosis (dd/mm/yyyy) ☐☐/☐☐/☐☐☐☐

Therapy received for previous malignancy

Surgery	☐	0=No 1=Yes	Hormonal therapy	☐	0=No 1=Yes	Chemo therapy	☐	0=No 1=Yes	Radic thera
Other therapy	☐	0=No 1=Yes	No therapy	☐	0=No 1=Yes			

Date of last therapy for previous malignancy (dd/mm/yyyy) ☐☐/☐☐/☐☐☐

Ethnicity ☐☐

1=White (European or American European)
2=White and Black Caribbean Mixed
3=White and Black African Mixed
4=White and Asian Mixed
5=Hispanic American
6=Turkish
7=Indian
8=Pakistani
9=Bangladeshi
10=Chinese
11=Japanese
12=Other Asian
13=Black Caribbean
14=Black African
15=Northern African
16=African American
17=Jewish Ashkenazi
18=Jewish Sephardi
19=Any Other Ethnic Background; please specify _____
77=Patient refused to give answer

Highest educational/professional qualification received

☐

1=Primary school
2=Secondary school (Please select an option _____)
3=Professional school (e.g. technical. Please specify type _____)
4=University (or equivalent)
5=Others, please specify _____
7=Do not wish to answer

Options for "Secondary school":

a. *UK: GSCE / O level*
b. *UK: A level*
c. *US: High school*
d. *B: Algemeen Secundair Onderwijs*
e. *GER: Hauptschule*
f. *GER: Realschule/Mittlere Reife*
g. *GER: Gymnasium/Abitur*
h. *CH: Realschule*
i. *CH: Sekundarschule*
j. *CH: Gymnasium / Matura*
k. *F: college*
l. *F: lycée/baccalaureate*
m. *I: scuola secondaria di primo grado*

Net household income (average) per month

☐☐

1=<1.000 €
2=1.000-<2.000€
3=2.000-<3.000€
4=3.000-<4.000€
5=4.000-<5.000€
6=5.000-<6.000€
7=6.000-<7.000€
8=7.000-<8.000€
9=8.000€ and higher
77= Do not wish to answer

Number of household members ☐☐

REQUITE
Validating Predictive Models and Biomarkers of Radiotherapy Toxicity to Reduce Side-Effects and Improve Quality-of-Life in Cancer Survivors

[redacted]

HEALTH PROFESSIONAL BREAST TOXICITY DATA (CTCAEv4.0)

To be completed by the Doctor or Research Nurse ONLY

Study Number RQ☐☐☐☐☐-☐

Patient Initials ☐☐☐☐

Date of Birth (dd/mm/yyyy) ☐☐/☐☐/☐☐☐☐

Date Completed (dd/mm/yyyy) ☐☐/☐☐/☐☐☐☐

Name + Signature of Person completing the CRF _____

Time Point

☐ Pre-radiotherapy (RT)
☐ End of RT
☐ 6 weeks after end of RT*
☐ 1 year after RT start
☐ 2 years after RT start
☐ 3 years after RT start*
☐ 4 years after RT start*
* if available

If information is **Not Known** fill boxes with 9's, if **Not Applicable** with 8's

Is there atrophy within the treated breast? ☐
- 0 = None
- 1 = Minimal asymmetry; minimal atrophy
- 2 = Moderate asymmetry; moderate atrophy
- 3 = Asymmetry >1/3 of breast volume; severe atrophy

Is there any nipple retraction of the treated breast? ☐
- 0 = None
- 1 = Asymptomatic; asymmetry with slight retraction and/or thickening of the nipple areolar complex
- 2 = Symptomatic; asymmetry of nipple areolar complex with moderate retraction and/or thickening of the nipple areolar complex

Is there oedema of the treated breast? ☐
- 0 = None
- 1 = Swelling or obscuration of anatomic architecture on close inspection
- 2 =Readily apparent obscuration of anatomic architecture; obliteration of skin folds; readily apparent deviation from normal anatomic contour; limiting instrumental activities of daily living (ADL)
- 3= Gross deviation from normal anatomic contour; limiting self care ADL

Is there any skin ulceration? ☐
- 0 = None
- 1 = Combined area of ulcers <1cm nonblanchable erythema of intact skin with associated warmth or edema
- 2 = Combined area of ulcers 1-2cm; partial thickness skin loss involving skin or subcutaneous fat
- 3 = Combined area of ulcers >2cm; full thickness skin loss involving damage to or necrosis of subcutaneous tissue that may extend down to fascia
- 4 = Any ulcer size with extensive destruction, tissue necrosis, or damage to muscle, bone, or supporting structures with or without full thickness skin loss

Study Number RQ☐☐☐☐☐-☐

Is there any telangiectasia of the tumour bed?
0 = None
1 = Telangiectasia covering <10% of the treated breast
2 = Telangiectasia covering >10% of the treated breast; associated with psychosocial impact ☐

Is there any telangiectasia outside the tumour bed?
0 = None
1 = Telangiectasia covering <10% of the treated breast
2 = Telangiectasia covering >10% of the treated breast; associated with psychosocial impact ☐

Is there any skin induration (fibrosis) of the tumour bed?
0 = None
1 = Mild induration, able to move skin parallel to plane (sliding) and perpendicular to skin (pinching up)
2 = Moderate induration, able to slide skin, unable to pinch skin, limiting instrumental activities for daily living (ADL)
3 = Severe induration; unable to slide or pinch skin; affecting activities for daily living; limiting self care ADL ☐

Is there any skin induration (fibrosis) outside the tumour bed?
0 = None
1 = Mild induration, able to move skin parallel to plane (sliding) and perpendicular to skin (pinching up)
2 = Moderate induration, able to slide skin, unable to pinch skin, limiting instrumental activities for daily living (ADL)
3 = Severe induration; unable to slide or pinch skin; affecting activities for daily living; limiting self care ADL ☐

Erythema
0 = None
1 = Faint erythema or dry desquamation
2 = Moderate to brisk erythema; patchy moist desquamation, mostly confined to skin folds and creases; moderate oedema
3 = Moist desquamation in areas other than skin folds and creases; bleeding induced by minor trauma or abrasion
4 = Life-threatening consequences; skin necrosis or ulceration of full thickness dermis; spontaneous bleeding from involved site; skin graft indicated ☐

Arm lymphoedema
0 = None
1 = 5-10% inter limb discrepancy in volume or circumference at point of greatest visible difference; swelling or obscuration of anatomic architecture on close inspection
2 = >10-30% inter limb discrepancy in volume or circumference at point of greatest visible difference; Readily apparent obscuration of anatomic architecture; obliteration of skin folds; readily apparent deviation from normal anatomic contour; limiting instrumental activities of daily living (ADL)
3 = > 30% inter-limb discrepancy in volume; gross deviation from normal anatomic contour; limiting self care ADL ☐

Skin hyperpigmentation
0 = None
1 = Hyperpigmentation or depigmentation covering <10% body surface area (BSA); no psychosocial impact
2 = Hyperpigmentation or depigmentation covering >10% BSA; associated with psychosocial impact ☐

Pneumonitis
0 = None
1 = Asymptomatic; clinical or diagnostic observations only; intervention not indicated
2 = Symptomatic; medical intervention indicated; limiting instrumental activities of daily living (ADL)
3 = Severe symptoms; limiting self care ADL; oxygen indicated
4 = Life-threatening respiratory compromise; urgent intervention indicated (e.g., tracheotomy or intubation) ☐

