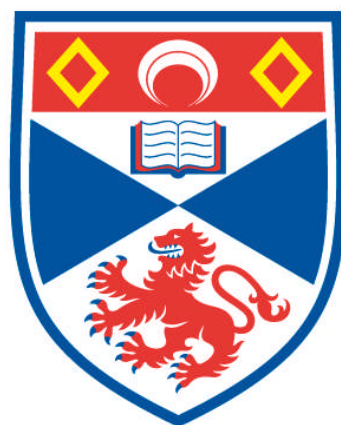


**DEVELOPMENT OF A PREDICTIVE DNA DOUBLE STRAND
BREAK ASSAY FOR THE IDENTIFICATION OF INDIVIDUALS
WITH HIGH NORMAL TISSUE RADIOSENSITIVITY**

Emma Jane Hay Brown

**A Thesis Submitted for the Degree of MD
at the
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Development of a predictive DNA double strand break assay for the
identification of individuals with high normal tissue radiosensitivity

A thesis submitted in fulfilment of the
requirements for the degree of

Doctor of Medicine
University of St Andrews

Emma Jane Hay Brown
30th April 2008

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Thesis Declaration

I, Emma Jane Hay Brown, hereby certify that this thesis, which is approximately 50,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

I was admitted as a research student in [month, year] and as a candidate for the degree of Doctor of Medicine in November 2003, the higher study for which this is a record was carried out in the University of St Andrews between 2003 and 2006.

Signature of candidateDate.....

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Medicine in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Signature of supervisor.....Date.....

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Abbreviations

AUC	Area Under the Curve
BMI	Body Mass Index
BNI	Binucleated Index
CFGE	Constant Field Gel Electrophoresis
CPT	Cell Preparation Tube
CTV	Clinical Target Volume
CV	Coefficient of Variation
DOR	Diagnostic Odds Ratio
DSB	Double strand break
DVH	Dose Volume Histogram
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GTV	Gross Tumour Volume
Gy	Gray
LDR	Low Dose Rate
LMP	low melting point (agarose)
MPC	Magnetic Particle Concentrator
MTD	Maximum Tolerated Dose
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NFR	Normalised Fluorescence Ratio
NTCP	Normal Tissue Complication Probability
PBL	Peripheral blood lymphocyte
PBS	Phosphate Buffered Saline
PE	Phycoerytherin
PFA	Paraformaldehyde
PFGE	Pulsed Field Gel Electrophoresis
PI	Propidium iodide
PST	PBS/donkey serum/Triton X-100
PTV	Planning Target Volume
ROC	Receiver Operator Characteristic
ROS	Reactive Oxygen Species
SF2	Surviving Fraction at 2Gy
SNP	Single Nucleotide Polymorphism
TAE	Tris/acetic acid/EDTA
TCP	Tumour Control Probability
γ H2AX	Phosphorylated histone H2AX

Summary

A genetically determined high level of intrinsic normal tissue radiosensitivity may account for the 5% of patients who experience unexpectedly severe normal tissue side effects following radiotherapy. The pre-treatment identification of these individuals by a diagnostic test or “predictive assay “ may allow appropriate modification of treatment plans and improve the therapeutic index of radiotherapy.

Results from studies of cell-based assays measuring the response of a single cell type taken from patients to in vitro irradiation have been inconsistent, leading to the opinion of many that they are of no value in the prediction of normal tissue radiosensitivity.

A systematic review of the literature presented here, however, suggests that poor methodology of study design often with inadequate control for those factors other than normal tissue radiosensitivity which influence radiotherapy toxicity and lack of reporting of assay precision means that it is difficult to form any conclusions, positive or negative about the diagnostic accuracy of the cell-based assays studied so far. Analysis of individual patient data extracted from these studies suggests that at least some of these assays may possess some discriminatory value.

This finding justified an attempt to develop a novel cell-based assay based on the kinetics of radiation-induced γ H2AX in peripheral blood lymphocytes. Assay failure rate was high and intra- and inter-sample assay reproducibility was poor for quantification by microscopy but were better for flow cytometric analysis. A study of 8 volunteers, however, demonstrated that intra-individual variation was higher than inter-individual variation in assay results, strongly suggesting that poor assay reproducibility due to technical or biological factors may limit the assay’s potential to identify radiosensitive individuals. This suspicion needs to be confirmed in a clinical study of patients of known radiosensitivity. As blood sample storage conditions affect assay results these will need to be standardized to prevent confounding of results.

1. Introduction

The medical use of ionising radiation to treat disease (radiotherapy) is most often used as part of cancer treatment. Approximately half of all patients diagnosed with cancer will receive radiotherapy during the course of their illness.

It has been estimated that approximately 5% of patients treated with radiotherapy experience unexpectedly severe side effects that could not have been predicted from the patient-related and treatment-related factors known to influence radiation toxicity. It is thought that the normal tissues of these individuals possess an intrinsically high level of sensitivity to radiation damage and that this may be genetically determined. If it were possible to measure an individual's normal tissue radiosensitivity before treatment it may be possible to predict their likely radiation toxicity and modify their treatment accordingly. At present no such predictive assay of normal tissue radiosensitivity exists in clinical practice despite over a decade of research interest. The majority of studies published so far have examined the role of functional cell-based assays - giving a test dose of radiation in vitro to a tissue sample from the individual in question and examining the response. As results have been perceived to be disappointing interest has moved away from these cell-based assays to assays based on genotyping – or “radiogenomics”.

Inadequate study design may have contributed to the apparent failure of cell-based assays to deliver a clinically useful diagnostic test. The aims of this project were therefore to evaluate the current evidence regarding functional cell-based assays in the predictive testing of normal tissue radiosensitivity to determine if the perception that they are of no clinical utility is justified and, if then deemed appropriate, to develop a novel functional cell-based assay in a methodical and systematic fashion which might have a potential role as a predictive assay for normal tissue radiosensitivity in the clinic

1.1 Radiotherapy - clinical importance.

As the population of Scotland ages, with a corresponding rise in cancer incidence, the number of patients being treated with radiotherapy annually is set to increase. Over 7000

patients were treated with radiotherapy in Scotland in 1999 and it is projected that by 2011 this will increase to 16 500 (Scottish Executive 2004).

Radical radiotherapy may be used in some cancer types as an alternative to surgery to achieve long term tumour control and cure. Long-term tumour control can often be achieved with acceptable cosmetic and functional results, which may be superior to those following radical surgical resection. Increasingly chemotherapy or other biological targeted therapies are being administered in combination with radiotherapy in an attempt improve the chances of local tumour control and survival over those achievable with radiotherapy alone.

Adjuvant radiotherapy is used in combination with surgery to improve the chance of long term tumour control. Radiation is delivered pre- or post-operatively to eradicate microscopic residual disease in or around the tumour bed that may be left behind by the surgeon.

Palliative radiotherapy also has a key role in the relief of distressing symptoms such as pain, breathlessness and bleeding when the cancer cannot be cured. Palliation is the most common indication for radiotherapy and can be very effective – for example, 80% of patients with pain secondary to metastatic tumour deposit in bone will experience relief after palliative radiation treatment (Hoskin, Yarnold, 2001).

1.2 Principles of radiotherapy planning and delivery

The ultimate aim of radiotherapy is to deliver a clinically effective dose of ionising radiation to a tumour to kill or limit the proliferation of tumour cells that would normally multiply causing the cancer to survive and grow.

Radiotherapy planning is the process by which the target volume for treatment is defined within the patient and the optimal arrangement of radiation beams or radioisotope sources which will distribute a homogenous radiation dose within this target whilst minimising the dose to normal tissue determined. This goal can be achieved using different levels of technical complexity. The underlying principles of all radiotherapy planning and delivery techniques no matter how complex are the same (Figure 1.1)

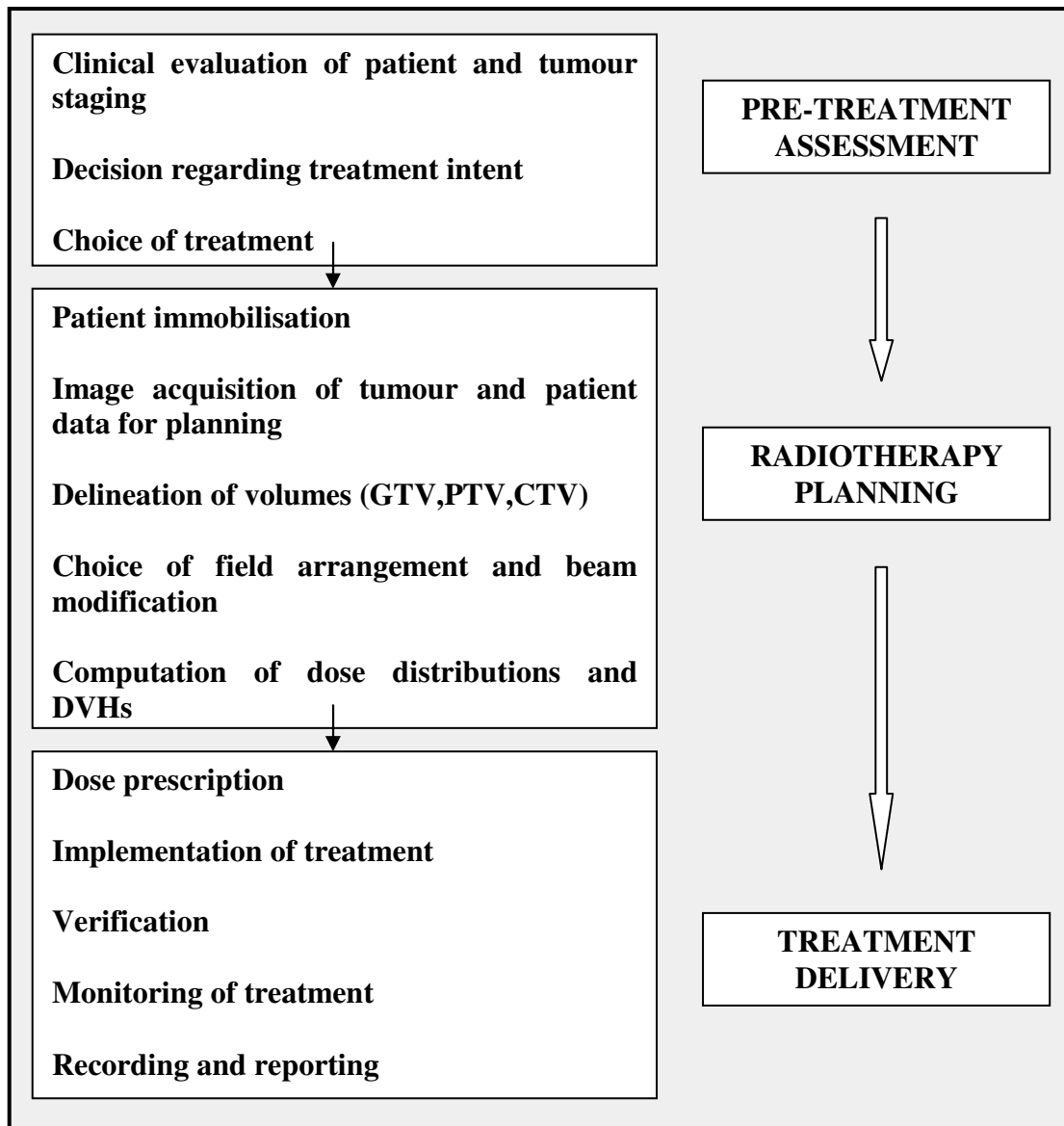


Figure 1.1 Basic principles of radiotherapy planning (adapted from Dobbs, 1999)

The tumour volume to be irradiated is defined according to international definitions for tumour and normal tissue volumes (Figure 1.2) (ICRU 1993; ICRU 1999)

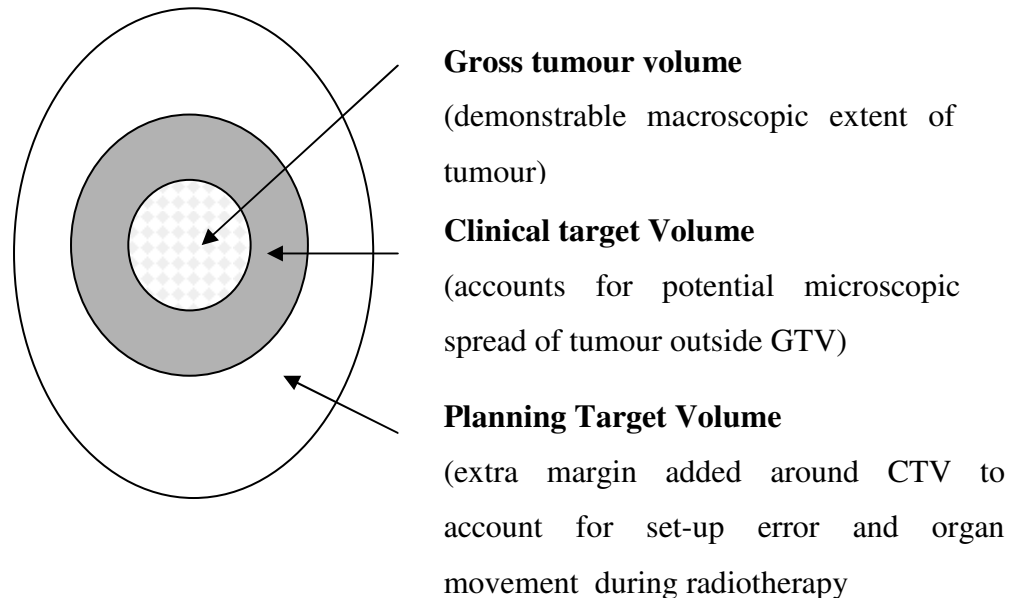


Figure 1.2 Definitions of target volumes for radiotherapy planning

Organs at risk are defined as those normal tissues likely to be irradiated during the course of treatment.

Radiotherapy planners must then determine the beam energy, arrangement of radiation beams, and required beam modification, required to achieve the aim of delivering the prescribed dose in a uniform distribution to encompass the entire PTV, whilst minimising the dose to the organs at risk. This part of the planning process has been greatly assisted by the development of sophisticated radiotherapy planning software.

The radiation dose to be delivered to the PTV will vary according to the tumour type, and aim of treatment. Treatment is usually fractionated with fractions delivered on a daily basis over a number of weeks. The radiotherapy prescription must specify the total dose, number and size of each fraction, overall time over which the treatment is to be delivered and the point to which the prescribed dose is to be delivered.

For different tumour types there are generally accepted standard radiotherapy schedules usually defined by clinical experience or evidence from randomised controlled trials. Radiation dose is defined as the amount of energy absorbed per unit mass and is measured in Gray, where 1 Gray is equal to 1 Joule of energy absorbed per kilogram of tissue. As an example, radical radiotherapy to the lung may be delivered as 55Gy in 20 fractions over 4 weeks. Palliative radiotherapy is usually a lower dose delivered more quickly e.g. 20Gy in 5 fractions over 5-7 days.

The technological systems for planning and delivery of radiation therapy are becoming increasingly more sophisticated. 3-D conformal radiotherapy allows precise shaping of the radiation beam around the target. Intensity Modulated radiotherapy (IMRT) allow shaping of the radiation dose around critical normal structures, and Image-guided radiotherapy (IGRT) improves precision of radiation delivery to the PTV by allowing for patient and/or organ motion during the course of treatment delivery.

1.3 Normal Tissue Toxicity

Despite technological improvements in radiotherapy delivery it will always be impossible to treat a cancer without simultaneously irradiating surrounding normal tissue. Often the volume of normal tissue within the planning target volume will exceed the gross tumour volume. Radiation beams must traverse normal tissue below the patient's surface to reach deep-seated tumours. The unavoidable irradiation of normal tissues causes the side effects associated with radiotherapy.

The pathological processes that lead to radiation injury begin immediately after radiation exposure but may not become clinically apparent for days, weeks, months or even years. By convention, radiation effects on normal tissues are usually divided into acute and late reactions.

Acute effects are those which appear within 90 days of the start of a course of radiotherapy as an acute effect (Cox, Stetz et al. 1995) They are usually transient but can cause significant morbidity.

Acute effects typically involve rapidly renewing tissues with a hierarchical cell lineage such as the skin, lining of the GI tract and haematopoietic system. These tissues are composed of a stem cell compartment in which the cells divide and give rise to differentiating daughter cells. Acute radiation reactions occur when damage to the stem cell compartment caused by radiation means that differentiated cells in the tissue lost during normal tissue turnover are not replaced at a sufficient rate. The time of onset of acute reactions therefore is determined by the lifespan of the differentiated cells. On completion of radiotherapy or even sometimes during it, compensatory proliferation of the remaining stem cells is followed by replacement of the functional cells and recovery.

If acute toxicity is very severe a course of radical or adjuvant radiotherapy treatment may have to be abandoned or interrupted impacting detrimentally on the long term probability of tumour control and cure (Hendry, Bentzen et al. 1996). Acute severely symptomatic toxicity following palliative radiotherapy is obviously undesirable given that the aim of treatment is to improve symptoms and quality of life.

Late normal tissue effects are those which appear more than 90 days after completion of radiotherapy and may not appear for months or years after radiation exposure. Once established, late reactions tend to be irreversible and their severity can progress with time resulting in increasing functional loss and cosmetic changes. Functional loss can be severe and have a major impact on an individual's quality or even duration of life. Examples include hemi or quadriplegia in the case of spinal cord damage, loss of upper limb function with brachial plexus damage, focal neurological deficit due to brain necrosis, severe dyspnoea due to radiation induced pulmonary fibrosis and renal failure due to radiation nephropathy. Cosmetic disfigurement such as visible skin changes on the hands or face, or retardation of bone or muscle growth in the case of children can result in significant psychological or social morbidity. The clinical importance of late normal tissue toxicity is growing as long-term survival after cancer therapy continues to improve.

1.3.1 Clinical features of radiotherapy toxicity.

Acute and late toxicities vary in different organs. The pathophysiology underlying the manifestation of radiotherapy toxicity is incompletely understood, particularly for late damage. Some examples of normal tissue toxicities of clinical importance are given below:

a) Skin and submucosa

Acute skin toxicity is a well-recognised complication of radiotherapy and before the advent of modern megavoltage linear accelerators was frequently the dose-limiting toxicity encountered in clinical practice. It is still a frequent complication of treatment of breast, head and neck and ano-genital malignancies.

Erythema develops in the 2nd or 3rd week of a fractionated course of radiotherapy followed by dry, then moist desquamation due to depletion of the basal stem cell population and failure to replace functional cells (Archambeau, Pezner et al. 1995). Moist desquamation may lead to ulceration. Acute skin toxicity is accompanied by pruritis, hypersensitivity and pain, is distressing for the patient, may require intensive nursing input and can also lead to breaks in or curtailment of treatment. It may begin to heal by the end of treatment or may not resolve for several weeks after the completion of therapy.

Late changes in the skin and submucosa are characterised by atrophy, fibrosis and telangiectasia, which are thought to be a result of vascular injury with endothelial cell loss, vessel dilation and increased blood flow in remaining vessels. Marked fibrosis has obvious cosmetic implications and depending on the site can result in impairment of function.

b) Oral Mucosa

Acute radiation toxicity in the oral and pharyngeal mucosa is a significant complication in treatment for head and neck cancer. As with radiation dermatitis, mucositis also results from loss of functional cells from the mucous membrane lining of the oral cavity and pharynx. Severe confluent mucositis is painful and can lead to diminished oral intake often requiring hospital admission for enteral feeding. Temporary treatment interruption may be required.

c) GI tract

In the treatment of pelvic and abdominal cancers substantial parts of the GI tract are often included in the irradiated volume. Acute toxicity is due to depletion of mucosal pre-cursor cells such as the intestinal crypt cells and lack of replacement of functional intestinal villus cells. Clinical symptoms may include diarrhoea, tenesmus, increased passage of mucus per

rectum, nausea and gastritis. If severe, treatment may have to be temporarily interrupted and can occasionally be life-threatening.

The resulting breakdown in the mucosal barrier in the GI tract results in inflammation which may subside rapidly once treatment is completed, or give rise to waves of ongoing inflammation with induction of necrosis, vascular sclerosis and fibrosis (Hauer-Jensen, Richter et al. 1998). Late effects may be consequential to this on-going inflammation and vascular damage and include fibrosis and ischaemia in the submucosa and muscle wall of the bowel, along with development of telangiectasia and other vascular abnormalities. Clinical symptoms of late bowel toxicity include increased stool frequency, urgency, spotting of blood and faecal leakage. Occasionally mucosal ulceration, severe bleeding, pain, fistulation, stricture formation and severe incontinence can occur (O'Brien 2001)

d) Brain

Cerebral oedema with increased intracranial pressure and accompanying headache and nausea can occur during radiotherapy. The most important and potentially devastating consequences of normal tissue damage in the brain tend to occur a few months to several years after radiotherapy. Transient demyelination in the CNS can occur in the first 6 months causing “somnolence syndrome” characterised by drowsiness, lethargy and anorexia (Faithfull and Brada 1998). Transient memory impairment has also been reported as an delayed acute effect of cranial irradiation (Armstrong, Ruffer et al. 1995; Vigliani, Sichez et al. 1996). Features of late radiation damage to the brain occurring 6 months to several years following treatment are demyelination and necrosis leading to permanent and sometimes progressive neurological and cognitive deficit. In the first year after radiotherapy histological changes are mostly limited to the white matter, with increasing grey matter changes and pronounced vascular lesions developing later. Histological changes include necrosis, glial atrophy and vasculopathies with telangiectasia and haemorrhage (Van der Kogel 1991).

e) Spinal cord

Radiation toxicity to the spinal cord is similar to that seen in the brain in terms of timing and histology. Early transient demyelination results in Lhermitte’s syndrome which occurs

several months following treatment and can last several months. Clinical features are of shock-like sensations radiating to the hands and feet when the neck is flexed.

Late radiation damage includes a permanent demyelination and necrosis of white matter which can begin 6-18 months post-treatment. A later manifestation with a latent period of 1-4 years is progressive vascular damage with telangiectasia, haemorrhages and on-going necrosis. The clinical features of both of these processes are neural dysfunction with severe functional loss and permanent paraplegia

f) Lung

The lung is very sensitive to radiation damage. It is frequently irradiated as part of treatment for lung, breast and oesophageal cancers and lymphoma.

Acute radiation toxicity becomes apparent 1-3 months post-treatment as “pneumonitis” manifested by cough, breathlessness, fever and occasionally chest pain. Histological changes are of inflammation with oedema and inflammatory cell infiltrate including alveolar macrophages (McDonald, Rubin et al. 1995). Type II pneumocytes are increased and there are a reduced number of parenchymal cells. The alveoli fill with fibrinous exudates and gas exchange is impaired. Radiological changes are of pulmonary infiltrates within the irradiated volume.

Pneumonitis resolves but may then be followed by late radiation toxicity consisting of chronic inflammation and fibrosis that may continue to develop for many years after radiotherapy. Histologically there is evidence of vascular damage and collagen deposition (McDonald, Rubin et al. 1995). The patient may experience no symptoms if the irradiated volume was small, but if a large lung volume has been damaged they may be permanently and severely breathless due to diminished gas exchange.

1.3.2 Grading systems

Evaluation of treatment outcome following radiotherapy must not only assess tumour control, but also the frequency and severity of side effects resulting from treatment. Thorough and standardised documentation of normal tissue effects is vital, both in day to day assessment of patients in the clinic, and in the research setting when evaluating new

radiotherapy regimens. Several attempts have been made to devise systems for accurate reporting and grading of radiation normal tissue toxicity, none of which has gained general acceptance as the “gold standard”.

The ideal toxicity scoring system should be

- comprehensive – it should be possible to score any relevant adverse effect
- reproducible – intra- and inter- observer variation in scoring should be low
- sensitive – the system should be able to detect small increases or decreases in rates of adverse effects

In addition the ideal system should be easy to use, clinically relevant and ensure that information is of sufficient quality to be of use to both clinicians and radiobiologists wishing to assess treatment outcomes. A number of systems have been devised and are in clinical use. These include:

- RTOG/EORTC Acute Radiation Morbidity Scoring criteria and Late Morbidity scoring criteria (Cox, Stetz et al. 1995).
- LENT/SOMA (Late Effects on Normal Tissues/ Subjective Objective Management Analytic) scales (Rubin, Constine et al. 1995; Rubin, Constine et al. 1995; Denekamp, Bartelink et al. 1996; Denekamp, Bartelink et al. 1996; Dorr and Hendry 2001)
- NCI CTC (Common Toxicity Criteria) system (Trotti, Byhardt et al. 2000; Trotti 2002; Trotti, Colevas et al. 2003; Trotti and Bentzen 2004)
- UCLA index
- Franco-Italian Glossary.

Many centres have devised their own scoring systems or modified those above.

1.4 What determines the radiation dose prescribed in today’s clinical practice?

The aim of radiotherapy is to achieve the clinically desired effect, whether it be tumour cure or palliation of symptoms, without causing treatment related complications. There is evidence of a dose response relationship for tumour control probability in experimental

animal tumour systems and in clinical practice (Fletcher 1972; Suit 1982; Steel and Peacock 1989; Withers 1992; Okunieff, Morgan et al. 1995; Zelefsky, Leibel et al. 1998; Kuban, Pollack et al. 2003; Bradley, Graham et al. 2005; Belderbos, Heemsbergen et al. 2006). Equally increasing radiation dose also increases the probability of normal tissue complications (Bentzen 1994; Bentzen 2002). The radiotherapy schedules in clinical practice have been developed to balance tumour control probability with normal tissue complication probability to try to maximise tumour control whilst keeping the risk of severe normal tissue toxicity in the treated population at an acceptable level. What constitutes an “acceptable” level of risk depends on the specific toxicity and its effect on the functioning of the patient. Spinal cord damage has a potentially devastating effect on the patient and so even a small risk is unacceptable, whilst a slightly higher risk of damage that is primarily cosmetic may be tolerated.

Few prospective dose-escalation studies have been performed to determine the maximum tolerated radiation dose (MTD) in any given tissue and there is little quantitative data on normal tissue tolerance, particularly for those tissues where damage can lead to a catastrophic functional outcome, such as the spinal cord. MTD-finding studies are difficult to conduct as it is the late irreversible and severe effects on normal tissues rather than acute reversible toxicities that are generally dose limiting. A number of dose escalation studies aiming to define MTD in the modern radiotherapy era do exist, but in general the dose- effect relationships for toxicity of individual normal tissues have been derived empirically from clinical observation, retrospective data and consensus opinion. (Emami, Lyman et al. 1991). This has led to the development of parameters attempting to define the risk of normal tissue toxicity for a given radiotherapy schedule such as the $TD_{5/5}$ i.e. the dose that results in a 5% chance of a specific normal tissue toxicity within 5 years of radiotherapy delivery (Emami, Lyman et al. 1991)., and the normalised dose-response gradient i.e. the percentage of increase in toxicity for a 1% increase in dose (Brahme 1984; Bentzen and Tucker 1997). These population-based assessments of risk of normal tissue toxicity with dose form the basis for prescribing radiotherapy in clinical practice.

1.4.1 Factors influencing the risk of and severity of radiation toxicity

The risk of normal tissue toxicity is not only defined by the prescribed radiation dose. There are other features of the treatment itself and patient-related factors that are known influence the risk of developing normal tissue complications due to radiotherapy.

1.4.1.1 Treatment-related factors

Dose per fraction

It is recognised that delivering the total radiation dose in multiple small fractions rather than fewer larger fractions results in a reduction in severity of late effects. Late effects are more sensitive to changes in fraction size than acute effects (Thames, Withers et al. 1982).

Overall treatment time

Acute effects are sensitive to changes in the overall treatment time but late effects less so – it has been demonstrated that a reduction in the overall treatment time in head and neck cancer by treating with two or three fractions of radiotherapy per day (accelerated fractionation) increases the risk and severity of acute effects, with a decrease in some late toxicity end-points (Dische, Saunders et al. 1997; Bourhis, Calais et al. 2004).

Volume of irradiated tissue

The risk of organ dysfunction and symptomatic toxicity is related to the volume of tissue receiving a high radiation dose (Withers, Taylor et al. 1988). The functional structure of the organ and its functional reserve determines its susceptibility to this volume effect. An organ with serial organisation of functional subunits will fail if even a small section is damaged – an above-tolerance dose of radiation to even a small volume of the spinal cord can result in overall organ dysfunction and paralysis. An organ with parallel arrangement of functional subunits, such as the lung, can tolerate radiation damage to a small volume. For example, lung tissue has a low radiation tolerance but a high dose of radiation to a small volume of lung can be tolerated as the larger volume of independently functioning undamaged lung can compensate. However, a low dose of radiation to a large volume of lung can reduce overall gas exchange significantly resulting in potentially fatal respiratory failure.

Dose homogeneity

Despite careful attention to beam modification and arrangement patient outline and tumour position may mean that there are unavoidable “hot-spots” within the irradiated volume which will receive a higher dose and dose per fraction than other areas. As a consequence this tissue will be at a higher risk of complications.

Concurrent chemotherapy

The concurrent administration of chemotherapy with radiotherapy has been shown in randomised controlled trials and large meta-analyses to improve local control and survival in some tumour types, particularly in cervical and head and neck cancer (Pignon, Bourhis et al. 2000; Green, Kirwan et al. 2005). There is also clear randomised controlled evidence that concurrent chemotherapy increases the risk and severity of acute radiation reactions although the effect on late toxicity is less clear (Bourhis, Calais et al. 2004; Denis, Garaud et al. 2004; Green, Kirwan et al. 2005).

1.4.1.2 Patient-related factors

Co-morbidity

Patient comorbidities can influence the development of radiation normal tissue toxicity. Co-morbidities that affect the vascular system especially diabetes and uncontrolled hypertension appear to increase the risk of radiation toxicity (Turesson, Nyman et al. 1996; Herold, Hanlon et al. 1999). A systematic review has identified that connective tissue disease is associated with an increased risk of late radiation toxicity (Holscher, Bentzen et al. 2006)

Smoking

Smoking during therapy can increase the risk and severity of both acute and late normal tissue radiation toxicity (Johansson, Bjermer et al. 1998; van der Voet, Keus et al. 1998; Eifel, Jhingran et al. 2002; Twardella, Popanda et al. 2003; Wells, Macmillan et al. 2004)

Body Mass Index

Wells et al (2004) found that a high body mass index was predictive of increased skin toxicity in patients treated for breast and head and neck cancer

Haemoglobin

Haemoglobin level during radiotherapy may influence the development of normal tissue toxicity. There is some evidence suggesting that a low haemoglobin during treatment is associated with a lower risk of normal tissue toxicity but this has not been a consistent finding by all groups (Bentzen and Overgaard 1994; Henke, Bechtold et al. 2000; Daly, Poulsen et al. 2003).

Genetic syndromes

Some groups of patients may have a genetic susceptibility to radiation normal tissue damage – rare but recognised genetic syndromes associated with increased normal tissue radiosensitivity include Ataxia-telangiectasia, Blooms', Fanconi's anaemia and Nijmegen Breakage syndrome. (Rogers, Plowman et al. 2000; Gatti 2001; Alter 2002; McMillan 2002)

1.5 Individual intrinsic normal tissue radiosensitivity

Patients who are apparently matched in terms of the factors known to influence radiotherapy toxicity will exhibit a wide spectrum of incidence and severity of normal tissue toxicity for a given radiotherapy dose and fractionation regimen.(Turesson 1989; Turesson 1990; Withers 1992). Some patients will exhibit greater than average normal tissue reaction whilst others will exhibit less toxicity for the same given dose. The frequency of differing severities of normal tissue toxicity forms an approximate Gaussian distribution (Burnet, Johansen et al. 1998). Analysis of the clinical radiotherapy fractionation studies performed in Gothenburg has estimated that approximately 80% of the inter-individual variation in observed normal tissue reactions is due to differences in individual intrinsic normal tissue radiosensitivity rather than extrinsic factors such as differences in delivered dose, co-morbidities, smoking or other confounding factors. (Turesson 1989; Turesson and Thames 1989; Turesson 1990; Turesson, Nyman et al. 1996). Intra-patient correlation in severity of normal tissue toxicity in separately treated areas is further evidence that individuals possess an intrinsic level of normal tissue radiosensitivity (Tucker, Turesson et al. 1992; Bentzen, Overgaard et al. 1993) and suggests this may be genetically determined. Further weight to the idea that intrinsic tissue radiosensitivity is genetically determined comes from the existence of the inherited

syndromes mentioned above, a component of which is enhanced radiation sensitivity of normal tissue

If the biological determinants of intrinsic normal tissue radiosensitivity could be identified they could potentially form the basis of a diagnostic test or “predictive assay” to identify individuals at risk of severe normal tissue damage.

1.6 Predictive assays for normal tissue radiosensitivity

1.6.1 Clinical value of a predictive assay of normal tissue radiosensitivity

If a high precision assay of normal tissue radiosensitivity existed which could accurately and reliably predict an individual’s risk of developing severe normal tissue radiation toxicity, how could its results be incorporated into clinical practice and used to improve the therapeutic index of radiotherapy?

As discussed above, an apparently similar group of patients treated with the same dose of radiotherapy will experience a wide range of severity of normal tissue toxicity due to a presumed spectrum of intrinsic normal tissue sensitivity (Figure 1.3). In clinical radiotherapy the radiation dose delivered in standard treatment regimens is limited in order to maintain the population risk of unacceptable normal tissue toxicity at approximately 5% or less. The population risk of toxicity is determined by the minority of patients whose tissues are particularly radiosensitive (Group A in Figure 1.3). This minority who form the tail of the Gaussian distribution of radiation sensitivity therefore define the tolerance dose for the whole population. The “normally” radiation sensitive majority and the “radiation-resistant” minority could potentially tolerate a higher dose of radiation than is delivered in standard radiotherapy regimens before developing toxicity. They will therefore effectively be “under-dosed” in order to try to prevent damage to the sensitive minority.

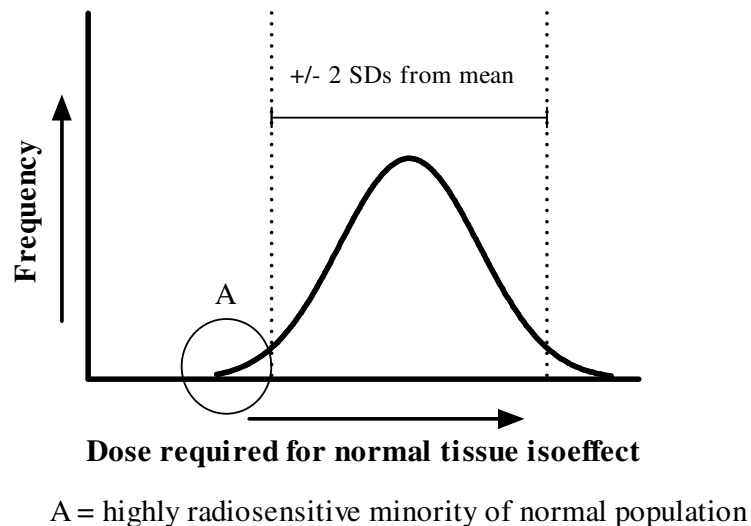


Figure 1.3 Frequency distribution of normal tissue response amongst patients treated with an identical radiotherapy schedule

If it were possible to measure an individual's propensity to develop normal tissue damage prior to starting radiotherapy then theoretically their radiation treatment could be modified with the aim of preventing serious toxicity in those who are radiosensitive. A strategy using normal tissue radiosensitivity testing and prospective prediction of normal tissue response might also permit safe dose escalation in appropriate "non-sensitive" individuals which should result in improved rates of tumour control. In this way treatment could be "biologically" individualised and the therapeutic ratio of radiotherapy could be improved. The results from a predictive assay of normal tissue radiosensitivity could therefore potentially be used to:

- a) Screen for the minority of individuals with very high normal tissue radiosensitivity and treat these with a reduced radiation dose or offer an alternative to radiotherapy such as surgical resection.
- b) Screen out the radiosensitive minority and escalate the dose in the remainder.
- c) Completely individualise the dose prescription so that patients are treated with different radiotherapy doses according to their individually quantified normal tissue tolerance – this should result in the same level of normal tissue complications in all patients

A number of authors have modelled the potential impact of normal tissue radiosensitivity testing on outcome after radiotherapy (Norman, Kagan et al. 1988; West and Hendry 1992; Tucker, Geara et al. 1996; Bentzen 1997; MacKay, Niemierko et al. 1998; Mackay and Hendry 1999) . In most cases a clinically useful improvement in tumour control probability without a corresponding increase in normal tissue radiation toxicity has been predicted.

Tucker et al (Tucker, Geara et al. 1996) have estimated the potential for individualising dose prescription in order to attain a uniform 15% risk of severe late damage to mucous membrane and bone, based on results from a study correlating fibroblast radiosensitivity in vitro and late normal tissue complications. They estimate that for the 8 patients in the study who were sensitive to radiation, an average dose reduction of 13.1Gy would have been necessary to reduce the Normal Tissue Complication Probability (NTCP) to 15%. In 20 of the 21 remaining cases, a dose escalation averaging 7.7Gy could be tolerated whilst still maintaining the risk of severe late damage at 15% for each individual. The resulting effect on tumour control probability is not modelled but the authors assume that these dose modifications would result in higher Tumour Control Probability (TCP) than observed in reality as there would have been twice as many dose increases as decreases.

If a precise and accurate predictive assay was available with high positive predictive power MacKay et al have estimated that by selecting an appropriate assay cut-off point that it would be possible to increase the dose to 95% of patients (MacKay, Niemierko et al. 1998). Recognising that such an idealised highly precise assay is potentially unachievable, that in vitro assays do not necessarily account for in vivo factors that may modify normal tissue response and that the relationship between normal tissue and tumour radiosensitivity is unresolved, MacKay et al then went on to re-model the potential impact of normal tissue radiosensitivity testing incorporating these inherent sources of imprecision (Mackay and Hendry 1999). This model was again based on the radiosensitivity of fibroblasts. Modelling was performed using a hypothetical population of 10 000 patients with a log normal distribution of fibroblast radiosensitivities created by a random number generator. An idealised perfect correlation between predictive assay result and clinical outcome was assumed and then the inherent variability and uncertainties likely in clinical practice fitted into the model to assess how they impacted clinical utility of the assay.

In the context of an ideal predictive assay, the predicted gain in population TCP resulting from tailoring individual patient dose to attain a NTCP of 5% is 30% and is predicted to be highest when individuals have a higher sensitivity and the distribution of radiosensitivity in the population is greatest. When the distribution of radiosensitivity in the population is high, the spread of doses required to achieve the isoeffect of 5% NTCP is wide, with a small tail of very high doses achievable of up to 200Gy. In reality, the inherent inaccuracies in the fibroblast radiosensitivity data used to generate the model have probably resulted in an overestimate of the spread of intrinsic radiosensitivity in the population. If radiosensitivity is modelled with a narrower distribution within the population, possibly more reflective of reality, the range of doses predicted to give a NTCP of 5% is narrower and the high dose tail much smaller.

In reality results of predictive assays measuring a biological endpoint such as normal tissue radiosensitivity are likely to possess an inherent variability or “noise” – i.e. the same test performed on repeated occasions on the same individual may give differing results. MacKay and Hendry demonstrated that when assay noise is fitted into their model no gain in TCP could be achieved if the assay coefficient of variation was greater than 50% of the inter-individual biological variation. They conclude that a crucial factor in gaining a therapeutic advantage by individualisation of radiotherapy dose is the development of an accurate and reliable assay.

In another study modelling the potential effect of individualising dose prescription according to normal tissue radiosensitivity Bentzen comes to a different conclusion (Bentzen 1997). He also bases his model on published results of in vitro assays of fibroblast radiosensitivity and argues that the low prevalence of highly radiosensitive individuals in the population reduces the positive predictive value of any screening test. Even if successfully identified, the removal of this radiosensitive minority, who form the left hand tail of the Gaussian distribution of normal tissue radiosensitivity, is unlikely to allow a significant dose increase to the remaining patients because of the sigmoid shape of the dose-response curve for normal tissue toxicity. In his model, individualisation of dose based on information about normal tissue radiosensitivity without simultaneous information about tumour sensitivity leads to a virtually symmetrical distribution of dose changes in his simulation that could lead to a reduction rather than an increase in tumour

control probability. In the simulation the individualisation of dose increased the average dose received by the population as a whole by 1.4Gy, but tumour control dropped by 3%. Because of the non-linearity of the dose response curve for TCP, a positive dose increment of a given size will increase the TCP by a smaller amount than the same dose decrement would decrease TCP. These conclusions have been criticised by other authors who feel that some of the assumptions used in Bentzen's model are based on too small a data set and are therefore inaccurate (MacKay, Niemierko et al. 1998; Mackay and Hendry 1999).

An alternative to complete individualisation of dose prescription is to use predictive testing of normal tissue radiosensitivity to split the population into three groups – high, average and low radiosensitivity - and to treat each group differently (Mackay and Hendry 1999) – (Table 1). This would avoid the potential problems likely to be encountered whilst trying to individualise dose prescriptions using an assay that is less than completely reliable.

Table 1.1 Theoretical division of population into 3 groups according to normal tissue radiosensitivity and the dose to which each group could be treated to maintain NTCP<5%

	CV = 0.2			CV = 0.1		
Radiosensitivity	High	Average	Low	High	Average	Low
% Population	12	43	45	18	42	40
Prescribed dose (Gy)	56	66	78	64	70	78

Results are shown for 2 different populations with log normal distribution of SF2=0.36 and CV (coefficient of variation) of assay results across the population of 0.2 and 0.1. Before splitting into groups the whole population would have been treated with 60Gy (CV = 0.2) or 66Gy (CV = 0.1) in order to keep the population NTCP <5%. (Mackay and Hendry 1999)

MacKay and Hendry calculate that by splitting into groups and dosing as in Table 1.1, the overall population TCP increases by 22% if the assay CV is 0.2. As assay reliability decreases, the NTCP increases for the population as patients are placed in the wrong group. To compensate for this misclassification and to reduce the NTCP to an acceptable level the dose prescribed must also be reduced with a consequential reduction in TCP.

When the assay CV is 50% of the biological variation within the population it is still possible, however, to have a potential gain in TCP of 27%. This contrasts to the effect of assay unreliability on complete individualisation of dose prescription where no gain in TCP was expected with this level of assay unreliability. In fact, by dividing the population into groups in this way it is predicted that an increase in TCP of 11% is still possible even if the assay CV is the same as the biological CV. Tripartite separation of the population would therefore seem to be less sensitive to assay uncertainty than complete individualisation of radiotherapy dosing.

If tumour radiosensitivity is correlated with normal tissue radiosensitivity then the potential clinical gains resulting from normal tissue radiosensitivity testing are even greater (MacKay, Niemierko et al. 1998). However, whether such a relationship exists is debatable and if present is likely to be weak. (West and Hendry 1992; Geara, Peters et al. 1996; Bernier, Thames et al. 1998; West, Davidson et al. 1998).