Questions for the patient

Pain

Have you had any pain in your treated breast in the last two weeks? ☐ 1=Yes 0=No

If yes, how severe is the pain? ☐ 1=Mild 2=Moderate, limiting usual activities 3=Severe, stopping activities

Are you taking any medication for this pain? ☐ 0=No 1=Yes

If yes, please give name of medication and how often you take this.

drug name _____

dose and frequency _____

Swollen arm

Do you have a swollen arm? ☐ 0=No 1=Yes

If yes, does your swollen arm interfere with normal activity? ☐ 1=No 2=Limiting activity 3=Limiting self care

---

Mid-upper arm circumference

Left arm (mm) ☐☐☐

Right arm (mm) ☐☐☐

☐☐/☐☐/☐☐☐☐ Measuring Date (dd/mm/yyyy)

If information is **Not Known** fill boxes with 9's, if **Not Applicable** with 8's

A.3 Munich Chronotype Questionnaire

PATIENT QUESTIONNAIRE – Chronotype

REQUITE
Validating Predictive Models and Biomarkers of Radiotherapy Toxicity to Reduce Side-Effects and Improve Quality-of-Life in Cancer Survivors

Chrono

Study Number RQ□□□□□-□

Patient Initials □□□□

Date of Birth (dd/mm/yyyy) □□/□□/□□□□

Date Completed (dd/mm/yyyy) □□/□□/□□□□

Chronotype Questionnaire

for Non Shift Workers

Instructions: The following pages contain several questions about your sleep wake cycle.
Please only complete if you are currently retired/unemployed or work a regular work pattern between 06:00 and 20:00

Do **NOT** complete this questionnaire if you regularly work shifts outside of the above timeframe (06:00 – 20:00)

Munich Chronotype questionnaire for non-shift workers

WORK DAYS

1. Are you currently working? (Includes housewife/househusband) Yes ☐ No ☐
2. Do you work shifts? Yes ☐ No ☐

   If "no" continue to question 3.

   If “yes” then you do not need to complete any further questions.
3. Do you have a regular work schedule? Yes ☐ No ☐
4. How many days a week do you work? ……
5. What time do you go to bed? …….. (Please use 24 hour clock)
6. What time do you actually go to sleep? …….. (Please use 24 hour clock)
7. How many minutes do you take to fall asleep? ……..
8. What time do you wake up? …….. (Please use 24 hour clock)
9. Do you use an alarm clock (answer yes even if you are awake before your alarm clock) Yes ☐ No ☐
10. How many minutes after you wake up do you get up? …….

FREE DAYS

11. What time do you go to bed? …….. (Please use 24 hour clock)
12. What time do you actually go to sleep? …….. (Please use 24 hour clock)
13. How many minutes do you take to fall asleep? ……..
14. What time do you wake up? …….. (Please use 24 hour clock)
15. Do you use an alarm clock (answer yes even if you are awake before your alarm clock) Yes ☐ No ☐

    How many minutes after you wake up do you get up? …….

DAYLIGHT

16. How many hours/mins a day do you spend outdoors in daylight (complete for the current season) ……..

Comments – please use this section to inform us if there are any reasons why you are not able to get up when you choose e.g. Children/dependents, pets. Also please let us know if you take any medication which affects your sleep pattern e.g. sleeping tablets, steroids.

……
……
……
……
……
……

A.4 List of solutions

Reagent	Preparation/storage
PI:	20ml ddH20 and 100ul PI (2mg/ml kept in fridge)
Electrophoresis Buffer:	
Stock solutions:	10M NaOH [200g in 500ml], 200mM Na2EDTA [7.4g in 100ml]
Working solution:	600mls ice cold ddH20, 60mls 10M NaOH, 10mls 200mM NaEDTA then add ddH20 up to 2L.
Comet Lysis Buffer	
Stock solution:	2.5M NaCl [146.1g]; 100mM Na2EDTA [37.2g]; 10mM Tris [1.2g]; make up to 1L with ddH2O and set pH to 10.0 with 10M NaOH.
Working solution:	Add 1% Triton X-100.
Neutralisation buffer *:*	
Working solution:	0.4M Tris [48.5g]; make up to 1L with ddH2O. Set pH to 7.5 with concentrated HCl.
0.8 % LMP agarose	(0.8 g in 100 ml PBS) Heat in microwave until agarose completely melted.
1 % NMP agarose	(0.5 g in 50 m ddH20) Heat in microwave until agarose completely melted.
PBS	Solid tablets are constituted in deionised water at one tablet per 200ml. This creates a solution of concentration

	0.01M. Solution is autoclaved after the tablets have fully dissolved after which it is considered sterile.
BD FACS Lysing solution	Solution is diluted in a 10:1 ratio with distilled deionised water. This preparation is performed in a tissue culture hood to avoid contamination of solution.
RMPI Culture medium	Stored at 4C in a dark cold room, this is combined with activated Foetal bovine serum (FBS) (20%). 24 hours prior to use it is brought to room temperature. The FBS is stored at -20 degrees until added to the RPMI solution. This solution is optimised for the culture of lymphocytes in vitro.
Virkon	One tablet per 500ml water used in the decontamination of blood products. Solution can be disposed of 24hours after mixed with blood products.
RNA-ase	Store at room temperature with a new stock prepared monthly. Solution prepared at 10 mg/ml in 10 mM Tris-HCl.
10xTBE	890mM Tris-borate [pH 8.3], 20mM EDTA

DNA loading dye	6x concentration; 3.5g sucrose, 4ml 10xTBE, 0.025g bromophenol blue in a final volume of 10ml Dh20
Ethidium bromide	10mg/ml (dissolved in distilled water)

BIBLIOGRAPHY

Agency, I. A. E. (2010) *Radiation Biology a handbook for teachers and students,* [online], available: [accessed

Alapetite, C., Thirion, P., de la Rochefordiere, A., Cosset, J. M. and Moustacchi, E. (1999) 'Analysis by alkaline comet assay of cancer patients with severe reactions to radiotherapy: defective rejoining of radioinduced DNA strand breaks in lymphocytes of breast cancer patients', *Int J Cancer,* 83(1), 83-90.

Alongi, F., Fiorino, C., Cozzarini, C., Broggi, S., Perna, L., Cattaneo, G. M., Calandrino, R. and Di Muzio, N. (2009) 'IMRT significantly reduces acute toxicity of whole-pelvis irradiation in patients treated with post-operative adjuvant or salvage radiotherapy after radical prostatectomy', *Radiotherapy and Oncology,* 93(2), 207-212.

Amini, A., Kavanagh, B. D. and Rusthoven, C. G. (2016) 'Improved survival with the addition of radiotherapy to androgen deprivation: questions answered and a review of current controversies in radiotherapy for non-metastatic prostate cancer', *Ann Transl Med,* 4(1), 14.