Mathematical models are provisional and require appropriate caveats (Jones and Dale 1999) – their output is dependent on the quality of the data entered into the model and so results cannot be assumed to be generalisable, and they must be re-tested when new clinical data is available. They do however provide a useful tool for estimating possible outcomes of changes in therapy without conducting clinical trials which, in the case of normal tissue radiosensitivity testing, would not only be costly and time-consuming, but potentially dangerous with a concomitant risk of loss of tumour control and increased radiation toxicity. Mathematical modeling so far would seem to support the concept that normal tissue radiosensitivity testing could potentially improve the therapeutic index of radiation therapy if incorporated into clinical practice.

1.6.2 Development of a predictive assay of normal tissue radiosensitivity

The principles underpinning the development of any diagnostic test apply to the development of predictive assays of normal tissue radiosensitivity. The assay must be reliable and not subject to repeated assay failures. It must also be precise and accurate. Precision is a measure of assay reproducibility – an assay which is precise will give a similar result each time when repeated on the same sample or same individual. Diagnostic

accuracy is the ability of an assay to correctly diagnose the condition in question and is measured by assay sensitivity and specificity. Ideally an assay should be generalisable to a large population and could be performed in different laboratories with reproducible results. The results of a diagnostic assay should influence clinical decisions - to do this it must not only be reliable, precise and accurate, it must be able to produce results quickly in a clinically relevant time-frame. It must also be affordable and acceptable to patient and clinician without the need for unpleasant or dangerous invasive procedures otherwise it will not be adopted in routine clinical practice no matter how efficiently it performs.

A fundamental issue in the development of any diagnostic assay is determining which parameter should be measured and how to measure it reliably. One of the main problems in the development of a biological assay to predicate normal tissue toxicity has been the relative lack of knowledge of the molecular, cellular and tissue pathophysiology underlying acute and late radiation toxicity. Strategies tested so far have had mixed outcomes.

1.6.2.1 Functional cell-based assays

Assays that are based on sampling of living cells from an individual and examining their response to ex-vivo irradiation have until recently been the main focus of most work on predictive testing. The fundamental principle underlying these assays is that there is a relationship between in vitro cellular response to irradiation and normal tissue toxicity and that it is possible to test for this relationship using a single cell type sampled from an individual as a surrogate for the normal tissue in question. Assay end-points examined have included clonogenic cell survival, assays of radiation-induced DNA damage, apoptosis, and differentiation. Fibroblasts derived from skin biopsy and peripheral blood lymphocytes have been the most frequently tested surrogate tissue

Clonogenic cell survival.

Normal tissue toxicity may be a result of loss of proliferative capacity of crucial stem cells within the normal tissue due to death or permanent growth arrest induced by radiation. One of the first indications that there may be a relationship between in vitro cell response to radiation and normal tissue toxicity came when Burnet et al demonstrated a correlation

between fibroblast radiosensitivity as measured by a clonogenic cell survival assay and late skin toxicity in 6 patients treated with post-mastectomy chest wall radiation (Burnet, Nyman et al. 1992; Burnet, Nyman et al. 1994). Subsequent studies confirmed this relationship (Geara, Peters et al. 1993; Brock, Tucker et al. 1995; Johansen, Bentzen et al. 1996) whilst others did not (Russell, Grummels et al. 1998; Peacock, Ashton et al. 2000; Oppitz, Baier et al. 2001). Fibroblast radiosensitivity correlated with the development of late central nervous system complications after stereotactic radiosurgery for arterio-venous malformations in a small group of Canadian patients (Raaphorst, Malone et al. 2002). Late radiation effects after head and neck radiotherapy, however, did not correlate with fibroblast SF2 in a prospective study of 25 patients (Rudat, Dietz et al. 1999).

Similarly studies examining the relationship between acute radiotherapy toxicity and the clonogenic survival of skin fibroblasts have revealed conflicting results (Begg, Russell et al. 1993; Rudat, Dietz et al. 1997; Oppitz, Baier et al. 2001; El-Awady, Mahmoud et al. 2005).

Lymphocyte radiosensitivity measured by clonogenic survival has been correlated with the development of late effects after pelvic radiotherapy (West, Davidson et al. 2001).

Assays of radiation-induced DNA damage

Clonogenic survival assays are time consuming to perform. Fibroblast assays do not deliver results for at least 6 weeks post biopsy and even lymphocyte clonogenic survival assays can take 2-3 weeks to perform. In order to try to develop assays that deliver results within a shorter, clinically useful time frame several groups have examined the relationship between in vitro radiation-induced DNA damage and normal tissue damage.

There is a substantial body of evidence that suggests that DNA damage is a crucial type of cellular damage that can lead to cell death following irradiation (McMillan 2002). Microbeam irradiation of the nucleus results in much higher cell death than irradiation of the cytoplasm. The incorporation of radionucleotides with short-range emissions into DNA causes cell killing at much lower absorbed doses than when the same radionucleotides are incorporated into the cytoplasm. The number of chromosome or chromatid aberrations and the number of unrepaired DNA double-strand breaks following ionising radiation exposure both correlate well with cell death rates in cell culture. Cell death correlates best with the

level of DNA double-strand breaks (DSBs) rather than with other types of DNA damage. Cells are very sensitive to formation of DNA DSBs and even a single DSB can trigger the damage sensing process (Huang, Clarkin et al. 1996) and can lead to cell death if unrepaired (Bennett, Lewis et al. 1993). Maintaining the integrity of DNA therefore seems to be very important biologically, and the ability to detect and repair DNA damage appears to determine whether a cell will survive following radiation.

It follows then that an individual's ability to detect, process and repair DNA damage may in part determine their intrinsic normal tissue radiosensitivity and radiation tolerance and several studies have studied the relationship between in vitro DNA damage and repair and clinical radiosensitivity using assays of DNA damage such as the comet assay or pulsed-field gel electrophoresis (PFGE) or cytogenetic endpoints of DNA double-strand breaks such as the micronucleus assay, G2 chromatid radiosensitivity or studies of chromosome re-arrangements

A positive correlation has been found between cellular radiosensitivity as measured by the micronucleus assay and both early (Widel, Jedrus et al. 2003) and late radiotherapy toxicity (Nachtrab, Oppitz et al. 1998; Barber, Burrill et al. 2000; Lee, Allison et al. 2003; Widel, Jedrus et al. 2003). Rached et al however found no such relationship between micronucleus yield and acute radiotherapy toxicity (Rached, Schindler et al. 1998)

Chromosome aberrations or G2 chromatid damage induced by in vitro irradiation have been found to correlate with acute (Kearsley, Fang et al. 1998) and late radiation toxicity (Borgmann, Roper et al. 2002)(Borgmann, Roper et al. 2000; (Neubauer, Dunst et al. 1997; Barber, Burrill et al. 2000)

Ability to repair radiation-induced DNA damage in vitro as measured by the alkaline comet assay has been found by some groups to correlate with acute and late toxicity (Oppitz, Denzinger et al. 1999) whilst others have not demonstrated such a relationship (Popanda, Ebbeler et al. 2003; Twardella, Popanda et al. 2003) . Similar inconsistency of results has been found with assays of DNA DSB formation and repair measured by electrophoresis (Kiltie, Barber et al. 1999; Kiltie, Ryan et al. 1999; Dikomey, Brammer et

al. 2000; Dickson, Magee et al. 2002; El-Awady, Mahmoud et al. 2005; Lopez, Guerrero et al. 2005; Wang, Chen et al. 2005; Pinar, Lara et al. 2007)

Radiation-induced apoptosis

Crompton et al have demonstrated that the lymphocytes in blood samples from patients with high levels of both acute and late radiation toxicity undergo less radiation-induced apoptosis in vitro as measured by flow cytometry than those from patients with average levels of normal tissue toxicity (Crompton, Miralbell et al. 1999). The same group confirmed this finding in a prospective study demonstrating a relationship between increased late radiation toxicity and lower levels lymphocyte apoptosis after in vitro irradiation of peripheral blood samples (Ozsahin, Crompton et al. 2005). Another group however, could not correlate apoptotic rate in human peripheral blood lymphocytes after in vitro irradiation with rates of breast fibrosis, telangiectasia or breast retraction after radiotherapy for breast cancer (Barber, West et al. 2000)

1.6.2.2 Other strategies in development of predictive assays of normal tissue radiosensitivity:

As shown above, the results from functional cell-based assays of normal tissue radiosensitivity have been inconsistent. Authors have raised concerns that the assays lack reliability due to confounding external influences (West, Davidson et al. 2001). Even when a statistically significant relationship between an assay result and normal tissue toxicity has been demonstrated considerable overlap in assay results between patients with high and low normal tissue toxicity has led authors to conclude that cell based assays are not sufficiently discriminatory to be of any use in the clinic (West, McKay et al. 2005; Burnet, Elliott et al. 2006).

Given that intrinsic normal tissue radiosensitivity is thought to have a genetic basis attention has focussed instead on developing assays based on genotyping to measure an individual's risk of developing severe normal tissue toxicity – so called “radiogenomics”. A cell's genetic code is fixed and assay results should not be susceptible to the confounding environmental or biological confounders that might interfere with functional assays. Modern technology allows high throughput genotyping and it is hoped that by indentifying high risk single nucleotide polymorphisms (SNPs) in candidate genes, such as

those involved in DNA repair, free radical scavenging, cell cycle control and cytokine release, it may be possible to predict radiation sensitivity (Andreassen, Alsner et al. 2002). A tissue bank with linked data regarding patients demographics, comorbidity, tumour, and precise radiotherapy details and dosimetry has been set up in the EU to enable this process (GENEPI) (West, McKay et al. 2005). Initial results from genotyping assays have been promising but positive results have so far not been confirmed in appropriate validation studies (Andreassen, Alsner et al. 2003; Andreassen, Alsner et al. 2005; Andreassen, Alsner et al. 2006).

Other assays based on serum cytokine measurements during radiotherapy have shown a correlation between increased TGF- β at the end of radiotherapy and interleukin 6 levels during radiotherapy for lung cancer and the development of subsequent radiation pneumonitis (Anscher, Kong et al. 1997; Chen, Hyrien et al. 2005). These assays have already been tested to see if radiation dose can safely be escalated in those patients identified as having a low susceptibility to pneumonitis (Anscher, Marks et al. 2003). This study confirmed the principle that TGF- β could predict those at risk of pneumonitis – unfortunately whilst patients who were dose escalated had acceptable rates of pneumonitis they did suffer severe complications in other normal tissues which had not been predicted by the assay.

1.6.3 Have functional cell-based assays been proven to be of no clinical utility?

Although potentially susceptible to confounding external influences and of no proven clinical utility so far, functional cell-based assays do theoretically hold advantages over assays based on genotyping. Assays based on genotyping alone do not give information about epigenetic phenomena such as control of gene expression, post-translational modification and interaction of gene products themselves that ultimately determine the cellular and tissue response to radiation. Genotyping can only be performed in selected genes and not the whole genome. We have only limited knowledge of the molecular pathology underlying normal tissue radiation reactions, so selection of the genes for examination is limited to those that we believe are important such as those involved in DNA repair, ROS scavenging, cell cycle control and induction of fibrotic reactions. We know from gene expression studies using microarray technology that the expression of thousands of genes is altered by in vitro irradiation and the sequence and function of them

all is not yet known (Rodningen, Overgaard et al. 2005). It remains to be seen whether SNPs in a few of these candidate genes can reliably predict a highly radiosensitive phenotype.

In the meantime, functional assays have the advantage of not relying on an in depth knowledge of the molecular machinery governing radiation response. In effect the cell is a “black box” – we can put in a signal (the test dose of radiation) and measure the output signal (the assay result) without knowledge of the circuitry and wiring within. Is there sufficient evidence to be sure that function cell based assays have no role in predictive testing of normal tissue radiosensitivity?

As described above many groups have tried to develop functional cell-based assays of intrinsic normal tissue sensitivity based on study designs which correlate in vitro cellular radiosensitivity with severity of normal tissue reaction. These studies must collect standardised information on radiotherapy toxicity as well as radiation exposure and factors other than intrinsic normal tissue radiosensitivity known to modify normal tissue response so allowing potential confounding factors to be taken into account during data analysis. Twardella et al performed a systematic review of studies examining the relationship between cell based assays and normal tissue toxicity in patients receiving radiotherapy for breast cancer (Twardella and Chang-Claude 2002) . They found that of the 25 studies identified limitations in study design were frequently found, with potential sources of bias arising from misclassification of patients due to non-standardisation of assessment of treatment related side effects, selection bias by studying convenient patient groups rather than truly representative groups and confounding due to failure to adjust analysis for important factors influencing normal tissue reaction. An estimate of assay sensitivity and specificity was performed in only one of the studies identified. Given the methodological problems in assay design and testing reported by Twardella et al it is possible that the assumption that functional cell based assays are unhelpful in predictive testing of normal tissue sensitivity may be premature.

Similar concerns about study design and statistical analysis have also been raised about prognostic tumour marker studies. In this field, despite years of research and numerous published reports, very few clinically useful prognostic tumour markers have been

identified – as with predictive testing of normal tissue sensitivity studies looking at similar assay end points have yielded inconsistent results with methodological differences, poor study design, small sample sizes, non-standardised or non-reproducible assays, and inappropriate statistical analyses being thought to account for these inconsistencies (Fielding, Fenoglio-Preiser et al. 1992; Simon and Altman 1994; Hayes, Bast et al. 1996; Hall and Going 1999; Schilsky and Taube 2002).

A joint initiative between the National Cancer Institute (NCI) and European Organisation for the Research and Treatment of Cancer (EORTC) has identified poor study design and analysis, assay variability and inadequate reporting of studies as some of the major barriers to progress in this field. Following this the reporting recommendations for tumour marker prognostic studies have been published (REMARK) (McShane, Altman et al. 2005). These recommendations suggest the systematic reporting of key study features including quality control assessment procedures for the prevention of bias in assay and clinical measurement, statistical analysis and appropriate reporting of findings which should allow transparent and complete reporting with relevant information so that the usefulness of the data can be judged clearly by others. The same reporting standards are transferable to predictive assays of normal tissue radiosensitivity.

1.7 H2AX phosphorylation in human peripheral blood lymphocytes – a potential predictive assay of normal tissue sensitivity.

Predictive assays based on measuring DNA damage in cells irradiated in vitro using standard techniques have yielded inconsistent results as discussed. Recently much has been discovered about the molecular processes involved in the recognition of DSBs within a cell and the subsequent signalling processes leading to repair of DNA damage or to cell death. The question arises as to whether this greater understanding can lead to the discovery of molecular markers of DNA damage and repair that might be quantified and may result in the development of more sensitive and reliable assays of DNA damage and repair than previously used cell-based techniques.

1.7.1 DNA DSBs and γ H2AX induction

When a DSB is introduced into DNA the histone protein H2AX becomes rapidly phosphorylated at serine 139 within its COOH-terminal region involving hundreds to thousands of H2AX molecules in a megabase region on either side of the DSB (Rogakou, Pilch et al. 1998; Stiff, O'Driscoll et al. 2004). A commercially available monoclonal antibody to the phosphorylated form of H2AX (γ H2AX) has been developed and using immunocytochemistry it is possible to visualise these DNA DSBs as large foci within the cell nucleus (Rogakou, Boon et al. 1999). A single DSB is sufficient for the formation of a γ H2AX focus and there appears to be a 1:1 correspondence between the number of DSBs and γ H2AX foci after DNA damage induction (Sedelnikova, Rogakou et al. 2002). Immunofluorescent staining for γ H2AX foci can detect DSB induction at much lower doses than established DNA DSB assays and has been reported to be sensitive enough to detect DSBs in cells after doses as low as 0.001Gy (Rothkamm and Lobrich 2003).

The phosphorylation of H2AX is thought to recruit DNA repair factors to the site of the DNA DSB (Paull, Rogakou et al. 2000) and may also be involved in the amplification of DNA damage signals that activate the G2/M checkpoint to prevent damaged cells from entering mitosis (Fernandez-Capetillo, Chen et al. 2002). Inability to form γ H2AX foci has been correlated to radiosensitivity, genomic instability and other repair defects (Bassing, Chua et al. 2002; Celeste, Petersen et al. 2002; Kuhne, Riballo et al. 2004; Taneja, Davis et al. 2004). The mechanism by which γ H2AX is removed following DNA repair is incompletely understood. In some studies the kinetics of γ H2AX loss mirrors the kinetics of DNA DSB rejoining (Rothkamm and Lobrich 2003) whilst others have found that although the number of γ H2AX foci formed after irradiation correlates with the number of double strand breaks formed the kinetics of foci development and loss differ from those characteristic of double-strand break re-joining, and loss of γ H2AX may therefore be indicative of more than simple DSB re-joining..(MacPhail, Banath et al. 2003).

Clinically relevant ionizing radiation doses induce similar patterns of γ H2AX focus formation in radiosensitive and radioresistant human tumour cell lines and xenografted tumours, but radiosensitive tumour cells and xenografts retain γ H2AX for a greater duration than radioresistant cells and tumours (MacPhail, Banath et al. 2003; Taneja, Davis et al. 2004). There is evidence that the rate of loss of γ H2AX after irradiation of cells in

culture correlates with clonogenic survival at 2Gy (MacPhail, Banath et al. 2003) and the rate of γ H2AX disappearance is slower in radiosensitive tumour cells and radiosensitive murine normal tissue than radioresistant cell lines or normal tissue (Olive and Banath 2004). The techniques for immunofluorescent staining and γ H2AX quantification in cultured cells are quick and yield results in a number of days rather than many weeks. Quantification of γ H2AX induction and the kinetics of γ H2AX loss in normal human cells or tissues after a test dose of radiation could therefore potentially form the basis of a predictive assay of human normal tissue radiosensitivity.

Normal cells sampled from patients and utilised for predictive testing should be plentiful and easily and rapidly accessible by non-invasive means. For γ H2AX quantification the cells would ideally be non-cycling as γ H2AX foci are also induced at collision of replication forks during DNA replication (Furuta, Takemura et al. 2003). Human peripheral blood lymphocytes (PBLs) obtained by venepuncture in the clinic fulfil both these criteria and could be used as a surrogate tissue to test if there is a relationship between cellular γ H2AX induction and loss in vitro after irradiation and normal tissue radiosensitivity.

1.8 Aims

The aims of this project were therefore:

- To conduct a systematic review of the literature regarding functional cell-based assays in the predictive testing of normal tissue radiosensitivity to ascertain whether studies so far have been performed with sufficiently rigorous approach to assay quality control, avoidance of bias in study design, and statistical analysis of results, and to determine if the perception that cell-based assays are not helpful in predicting normal tissue radiosensitivity is really valid based on current literature.
- To develop a rapid, reliable cell-based assay measuring γ H2AX kinetics in human PBLs in a methodical and systematic fashion with appropriate attention to issues of precision and quality control, which might have a potential role as a predictive assay for normal tissue radiosensitivity in the clinic

2. Materials and methods

2.1 Materials

A list of the laboratory equipment, consumables, chemicals and reagents, kits and antibodies used can be found in Appendix 1.

2.2 Methods

The methods for the systematic review are described in Chapter 3.

2.2.1 Collection of peripheral blood samples from healthy volunteers

The School of Medicine did not have an active research ethics committee scrutinising research involving staff and students when this study commenced. Healthy volunteer subjects were recruited from within the department. The purpose of the study was explained to volunteers, and it was made clear that samples collected would not be used for any other purpose other than the research described. All subjects agreed to give at least 3 samples of peripheral blood on 3 separate occasions. Volunteers were informed that blood samples and data derived from samples would be anonymised and individual volunteers would not be made aware of their assay results. Samples would not be stored and would be destroyed after the completion of the study. Basic demographic details regarding sex, age and smoking status at the time of blood sampling were taken from volunteers but no information regarding medical history or current medication was sought. Details of the volunteers recruited are given in Table 6.1 (page 140).

Blood collection

Peripheral blood samples were collected from volunteer subjects by medically qualified staff using the BD Vacutainer system for venous blood collection. Samples for analysis by microscopy were collected into 5ml Lithium heparin tubes, whilst those for analysis by flow cytometry were collected into 8ml sodium citrate CPT tubes (Cell Preparation tubes, BD Biosciences).

2.2.2 Gamma source operation and dosimetry

In vitro irradiation was performed in a self-contained $^{137}\text{Caesium}$ gamma irradiator (IBL437C Cis Bio International, High Wycombe, UK).

Dosimetry

Calibration was performed using the Fricke-Frankenberg procedure for ferrous sulphate dosimetry (Frankenberg 1969). The dosimetry solution was prepared by diluting Fricke stock solution 1:10 with deionised water (sterilised). 4x15ml Polypropylene tubes were "conditioned" by filling with 10ml solution and irradiating for 600 seconds within the gamma source. The tubes were emptied and rinsed with deionised sterilised water. Each was then filled with 6 ml solution and irradiated for 300 seconds. During irradiation each tube was positioned within the 4-tube rack which was used for irradiation in the subsequent γH2AX experiments. The rack was rotated automatically within the chamber during irradiation to achieve equal distribution of dose to all tubes. A sample from each tube was then transferred to a quartz cuvette and extinction was measured using a UV spectrophotometer (304nm). The spectrophotometer was zeroed between each sample using the quartz cuvette filled with non-irradiated solution.