Andreassen, C. N., Rosenstein, B. S., Kerns, S. L., Ostrer, H., De Ruysscher, D., Cesaretti, J. A., Barnett, G. C., Dunning, A. M., Dorling, L., West, C. M., Burnet, N. G., Elliott, R., Coles, C., Hall, E., Fachal, L., Vega, A., Gomez-Caamano, A., Talbot, C. J., Symonds, R. P., De Ruyck, K., Thierens, H., Ost, P., Chang-Claude, J., Seibold, P., Popanda, O., Overgaard, M., Dearnaley, D., Sydes, M. R., Azria, D., Koch, C. A., Parliament, M., Blackshaw, M., Sia, M., Fuentes-Raspall, M. J., Ramon, Y. C. T., Barnadas, A., Vesprini, D., Gutierrez-Enriquez, S., Molla, M., Diez, O., Yarnold, J. R., Overgaard, J., Bentzen, S. M. and Alsner, J. (2016) 'Individual patient data meta-analysis shows a significant association between the ATM rs1801516 SNP and toxicity after radiotherapy in 5456 breast and prostate cancer patients', *Radiother Oncol,* 121(3), 431-439.

Anscher, M. S. (2010) 'Targeting the TGF-β1 Pathway to Prevent Normal Tissue Injury After Cancer Therapy', *The Oncologist,* 15(4), 350-359.

Arcangeli, G., Fowler, J., Gomellini, S., Arcangeli, S., Saracino, B., Petrongari, M. G., Benassi, M. and Strigari, L. (2011) 'Acute and late toxicity in a randomized trial of conventional versus hypofractionated three-dimensional conformal radiotherapy for prostate cancer', *Int J Radiat Oncol Biol Phys,* 79(4), 1013-21.

Ashman, J. B., Zelefsky, M. J., Hunt, M. S., Leibel, S. A. and Fuks, Z. (2005) 'Whole pelvic radiotherapy for prostate cancer using 3D conformal and intensity-modulated radiotherapy', *International Journal of Radiation Oncology* Biology* Physics,* 63(3), 765-771.

Azria, D., Ozsahin, M., Kramar, A., Peters, S., Atencio, D. P., Crompton, N. E., Mornex, F., Pelegrin, A., Dubois, J. B., Mirimanoff, R. O. and Rosenstein, B. S. (2008) 'Single nucleotide polymorphisms, apoptosis, and the development of severe late adverse effects after radiotherapy', *Clin Cancer Res,* 14(19), 6284-8.

Azria, D., Riou, O., Castan, F., Nguyen, T. D., Peignaux, K., Lemanski, C., Lagrange, J. L., Kirova, Y., Lartigau, E., Belkacemi, Y., Bourgier, C., Rivera, S., Noel, G., Clippe, S., Mornex, F., Hennequin, C., Kramar, A., Gourgou, S., Pelegrin, A., Fenoglietto, P. and Ozsahin, E. M. (2015) 'Radiation-induced CD8 T-lymphocyte Apoptosis as a Predictor of Breast Fibrosis After Radiotherapy: Results of the Prospective Multicenter French Trial', *EBioMedicine,* 2(12), 1965-73.

Baldwin, D. R., White, B., Schmidt-Hansen, M., Champion, A. R., Melder, A. M. and Guideline Development, G. (2011) 'Diagnosis and treatment of lung cancer: summary of updated NICE guidance', *BMJ,* 342, d2110.

Banchereau, J., Pascual, V. and O'Garra, A. (2012) 'From IL-2 to IL-37: the expanding spectrum of anti-inflammatory cytokines', *Nat Immunol,* 13(10), 925-931.

Barnett, G. C., Thompson, D., Fachal, L., Kerns, S., Talbot, C., Elliott, R. M., Dorling, L., Coles, C. E., Dearnaley, D. P., Rosenstein, B. S., Vega, A., Symonds, P., Yarnold, J., Baynes, C., Michailidou, K., Dennis, J., Tyrer, J. P., Wilkinson, J. S., Gomez-Caamano, A., Tanteles, G. A., Platte, R., Mayes, R., Conroy, D., Maranian, M., Luccarini, C., Gulliford, S. L., Sydes, M. R., Hall, E., Haviland, J., Misra, V., Titley, J., Bentzen, S. M., Pharoah, P. D., Burnet, N. G., Dunning, A. M. and West, C. M. (2014) 'A genome wide association study (GWAS) providing evidence of an association between common genetic variants and late radiotherapy toxicity', *Radiother Oncol,* 111(2), 178-85.

Barnett, G. C., West, C. M., Coles, C. E., Pharoah, P. D., Talbot, C. J., Elliott, R. M., Tanteles, G. A., Symonds, R. P., Wilkinson, J. S., Dunning, A. M., Burnet, N. G. and Bentzen, S. M. (2012) 'Standardized Total Average Toxicity score: a scale- and grade-independent measure of late radiotherapy toxicity to facilitate pooling of data from different studies', *Int J Radiat Oncol Biol Phys,* 82(3), 1065-74.

Barnett, G. C., West, C. M., Dunning, A. M., Elliott, R. M., Coles, C. E., Pharoah, P. D. and Burnet, N. G. (2009) 'Normal tissue reactions to radiotherapy: towards tailoring treatment dose by genotype', *Nat Rev Cancer,* 9(2), 134-42.

Beasley, M., Driver, D. and Dobbs, H. J. (2005) 'Complications of radiotherapy: improving the therapeutic index', *Cancer Imaging,* 5, 78-84.

Ben-David, M. A., Elkayam, R., Gelernter, I. and Pfeffer, R. M. (2016) 'Melatonin for Prevention of Breast Radiation Dermatitis: A Phase II, Prospective, Double-Blind Randomized Trial', *Isr Med Assoc J,* 18(3-4), 188-92.

Benhusein, G. M., Mutch, E., Aburawi, S. and Williams, F. M. (2010) 'Genotoxic effect induced by hydrogen peroxide in human hepatoma cells using comet assay', *Libyan J Med,* 5.

Bentzen, S. M., Constine, L. S., Deasy, J. O., Eisbruch, A., Jackson, A., Marks, L. B., Ten Haken, R. K. and Yorke, E. D. (2010) 'Quantitative Analyses of Normal Tissue Effects in the Clinic (QUANTEC): an introduction to the scientific issues', *Int J Radiat Oncol Biol Phys,* 76(3 Suppl), S3-9.

Bjarnason, G. A. and Jordan, R. (2002) 'Rhythms in Human Gastrointestinal Mucosa and Skin', *Chronobiol Int,* 19(1), 129-140.

Bjarnason, G. A., Mackenzie, R. G., Nabid, A., Hodson, I. D., El-Sayed, S., Grimard, L., Brundage, M., Wright, J., Hay, J., Ganguly, P., Leong, C., Wilson, J., Jordan, R. C., Walker, M., Tu, D. and Parulekar, W. (2009a) 'Comparison of toxicity associated with early morning versus late afternoon radiotherapy in patients with head-and-neck cancer: a prospective randomized trial of the National Cancer Institute of Canada Clinical Trials Group (HN3)', *Int J Radiat Oncol Biol Phys,* 73(1), 166-72.

Bjarnason, G. A., Mackenzie, R. G., Nabid, A., Hodson, I. D., El-Sayed, S., Grimard, L., Brundage, M., Wright, J., Hay, J., Ganguly, P., Leong, C., Wilson, J., Jordan, R. C., Walker, M., Tu, D., Parulekar, W. and National Cancer Institute of Canada Clinical Trials, G. (2009b) 'Comparison of toxicity associated with early morning versus late afternoon radiotherapy in patients with head-and-neck cancer: a prospective randomized trial of the National Cancer Institute of Canada Clinical Trials Group (HN3)', *Int J Radiat Oncol Biol Phys,* 73(1), 166-72.

Bolus, N. E. (2001) 'Basic review of radiation biology and terminology', *J Nucl Med Technol,* 29(2), 67-73; test 76-7.

Bourton, E. C., Plowman, P. N., Smith, D., Arlett, C. F. and Parris, C. N. (2011) 'Prolonged expression of the gamma-H2AX DNA repair biomarker correlates with excess acute and chronic toxicity from radiotherapy treatment', *Int J Cancer,* 129(12), 2928-34.

Brolinson, P. G. and Elliott, D. (2007) 'Exercise and the immune system', *Clin Sports Med,* 26(3), 311-9.

Brunt, A. M., Wheatley, D., Yarnold, J., Somaiah, N., Kelly, S., Harnett, A., Coles, C., Goodman, A., Bahl, A., Churn, M., Zotova, R., Sydenham, M., Griffin, C. L., Morden, J. P. and Bliss, J. M. (2016) 'Acute skin toxicity associated with a 1-week schedule of whole breast radiotherapy compared with a standard 3-week regimen delivered in the UK FAST-Forward Trial', *Radiother Oncol,* 120(1), 114-8.

Brush, J., Lipnick, S. L., Phillips, T., Sitko, J., McDonald, J. T. and McBride, W. H. (2007) 'Molecular mechanisms of late normal tissue injury', *Semin Radiat Oncol,* 17(2), 121-30.

Cahlon, O., Hunt, M. and Zelefsky, M. J. (2008) 'Intensity-Modulated Radiation Therapy: Supportive Data for Prostate Cancer', *Semin Radiat Oncol,* 18(1), 48-57.

Cameron, I. L. and Greulich, R. C. (1963) 'EVIDENCE FOR AN ESSENTIALLY CONSTANT DURATION OF DNA SYNTHESIS IN RENEWING EPITHELIA OF THE ADULT MOUSE', *J Cell Biol,* 18(1), 31-40.

Chang, J. Y., Senan, S., Paul, M. A., Mehran, R. J., Louie, A. V., Balter, P., Groen, H. J., McRae, S. E., Widder, J., Feng, L., van den Borne, B. E., Munsell, M. F., Hurkmans, C., Berry, D. A., van Werkhoven, E., Kresl, J. J., Dingemans, A. M., Dawood, O., Haasbeek, C. J., Carpenter, L. S., De Jaeger, K., Komaki, R., Slotman, B. J., Smit, E. F. and Roth, J. A. (2015) 'Stereotactic ablative radiotherapy versus lobectomy for operable stage I non-small-cell lung cancer: a pooled analysis of two randomised trials', *Lancet Oncol,* 16(6), 630-7.

Chen, G., Zhou, M., Chen, L., Meng, Z. J., Xiong, X. Z., Liu, H. J., Xin, J. B. and Zhang, J. C. (2016) 'Cigarette Smoke Disturbs the Survival of CD8+ Tc/Tregs

Partially through Muscarinic Receptors-Dependent Mechanisms in Chronic Obstructive Pulmonary Disease', *PLoS One,* 11(1), e0147232.

Chen, Y., Rubin, P., Williams, J., Hernady, E., Smudzin, T. and Okunieff, P. (2001) 'Circulating IL-6 as a predictor of radiation pneumonitis', *International Journal of Radiation Oncology* Biology* Physics,* 49(3), 641-648.

Chen, Y., Zhang, F., Tsai, Y., Yang, X., Yang, L., Duan, S., Wang, X., Keng, P. and Lee, S. O. (2015) 'IL-6 signaling promotes DNA repair and prevents apoptosis in CD133+ stem-like cells of lung cancer after radiation', *Radiat Oncol,* 10, 227.

Chowdhury, D., Choi, Y. E. and Brault, M. E. (2013) 'Charity begins at home: non-coding RNA functions in DNA repair', *Nat Rev Mol Cell Biol,* 14(3), 181-189.

Cozzarini, C. (2015) 'Radiation Induced Lymphocyte Apoptosis: An Effective Way of “Tailoring” Radiotherapy to the Right Patients Only?', *EBioMedicine,* 2(12), 1852-1853.

Cozzarini, C., Fiorino, C., Di Muzio, N., Alongi, F., Broggi, S., Cattaneo, M., Montorsi, F., Rigatti, P., Calandrino, R. and Fazio, F. (2007) 'Significant reduction of acute toxicity following pelvic irradiation with Helical Tomotherapy in patients with localized prostate cancer', *Radiotherapy and Oncology,* 84(2), 164-170.

CRUK (2013) 'CRUK (2013) breast cancer statistics', [online], available: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/breast-cancer [accessed

CRUK (2014) 'Lung cancer statistics', [online], available: http://www.cancerresearchuk.org/cancer-info/cancerstats/types/lung/?script=true [accessed

Darby, S., McGale, P., Correa, C., Taylor, C., Arriagada, R., Clarke, M., Cutter, D., Davies, C., Ewertz, M., Godwin, J., Gray, R., Pierce, L., Whelan, T., Wang, Y. and Peto, R. (2011) 'Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials', *Lancet,* 378(9804), 1707-16.

De Meerleer, G., Vakaet, L., Meersschout, S., Villeirs, G., Verbaeys, A., Oosterlinck, W. and De Neve, W. (2004) 'Intensity-modulated radiotherapy as primary

treatment for prostate cancer: acute toxicity in 114 patients', *Int J Radiat Oncol Biol Phys,* 60(3), 777-87.

Deville, C., Both, S., Hwang, W. T., Tochner, Z. and Vapiwala, N. (2010) 'Clinical toxicities and dosimetric parameters after whole-pelvis versus prostate-only intensity-modulated radiation therapy for prostate cancer', *Int J Radiat Oncol Biol Phys,* 78(3), 763-72.

Dong, L., Cui, J., Tang, F., Cong, X. and Han, F. (2015) 'Ataxia telangiectasia-mutated gene polymorphisms and acute normal tissue injuries in cancer patients after radiation therapy: a systematic review and meta-analysis', *Int J Radiat Oncol Biol Phys,* 91(5), 1090-8.

Donovan, J. L., Hamdy, F. C., Lane, J. A., Mason, M., Metcalfe, C., Walsh, E., Blazeby, J. M., Peters, T. J., Holding, P., Bonnington, S., Lennon, T., Bradshaw, L., Cooper, D., Herbert, P., Howson, J., Jones, A., Lyons, N., Salter, E., Thompson, P., Tidball, S., Blaikie, J., Gray, C., Bollina, P., Catto, J., Doble, A., Doherty, A., Gillatt, D., Kockelbergh, R., Kynaston, H., Paul, A., Powell, P., Prescott, S., Rosario, D. J., Rowe, E., Davis, M., Turner, E. L., Martin, R. M. and Neal, D. E. 'Patient-Reported Outcomes after Monitoring, Surgery, or Radiotherapy for Prostate Cancer', *New England Journal of Medicine,* 0(0), null.

Douris, N., Kojima, S., Pan, X., Lerch-Gaggl, A. F., Duong, S. Q., Hussain, M. M. and Green, C. B. (2011) 'Nocturnin regulates circadian trafficking of dietary lipid in intestinal enterocytes', *Curr Biol,* 21(16), 1347-55.