Dose (D) was calculated by $D = \text{Extinction at 304nm} \times 281/0.97 \text{ Gy}$ (0.97 = correction factor for temperature 22°C)

This was repeated for 600, 900 and 1200 seconds. The mean value for each time point was plotted against expected dose using half life based on the manufacturer's dosimetry on manufacture. The mean measured dose rate in Gray/minute was calculated and the projected dose rate over the next 12 months tabulated assuming decrease in dose rate over time due to decay of the source calculated. For calculations the half-life of $^{137}\text{caesium}$ was assumed to be 30.6 years.

The measured dose rate was 3.297Gy/minute on 25/1/2005. See Appendix 2 for results and theoretical isodose distribution within the irradiation chamber.

Sample irradiation

Muntjac fibroblasts were grown on coverslips in Petri dishes and irradiated by placing the Petri dish in a custom made Perspex rack in the centre of the irradiation chamber. For irradiation of lymphocytes in suspension, 15ml sample tubes were placed in the custom built Perspex rack in the irradiation chamber. The cells were exposed to the gamma source for the appropriate number of seconds required to achieve the desired total dose. Control samples were mock-irradiated by taking them from the incubator at the same time as the cells to be irradiated and transporting them to the gamma source together. Control samples were left outside the irradiator on the bench whilst irradiation took place.

2.2.3 Immunomagnetic isolation of CD4 and CD8 positive peripheral blood lymphocytes

Human peripheral blood lymphocytes were isolated using a CD4 and a CD8 positive isolation kit in combination (Dyna, Oslo, Norway). Each kit contains Dynabeads, 4.5micrometre magnetisable polystyrene beads coated with primary monoclonal antibody specific for either the CD4 or CD8 membrane antigen on human peripheral blood lymphocyte subsets. Each kit also contains either DETACHaBEAD CD4 or DETACHaBEAD CD8 as appropriate. DETACHaBEAD is a polyclonal anti-Fab antibody specific for either the CD4 or CD8 antibody on the Dynabeads and when added to bead-bound cells competes with the antibody/antigen at the cell surface and releases the antibody and bead from the cell. The manufacturers confirmed that it was possible to mix reagents from both kits together and isolate both CD4 positive and CD8 positive cells simultaneously in the same sample tube. The magnetic separation device used throughout was Dynal Magnetic Particle Concentrator (MPC) MPC –L (Dyna, Oslo, Norway). Cell isolation technique was performed as per the manufacturer's instructions.

Dynabeads washing procedure

The Dynabeads were resuspended in the vial. 180 microlitres each of the CD4 and CD8 beads were transferred to a 15ml polypropylene tube (36 microlitres of each per ml whole blood to be processed – 5ml blood sample). 1ml PBS/1%FCS added and thoroughly mixed with beads. The tube was placed in the magnet for 30 seconds and supernatant pipetted off and discarded. The washed beads were then resuspended in 360 microlitres of PBS/2%FCS.

Separation of CD4 and CD8 positive cells

5ml of whole blood was added to the pre-washed beads and mixed by inversion of the tube three times. The tube was then incubated at 4°C for 30 minutes on a rotary mixer. The CD4 and CD8 positive cells were isolated by placing the tube in the MPC for 3 minutes. The supernatant was discarded whilst the bead/cell rosettes were still attached to the test tube wall by the MPC. The rosettes were then washed 4-5 times in PBS/2%FCS using the MPC and then resuspended in 250 microlitres RPMI1640/1%FCS.

Bead detachment

25 microlitres each of DETACHaBEAD CD4 and DETACHaBEAD CD8 were added to the prepared cell suspension and incubated at room temperature for 60 minutes. The tube was then placed in the MPC for 2 minutes and the supernatant containing the released cells was transferred to a fresh tube. To obtain residual cells the Dynabeads were washed 3 times in 500 microlitres RPMI/1% FCS and the supernatant collected each time. The detached cells were then washed to remove DETACHaBEAD by re-suspending in 10ml RPMI/1%FCS and then centrifuging at 300g for 8 minutes. The cells were then counted using the pre-calibrated Coulter counter (Beckman Coulter) and resuspended in RPMI/1%FCS at a concentration of 1×10^5 /ml for use in the γ H2AX immunofluorescence staining procedures

2.2.4 Immunostaining procedure for focus analysis by microscopy

Peripheral blood lymphocyte suspensions or Muntjac fibroblasts on coverslips were irradiated or “mock-irradiated” and then returned to the 37°C incubator for the pre-determined interval to allow formation of γ H2AX. 0.4ml of the lymphocyte suspension was then placed in the chamber of a cytopspin funnel and cyto-centrifuged onto a clean glass slide at 800rpm for 10 minutes. The slides were not allowed to dry but were immediately immersed in freshly prepared 4% paraformaldehyde (PFA) for 10 minutes. For the Muntjac fibroblasts, culture medium was removed from the Petri dish after incubation, and the coverslips were washed in PBS x1 before fixation.

After fixation in PFA, the slides were washed twice in PBS for 5 minutes before immersion in 0.2% Triton in PBS for 10 minutes. After two more 5 minute washes in PBS

slides were placed in blocking solution (0.1M glycine/1% donkey serum in PBS) and stored at 4°C overnight.

The following day the blocking solution was removed and slides were placed on a tray in a humidified chamber. 100 microlitres of a 1:500 dilution of mouse anti- γ H2AX antibody in 0.5% donkey serum/PBS (Upstate, USA) was pipetted carefully onto the cell layer. Laboratory film was carefully laid over each slide to ensure even distribution of the antibody solution over the cell layer and to prevent drying during incubation. The slides were incubated at room temperature for 1 hour. The film was removed and the slides placed in a rack and washed in 0.5% donkey serum/PBS for 15 minutes x 4 over 1 hour. Slides were then placed in the humidified chamber and, in the dark-room, 100 microlitres of a 1:500 dilution of FITC-tagged donkey anti-mouse secondary antibody (Jackson ImmunoResearch) in 0.5% donkey serum/PBS was pipetted over each cell layer and a piece of laboratory film placed over each as before. The slides were incubated at room temperature in the dark for 1 hour before being uncovered and washed in PBS for 15 minutes x 4 over 1 hour in the dark. Without allowing the cells to dry and working in the dark, the slides were mounted with coverslips and 12 microlitres of MOWIOL-DAPI and left to dry in the dark at 4°C overnight before microscopy.

2.2.5 Fluorescence microscopy, digital image capture and image analysis

For image acquisition, an upright fluorescence microscope (Zeiss Axioplan) with a CCD digital camera interfaced with the Cytovision System (Applied Imaging, Sunderland, Tyne & Wear, UK) was used. Control of the camera, image acquisition, and image analysis were performed by the system software.

Throughout the study the same control settings for exposure, brightness/darkness and z-stack and image thresholding were utilised for each image during image capture using the FITC filter: Brightness 100, darkness 111, exposure 5 seconds, z-stack 5 x 1.5micrometres, threshold 180.

2.2.6 Cell separation procedure using CPT tubes

The cell isolation procedure for blood samples collected in CPT tubes was performed according to the manufacturer's recommendations. CPT tubes were kept upright after blood collection. Prior to centrifugation the tubes were remixed by gentle inversion 5

times. The tubes were then centrifuged at room temperature in a horizontal rotor (Heraeus) for 20 minutes at 1500 x g

After centrifugation the monocyte layer was resuspended in plasma by inversion of the unopened container 5 times. The CPT tube was then opened and the entire contents of the tube above the gel layer were pipetted into a sterile 15ml polypropylene tube.

Sterile PBS at room temperature was added to bring the volume up to 15ml. The tube contents were mixed by inversion of the capped tube and then centrifuged at 1500 x g for 15 minutes. The supernatant was aspirated and the cell pellet was resuspended by tapping the tube. Sterile PBS was added to bring the volume up to 10ml and the contents were mixed by inversion of the capped tube. The tube was centrifuged for 10 minutes at 300 x g. The supernatant was aspirated, the cell pellet resuspended in 5ml RPMI/1% FCS. The cells were then counted using the pre-calibrated Coulter counter (Beckman Coulter) and then finally resuspended at a concentration of 1×10^6 cell/ml in RPMI/1% FCS. 2ml of cell suspension was pipetted into individual 15ml sterile polypropylene tubes and RPMI/1% FCS added to make up a total volume of 7ml. Each tube was gassed with 5% CO₂ in air and then placed in the incubator at 37°C prior to irradiation and γ H2AX quantification.

2.2.7 Immunofluorescent staining of PBLs for flow cytometric analysis

Following irradiation or mock-irradiation and the appropriate incubation period, tubes were removed from the incubator, cooled on ice and centrifuged at 1°C at 300 x g for 5 minutes. The supernatant was discarded and the pellet resuspended by tapping the tube before adding 2ml ice cold 70% ethanol. After thorough mixing by pipetting the sample was divided between 2 pre-labelled 1.5ml plastic safe-lock tubes (Eppendorf) and stored at -20°C for up to 15 days before immunostaining. One tube from each pair served as the negative control for flow cytometry to correct for autofluorescence and non-specific binding of the secondary antibody.

For immunostaining and analysis the samples were removed from the freezer and centrifuged at 300 x g for 5 minutes. The supernatant was discarded and the pellet resuspended in 1ml PBS before centrifugation, removal of the supernatant and re-suspension of the cell pellet in 1ml cold PBS/1% donkey serum/0.1% Triton X-100 (PST solution). The tubes were placed on ice for 30 minutes to allow cell rehydration and permeabilisation and blocking. Tubes were centrifuged at 300 x g for 5 minutes and the

supernatant discarded. For the negative controls the cell pellet was resuspended in 200 microlitres of PST, whilst the other cell pellet from the other member of the pair was resuspended in 200 microlitres of anti- γ H2AX mouse monoclonal antibody diluted to 1:500 in PST. The cells were incubated at room temperature for 2 hours before being washed twice in 1 ml PBS/0.5% donkey serum. The cell pellets were then resuspended in 200 microlitres of FITC-labelled donkey anti-mouse secondary antibody diluted to 1:500 in PST and incubated at room temperature for 1 hour in the dark. Cells were rinsed twice in PBS and then resuspended in 1ml PBS and transferred to pre-labelled 5ml polystyrene tubes for flow cytometric analysis

2.2.8 Flow cytometric analysis of γ H2AX staining

The cell sample was introduced through the sample injection port. Using Cell Quest software (Version 3, Becton Dickinson) a dot plot of FSC (forward scatter) vs. SSC side scatter was generated (Figure 2.1 A) and the FSC and SSC gains altered to produce a clearly discernable cell population corresponding to the lymphocytes within the sample. This population was gated and the mean fluorescence intensity of 10 000 cells was measured on the FL-1 channel and plotted on a log scale (Figure 2.1 B). FL-1 photomultiplier tube settings were kept at 500 throughout the study. For each sample the mean fluorescence intensity for the negative control corresponding to autofluorescence and non-specific binding of the secondary antibody were subtracted from the results from the corresponding fully stained irradiated or non-irradiated sample. γ H2AX levels were then calculated as the normalised fluorescence ratio (NFR) i.e. the ratio of mean fluorescence intensity in irradiated cells to that of non-irradiated controls with both values having been corrected for autofluorescence.

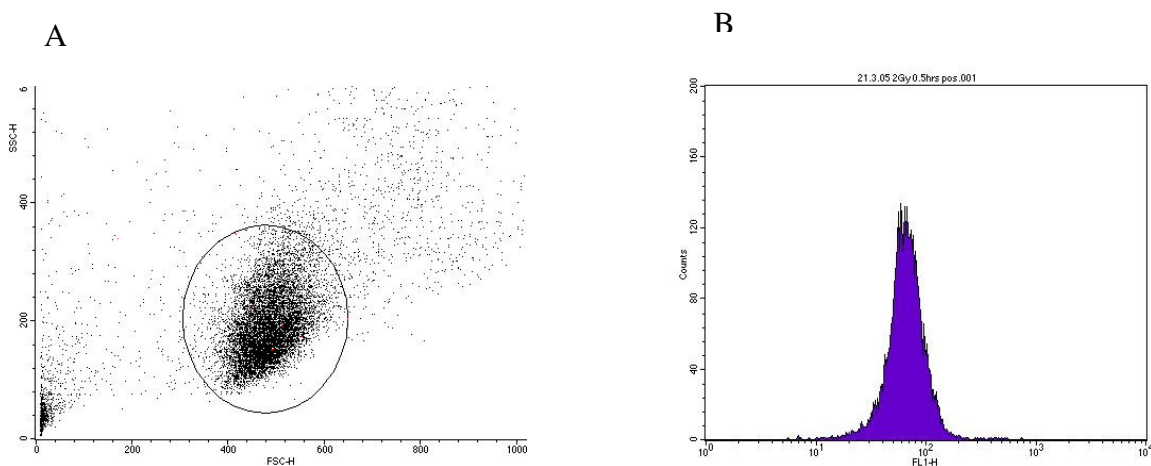


Figure 2.1 Flow cytometric analysis of γ H2AX stained PBLs

A. Dot plot of FSC vs. SSC showing a clearly defined cell population with has been gated (circle). **B.** Histogram of the fluorescence intensity of 10 000 cells in the gated population. Cell Quest software calculated the mean fluorescence intensity for the gated population and this value was used for subsequent calculations of γ H2AX induction.

2.2.9 Cell phenotyping

In order to confirm that the gated population did consist of human peripheral blood lymphocytes, cell immunophenotyping was performed. Different white blood cells can be identified by the expression of differing cell surface markers. All white blood cells are CD45 positive and monocytes are CD14 positive. Given that the cell separation procedure during centrifugation of the CPT tubes should have removed granulocytes, the remaining white cell population in the supernatant layer that underwent irradiation and γ H2AX staining procedure should contain a mixture of lymphocytes and monocytes. The gating procedure was designed to exclude monocytes from analysis so the gated population, if lymphocytes, should be CD45 positive confirming leucocyte origin and CD14 negative confirming that it does not contain monocytes. In addition the gated population should be CD3 and CD19 positive confirming a mix of T- and B-lymphocytes.

For the fixed sample phenotyping, peripheral blood was collected in a sodium citrate CPT tube and the mononuclear cell fraction separated and washed. 1×10^5 cells were transferred into each of 6 x 15ml sterile polypropylene tubes and fixed in 70% ethanol for 10 minutes. Following fixation the cells were washed in PBS, centrifuged at 300 x g and the cell pellet resuspended in 100 microlitres of PBS alone. To each was added:

Tube 1: nil – unstained control

Tube 2: 20 microlitres of FITC-conjugated mouse IgG1 isotype control

Tube 3: 20 microlitres of FITC- conjugated mouse anti-human CD3 antibody

Tube 4: 20 microlitres of PE-conjugated mouse anti-human CD19 antibody

Tube 5: 20 microlitres each of both anti CD3 and CD19 antibodies

Tube 6: 20 microlitres of Simultest LeucoGATE (FITC-labelled anti CD45 and PE-labelled anti-CD14).

Each tube was incubated for 30 minutes at room temperature in the dark before the addition of 1ml PBS, mixing, centrifugation at 300 x *g* for 5 minutes, aspiration of supernatant and re-suspension in 1 ml of PBS. This washing procedure was repeated before analysing the samples on the flow cytometer. For each sample the presumed lymphocyte population was gated and the green (FITC) fluorescence measured on the FL-1 channel and plotted against similar measurement of red (PE) fluorescence on the FL-2 channel to confirm leucocyte phenotyping. See Appendix 3 for results.

2.2.10 Flow cytometer performance monitoring with CaliBrite beads

BD CaliBrite beads are designed for use with FACSCComp software to adjust instrument settings, set fluorescence compensation, and check instrument sensitivity. Using the 2 colour kit, two sample tubes were prepared. Tube A contained 1 drop of unlabelled bead suspension in 1ml sheath fluid. Tube B contained 1 drop each of unlabelled, FITC-labelled and PE-labelled beads in 3ml sheath fluid. Both tubes were stored on ice in the dark before use. The initial adjustment of instrument setting was performed with the Becton Dickson engineer – following the instructions accompanying the FACSCComp software Tube A was used to adjust the photomultiplier tube settings and Tube B was used to adjust fluorescence compensation to optimise discrimination between green and red fluorescent signals using the FL-1 and FL-2 channels and to perform a sensitivity test for all channels. The beads were used again after 6 months to repeat the sensitivity test but a further adjustment of compensation was not performed.

2.2.11 Measurement of DNA double-strand break re-joining in human peripheral blood lymphocytes

Mononuclear cells were separated from whole blood, washed and resuspended in 10 ml RPMI/1% FCS as per the protocol in Section 2.2.6. The cell suspension was transferred into a 25cm² tissue culture flask and incubated at 37°C for 1 hour to allow the monocytes to attach to the culture flask and therefore remove them from the analysed cell population. The cells were then transferred to a 15ml polypropylene tube, centrifuged, resuspended in RPMI/1%FCS, and counted, adjusting the final concentration of cells to 1.5×10^5 per ml. 1ml of cell suspension was transferred to each of the required number of 15ml polypropylene tubes. The tubes were gassed with 5% CO₂ in air and incubated at 37°C for 30 minutes. The cells were then cooled on ice and irradiated on ice. Samples for dose-response experiments were then kept on ice. Samples for measurement of DNA DSB repair quantification were transferred to a water bath at 37°C for the required repair time before being transferred to ice again for rapid cooling before lysis.

Once all samples were ready and cooled on ice, the tubes were centrifuged at 0°C and the medium aspirated. 80 microlitres of 0.8% low melting point agarose (LMP) in PBS at 37°C was added to each cell pellet and rapidly mixed and transferred to a gel plug mould and allowed to set on ice for 10 minutes. Plugs of agarose containing the cells were then expelled into ice-cold lysis solution and held for 30 minutes on ice before incubation at 37°C for 18 hours.

Plugs were recovered from the lysis solution and placed in comb wells in a 200ml 0.8% Ultrapure agarose gel in TAE (Tris/Acetic acid/EDTA buffer) which contained ethidium bromide (0.5 micrograms/ml) in a Bio-Rad Sub-Cell horizontal electrophoresis apparatus. Wells were sealed using 0.8% LMP agarose in PBS and the gel run in 0.5 TAE at 0.6V/cm, 8V, constant current, for 96 hours.

The gel was imaged and the image analysed using a gel documentation and analysis system (SynGene, Synoptics, Cambridge, UK). The fraction of DNA released from the wells during electrophoresis was used as a measure of the induced-double-strand breakage. DNA was quantified by ethidium bromide fluorescence, analysed using Syngene

Genetools software. The fraction of DNA released (FDR) was calculated from the relationship:

$$\text{FDR} = \frac{\text{DRi}}{(\text{DRi} + \text{DWi})} - \frac{\text{DRc}}{(\text{DRc} + \text{DWc})}$$

Where:

DRi = DNA released (irradiated sample)

DWi = DNA remaining in well (irradiated sample)

DRc = DNA released (unirradiated control sample)

DWc = DNA remaining in well (unirradiated control sample)

2.2.12 Detection of apoptosis in human peripheral blood lymphocytes after irradiation

The Annexin V-FITC apoptosis detection kit (BD Pharmingen) utilises FITC conjugated Annexin V as a highly sensitive probe for identifying apoptotic cells and when used with flow cytometry can quantitatively determine the population of cells within a population that are undergoing apoptosis. It binds to phosphatidylserine (PS) which is a membrane phospholipid translocated from the inner to the outer leaflet of the plasma membrane during apoptosis. Propidium iodide (PI) is a standard flow cytometric viability probe that can be used to distinguish viable from non-viable cells. Viable cells with intact membranes exclude PI whereas the membranes of dead and damaged cells are permeable to PI. Cells that stain positive for Annexin V-FITC but are negative for PI are undergoing apoptosis. Cells that are positive for both Annexin V-FITC and PI are in the end-stage of apoptosis, necrotic or dead. Cells that are negative for both are alive and not undergoing measureable apoptosis.

Peripheral blood was collected in a CPT tube and peripheral blood mononuclear cells were isolated, resuspended in RPMI/1% FCS, irradiated and incubated at 37°C for the specified time. The cells were then washed twice in cold PBS and resuspended in 1x binding buffer (supplied as x10 concentrate in kit) at a concentration of 1×10^6 /ml. 100 microlitres was transferred to a 5ml polystyrene tube. 5 microlitres each of Annexin V-FITC and PI were added and gently mixed. The cells were then incubated at room temperature in the dark for 15 minutes. 400 microlitres of x1 binding buffer was added to the tube and the sample was analysed by flow cytometry. After gating the lymphocyte population FITC fluorescence (FL-1) was plotted against PI fluorescence (FL-2) and a density dot plot generated. The dot

plot was divided into quadrants and the proportion of cells in each quadrant compared. This was performed for irradiated and non-irradiated cells.

2.2.13 Statistical analysis

Statistical analyses and graph drawing were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

ROC curve construction and analysis and forest plot construction were performed using Stats Direct software version 2.6.6 (StatsDirect Ltd, Altrincham, UK).

Details of the statistical tests used are reported within each chapter.

3. Systematic review of the current evidence base relating to functional cell-based predictive assays of normal tissue radiosensitivity.

3.1 Methodology of diagnostic test development and assessment of clinical utility

How do the normal processes involved in the development of a diagnostic test relate to predictive assays of normal tissue radiosensitivity?

A diagnostic test has two purposes – to provide reliable information about a patient's condition and to influence the health care provider's plan for managing the patient. A test can only serve this purpose if the health care provider knows how to interpret it. This information is acquired through an assessment of the test's precision and diagnostic accuracy, which is simply the test's ability to discriminate between different states of health. Often the clinical question can be dichotomised - the presence or absence of high normal tissue radiosensitivity in the case of predictive testing for normal tissue radiosensitivity. If the test result does not differ in the two health states the test has negligible accuracy and if the test results do not overlap for the two health states the test has perfect accuracy. Most test accuracies fall between these extremes.

Fryback and Thornbury 1991 describe a working model for assessing the efficacy of diagnostic tests in clinical medicine and propose a six-level hierarchical model:

Level 1: Technical Efficiency

Optimisation of assay parameters within the laboratory and examination of assay precision measured by features such as within- and between-sample reproducibility.

Level 2: Diagnostic accuracy efficacy

The test's sensitivity, specificity i.e. it's ability to discriminate between radiosensitive and non-radiosensitive individuals.

Level 3: Diagnostic thinking efficacy

The difference in the clinician's estimated probabilities of a diagnosis (high normal tissue radiosensitivity) before versus after the test results are known.

Level 4: Therapeutic efficacy

The percentage of time that therapy planned before the diagnostic test is altered by the result of the test, e.g. radiotherapy is not given or dose reduced for those with a positive test, or dose-escalated for those with a negative test.

Level 5: Patient outcome efficacy

e.g. the improvement in an individual's quality of life gained by avoiding radiation toxicity in a radiosensitive patient or the control of a tumour achieved by dose-escalation in a patient with a negative test.

Level 6: Societal efficacy

The cost-effectiveness of the test from society's point of view e.g. improved population control rate of cancer or reduced cost of treating radiation toxicity.

In this model a key feature is that for a diagnostic test to be efficacious at a higher level it must be efficacious at all lower levels. Equally, if the test is efficacious at one level it does not follow that it will be efficacious at all higher levels. Strategies for developing an efficacious diagnostic test must therefore start at level 1 and test efficacy at each level before working up to the next. Levels 1 to 2 are important in the pre-clinical testing of assay performance. No predictive assay of normal tissue radiosensitivity has so far been tested at level 3 or above.

3.1.1 Technical efficacy

Level 1 testing of technical efficacy requires a measurement of assay precision or reproducibility once the technical parameters of the assay have been optimised. The technical performance of the assay can be estimated by the measurement of intra-sample and inter-sample reproducibility using samples from the same individual. For assays with subjective endpoints such as scoring of chromosomal aberrations an assessment of inter-scorer reproducibility is also necessary. Whether sample handling, such as storage of samples before testing, affects assay results should also be assessed and handling conditions optimised to avoid adding an uncontrolled source of assay variation into subsequent studies.

3.1.2 Diagnostic accuracy

To determine diagnostic accuracy (Level 2), the ability of the assay to discriminate between health conditions must be compared with the gold standard assay already in clinical use. No “gold standard” predictive test for normal tissue radiosensitivity currently exists so the gold standard must be a clinical assessment of normal tissue radiosensitivity. This is achieved by measuring normal tissue toxicity using a standardised tool such as one of the grading systems (e.g. RTOG/EORTC) described in Chapter 1. This must then be adjusted to account for those factors other than intrinsic normal tissue radiosensitivity known to influence the development of normal tissue toxicity. These confounding factors include the treatment related factors and patient related factors described in Section 1.4.1.

As the development of late toxicities increases over time following radiotherapy, the time elapsed between the completion of radiotherapy and the measurement of late toxicity must also be taken into account otherwise differing severities of late toxicity between individuals may simply be due to one individual having had more time to manifest side effects rather than a difference in intrinsic normal tissue radiosensitivity. Only after adjustment for these confounders of normal tissue reaction can an estimation of an individual’s normal tissue radiosensitivity be made with any degree of certainty and even then it is difficult to account for imprecision in radiation dose homogeneity caused by variations in shape and size of individual patients and other currently unknown factors (other than intrinsic normal tissue radiosensitivity) which may influence normal tissue radiation reaction.

3.1.3 Ideal study design in assessment of diagnostic accuracy of predictive assays of normal tissue radiosensitivity

The choice of study design is of primary importance in reducing bias in diagnostic test research. In a prospective cohort study the predictive assay is performed before radiotherapy, thus ensuring that radiotherapy itself does not influence the assay result. The researcher can strictly define the eligibility criteria for the study, the type of data to be collected and by whom, the method of data collection and the analysis techniques. With appropriate design, the study therefore includes an unselected group of homogenous patients receiving a homogenous radiotherapy regimen with standardised collection of information on toxicity by direct inspection by the researcher and prospective collection of

information on possible confounding factors. Statistical techniques such as multiple regression analysis can be used to correct for the influence of other recognised confounders and determine if assay result is an independent prognosticator of normal tissue reaction and therefore radiosensitivity. The quality of data from a prospective cohort study of radiosensitivity is high but the power of this study design to achieve statistically significant results in normal tissue radiosensitivity testing is limited by the low frequency of severe radiation toxicity amongst unselected patients, unless very large numbers of patients are recruited to the study. Although it is straightforward to collect data regarding acute toxicity rapidly, prospective cohort studies have limited potential when examining assays which predict late normal tissue damage which may take many years to manifest. Firstly it would take many years to obtain results from the study, and secondly the attrition of patients due to disease progression or other factors during this time means that the cohort size needs to be even larger than for studies using acute tissue toxicity as an end-point in order to generate sufficient data to potentially achieve meaningful results.

By performing the assay and measuring late toxicity in patients previously treated with radiotherapy in the past, retrospective cohort studies may help overcome the problems associated with assessing predictive assays of late normal tissue radiosensitivity in a prospective study. Collecting accurate information about patients and treatment retrospectively can be difficult, but if patients were treated as part of a clinical trial of radiotherapy they will usually form a relatively homogenous group with respect to tumour type and radiotherapy schedule and technique, and information about other confounding factors such as concurrent chemotherapy or comorbidity may already have been collected or built into the trial inclusion and exclusion criteria. The generalisability of assays assessed in retrospective studies may be limited by the fact that a selection process unavoidably occurs with time so that the patients available for study many years after radiotherapy delivery may not be truly representative of the original cohort and therefore the population as a whole.

Retrospective case control studies dichotomise the patient population and seek to compare assay results in a group of patients with known high clinical radiosensitivity (cases) and those known low or average clinical radiosensitivity (controls). In order to correct for the influence of confounding factors other than intrinsic normal tissue radiosensitivity on the

development of radiation toxicity the cases and controls must be matched with respect to known confounders and both cases and controls must be drawn from the same population (i.e. same group of patients treated within the same centre during the same specified time period). Failure to match cases and controls or drawing cases and controls from separate populations will introduce bias and make the results of the study difficult to interpret.

Scoring of toxicity should be standardised throughout the study with specification of scoring system used, the individual/s responsible for grading toxicity and an assessment of inter-scorer reproducibility performed, if relevant. As different cell-based assays may predict specific radiation responses in different tissues, homogeneity in toxicity assessment should be employed measuring specific early and late end-points in specific tissues separately for separate analysis rather than heterogeneous endpoints in a variety of different tissues which may lead to masking of a relationship between the assay result and a specific tissue endpoint.

Blinding of the laboratory researchers performing the assay to the normal tissue responses of the patients, and of the clinical researchers scoring toxicity to the assay result may also reduce potential sources of bias.

3.1.4 Statistical methods employed in assessment of diagnostic accuracy.

Whilst it is important to determine if there is a statistically significant correlation between assay result and normal tissue response after adjusting for confounders or if there is a statistically significant difference in mean or median assay results from cases and controls, it is the power of the assay to discriminate between radiosensitive and non-radiosensitive individuals that is important in diagnostic accuracy assessment. Outcome measures used in determining diagnostic test accuracy are:

- Sensitivity: the rate of correct identification of patients with high normal tissue radiosensitivity by the predictive assay
- Specificity: the rate of correct identification of patients with normal or low normal tissue radiosensitivity by the predictive assay.

In order to calculate these parameters radiosensitivity data has to be dichotomised which does lead to a loss of information and statistical power. It also requires a definition of a cut-point in toxicity grading to divide clinically highly radiosensitive from non-clinically radiosensitive individuals – given that clinical radiosensitivity is most likely a continuously varying characteristic the choice of cut-point is arbitrary. Nevertheless the sensitivity and specificity of a diagnostic assay are valuable measurements of its clinical utility and are the accepted methods for defining the discriminatory power of a diagnostic test.

In order to calculate sensitivity and specificity each individual needs to be classified as a true positive (radiosensitive) or true negative (not radiosensitive) according to the “gold standard” (in this case clinical assessment of normal tissue toxicity after adjustment for confounders) and then classified as positive or negative according to the predictive assay under investigation.

A diagnostic test 2 by 2 table is then constructed:

	Clinically radiosensitive	Clinically non-radiosensitive
Predictive assay positive	a (true positive)	b (false positive)
Predictive assay negative	c (false negative)	d (true negative)

$$\text{Sensitivity} = a/(a+c)$$

$$\text{Specificity} = d/(b+d)$$

In order to calculate sensitivity and specificity, a cut off for the assay results must be chosen to define what constitutes a positive and what constitutes a negative test. Receiver Operator Characteristic (ROC) curves have been used since the 1970s as a way of assessing whether or not a diagnostic test has useful discriminatory power and can be used to help define appropriate cut-offs for positive and negative tests and to compare the discriminatory power of different tests (Zweig and Campbell 1993).

To construct a ROC curve all cut-off points that give a unique pair of values for sensitivity and specificity are considered, sensitivity versus 1-specificity are plotted and the data points connected by lines to generate the curve (Figure 3.1). If a test has any discriminatory power the curve will lie to the left of the diagonal of the graph. The area under the curve gives an estimate of the discriminatory power of a test. If the test has perfect discriminatory power and possessed a cut off that would result in 100% sensitivity and 100% specificity then the ROC curve would go across the top of the grid area and the AUC would be 1. If the test possesses no discriminatory power then 100% sensitivity can only be achieved with 0% specificity and vice versa – the ROC curve would follow the diagonal on the grid and the AUC would be 0.5. The closer the AUC for the ROC curve is to 1 the better the assay performance.

The ROC curve can also be used to choose optimal cut-offs values for a test depending on the clinical implications of false positive and false negative results and therefore the relative requirements for sensitivity versus specificity.

The diagnostic odds ratio (ad/bc) is a summary statistic of diagnostic assay performance often used when combining studies of diagnostic accuracy in a systematic review. Its value is often reasonably constant no matter the choice of cut-off values for the diagnostic threshold, although a single diagnostic odds ratio corresponds to a single set of sensitivities and specificities chosen from the ROC curve. In the case of predictive assays of normal tissue radiosensitivity it describes the odds of a positive test in participants with high normal tissue radiosensitivity versus the odds of a positive test in those with normal tissue radiosensitivity. The value of this statistic in systematic reviews is that it combines both the positive and negative predictive capabilities of an assay in one value, but its meaning is difficult to apply directly to clinical practice (Deeks 2001) .

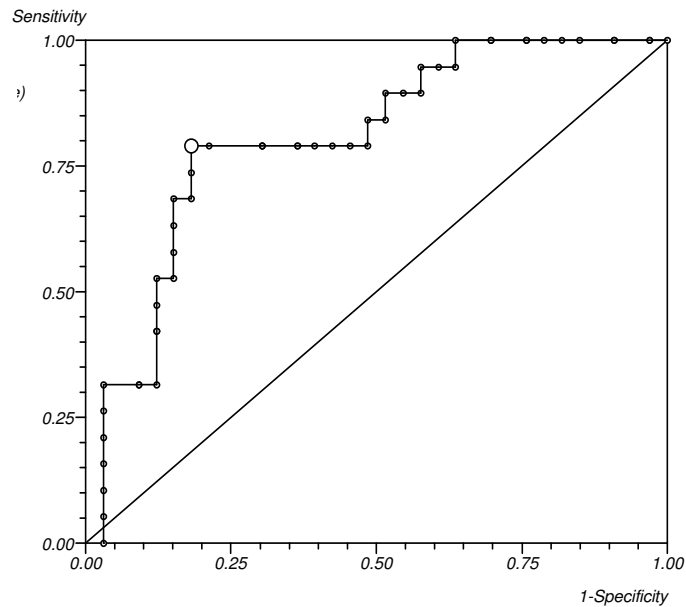


Figure 3.1 ROC curve for % reduction in Binucleated index after radiation in human fibroblasts and risk of wound healing complications after post-operative radiotherapy for soft-tissue sarcoma. AUC is 0.805 (95% CI 0.432-1) (Data for analysis extracted from (Akudugu, Bell et al. 2006))

3.1.5 Hypothesis generating and validation data sets

If a study establishes a relationship between a particular predictive assay result and an individual's normal tissue radiosensitivity, then this relationship should be validated by testing the assay's discriminatory ability on a separate independent data set to avoid recursive reasoning.

3.1.6 Rationale for a systematic review of the current literature pertaining to functional cell-based assays in the predictive testing of normal tissue radiosensitivity.

Many researchers have tried to develop a predictive assay of normal tissue radiosensitivity based on the measurement of the response of cells sampled from an individual to a test dose of radiation in vitro. As discussed in section 1.6 the results from these studies have been inconsistent leading to the opinion of many researchers that their ongoing investigation is not worthwhile and a move towards other strategies. Problems with the methodology of the studies involving patients with breast cancer have been identified (Twardella and Chang-Claude, 2002) and the question arises as to whether or not the current evidence base is sufficient to disregard cell-based assays entirely or whether there

is evidence that difficulties in study design and reporting may have resulted in bias and masked their potential clinical utility. A systematic review of the literature was therefore performed in an attempt to answer the following questions:

- Is there evidence from the literature that intrinsic radiosensitivity of normal tissue and consequent increased susceptibility to radiotherapy side effects can be predicted by functional cell-based laboratory assays?
- Have clinical studies assessing potential assays of normal tissue radiosensitivity been performed and reported with a sufficiently rigorous approach to assay feasibility and quality control and been reported in sufficient detail to allow adequate assessment of study quality and generalisability of results?
- Is the generally held belief that cell-based assays are not helpful in predicting normal tissue radiosensitivity justified based on current literature?

3.2 Method

3.2.1 Identification of relevant studies

The literature was searched according to the following strategy:

- Medline/EMBASE search - 1966-present (including articles published online before 28th February 2008). Search Strategy –“ radiotherapy AND radiation effect/s or radiation injury or radiation tolerance” (NB MeSH headings such as “assay” “test” “diagnostic test” “predictive” etc do not reliably pick up all of the predictive assay studies)
- Reference lists from papers
- Hand searching of “Radiotherapy and Oncology” and "International Journal of Radiation Oncology Biology and Physics” journals.
- Reverse citation tracking for electronic resources (“cited by” links)

Inclusion criteria

- Any study comparing cellular radiosensitivity as measured by the assay under investigation with severity of acute and/or late normal tissue toxicity following radiotherapy.
- All potential functional cell-based assays (i.e. measures response of a living cell after test dose of radiation in vitro) and assay techniques
- All types of tissue sample (e.g. skin, blood et c)
- Patients may have received radiotherapy (with or without synchronous chemotherapy) at any site, for any type of malignancy

Exclusion criteria

- Studies published in abstract form only
- Papers reporting data presented already elsewhere, or reports of pilot studies where the full data is presented in another later publication.
- Any study of a non- functional cell-based assay (e.g. genotyping)
- Studies comparing highly radiosensitive patients to healthy “controls” who have not had radiotherapy and are therefore of unknown radiosensitivity
- Studies comparing assays of radiosensitivity in cultured cell lines only without direct link to clinical data
- Case reports of assay results in individual unusually radiosensitive patients

3.2.2 Data Extraction

All studies that fulfilled the inclusion criteria were analysed according to the data extraction protocol in Appendix 4.2. which is based on REMARK recommendations (McShane, Altman et al. 2005).

3.2.3 Development of a scoring system for study “quality”

In order to help filter the available data and determine if there is any evidence of potential assay efficacy in the literature, an assessment of quality of study design and conduct was made in order to allow appropriate weighting to higher quality studies.

The scoring system is described in Appendix 4.3. A potential total score of 100 was possible. The heaviest weighting in the scoring system is given to appropriate study design

(ensuring homogeneity of patients and treatment), techniques for dealing with confounding factors, and appropriate choice and reporting of statistical methods. Clearly this scoring system is crude and has not been validated in other studies of diagnostic assays but does allow some objective measurement of how “convincing” the result of a particular study might be.

3.2.4 Extraction of individual patient data, construction of ROC curves and calculation of diagnostic odds ratio.

In an attempt to generate homogeneity of statistical analysis of the reviewed studies and a more meaningful comparison of the clinical utility of the assays under investigation, assay results for individual patients along with information about their normal tissue toxicity were extracted from the study reports where possible. Data was extracted directly from tables within the study report or, where individual patient data was presented as points on a scatter-plot, the assay result was estimated by measurements performed on a digital image of the relevant plot after digital calibration using the Mouseyes image digitisation program version 3.1 <http://www.hop.man.ac.uk/staff/rtaylor> . If the study in question had not been performed as a case-control study, a cut-off in normal tissue toxicity grading was chosen in order to divide patients into a radiosensitive group and a non-radiosensitive group for the purpose of ROC construction e.g. the majority of studies used the CTC or RTOG/EORTC grading systems and for the purpose of this analysis patients were divided into grades 0-2 toxicity (normal radiosensitivity) and grade 3-4 toxicity (high radiosensitivity). ROC curves were constructed for each assay using StatsDirect statistical software. Assay cut-offs (i.e. defining a positive versus a negative test) were chosen to try to optimise sensitivity and specificity with a 1:1 weighting of sensitivity: specificity. Once cut-off were chosen, assay sensitivity and specificity and corresponding diagnostic odds ratios and their 95% confidence intervals were calculated and presented graphically in Forest plots. Funnel plots of Sample size and quality index score against the log diagnostic odds ratio were constructed and visually inspected for asymmetry to assess for evidence of bias in results arising from publication bias or low quality of study design.

3.3 Results

64 studies were identified which fulfilled the inclusion criteria, published between 1993 and 2007 and are listed in Appendix.4.1. In many papers several assays or different assay end-points were compared with different toxicity end-points. In total 98 comparisons between assay results and specific normal tissue toxicity endpoints were reported. 47 comparisons were reported as showing a positive relationship between the assay result and normal tissue radiosensitivity, whilst in 51 no such relationship could be detected.

3.3.1 Assay under investigation and cell types used.

The majority of the assays investigated can broadly be divided into assays of clonogenic cell survival or assays of DNA damage whether measured by cytogenetic damage or direct assessment of DNA breaks (Table 3.1). A smaller number of studies reported assays of radiation-induced apoptosis, cell cycle arrest, gene expression profiling and spontaneous differentiation. One study examined DNA damage in keratinocytes and all other studies used skin derived fibroblasts or peripheral blood lymphocytes as the surrogate tissue for investigation.

3.3.2 Study design.

The majority of studies employed a case-control design (Table 3.2). There were an equal number of prospective and retrospective cohort studies. Five studies employed a mixed prospective and retrospective cohort design. Four of these examined acute toxicity prospectively and late toxicity in a different population retrospectively (Geara, Peters et al. 1993; Barber, Burrill et al. 2000; Slonina, Klimek et al. 2000; Alsbeih, El-Sebaie et al. 2004). The fifth began as a prospective cohort study but recruited patients retrospectively due to the low rates of toxicity apparent in the prospective cohort (Oppitz, Schulte et al. 2002).

Prospective cohort studies recruited the largest number of patients with a median sample size of 82. In retrospective studies the sample size tended to be small with the exception of 9 studies which included 50 or more patients.(Peacock, Eady et al. 1989; Russell, Grummels et al. 1998; West, Davidson et al. 1998; Crompton, Miralbell et al. 1999; Russell, Lara et al. 2000; Oppitz, Baier et al. 2001; Dickson, Magee et al. 2002; Hoeller, Borgmann et al. 2003; De Ruyck, Van Eijkeren et al. 2005)

Table 3.1 Categories of predictive assays and cell types investigated

Broad assay category	Assay	Number of papers reporting	Cell type (number of papers)
Survival	Clonogenic survival	24	Fibroblast (19), lymphocyte (5)
	MTT	1	Lymphocyte (1)
DNA damage	Cytogenetic - micronucleus	7	Fibroblast (2), lymphocyte (4), both (1)
	Cytogenetic – chromosomal	6	Lymphocyte (6)
	Alkaline comet (repair)	6	Lymphocyte (4), fibroblast (1), both (1)
	DSB repair (CF or PF gel electrophoresis)	6	Fibroblast (5), keratinocyte (1)
	DSB induction (CF or PF gel electrophoresis)	4	Lymphocyte (4)
	Radiation induced repair-related foci formation	1	Fibroblast (1)
Apoptosis	Radiation-induced apoptosis	4	Lymphocyte (4)
Cell cycle	Radiation-induced cell cycle arrest	3	Lymphocyte (3)
Differentiation	Spontaneous in vitro fibroblast differentiation	2	Fibroblast (2)
Gene expression	Microarray, Western blotting, reverse transcription PCR	5	Lymphocytes (5)

Table 3.2 Study design and sample size for identified studies of predictive assays of normal tissue radiosensitivity

Study design	Number	Median sample size (range)
Prospective cohort	15	83 (24-393)
Retrospective cohort	14	31.5 (6-93)
Retrospective case/control	30	29 (7-96)
Mixed prospective/retrospective cohort	5	27 (11-123)

3.3.3 Reporting of tumour and patient characteristics.

46 studies included patients in whom a solitary tumour site had been irradiated, whilst the remaining 18 included patients in who had received treatment for tumours in multiple difference sites. The majority of studies (45%) examined normal tissue toxicity following breast or chest wall radiotherapy.