Dunn, J. S. and Taylor, C. E. (2014) 'Cardiovascular reactivity to stressors: effect of time of day?', *Chronobiol Int,* 31(2), 166-74.

Early Breast Cancer Trialists' Collaborative, G. (2005) 'Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials', *The Lancet,* 366(9503), 2087-2106.

Emami, B., Lyman, J., Brown, A., Coia, L., Goitein, M., Munzenrider, J. E., Shank, B., Solin, L. J. and Wesson, M. (1991) 'Tolerance of normal tissue to therapeutic irradiation', *Int J Radiat Oncol Biol Phys,* 21(1), 109-22.

Fachal, L., Gomez-Caamano, A., Barnett, G. C., Peleteiro, P., Carballo, A. M., Calvo-Crespo, P., Kerns, S. L., Sanchez-Garcia, M., Lobato-Busto, R., Dorling, L., Elliott, R. M., Dearnaley, D. P. and Sydes, M. R. (2014) 'A three-

stage genome-wide association study identifies a susceptibility locus for late radiotherapy toxicity at 2q24.1', *Nat Genet,* 46(8), 891-4.

Foro, P., Algara, M., Lozano, J., Rodriguez, N., Sanz, X., Torres, E., Carles, J., Reig, A., Membrive, I., Quera, J., Fernandez-Velilla, E., Pera, O., Lacruz, M. and Bellosillo, B. (2014) 'Relationship between radiation-induced apoptosis of T lymphocytes and chronic toxicity in patients with prostate cancer treated by radiation therapy: a prospective study', *Int J Radiat Oncol Biol Phys,* 88(5), 1057-63.

Fowler, J. F. (1988) 'Intervals between multiple fractions per day. Differences between early and late radiation reactions', *Acta Oncol,* 27(2), 181-3.

Fu, L., Pelicano, H., Liu, J., Huang, P. and Lee, C. (2002) 'The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo', *Cell,* 111(1), 41-50.

Gomes, A. M., Barber, R. C. and Dubrova, Y. E. (2015) 'Paternal irradiation perturbs the expression of circadian genes in offspring', *Mutat Res,* 775, 33-7.

Guan, L., Suggs, A., Ahsanuddin, S., Tarrillion, M., Selph, J., Lam, M. and Baron, E. (2016) '2016 Arte Poster Competition First Place Winner: Circadian Rhythm and UV-Induced Skin Damage: An In Vivo Study', *J Drugs Dermatol,* 15(9), 1124-30.

Gupta, D., Shukla, P., Munshi, A. and Aggarwal, J. P. (2012) 'Cardioprotective radiotherapy: the circadian way', *Med Hypotheses,* 78(3), 353-5.

Hamdy, F. C., Donovan, J. L., Lane, J. A., Mason, M., Metcalfe, C., Holding, P., Davis, M., Peters, T. J., Turner, E. L., Martin, R. M., Oxley, J., Robinson, M., Staffurth, J., Walsh, E., Bollina, P., Catto, J., Doble, A., Doherty, A., Gillatt, D., Kockelbergh, R., Kynaston, H., Paul, A., Powell, P., Prescott, S., Rosario, D. J., Rowe, E. and Neal, D. E. (2016) '10-Year Outcomes after Monitoring, Surgery, or Radiotherapy for Localized Prostate Cancer', *New England Journal of Medicine,* (375), 1415-1424.

Haviland, J. S., Owen, J. R., Dewar, J. A., Agrawal, R. K., Barrett, J., Barrett-Lee, P. J., Dobbs, H. J., Hopwood, P., Lawton, P. A., Magee, B. J., Mills, J., Simmons, S., Sydenham, M. A., Venables, K., Bliss, J. M. and Yarnold, J. R. (2013) 'The UK Standardisation of Breast Radiotherapy (START) trials of radiotherapy hypofractionation for treatment of early breast cancer: 10-year

follow-up results of two randomised controlled trials', *The Lancet Oncology,* 14(11), 1086-1094.

Herbert, T. B. and Cohen, S. (1993) 'Depression and immunity: a meta-analytic review', *Psychol Bull,* 113(3), 472-86.

Hickey, B. E., James, M. L., Lehman, M., Hider, P. N., Jeffery, M., Francis, D. P. and See, A. M. (2016) 'Fraction size in radiation therapy for breast conservation in early breast cancer', *Cochrane Database Syst Rev,* 7, Cd003860.

Hida, A., Kitamura, S., Katayose, Y., Kato, M., Ono, H., Kadotani, H., Uchiyama, M., Ebisawa, T., Inoue, Y., Kamei, Y., Okawa, M., Takahashi, K. and Mishima, K. (2014) 'Screening of clock gene polymorphisms demonstrates association of a PER3 polymorphism with morningness-eveningness preference and circadian rhythm sleep disorder', *Sci Rep,* 4, 6309.

Hope, A. J., Lindsay, P. E., El Naqa, I., Alaly, J. R., Vicic, M., Bradley, J. D. and Deasy, J. O. (2006) 'Modeling radiation pneumonitis risk with clinical, dosimetric, and spatial parameters', *Int J Radiat Oncol Biol Phys,* 65(1), 112-24.

Hsu, F. M., Hou, W. H., Huang, C. Y., Wang, C. C., Tsai, C. L., Tsai, Y. C., Yu, H. J., Pu, Y. S. and Cheng, J. C. (2016) 'Differences in toxicity and outcome associated with circadian variations between patients undergoing daytime and evening radiotherapy for prostate adenocarcinoma', *Chronobiol Int,* 33(2), 210-9.

Huang, E. X., Hope, A. J., Lindsay, P. E., Trovo, M., El Naqa, I., Deasy, J. O. and Bradley, J. D. (2011) 'Heart irradiation as a risk factor for radiation pneumonitis', *Acta Oncol,* 50(1), 51-60.

Ijiri, K. and Potten, C. (1990) 'The circadian rhythm for the number and sensitivity of radiation-induced apoptosis in the crypts of mouse small intestine', *Int J Radiat Biol,* 58(1), 165-175.

Joiner, M. a. K., A (eds.) (2009) *Basic clinical radiobiology* 4th ed.*,* London: Hodder Arnold.

Jones, S. E., Tyrrell, J. and Wood, A. R. (2016) 'Genome-Wide Association Analyses in 128,266 Individuals Identifies New Morningness and Sleep Duration Loci', *PLoS Genet,* 12(8), e1006125.

Kapur, G., Windsor, P. M. and McCowan, C. (2010) 'The effect of aerobic exercise on treatment-related acute toxicity in men receiving radical external beam radiotherapy for localised prostate cancer', *Eur J Cancer Care (Engl),* 19(5), 643-7.

Khor, R., Duchesne, G., Tai, K. H., Foroudi, F., Chander, S., Van Dyk, S., Garth, M. and Williams, S. (2013) 'Direct 2-arm comparison shows benefit of high-dose-rate brachytherapy boost vs external beam radiation therapy alone for prostate cancer', *Int J Radiat Oncol Biol Phys,* 85(3), 679-85.

Kong, F.-M., Zhao, J., Wang, J. and Faivre-Finn, C. (2014) 'Radiation dose effect in locally advanced non-small cell lung cancer', *J Thorac Dis,* 6(4), 336-347.