Table 3.3 Tumour sites irradiated in predictive assay studies

Tumour site	Number of studies (%)
Breast	29 (45.3%)
Cervix/endometrium	5 (7.8%)
Head and Neck	5 (7.8%)
Prostate	4 (6.3%)
Soft tissue sarcoma (site not specified)	2 (3.1%)
Brain (arteriovenous malformation)	1 (1.5%)
Mixed	18 (28.1%)

Tumour staging was reported in only 25 (39%) of studies.

35 studies (55%) did not report patient demographics. In those studies where some demographics were reported these tended to be restricted to age and gender. Only 6 studies reported smoking status (Kiltie, Barber et al. 1999; Kiltie, Ryan et al. 1999; Rudat, Dietz et

al. 1999; Widel, Jedrus et al. 2003; Wiebalk, Schmezer et al. 2007), 2 reported body mass index (Popanda, Ebbeler et al. 2003; Wiebalk, Schmezer et al. 2007) and 1 reported haemoglobin level (Rudat, Dietz et al. 1999), all of which are thought to influence the severity of radiation morbidity (see section 1.4.1).

Only 18 studies (28%) either clearly excluded patients or documented the proportion of patients who were receiving or had received chemotherapy or other systemic therapy, or accounted for the confounding effect of chemotherapy by case-control matching.

3.3.4 Reporting of radiotherapy details.

The majority of, but not all, studies reported the total radiation dose and fractionation used in the treatment of the study subjects. Only half of the studies reported beam quality including energy and just above a quarter reported radiotherapy planning technique. The skin dose during radiotherapy was recorded in two studies of skin toxicity (Begg, Russell et al. 1993; Burnet, Nyman et al. 1994).

Table 3.4 Reporting of radiotherapy details

	Number reporting (%)
Radiotherapy dose	54 (84%)
Radiotherapy fractionation	47 (73%)
Beam quality	32 (50%)
Planning technique	18 (28%)

3.3.5 Recording and reporting of radiotherapy toxicity.

37 studies recorded acute radiotherapy toxicities, 38 late toxicity and 16 recorded both. The most frequently used toxicity grading system was the RTOG/EORTC system for both acute and late toxicity. Some studies examining both acute and late effects used different systems for different end-points. A number of studies used scoring systems that had been developed locally in that particular department, or a system that had been developed for the purposes of a previous clinical trial rather than an internationally recognised system e.g. the scoring system used by Peacock et al, 2000 which had been developed for the START trial comparing cosmetic results from different radiotherapy schedules in breast

cancer. In 3 studies grading of late fibrosis was adjusted to account for radiation dose, dose per fraction and follow-up time to give a score for “excess risk of fibrosis” which was then used as the toxicity end-point for analysis (Johansen, Bentzen et al. 1994; Johansen, Bentzen et al. 1996; Herskind, Bentzen et al. 1998; Dikomey, Brammer et al. 2000). In 3 studies toxicity was recorded as severe or not severe depending on clinical judgement with no further details given and 5 studies did not report how toxicity was graded at all.

Table 3.5 Radiotherapy toxicity grading systems utilised in the identified studies.

Toxicity grading system	Number of studies
RTOG/EORTC	19
CTC (NCI)	8
LENTSOMA	7
Franco-Italian Glossary	2
“Excess risk of fibrosis”	4
Reflectance spectrography	3
Burnet's modification of RTOG	3
START	1
WHO	1
Readmission for wound packing	2
Departmental scoring system	7
“Clinical judgement”	3
Not reported	5

The median follow-up time between radiotherapy and scoring of late toxicity was not reported in 9 of the 45 studies which examined late toxicity. In 5 studies it was clear that duration of follow up had been accounted for in the analysis (Johansen, Bentzen et al. 1994; Johansen, Bentzen et al. 1996; Herskind, Bentzen et al. 1998; Russell, Grummels et al. 1998; Dikomey, Brammer et al. 2000; Peacock, Ashton et al. 2000) whilst in the remaining studies it was not clear if varying duration of follow up in the cohort or between cases and controls may have confounded the study outcome.

In 13 studies only one clinician was responsible for scoring of toxicity. In 17 studies there was more than one toxicity scorer. In only 8 of these studies had an assessment of inter-scorer reproducibility been undertaken. The number of persons responsible for scoring toxicity was not reported in 34 studies.

In 13 studies the person scoring toxicity was blinded to the laboratory assay result. Whether or not toxicity scorers were blinded was not reported for the other studies.

3.3.6 Details of the laboratory assay under investigation.

All studies reported assay technique in detail or referenced the protocol in another publication.

Biological sample

In 24 studies a skin biopsy was required to obtain cells for investigation. 32 studies isolated lymphocytes from a sample of peripheral blood and 4 studies used both. No more invasive techniques to obtain tissue were utilised.

Sample handling

15 studies commented on whether the biological sample had been stored before analysis and for how long. 8 of these reported the cryopreservation of lymphocytes, 1 commented that samples had been sent to the laboratory in the post and the others recorded maximum duration of sample storage at room temperature before analysis. It was not clear from any report as to whether samples from the whole cohort had received similar treatment and if cases and control samples had been treated identically.

Time to obtain results

To be of any clinical utility a laboratory assay must be able to generate results within 2 weeks of tissue sampling, ideally faster. Although no study specifically reported the time taken between sampling and generation of results in the laboratory this information could be derived from the protocol in most studies. In 30 studies results were not available for at least 2 weeks following sampling (usually 4-6 weeks in the case of skin biopsies requiring fibroblast culture). In 32 studies results were available within 2 weeks, often within one week. In 2 studies it was not possible to derive this information.

Assay failure rate.

The assay failure rate (the percentage of biological samples from which no assay result could be generated) was reported or could be derived in only 16 studies (25%). The median assay failure rate was 18.5% (range 1.5-71%). Most failures were associated with failure to obtain viable lymphocytes for testing after cryopreservation.

Quality control and assay reproducibility.

No study reported on day to day laboratory quality control procedures. In 14 studies, all utilising fibroblasts from skin biopsies, intra-sample reproducibility was assessed. The coefficient of variation (CV) was not reported for 3 studies. For the remaining 11 studies the median reported intra-sample CV was 13% (range 4.4-53). Inter-sample reproducibility (repeating the assay using repeated samples from the same individual) was reported for 20 studies. For 7 of these studies the CV was not reported. Where figures are given the median CV is 15% (range 2.5-40%). In 5 studies references are given for previous reports from the same laboratory where intra- and inter-sample reproducibility had already been assessed (West, Elyan et al. 1995; West, Davidson et al. 1998; Barber, Burrill et al. 2000; West, Davidson et al. 2001; Lopez, Guerrero et al. 2005)

Scoring of subjective assay end-points

Laboratory assays had subjective endpoints in 50 of the studies reported. No study reported an assessment of intra-scorer reproducibility. In 46 studies there was no comment as to whether the scoring was performed by more than one individual and whether inter-scorer reproducibility had been assessed. In 4 studies inter-scorer comparisons were performed but not reported.

In 4 studies the scorer was blinded to the toxicity data pertaining to the relevant patient. In 41 studies there was no comment on blinding of the assay scorer.

3.3.7 Treatment of confounding factors and statistical analysis

In only 17 studies a deliberate strategy had been employed to control for at least some of the confounding factors other than intrinsic normal tissue radiosensitivity that might influence the development of normal tissue radiation toxicity in a given individual. In 5 studies matching of cases and controls was performed (Peacock, Eady et al. 1989; Borgmann, Roper et al. 2002; Leong, Chao et al. 2003; Tell, Edgren et al. 2003; Rieger,

Hong et al. 2004) with differing levels of stringency and therefore effectiveness. Peacock et al matched cases and controls stringently according to radiation dose, year of follow-up, width of radiation field, thickness of lung in field, breast size, radiotherapy boost, treating centre, radiotherapy field separation, axillary radiotherapy, tamoxifen, adjuvant chemotherapy and timing of chemotherapy in relation to the radiotherapy. On the other hand Leong et al matched only for gender, age, tumour site, tumour stage and concurrent medication, and not all cases were matched with appropriate controls.

Kiltie et al used multiple regression analysis including those patient and treatment related factors known to influence toxicity (but not specifying which ones) to determine if the predictive assay result was an independent prognostic factor for the development of radiation toxicity (Kiltie, Ryan et al. 1999; Twardella, Popanda et al. 2003). Twardella et al (2003) performed Cox proportional hazards analysis to determine which patient- and treatment-related factors influenced radiation toxicity but analysed assay result separately and did not incorporate it into the model with the potential confounders. Univariate analysis examining whether or not toxicity was influenced by chemotherapy, age and beam quality as well as the predictive assay result was utilised by Hoeller et al, 2003, analysing each separately and not in one combined multivariate model.

Other groups incorporated a correction factor for at least some confounders into their radiation morbidity scoring system - the system for measuring excess risk of fibrosis developed in Aarhus accounts for radiation dose, fraction size and duration of follow up (Johansen, Bentzen et al. 1994; Johansen, Bentzen et al. 1996; Herskind, Bentzen et al. 1998; Dikomey, Brammer et al. 2000) as does the system used for grading risk of fibrosis utilised by Russell et al, (1998 and 2000).

In 2 studies ROC curves were constructed from assay results and assay sensitivity and specificity calculated (Mariano Ruiz de Almodovar, Guirado et al. 2002; Wang, Chen et al. 2005). For an assay of DNA DSB induction in peripheral blood lymphocytes The Spanish group calculated a sensitivity of 33% and specificity of 95% for predicting acute skin toxicity in breast radiotherapy with a ROC AUC of 0.675 (95% CI 0.534-0.817) Wang et al calculated a sensitivity of 51%, specificity 94% and ROC AUC 0.715 (95% CI 0.557-

0.835) for an assay predicting acute toxicity in head and neck cancer patients again by the measurement DSB induction in lymphocytes.

For case control studies comparison of means or medians was the most commonly used statistical method to establish whether the assay result differed between radiosensitive and non-radiosensitive patients and was reported in 25 studies using either a the student's t-test, Mann-Whitney U-test or ANOVA to look for statistically significant differences between groups. In cohort studies, non-parametric correlation (usually Spearman) was used to test for a relationship between assay result and grade of radiation toxicity (16 studies), although linear regression was used in 3. Actuarial analysis with calculation of Kaplan-Meier survival curves, stratification by assay result and log-rank test to examine for a statistically significant difference in risk of developing toxicity was used in 5 studies. Statistical methods and results were not reported in 8 studies.

3.3.8 Hypothesis generating and validation data sets

One of the two studies of gene expression profiling (Svensson, Stalpers et al, 2006) included details of attempted validation of the trained classifier on a separate very small cohort of patients with limited success. The other study reported details of the training set only (Rieger, Hong et al 2004).

Four other studies were validation studies from previously published hypothesis generating data sets. Peacock et al 2000 were attempting to validate the initial report of a correlation between fibroblast cell survival and late effects of radiation in breast cancer patients published by Burnet et al. The study by Dickson et al, 2002 was an attempt to validate the previous report from the same group of a relationship between DNA DSB repair and late radiation toxicity in breast cancer patients (Kiltie, Ryan et al, 1999). Both of these validation studies could not confirm the findings of the initial studies. West et al confirmed their initial reports that lymphocyte radiosensitivity correlated with radiation toxicity in a prospective study (West, Davidson et al, 2001). The prospective study by Ozsahin et al was performed to validate the previous retrospective study proposing a relationship between radiation-induced lymphocyte apoptosis and radiation toxicity, and seem to confirm the initial findings (Ozsahin, Crompton et al, 2005).

One group reported three prospective studies of lymphocyte DNA damage assays in the prediction of toxicity following breast radiotherapy. It was difficult to determine from the reports whether the patient groups in these studies overlapped or formed early hypothesis-generating and later validation sets. (Lopez, E., R. Guerrero, et al. 2005; Mariano Ruiz de Almodovar, J., D. Guirado, et al., 2002; Pinar, B., P. C. Lara, et al. ,2007).

The other reports of a positive relationship between assay result and toxicity have not so far been followed by confirmatory validation studies from the same group.

3.3.9 Summary of the results from identified studies

There is clearly significant heterogeneity in the identified studies with respect to assay type, surrogate tissue analysed, tumour sites irradiated, radiotherapy scheduling, treatment of confounding factors and statistical analysis, with some studies better designed and reported than others. The majority involve the study of clonogenic cell survival or DNA damage in fibroblasts or lymphocytes. In order to try to ascertain if there is any clear pattern of positive and negative studies to suggest clinical utility or lack of it in the prediction of normal tissue radiosensitivity with any particular type of assay or cell type, individual comparisons between assay end-point and a particular toxicity end-point were extracted from each paper. These comparisons were broadly divided into two categories – those involving assays of clonogenic cell survival and those involving assays of DNA damage. The DNA damage category was not subdivided further. Each category was then subdivided into those studies based on fibroblasts versus those based on lymphocytes and then further into those examining acute versus late toxicity. The proportion of positive versus negative studies (based on the reported p-values) for each category was then calculated and an estimation of the influence of quality of study design and conduct was made by comparing the median quality index score for positive and negative studies within each subgroup.

Table 3.6 Assays of clonogenic cell survival – Fibroblasts and acute radiation toxicity**Negative result**

Reference	Assay end-point	Quality score	Toxicity
Akudugu, 2006	SF2.4	35	wound healing complication
Alsbeih, 2004	SF2	17	any
Begg, 1993	SF2, D10	45	skin erythema
Brock, 1995	SF2	39	skin erythema
Burnet, 1994	D0.01	35	skin erythema
Djuzenova 2004	SF2	21	skin
El-Adawy, 2005	SF2	38	skin
Geara, 1993	SF2	20	skin/mucosa
Johansen, 1996	SF3.5	60	skin erythema
Loeffler, 1990	D0, D, D10, n	31	skin
Oppitz, 2001	SF2	23	any
Oppitz, 2002	SF2	47	skin
Rudat, 1997	SF2	43	skin and mucosa
Rudat, 1999	SF2	55	skin and mucosa
Median quality score		36.5	

Positive result

Reference	Assay end-point	Quality score	Toxicity
Akudugu, 2006	%BNI	35	wound healing complication
Loeffler, 1990	D0, D, D10, n	31	skin
Oppitz, 2001	SF2	23	any
Median quality score		31	

Table 3.7 Assays of clonogenic cell survival – Fibroblasts and late radiation toxicity**Negative result**

Reference	Assay end-point	Quality score	Toxicity
Borgmann, 2002	D0.01	58	skin,mucosa,salivary glands, subcut
Brock, 1995	SF2	39	telangiectasia
Johansen, 1996	SF3.5	60	telangiectasia
Peacock, 2000	D0.01	77	skin, subcut
Rudat, 1999	SF2	55	laryngeal oedema/fibrosis/bone
Russell, 1998	SF2	70	fibrosis
Median quality score		59	

Positive result

Reference	Assay end-point	Quality score	Toxicity
Alsbeih, 2000	SF2	11	any
Alsbeih, 2004	SF2	17	any
Burnet, 1994	D0.01	35	telangiectasia
Geara, 1993	SF2	20	skin/fibrosis/mucosa/ bone/laryngeal oedema
Johansen, 1994	SF3.5	56	fibrosis
Johansen, 1996	SF3.5	60	fibrosis
Oppitz, 2001	SF2	23	any
Raaphorst,2002	SF2	41	radiation necrosis
Median quality score		29	

Table 3.8 Assays of clonogenic cell survival – Lymphocytes and acute radiation toxicity

Negative result

Reference	Assay end-point	Quality score	Toxicity
Geara, 1993	SF2	20	skin/mucosa
Oppitz, 2002	SF2	47	skin
West 1995	SF2 and LDR sparing	23	skin
Median quality score		23	

No positive results

Table 3.9 Assays of clonogenic cell survival – Lymphocytes and late radiation toxicity**Negative result**

Reference	Assay end-point	Quality score	Toxicity
Geara, 1993	SF2	20	skin/subcut/mucosa/bone/larynx
West 1995	SF2 and LDR sparing	23	any affected tissue
Median quality score		21.5	

Positive result

Reference	Assay end-point	Quality score	Toxicity
West 1998	SF2	35	any affected tissue
West 2001	SF2	53	most severe toxicity in any tissue
Ramsay, 1995	SF2 (MTT)	25	skin subcutaneous
Median quality score		35	

Table 3.10 Assays of DNA damage – Fibroblasts and acute radiation toxicity**Negative result**

Reference	Assay end-point	Quality score	Toxicity
Djuzenova 2004	%MRE11pos nuclei	21	skin
Djuzenova 2004	%Rad51 pos nuclei	21	skin
Oppitz, 2002	DNA repair - alkaline comet assay	47	skin
El-Adawy, 2005	DNA repair - residual DSBs (CFGE)	38	skin
Akudugu, 2004	micronucleus	45	wound healing complication
Slonina, 2000	micronucleus	11	mucosa /skin
Median quality score		29.5	

Positive result

Reference	Assay end-point	Quality score	Toxicity
Djuzenova, 2004	%Rad50 pos nuclei	21	skin
Nachtrab, 1998	micronucleus	5	mixed
Oppitz, 1999	DNA repair - alkaline comet assay	15	mixed
Median quality score		15	

Table 3.11 Assays of DNA damage – Fibroblasts and late radiation toxicity**Negative result**

Reference	Assay end-point	Quality score	Toxicity
Slonina, 2000	micronucleus	11	rectum/bladder
Borgmann, 2002	DNA repair - residual DNA damage (CFGE)	58	skin,mucosa,salivary glands, subcut
Dikomey, 2000	DNA repair - residual DNA damage (CFGE)	55	fibrosis
Dickson, 2002	DNA repair - residual DNA damage (PFGE)	61	fibrosis and SOMA score
Median quality score		56.5	

Positive result

Reference	Assay end-point	Quality score	Toxicity
Kiltie 1999 (1)	DNA repair - residual DNA damage (PFGE)	50	fibrosis , LENT score
Nachtrab, 1998	micronucleus	5	mixed
Oppitz, 1999	DNA repair - alkaline comet assay	15	mixed
Median quality score		15	

Table 3.12 Assays of DNA damage – Lymphocytes and acute radiation toxicity**Negative result**

Reference	Assay end-point	Quality score	Toxicity
Lopez, 2005	DNA DSB induction (PFGE)	53	skin
Twardella, 2003	DNA repair - alkaline comet assay	53	skin
Wang, 2005 (2)	DNA repair - alkaline comet assay	32	skin
Popanda, 2003	DNA repair - alkaline comet assay	28	skin
Rached, 1998	micronucleus	13	skin/mucosa/bowel
Slonina, 2000	micronucleus	11	mucosa /skin
Median quality score		30	

Positive result

Reference	Assay end-point	Quality score	Toxicity
Kearsley, 1998	Chromosome aberrations	1	mucosa /skin
Neubauer, 1997	chromosome rearrangements	16	mixed
Wang, 2005 (1)	DNA DSB induction (PFGE)	52	skin
Ruiz de Almodovar 2002	DNA DSB induction (PFGE)	52	skin
Alapetite, 1999	DNA repair - alkaline comet assay	22	skin, oesophagus
Oppitz, 2002	DNA repair - alkaline comet assay	47	skin
Barber, 2000 (1)	G2 assay	65	skin
Widel, 2003	micronucleus	33	any
Median quality score		40	

Table 3.13 Assays of DNA damage – Lymphocytes and late radiation toxicity**Negative result**

Reference	Assay end-point	Quality score	Toxicity
Lopez, 2005	DNA DSB induction (PFGE)	53	skin
Hoeller, 2003	lethal chromosome aberrations	63	time to fibrosis and fibrosis
Slonina, 2000	micronucleus	11	rectum/bladder
Median quality score		53	

Positive result

Reference	Assay end-point	Quality score	Toxicity
Neubauer, 1997	complex chromosome rearrangements	16	mixed
Pinar, 2007	DNA DSB induction (PFGE)	50	skin subcutaneous
Alapetite, 1999	DNA repair - alkaline comet assay	22	fibrosis, telangiectasia, lung dysfunction, cardiac disease
Deeley, 1989	DNA repair - nuclear lysate sedimentation	12	bowel or bladder
Borgmann, 2002	DNA repair - residual DNA damage (CFGE)	58	skin,mucosa,salivary glands, subcutaneous
De Ruyck,2005	G2 assay	39	any
Widel, 2003	micronucleus	33	any
Barber, 2000 (1)	micronucleus	65	fib,telang, retraction, pain
Lee, 2003	micronucleus	67	GI/GU
Median quality score		39	

Table 3.14 Summary of outcome and quality score for assays other than those examining clonogenic cell survival or DNA damage and repair

Reference	Assay category	Assay end-point	Cell type	Outcome	Quality score	Toxicity assessed
Barber 2000 (2)	Apoptosis	% apoptosis	lymphocyte	neg	42	Late
Ozsahin, 2005	Apoptosis	% apoptosis	lymphocyte	neg	44	Acute
Ozsahin, 2005	Apoptosis	% apoptosis	lymphocyte	pos	44	Late
Kilzilian-Martel, 2003	Apoptosis	Apoptotic fraction	leucocytes	pos	5	Mixed
Crompton, 1999	Apoptosis	% apoptosis	lymphocyte	pos	25	Mixed
Perez, 2007	Cell cycle	% cells in G2/M arrest	lymphocyte	pos	1	Late - bladder, bone, skin
Lavin 1994	Cell cycle	% G2 18 hours post 3Gy	lymphocyte	pos	15	mixed acute and late
Tell, 2003	Cell cycle	% cells in G2 after 8Gy	lymphocyte	neg	36	acute - pulmonary injury
Svensson, 2006	Gene expression profiling	gene expression classifier	lymphocyte	pos	32	late - bladder rectum
Hummerlich, 2006	Gene expression profiling	increased or decreased gene expression	lymphocyte	neg	32	Acute - Bone marrow, GI, GU
Reiger, 2004	Gene expression profiling	gene expression classifier	lymphocyte	pos	51	acute - any
Leong, 2003	DNA repair protein expression levels	western blot quantification	lymphocyte	neg	16	mixed acute and late

Table 3.15 summarises the percentage of positive versus negative study results for assays of clonogenic cell survival and DNA damage and repair in fibroblast and lymphocytes for prediction of acute and late radiation toxicity.