Kong, F. M., Hayman, J. A., Griffith, K. A., Kalemkerian, G. P., Arenberg, D., Lyons, S., Turrisi, A., Lichter, A., Fraass, B., Eisbruch, A., Lawrence, T. S. and Ten Haken, R. K. (2006) 'Final toxicity results of a radiation-dose escalation study in patients with non-small-cell lung cancer (NSCLC): predictors for radiation pneumonitis and fibrosis', *Int J Radiat Oncol Biol Phys,* 65(4), 1075-86.

Konturek, P. C., Brzozowski, T. and Konturek, S. J. (2011) 'Gut clock: implication of circadian rhythms in the gastrointestinal tract', *J Physiol Pharmacol,* 62(2), 139-50.

Labrecque, N. and Cermakian, N. (2015) 'Circadian Clocks in the Immune System', *J Biol Rhythms,* 30(4), 277-90.

Lacombe, J., Azria, D., Mange, A. and Solassol, J. (2013) 'Proteomic approaches to identify biomarkers predictive of radiotherapy outcomes', *Expert Rev Proteomics,* 10(1), 33-42.

Lamarche, B. J., Orazio, N. I. and Weitzman, M. D. (2010) 'The MRN complex in Double-Strand Break Repair and Telomere Maintenance', *FEBS Lett,* 584(17), 3682-3695.

Lane, J. A., Donovan, J. L., Davis, M., Walsh, E., Dedman, D., Down, L., Turner, E. L., Mason, M. D., Metcalfe, C., Peters, T. J., Martin, R. M., Neal, D. E., Hamdy, F. C. and Protec, T. s. g. (2014) 'Active monitoring, radical prostatectomy, or radiotherapy for localised prostate cancer: study design and diagnostic and baseline results of the ProtecT randomised phase 3 trial', *Lancet Oncol,* 15(10), 1109-18.

Levi, F., Focan, C., Karaboue, A., de la Valette, V., Focan-Henrard, D., Baron, B., Kreutz, F. and Giacchetti, S. (2007) 'Implications of circadian clocks for the rhythmic delivery of cancer therapeutics', *Adv Drug Deliv Rev,* 59(9-10), 1015-35.

Li, P., Du, C. R., Xu, W. C., Shi, Z. L., Zhang, Q., Li, Z. B. and Fu, S. (2013) 'Correlation of dynamic changes in gamma-H2AX expression in peripheral blood lymphocytes from head and neck cancer patients with radiation-induced oral mucositis', *Radiat Oncol,* 8, 155.

Luchtenborg, M., Riaz, S. P., Lim, E., Page, R., Baldwin, D. R., Jakobsen, E., Vedsted, P., Lind, M., Peake, M. D., Mellemgaard, A., Spicer, J., Lang-Lazdunski, L. and Moller, H. (2014) 'Survival of patients with small cell lung cancer undergoing lung resection in England, 1998-2009', *Thorax,* 69(3), 269-73.

Macmillan (2017) 'Cancer Survivorship Research Group', [online], available: http://www.macmillan.org.uk/about-us/what-we-do/evidence/research-funding/our-partnerships/macmillan-survivorship-research-group.html [accessed

Maestroni, G. J. (2001) 'The immunotherapeutic potential of melatonin', *Expert Opin Investig Drugs,* 10(3), 467-76.

Marks, L. B., Yorke, E. D., Jackson, A., Ten Haken, R. K., Constine, L. S., Eisbruch, A., Bentzen, S. M., Nam, J. and Deasy, J. O. (2010) 'Use of normal tissue complication probability models in the clinic', *Int J Radiat Oncol Biol Phys,* 76(3 Suppl), S10-9.

Mason, M. D., Parulekar, W. R., Sydes, M. R., Brundage, M., Kirkbride, P., Gospodarowicz, M., Cowan, R., Kostashuk, E. C., Anderson, J., Swanson, G., Parmar, M. K., Hayter, C., Jovic, G., Hiltz, A., Hetherington, J., Sathya, J., Barber, J. B., McKenzie, M., El-Sharkawi, S., Souhami, L., Hardman, P. D., Chen, B. E. and Warde, P. (2015) 'Final Report of the Intergroup Randomized Study of Combined Androgen-Deprivation Therapy Plus Radiotherapy Versus Androgen-Deprivation Therapy Alone in Locally Advanced Prostate Cancer', *J Clin Oncol,* 33(19), 2143-50.

Matsumura, R. and Akashi, M. (2017) 'Multiple circadian transcriptional elements cooperatively regulate cell-autonomous transcriptional oscillation of Period3, a mammalian clock gene', *J Biol Chem,* 292(39), 16081-16092.

Mehta, V. (2005) 'Radiation pneumonitis and pulmonary fibrosis in non-small-cell lung cancer: pulmonary function, prediction, and prevention', *Int J Radiat Oncol Biol Phys,* 63(1), 5-24.

Met_Office (2014) [online], available: http://www.metoffice.gov.uk/learning/learn-about-the-weather/how-weather-works/seasons [accessed 03/09/2014].

Miller, S. C., Pandi-Perumal, S. R., Esquifino, A. I., Cardinali, D. P. and Maestroni, G. J. (2006) 'The role of melatonin in immuno-enhancement: potential application in cancer', *Int J Exp Pathol,* 87(2), 81-7.

Mishra, S. I., Scherer, R. W., Snyder, C., Geigle, P. M., Berlanstein, D. R. and Topaloglu, O. (2012) 'Exercise interventions on health-related quality of life for people with cancer during active treatment', *Cochrane Database Syst Rev,* (8), Cd008465.

Morikawa, L. K. and Roach, M., III (2011) 'Pelvic Nodal Radiotherapy in Patients With Unfavorable Intermediate and High-Risk Prostate Cancer: Evidence, Rationale, and Future Directions', *International Journal of Radiation Oncology • Biology • Physics,* 80(1), 6-16.

Mumbrekar, K. D., Fernandes, D. J., Goutham, H. V., Sharan, K., Vadhiraja, B. M., Satyamoorthy, K. and Bola Sadashiva, S. R. (2014) 'Influence of double-strand break repair on radiation therapy-induced acute skin reactions in breast cancer patients', *Int J Radiat Oncol Biol Phys,* 88(3), 671-6.

NIH (2016) 'Common Terminology Criteria for Adverse Events (CTCAE) V4.0 protocol', [online], available: https://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm#ctc_40 [accessed 17/05/2016].

Noh, J. M., Choi, D. H., Park, H., Huh, S. J., Park, W., Seol, S. W., Jeong, B. K., Nam, S. J., Lee, J. E. and Kil, W. H. (2014) 'Comparison of acute skin reaction following morning versus late afternoon radiotherapy in patients with breast cancer who have undergone curative surgical resection', *J Radiat Res,* 55(3), 553-8.

Nuradh Joseph , T. R., Alessandro Cicchetti, Riccardo Valdagni ,Jenny Chang-Claude ,Susan Davidson, Kerstie Johnson, Chris Talbot, Adam Webb, Alan McWilliam, Catharine West, Ananya Choudhury (2016) 'A higher whole pelvic integral dose is associated with worsening fatigue and functional outcome in

prostate cancer patients treated with intensity modulated radiotherapy', in *NCRI*, LIVERPOOL, UK,

Ohri, N., Dicker, A. P. and Showalter, T. N. (2012) 'Late toxicity rates following definitive radiotherapy for prostate cancer', *Can J Urol,* 19(4), 6373-80.