The heterogeneity of design and statistical analysis of the studies used to compile this table means it is not possible to make firm conclusions about the relative efficacy of clonogenic cell survival and DNA damage assays in these cell types. There are two features worth noting. The majority (82%) of studies examining the role of fibroblast survival assays in the prediction of acute radiation toxicity were negative. In contrast, the majority (75%) of studies examining the role of DNA damage and repair assay in lymphocytes in the prediction of late radiation toxicity were positive. The low quality of the studies means it is not possible to conclude that this means that clonogenic cell survival assays in fibroblast have no role in the prediction of acute radiotherapy toxicity and conversely that lymphocyte DNA damage-based assay do have a role in the prediction of late toxicity - this asymmetry in positive and negative studies in these two groups may simply be due to chance or even publication bias in the case of lymphocyte assays, with positive studies more likely to be published than ones with negative findings.

For other assays, end-points and cell type, positive and negative studies appeared to be equally distributed.

The quality scores for all groups were low, the highest scoring group being the studies of fibroblast clonogenic cell-survival and late radiation toxicity which contained the two highest scoring studies (Peacock et al, 2000, and Russell et al 1998). Negative studies examining fibroblast clonogenic cell survival in the prediction of late radiation toxicity had a significantly higher quality score than studies with positive results. Although it is not possible to form definite conclusions on the basis of a non-validated quality index score this finding suggest that better quality studies with more robust conclusions are more likely to show no relationship between fibroblast clonogenic survival and late radiotherapy toxicity and raises the possibility that such a relationship may not exist.

Table 3.15 Summary of positive versus negative outcomes for studies of cell-based predictive assays of normal tissue radiosensitivity and comparison of quality scores for positive and negative studies.

Assay	Cell	Toxicity	% of studies positive	% studies negative	Significant difference in quality scores (QS) between positive and negative studies?	p-value for QS difference (Mann-Whitney U)
Clonogenic cell survival	Fibroblast	Acute	18 (3/17)	82 (14/17)	No	0.41
	Fibroblast	Late	53 (8/15)	47 (7/15)	Yes – quality score in negative studies higher then positive (median diff = 25, 96% CI 44-4)	0.02
	Lymphocyte	Acute	0 (0/3)	100 (3/3)	No	N/A
	Lymphocyte	Late	60 (3/5)	40 (2/5)	No	0.2
DNA damage/repair	Fibroblast	Acute	33 (3/9)	67 (6/9)	No	0.15
	Fibroblast	Late	37.5 (3/8)	62.5 (5/8)	No	0.25
	Lymphocyte	Acute	57 (8/14)	43 (6/14)	No	0.83
	Lymphocyte	Late	75 (9/12)	25 (3/12)	No	>0.99

3.3.10 Extraction of individual patient data, ROC curve construction and calculation of diagnostic odds ratios

It was possible to extract individual patient data, construct ROC curves and calculate diagnostic odds ratios for a total of 67 comparisons of assay result and specific normal tissue toxicity end-points. For each a ROC curve was constructed and assay cut-off points chosen to maximise assay sensitivity and specificity (sensitivity: specificity weighting = 1:1). Once these cut offs were chosen, a diagnostic 2 by 2 table was constructed for each comparison and the diagnostic odds ratio along with 95% confidence intervals calculated.

No attempt at pooling data by meta-analysis was made. As in systematic reviews of randomised controlled trials meta-analysis should only be considered when studies have recruited patients from similar populations, have used comparable experimental procedures and are unlikely to be biased in order to avoid generating a misleading pooled estimate (Deeks, 2001). As is clear from earlier sections none of these three criteria were fulfilled by the studies included in this systematic review.

For some studies several separate assay techniques were compared with a single or multiple toxicity end-points e.g. Djuzenova compared MRE11, Rad 50 and Rad 51 focus formation with acute toxicity in three separate analyses and a diagnostic odds ratio and data point has been generated for each of these comparisons. The data are presented on forest plots in Figures 3.2 (acute toxicity) and 3.3 (late toxicity). The area of each data point on the chart represents the overall quality index for each study, with the data points with the largest areas representing the higher quality studies. Funnel plots (log odds ratio versus quality score and sample size) were constructed and inspected to assess for the impact of bias (publication and study design) on study outcome (Egger et al, 1997). Bias due to publication bias or poor study design should result in asymmetry of the inverted funnel generated by these plots.

The score for appropriate statistical analysis was not included in the final quality score for the forest plot or funnel plot as the relevance of this parameter in study quality had been negated by the separate analysis of individual patient data.

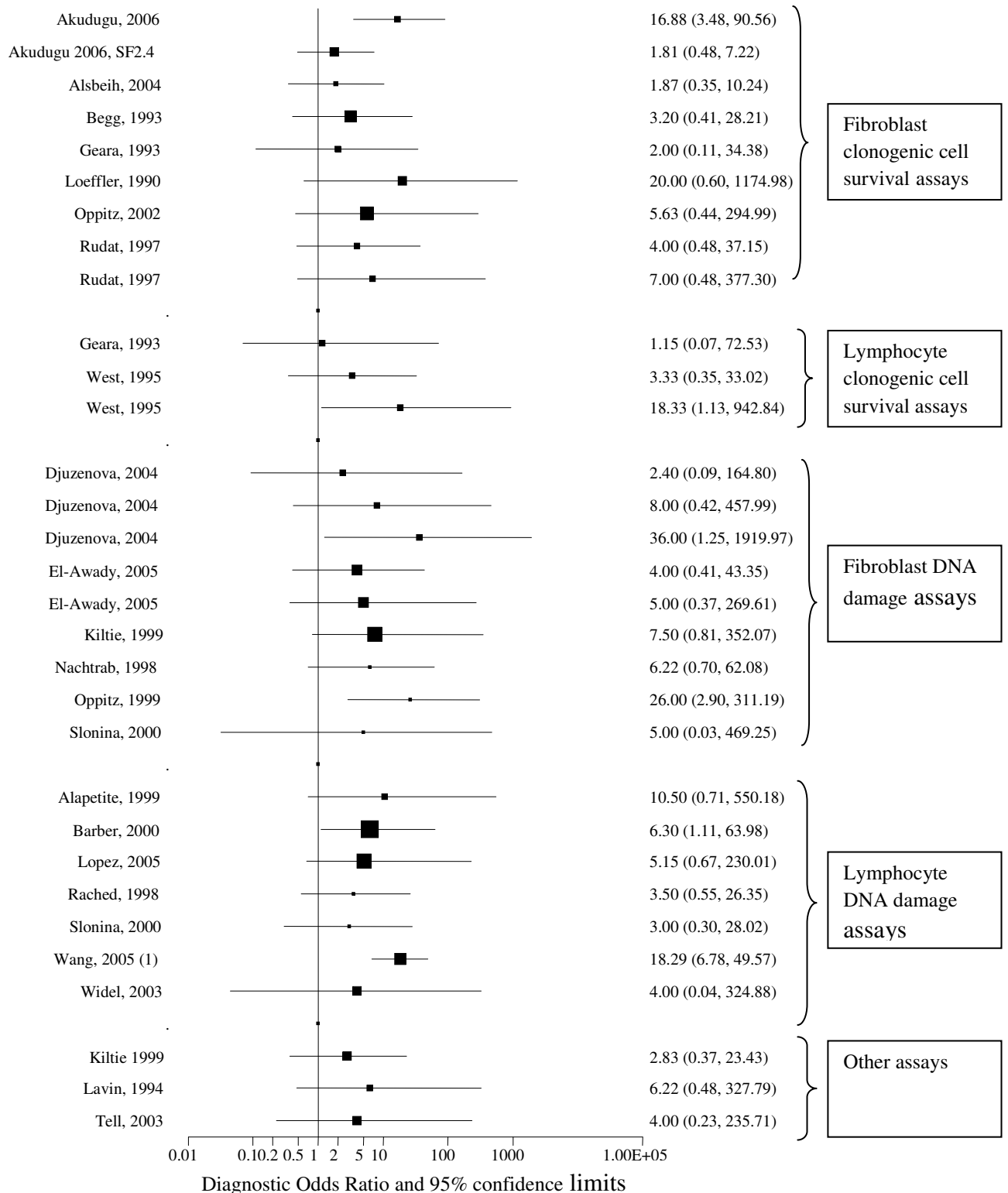


Figure 3.2 Forest plot of diagnostic odds ratio +/- 95% confidence intervals for individual comparisons of assay result and acute radiation toxicity end-points.

Area of data point corresponds to quality index value.

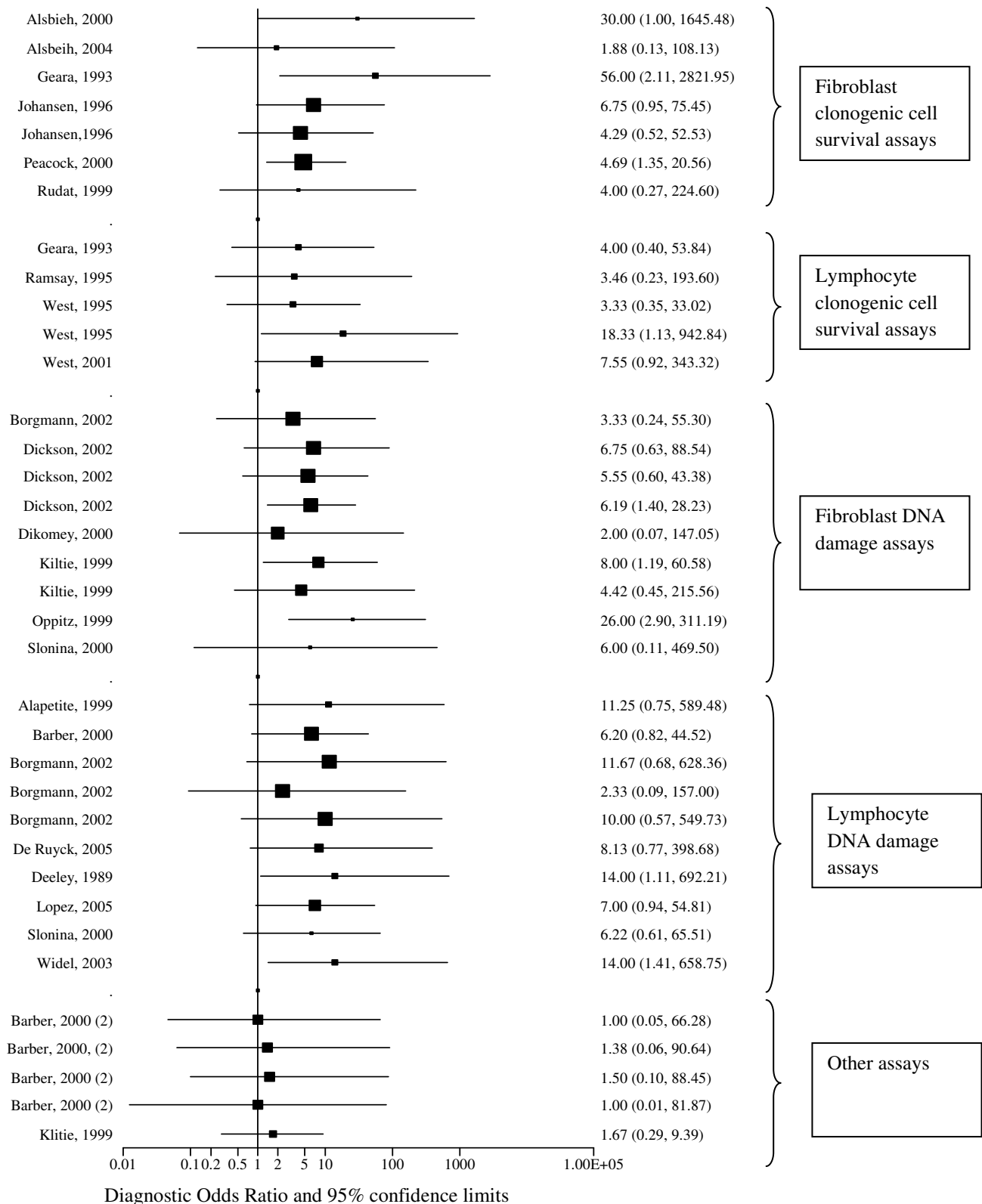


Figure 3.3 Forest plot of diagnostic odds ratio +/- 95% confidence intervals for individual comparisons of assay result and late radiation toxicity end-points.

Area of data point corresponds to quality index value.

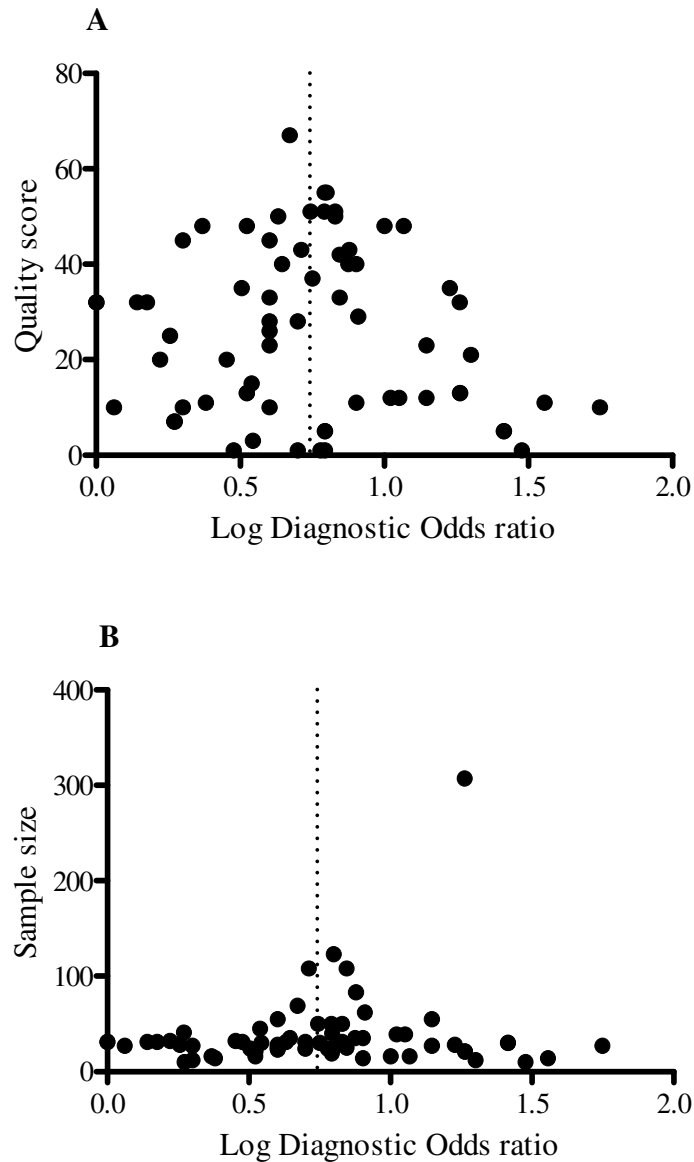


Figure 3.4 Funnel plot of effect (log DOR) vs. quality index (A) and sample size (B) to test for presence of bias in systematic review.

The dotted line represents the mean log of the diagnostic odds ratio. Asymmetry of the "funnel" is indicative of bias.

Results

For prediction of acute and late radiation toxicity the diagnostic odds ratios calculated have very wide confidence intervals most of which cross the value of 1. This means that for these assays a positive result could be just as likely in a non-radiosensitive as a

radiosensitive individual and therefore these assays had no discriminatory value within the studies in which they were investigated.

Assays showing at least some sign of discriminatory power are those where the 95% confidence intervals do not cross 1. The most promising of these will have a high odds ratio with narrow confidence intervals. In the prediction of acute toxicity five comparisons generated DOR where the lower 95% confidence limit was more than 1. One of these was an assay of lymphocyte clonogenic cell survival (West, 1995), another was for a measure of fibroblast clonogenic potential (binucleated index, Akudugu, 2006) whilst the other three were assays of DNA damage. The narrowest confidence limits and the higher quality scores were for 2 assays of DNA damage in lymphocytes - the G2 assay of chromosomal sensitivity and acute skin toxicity with a DOR of 6.3 (1.11-63.98) (Barber, 2000 (1)) and DNA damage induction and acute toxicity in head and neck radiotherapy with a DOR of 18.29 (6.78-49.57) (Wang et al 2005, 1), and for the cytokinesis-blocked binucleated index assay in fibroblasts (DOR 16.88, 3.48-90.56, Akudugu, 2006)

For late toxicity there were 8 assays whose lower 95% confidence limit was greater than 1. Two were assays of fibroblast clonogenic survival, and included the study which attained the highest quality rating (Peacock et al. 2000). The other studies examined assays of lymphocyte clonogenic survival (West, 1995), fibroblast DNA damage (Dickson et al, 2002; Kiltie et al, 1999), and lymphocyte DNA damage (Deeley et al, 1999; Widel et al, 2003). The narrowest confidence intervals and best quality scores were for the study of Peacock et al and for the comparison of DNA damage repair in fibroblasts and the modified LENTSOMA score (Dickson et al, 2002).

The DORs calculated from these studies must be interpreted with caution - the overall low quality of design and reporting of the studies from which they were derived means that their validity is uncertain and that they cannot be generalised to the wider population.

Inspection of the funnel plots in Figure 3.4 shows no obvious asymmetry suggesting no major effect of study design, sample size or publication bias on results.

3.4 Conclusion

- The studies of functional cell-based assays identified from the literature in this systematic review are heterogeneous with respect to basic study design, the assay parameters investigated, toxicity end-point and scoring, treatment of confounding factors and statistical analysis. There is poor reporting of assay reproducibility and treatment of potential sources of bias such as blinding of scorers and inter-scorer comparisons. The factors other than normal tissue radiosensitivity known to influence normal tissue toxicity that might confound the study results are in general poorly reported.
- This poor reporting and often inadequate study design makes it difficult to draw any conclusions about the presence or absence of potential clinical utility of functional cell-based assays in the predictive testing of normal tissue radiosensitivity.
- Comparison of relative diagnostic efficacy for different assays is difficult - some information is gained from the comparison of diagnostic odds ratios from individual patient data, but limited by the quality of the studies from which the data is drawn.
- In the current evidence base the assay which is most informative with a diagnostic odds ratio of 18.29 and narrow 95% confidence interval is an assay of DNA damage induction in lymphocytes in the prediction of acute toxicity in head and neck cancer (Wang et al, 2005 (1)). The hypothesis-generating study from which these figures were derived, however, has not been validated in a follow up study.
- Certainly no assay has emerged from studies so far that has proven its precision and diagnostic accuracy to be sufficiently robust to be used in clinical decision-making
- It is possible that heterogeneous study design and insufficient control of confounding factors have led to potentially useful cell based assays have been overlooked.

4. γ H2AX induction and loss as a potential assay of normal tissue radiosensitivity - quantification of γ H2AX foci by microscopy

The heterogeneity and poor quality of the majority of the studies included in the systematic review means that it is not possible to come to any firm conclusions about the potential discriminatory power and clinical utility of functional cell-based assays in the identification of individuals with high normal tissue radiosensitivity, and it is entirely possible that the potential discriminatory power of some assays may have been masked. The highest diagnostic odds ratio was derived for an assay of DNA DSB induction in lymphocytes. Although this result has not been validated it justifies the investigation of the diagnostic potential of a novel cell-based assay which utilises recent progress in the understanding of the molecular processes of the cellular response to DNA damage.

The rate of loss of γ H2AX after irradiation of 10 cell lines correlates with clonogenic survival at 2Gy with the most radiosensitive cell lines demonstrating slower rates of loss. (MacPhail, Banath et al. 2003). The rate of γ H2AX disappearance is slower in radiosensitive tumour cells both in culture and tumour xenografts as well as radiosensitive murine normal tissue (Olive and Banath 2004; Taneja, Davis et al. 2004). The quantification of γ H2AX induction and kinetics of γ H2AX formation and loss in human peripheral blood lymphocytes after a test dose of radiation could therefore potentially form the basis of a predictive assay of human normal tissue radiosensitivity.

Two methods for the quantification of γ H2AX foci in cultured cell lines have been published in the literature – direct visualisation and quantification of foci by microscopy and indirect quantification by flow cytometry. Both techniques could potentially be applied to isolated human peripheral blood lymphocytes (PBLs).

Counting of γ H2AX foci by direct visualisation by microscopy has been the most frequently reported technique for the quantitative evaluation of γ H2AX induction and loss by various cytotoxic agents, including ionising radiation (Sedelnikova, Rogakou et al. 2002; Rothkamm and Lobrich 2003). Whilst this technique has been applied to cell cultures grown in an adherent monolayer it had not been described as a technique for quantifying γ H2AX in cells in suspension such as human peripheral blood lymphocytes. In

order to explore the potential of γ H2AX quantification by microscopy in human PBLs as a predictive assay of normal tissue sensitivity, a technique for the immunostaining of isolated human PBLs using the commercially available monoclonal antibody was developed. The optimal method for quantification of foci was then investigated and the precision of the assay assessed - as in any diagnostic test, assay development must start at Level 1 of Fryback and Thornbury's model with assessment of the technical efficacy.