Oppitz, U., Denzinger, S., Nachtrab, U., Flentje, M. and Stopper, H. (1999) 'Radiation-induced comet-formation in human skin fibroblasts from radiotherapy patients with different normal tissue reactions', *Strahlenther Onkol,* 175(7), 341-6.

Ozsahin, M., Crompton, N. E., Gourgou, S., Kramar, A., Li, L., Shi, Y., Sozzi, W. J., Zouhair, A., Mirimanoff, R. O. and Azria, D. (2005) 'CD4 and CD8 T-lymphocyte apoptosis can predict radiation-induced late toxicity: a prospective study in 399 patients', *Clin Cancer Res,* 11(20), 7426-33.

Pavy, J. J., Denekamp, J., Letschert, J., Littbrand, B., Mornex, F., Bernier, J., Gonzales-Gonzales, D., Horiot, J. C., Bolla, M. and Bartelink, H. (1995) 'EORTC Late Effects Working Group. Late effects toxicity scoring: the SOMA scale', *Radiother Oncol,* 35(1), 11-5.

Pilch, D. R. (2003) 'Characteristics of gamma-H2AX foci at DNA double-strand breaks sites', *Biochem. Cell Biol.,* 81, 123-129.

Pinar, B., Lara, P. C., Lloret, M., Bordon, E., Nunez, M. I., Villalobos, M., Guerrero, R., Luna, J. D. and Ruiz de Almodovar, J. M. (2007) 'Radiation-induced DNA damage as a predictor of long-term toxicity in locally advanced breast cancer patients treated with high-dose hyperfractionated radical radiotherapy', *Radiat Res,* 168(4), 415-22.

Radojcic, M. and Crompton, N. E. (2001) 'Age dependence of T-lymphocyte apoptosis induced by high-energy proton exposure', *Radiat Environ Biophys,* 40(2), 131-5.

Reiter, R. J., Tan, D. X., Mayo, J. C., Sainz, R. M., Leon, J. and Czarnocki, Z. (2003) 'Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans', *Acta Biochim Pol,* 50(4), 1129-46.

Rodemann, H. P. and Bamberg, M. (1995) 'Cellular basis of radiation-induced fibrosis', *Radiother Oncol,* 35(2), 83-90.

Roenneberg, T., Wirz-Justice, A. and Merrow, M. (2003) 'Life between clocks: daily temporal patterns of human chronotypes', *J Biol Rhythms,* 18(1), 80-90.

Roeser, K., Obergfell, F., Meule, A., Vogele, C., Schlarb, A. A. and Kubler, A. (2012) 'Of larks and hearts--morningness/eveningness, heart rate variability and cardiovascular stress response at different times of day', *Physiol Behav,* 106(2), 151-7.

Rube, C. E., Uthe, D., Schmid, K. W., Richter, K. D., Wessel, J., Schuck, A., Willich, N. and Rube, C. (2000) 'Dose-dependent induction of transforming growth factor beta (TGF-beta) in the lung tissue of fibrosis-prone mice after thoracic irradiation', *Int J Radiat Oncol Biol Phys,* 47(4), 1033-42.

Sancar, A., Lindsey-Boltz, L. A., Gaddameedhi, S., Selby, C. P., Ye, R., Chiou, Y.-Y., Kemp, M. G., Hu, J., Lee, J. H. and Ozturk, N. (2014) 'Circadian Clock, Cancer, and Chemotherapy', *Biochemistry,* 54(2), 110-123.

Saunders, M., Dische, S., Barrett, A., Harvey, A., Griffiths, G. and Palmar, M. (1999) 'Continuous, hyperfractionated, accelerated radiotherapy (CHART) versus conventional radiotherapy in non-small cell lung cancer: mature data from the randomised multicentre trial. CHART Steering committee', *Radiother Oncol,* 52(2), 137-48.

Saunders, M. I., Dische, S., Barrett, A., Parmar, M. K., Harvey, A. and Gibson, D. (1996) 'Randomised multicentre trials of CHART vs conventional radiotherapy in head and neck and non-small-cell lung cancer: an interim report. CHART Steering Committee', *Br J Cancer,* 73(12), 1455-62.

Scheller, J., Chalaris, A., Schmidt-Arras, D. and Rose-John, S. (2011) 'The pro- and anti-inflammatory properties of the cytokine interleukin-6', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1813(5), 878-888.

Schnarr, K., Boreham, D., Sathya, J., Julian, J. and Dayes, I. S. (2009) 'Radiation-induced lymphocyte apoptosis to predict radiation therapy late toxicity in prostate cancer patients', *Int J Radiat Oncol Biol Phys,* 74(5), 1424-30.

Scully, R. and Xie, A. (2013) 'Double strand break repair functions of histone H2AX', *Mutat Res,* 750(1-2), 5-14.

Serag, H., Banerjee, S., Saeb-Parsy, K., Irving, S., Wright, K., Stearn, S., Doble, A. and Gnanapragasam, V. J. (2012) 'Risk profiles of prostate cancers identified

from UK primary care using national referral guidelines', *Br J Cancer,* 106(3), 436-9.

Shirazi, A., Ghobadi, G. and Ghazi-Khansari, M. (2007) 'A radiobiological review on melatonin: a novel radioprotector', *J Radiat Res,* 48(4), 263-72.

Somaiah, N., Chua, M. L., Bourne, S., Daley, F., R, A. H., Nuta, O., Gothard, L., Boyle, S., Herskind, C., Pearson, A., Warrington, J., Helyer, S., Owen, R., Rothkamm, K. and Yarnold, J. (2016) 'Correlation between DNA damage responses of skin to a test dose of radiation and late adverse effects of earlier breast radiotherapy', *Radiother Oncol,* 119(2), 244-9.

Sterpone, S., Cornetta, T., Padua, L., Mastellone, V., Giammarino, D., Testa, A., Tirindelli, D., Cozzi, R. and Donato, V. (2010) 'DNA repair capacity and acute radiotherapy adverse effects in Italian breast cancer patients', *Mutat Res,* 684(1-2), 43-8.

Stone, H. B., Coleman, C. N., Anscher, M. S. and McBride, W. H. (2003) 'Effects of radiation on normal tissue: consequences and mechanisms', *The Lancet Oncology,* 4(9), 529-536.

Straub, J. M., New, J., Hamilton, C. D., Lominska, C., Shnayder, Y. and Thomas, S. M. (2015) 'Radiation-induced fibrosis: mechanisms and implications for therapy', *J Cancer Res Clin Oncol,* 141(11), 1985-94.

Stubblefield, J. J., Terrien, J. and Green, C. B. (2012) 'Nocturnin: at the crossroads of clocks and metabolism', *Trends Endocrinol Metab,* 23(7), 326-33.

Su, M., Yin, Z. H., Wu, W., Li, X. L. and Zhou, B. S. (2014) 'Meta-analysis of associations between ATM Asp1853Asn and TP53 Arg72Pro polymorphisms and adverse effects of cancer radiotherapy', *Asian Pac J Cancer Prev,* 15(24), 10675-81.

Talbot, C. J., Tanteles, G. A., Barnett, G. C., Burnet, N. G., Chang-Claude, J., Coles, C. E., Davidson, S., Dunning, A. M., Mills, J., Murray, R. J., Popanda, O., Seibold, P., West, C. M., Yarnold, J. R. and Symonds, R. P. (2012) 'A replicated association between polymorphisms near TNFalpha and risk for adverse reactions to radiotherapy', *Br J Cancer,* 107(4), 748-53.

Tamaru, T., Hattori, M., Honda, K., Nakahata, Y., Sassone-Corsi, P., van der Horst, G. T., Ozawa, T. and Takamatsu, K. (2015) 'CRY Drives Cyclic CK2-Mediated

BMAL1 Phosphorylation to Control the Mammalian Circadian Clock', *PLoS Biol,* 13(11), e1002293.

Twardella, D., Popanda, O., Helmbold, I., Ebbeler, R., Benner, A., von Fournier, D., Haase, W., Sautter-Bihl, M. L., Wenz, F., Schmezer, P. and Chang-Claude, J. (2003) 'Personal characteristics, therapy modalities and individual DNA repair capacity as predictive factors of acute skin toxicity in an unselected cohort of breast cancer patients receiving radiotherapy', *Radiother Oncol,* 69(2), 145-53.

UK, C. R. (2014) 'Cancer Research UK statitistics'.

UK, P. C. 'Prostate Cancer UK Facts and Figures on Prostate Cancer', [online], available: http://prostatecanceruk.org/prostate-information/about-prostate-cancer [accessed April 2016].

Van Cauter, E., Leproult, R. and Kupfer, D. J. (1996) 'Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol', *J Clin Endocrinol Metab,* 81(7), 2468-73.

van Esseveldt, K. E., Lehman, M. N. and Boer, G. J. (2000) 'The suprachiasmatic nucleus and the circadian time-keeping system revisited', *Brain Res Brain Res Rev,* 33(1), 34-77.

van Oorschot, B., Hovingh, S., Dekker, A., Stalpers, L. J. and Franken, N. A. (2016) 'Predicting Radiosensitivity with Gamma-H2AX Foci Assay after Single High-Dose-Rate and Pulsed Dose-Rate Ionizing Irradiation', *Radiat Res,* 185(2), 190-8.

van Oorschot, B., Hovingh, S. E., Moerland, P. D., Medema, J. P., Stalpers, L. J., Vrieling, H. and Franken, N. A. (2014) 'Reduced activity of double-strand break repair genes in prostate cancer patients with late normal tissue radiation toxicity', *Int J Radiat Oncol Biol Phys,* 88(3), 664-70.

Venkateswarlu, R., Tamizh, S. G., Bhavani, M., Kumar, A., Alok, A., Karthik, K., Kalra, N., Vijayalakshmi, J., Paul, S. F., Chaudhury, N. K. and Venkatachalam, P. (2015) 'Mean frequency and relative fluorescence intensity measurement of gamma-H2AX foci dose response in PBL exposed to gamma-irradiation: An inter- and intra-laboratory comparison and its relevance for radiation triage', *Cytometry A,* 87(12), 1138-46.

von Schantz, M. (2008) 'Phenotypic effects of genetic variability in human clock genes on circadian and sleep parameters', *J Genet,* 87(5), 513-9.

Wang, Y. M., Jin, B. Z., Ai, F., Duan, C. H., Lu, Y. Z., Dong, T. F. and Fu, Q. L. (2012) 'The efficacy and safety of melatonin in concurrent chemotherapy or radiotherapy for solid tumors: a meta-analysis of randomized controlled trials', *Cancer Chemother Pharmacol,* 69(5), 1213-20.

Werbrouck, J., De Ruyck, K., Beels, L., Vral, A., Van Eijkeren, M., De Neve, W. and Thierens, H. (2010) 'Prediction of late normal tissue complications in RT treated gynaecological cancer patients: potential of the gamma-H2AX foci assay and association with chromosomal radiosensitivity', *Oncol Rep,* 23(2), 571-8.

West, C., Azria, D., Chang-Claude, J., Davidson, S., Lambin, P., Rosenstein, B., De Ruysscher, D., Talbot, C., Thierens, H., Valdagni, R., Vega, A. and Yuille, M. (2014) 'The REQUITE Project: Validating Predictive Models and Biomarkers of Radiotherapy Toxicity to Reduce Side-effects and Improve Quality of Life in Cancer Survivors', *Clin Oncol (R Coll Radiol),* 26(12), 739-42.

Westbury, C. B. and Yarnold, J. R. (2012) 'Radiation fibrosis--current clinical and therapeutic perspectives', *Clin Oncol (R Coll Radiol),* 24(10), 657-72.

WHO (2016) 'GPAQ questionnaire analysis', [online], available: http://www.who.int/chp/steps/GPAQ%20Instrument%20and%20Analysis%20Guide%20v2.pdf [accessed

Widmark, A., Klepp, O., Solberg, A., Damber, J. E., Angelsen, A., Fransson, P., Lund, J. A., Tasdemir, I., Hoyer, M., Wiklund, F. and Fossa, S. D. (2009) 'Endocrine treatment, with or without radiotherapy, in locally advanced prostate cancer (SPCG-7/SFUO-3): an open randomised phase III trial', *Lancet,* 373(9660), 301-8.

Wilt, T. J., Brawer, M. K., Jones, K. M., Barry, M. J., Aronson, W. J., Fox, S., Gingrich, J. R., Wei, J. T., Gilhooly, P., Grob, B. M., Nsouli, I., Iyer, P., Cartagena, R., Snider, G., Roehrborn, C., Sharifi, R., Blank, W., Pandya, P., Andriole, G. L., Culkin, D. and Wheeler, T. (2012) 'Radical Prostatectomy versus Observation for Localized Prostate Cancer', *New England Journal of Medicine,* 367(3), 203-213.

Wolff, R. F., Ryder, S., Bossi, A., Briganti, A., Crook, J., Henry, A., Karnes, J., Potters, L., de Reijke, T., Stone, N., Burckhardt, M., Duffy, S., Worthy, G. and

Kleijnen, J. (2015) 'A systematic review of randomised controlled trials of radiotherapy for localised prostate cancer', *Eur J Cancer,* 51(16), 2345-2367.

Xiong, T., Turner, R. M., Wei, Y., Neal, D. E., Lyratzopoulos, G. and Higgins, J. P. (2014) 'Comparative efficacy and safety of treatments for localised prostate cancer: an application of network meta-analysis', *BMJ Open,* 4(5), e004285.

Zetner, D., Andersen, L. P. and Rosenberg, J. (2016) 'Melatonin as Protection Against Radiation Injury: A Systematic Review', *Drug Res (Stuttg)*.

Zhang, Y., Liu, Z., Wang, M., Tian, H., Su, K., Cui, J., Dong, L. and Han, F. (2016) 'Single Nucleotide Polymorphism rs1801516 in Ataxia Telangiectasia-Mutated Gene Predicts Late Fibrosis in Cancer Patients After Radiotherapy: A PRISMA-Compliant Systematic Review and Meta-Analysis', *Medicine (Baltimore),* 95(14), e3267.

Zhu, H., Han, S. Y., Li, X. G., Zhou, X. G. and Zhang, Q. F. (2013) 'DNA damage in peripheral blood lymphocytes of ovarian cancer patients after radiotherapy', *Eur J Gynaecol Oncol,* 34(5), 450-2.