4.1 Quantification of γ H2AX foci by microscopy in irradiated mammalian fibroblasts

A standard immuno-fluorescent staining technique was firstly used to confirm that it was possible to induce and detect discrete foci of γ H2AX in mammalian cells (Muntjac fibroblast) in cell culture in our laboratory. Muntjac fibroblasts were selected due to their tendency to enter growth arrest when grown to confluence in culture so reducing background γ H2AX staining due to DNA breaks during replication fork collision. Cells were cultured on glass cover slips in MEM supplemented with 10% FCS, and allowed to grow to confluence at which point they entered growth arrest (G0). The cover slips were irradiated (0.5Gy) in their growing medium at room temperature whilst the controls were “mock-irradiated”. Cover slips were then incubated at 37°C for 15 minutes to allow formation of γ H2AX before being washed and fixed in paraformaldehyde. The immunostaining procedure for γ H2AX described in section 2.2.4 was then performed.

Cover slips were mounted on glass slides and viewed on a Zeiss fluorescence microscope at x40 magnification. The number of discrete FITC-tagged foci in 100 nuclei was counted using a hand-held counter, and the mean number of discrete foci per cell calculated. The experiment was repeated four times.

Discrete foci were clearly visible in both irradiated and non-irradiated G0 Muntjac cells presumed to correspond to foci of γ H2AX. The foci were clearly separated and easily quantifiable by eye. There was a clear and statistically significant increase in the mean number of foci per cell in the irradiated cells ($p= 0.0286$, Mann-Whitney U test) (Figure 4.1).

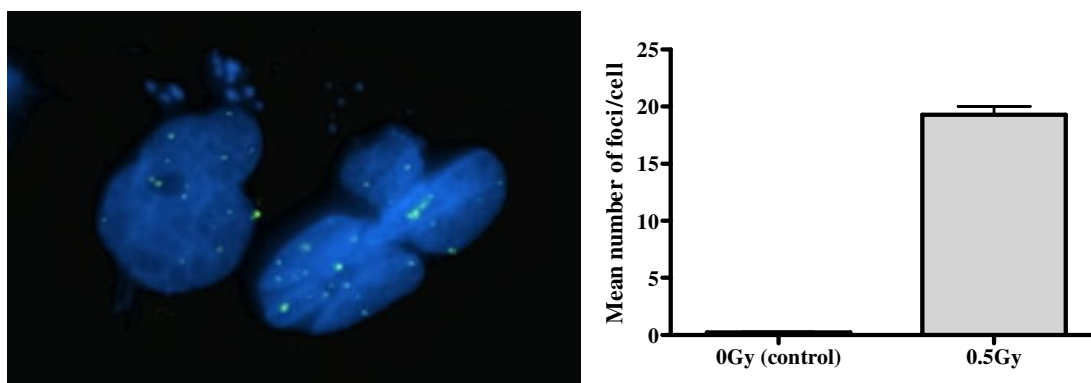


Figure 4.1 Irradiated Muntjac fibroblast after 0.5Gy γ -rays showing discrete, easily visualised and quantifiable foci of γ H2AX.

There was a statistically significant increase in the mean number of foci per cell in the irradiated compared to non-irradiated cells.

4.1.1 Immunofluorescent detection of γ H2AX in human peripheral blood lymphocytes.

Immunostaining and direct visualisation of γ H2AX foci seemed a feasible method for the quantification of in cultured mammalian cells grown on cover slips. The next step was to adapt this technique for use in human peripheral blood lymphocytes.

Human peripheral blood contains a mixed cell population of erythrocytes, lymphocytes, granulocytes, monocytes and platelets. In adults lymphocytes represent 35% of the total circulating blood leucocyte population and are present at a concentration of $2.5 \times 10^9/\text{L}$. 60% of the total circulating lymphocyte pool are T cells and 20% B-cells. 5% of total body pool of lymphocytes are circulating at any one time (Turgeon 2004).

In order to obtain sufficient numbers of human PBLs for immunostaining PBLs had to be isolated and concentrated from samples of whole blood. The cell separation method had to be quick and result in a reliably consistent pure population of non-activated (and therefore G0) PBLs. Contamination by erythrocytes which are subject to autofluorescence (Bidlack and Tappel 1973; el-Rahman, Hammouda et al. 1995) would hinder focus quantification.

There is some evidence that B-cells and T-cell populations may differ in radiosensitivity especially at low radiation dose (Prosser 1976; Louagie, Van Eijkeren et al. 1999; Vral,

Thierens et al. 2001; Schmitz, Bayer et al. 2003). In order to minimise any confounding influence on the assay results that might result from differing proportions of B and T cells in different individuals or within the same individual over time it was decided that a pure T-cell population should be used for investigation. In order to achieve a consistently pure population of human peripheral blood T-lymphocytes an immunomagnetic bead separation technique (Dynal) was employed using immunomagnetic beads to isolate a pure CD4 and CD8 positive T-cell population. The immunomagnetic bead separation process should result in an isolated cell population that is pure (>99%), with >90% of cells in G0 phase of the cell cycle, with no up regulation of activation markers or detectable proliferation (Friedl, Noble et al. 1995; Dynal 2000)

Once isolated the T lymphocytes were resuspended in RPMI/1%FCS, placed in 15ml polypropylene tubes, gassed with 5% CO₂/air and incubated at 37°C for 30 minutes. Samples were then irradiated with increasing radiation doses (0Gy, 0.2Gy, 0.4Gy, 0.6Gy, 0.8Gy and 1.0Gy) before being incubated at 37°C for 30 minutes to allow γ H2AX formation to take place. The PBLs were attached to glass slides by cytocentrifugation before being fixed and stained using the same technique as for the Muntjac fibroblasts. Slides were examined and photographed (Figure 4.2). Foci of γ H2AX were visible in irradiated cells only and appeared on inspection to increase in number as radiation dose increased, in a dose dependent fashion confirming that the immunostaining procedure was indeed detecting a radiation-induced phenomenon in keeping with DNA DSB formation.

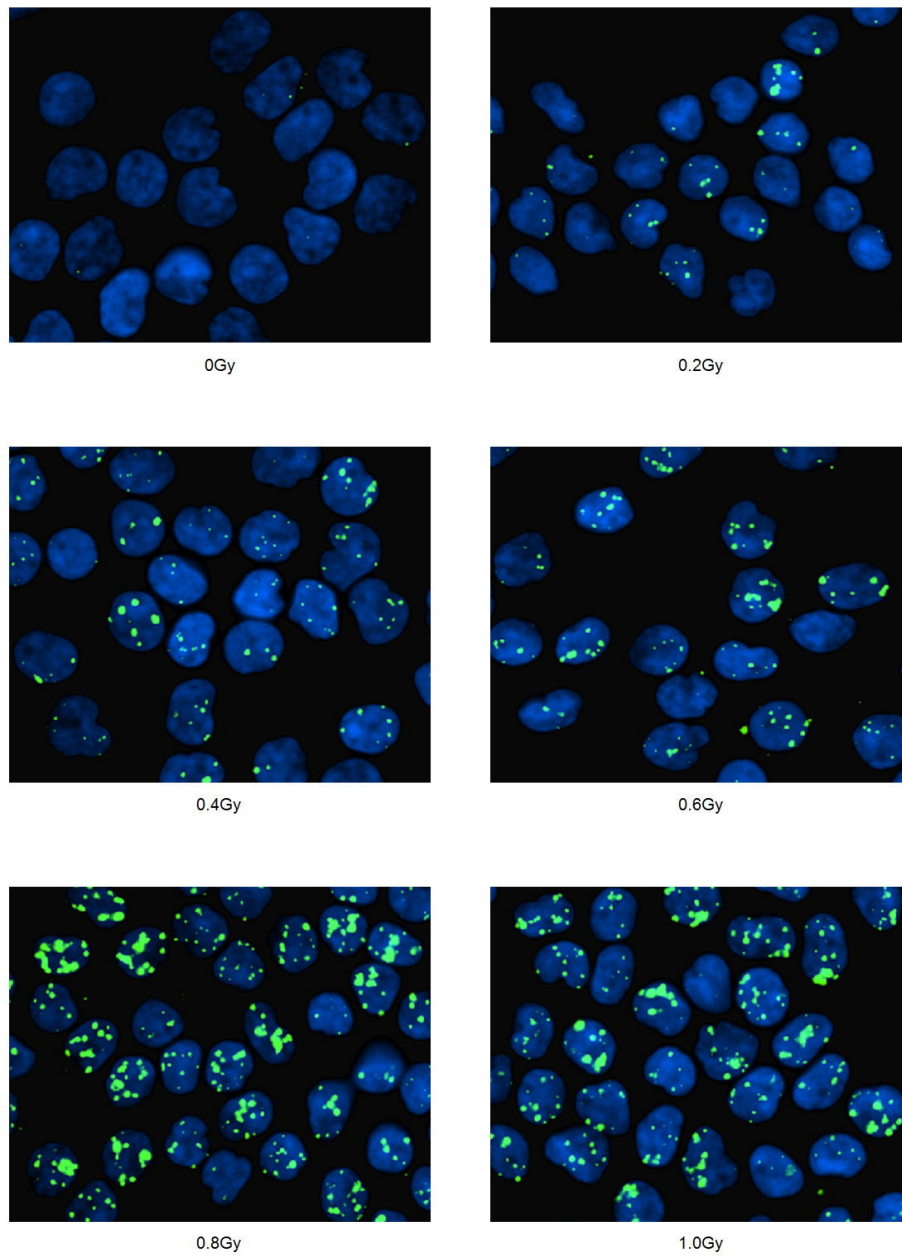


Figure 4.2 Cytospin preparations of human peripheral blood CD4 and CD8 T-lymphocytes fixed and stained for γ H2AX 30 minutes after irradiation and viewed at x100 magnification.

4.2 Techniques for quantification of γ H2AX foci in human PBLs by microscopy

Having demonstrated that it was possible to detect radiation-induced foci of γ H2AX in PBLs by immunocytochemistry, a robust and reliable method of quantifying γ H2AX had to be developed. Whilst direct “real-time” inspection and manual counting of foci in Muntjac fibroblasts had been relatively straightforward, a number of problems were encountered when attempting the same technique in human PBLs.

Human PBLs are significantly smaller than fibroblasts and need to be viewed at a higher power of magnification. Their nuclei are spherical and, despite cytocentrifugation at increased speed and times (data not shown), still retained a significant “depth” on the slide compared to fibroblast nuclei. This resulted in overlap of foci in the x and y planes making it difficult to distinguish between different foci. To avoid missing foci lying at different depths within the nucleus, each nucleus also had to be examined whilst focussing in and out through the z plane.

As a result analysis of each nucleus took considerably longer than with fibroblasts and consequently problems with the recognised phenomenon of “bleaching” of the FITC signal due to prolonged incident light exposure were then encountered (Longin, Souchier et al. 1993). Frequently the FITC signal from γ H2AX foci had completely disappeared after only 10-20 cells had been analysed, making further analysis impossible and casting doubt over the validity of the results from those nuclei already examined. This problem was not overcome by changing the anti-fading agent in the mounting medium from MOWIOL to Vectashield.

In order to circumvent this problem, digital images of 100 cells from each slide were obtained so that “data” could be captured more rapidly before bleaching had time to occur. Analysis of the digital images was then performed. To overcome the problem of the “rounding up” of the PBL nuclei on the slide, images were obtained through several slices of the nucleus using the Z-stack facility on the motorised microscope stage. Five slices were taken through each nucleus images taken at 5 micrometre intervals. Digital capture software (CytoVision, Applied Imaging) superimposed the images to create a 2-dimensional image of a 3-dimensional structure. A threshold level was selected for the images by visual inspection which resulted in the best discrimination of foci from

background and the images saved for later analysis. The threshold level was noted and used at the same setting for all further experiments.

4.2.1 Analysis of digital images

A reproducible and objective method of quantifying γ H2AX foci on each digitally captured image was required. Using the available equipment and software within the department a number of options were available:

- a) Direct visual quantification
- b) Automatic slide scanning and digital image analysis using CytoVision “SPOT” software
- c) Semi-automated “computer” quantification

a) Direct visual quantification

This consisted of counting the number of foci per cell using a hand-held counter by inspecting the images by eye. The advantages of this technique are that obvious non-specific background staining can be disregarded and a judgement can be made about the number of foci actually present when several foci overlap on the images. The disadvantages are that visual inspection is time-consuming and labour-intensive, and subjective assessment of what does and does not constitute a focus could lead to inter-observer variation in results if the technique is utilised by a number of workers in more than one laboratory.

b) Automatic slide scanning and digital image analysis using CytoVision “SPOT” software.

The Spot AX system (CytoVision, Applied Imaging) had already been installed in the department for use in the scanning, re-location, capture and analysis of FISH signals during chromosome analysis. The system consists of a fully motorised, automated microscope stage on a fluorescence microscope, with a motorised focus and fluorescent filter changer. The Spot AX software allows fully automated scanning and digital image capture with data being transferred to an analysis program (Review, Applied Imaging) which identifies and quantifies fluorescent FISH signals. During a visit by a product specialist from Applied Imaging, the possibility of adapting this system to capture and quantify γ H2AX foci was explored.

Various parameters in the Review programme can be altered to determine the level of sensitivity of focus detection, ability to discriminate between real signal and background “noise” and ability to discriminate between two separate foci lying close together. By altering the parameters which determine the minimum and maximum thresholds for spot area, distance between spots, and spot intensity, and the thresholds for recognising background debris, it was possible to adapt the program for automatic scanning and γ H2AX foci quantification in Muntjac fibroblast cells. (Figure 4.3). Unfortunately attempts to with human PBLs were less successful. Despite altering the thresholding for spot intensity, area, and separation the system regularly failed to count several foci per cell (Figure 4.4), failed to discriminate between overlapping foci (Figure 4.5) and often failed to distinguish between nuclei lying close to each other and counted all foci within a group of nuclei as belonging to one cell. (Figure 4.6). It was concluded that whilst the system could be adapted for foci quantification in large well separated nuclei it did not have sufficient discriminatory capacity or sensitivity for similar use in small, often clumped human PBLs.

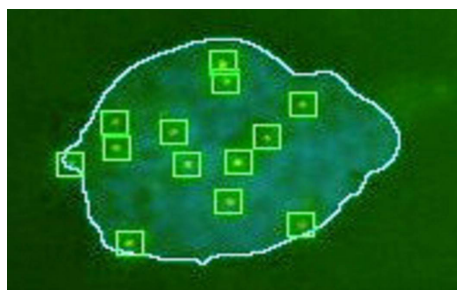


Figure 4.3 “SPOT” image analysis

The SPOT program has successfully identified and marked with a green square all foci within a muntjac fibroblast nucleus. The program recognises the DAPI mask and outlines a solitary nucleus for focus quantification

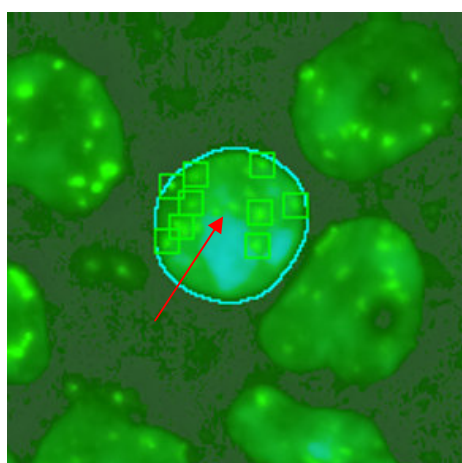


Figure 4.4 “SPOT” image analysis

Human lymphocyte nucleus – SPOT programme has failed to identify several obvious foci (arrowed)

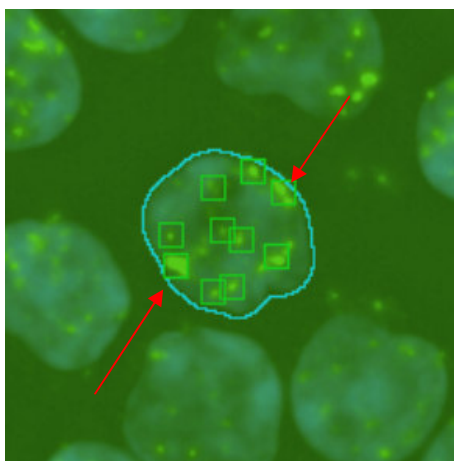


Figure 4.5 “SPOT” image analysis

Human lymphocyte nucleus – several partially overlapping but obviously discrete foci have been counted as one single focus (arrowed)

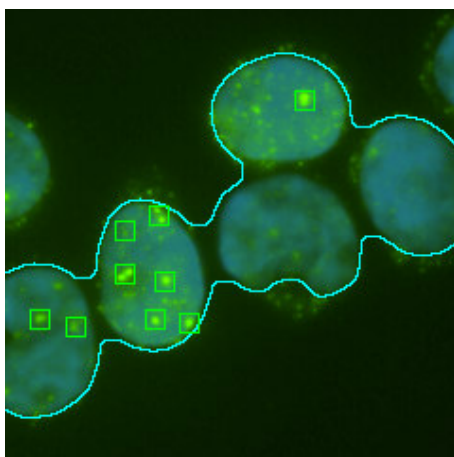


Figure 4.6 “SPOT” image analysis

The program has not discriminated between several close lying nuclei. They have been outlined as one cell and all foci within them counted together.

c) Semi-automated “computer” quantification

As a compromise between the time consuming and subjective manual focus quantification and the fully automated but less sensitive and less discriminatory “Spot” analysis a technique was developed that combined features of each.

The CytoVision software includes a facility that automatically outlines all discrete areas of FITC staining on a digital image (Figure 4.7). For each image a summary statistic containing the total number of regions outlined on the image and mean area of outlined regions (in pixels) is generated. The outlining facility is very sensitive and outlines all discrete FITC staining regions on the image, even very small areas of only a few pixels diameter, barely visible to the eye. This means that areas of obvious background staining between nuclei are outlined i.e. the outlining tool is very sensitive but not specific. It is possible to visually inspect the image after automatic outlining and to delete the foci between cells that are obviously background staining erroneously outlined by the software,

and to delete the foci outlined on partial images of cells. This can be repeated for a number of images so that a total of 100 complete cells have been analysed. It is then possible to calculate the total number of foci seen per 100 cells and therefore the mean number of foci per cell. The program also calculates the mean focus area for per image in pixels. If the results from non-irradiated control samples are subtracted then any erroneously included background staining overlying the DAPI stained nuclei should be accounted for.

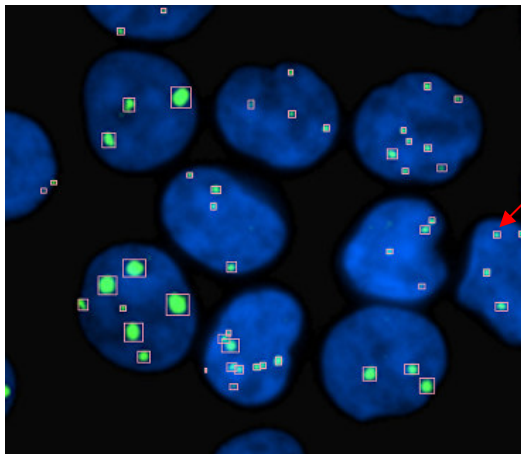


Figure 4.7 Semi-automated focus quantification

Cytovision software automatically outlines areas of FITC staining on the image (pink boxes). Any area of background staining between nuclei or areas outline in partial images of cells (arrowed) can be deleted by clicking the cursor over them. They will then not be included in the statistics regarding focus number and area generated for the image

The advantage of this method over manual scoring is that the use of the computer to identify foci removes subjectivity and increases sensitivity. Confounding effects of non-specific background speckling difficult to quantify by eye can theoretically be accounted for by subtracting the results from the non-irradiated control. Although it is time consuming removing the background staining between the cells, the final count is fully automated and information about focus size is also retrieved.

Using this hybrid technique it is therefore possible to generate data on:

- Mean number foci per cell
- Mean focus area
- Mean FITC coverage per cell by multiplying the mean focus area by the mean number of foci per cell – multiple foci overlapping result in one apparent large focus. A simple count of number of foci will not take this into account and may confound the results i.e. produce an erroneously low value for a cell with multiple foci that appear to merge into one. Calculating the mean FITC coverage per cell may correct for this.

4.3 Determination of the most precise technique for focus quantification.

For an assay to have any utility as a diagnostic test to guide clinical practice it must be reliable and precise. Reliability means that one can expect to obtain a result each time the assay is performed. If precise, an assay will generate reproducible results when performed on the same sample within the same “run” and on the same individual on multiple occasions over time.

In order to estimate assay precision for the various focus quantification techniques described above, an assay endpoint needed to be chosen and the ability of each technique to produce reproducible results for this end point assessed. The end point chosen was the quantification of γ H2AX at a specific time point in isolated PBLs after a test dose of radiation in vitro. The most appropriate radiation dose and time point were determined by measuring radiation dose response and kinetics of radiation-induced γ H2AX formation and loss.

4.3.1 Dose response of γ H2AX induction in human peripheral blood lymphocytes

Human peripheral blood lymphocytes were isolated from a venous blood sample freshly drawn from a healthy volunteer (Subject 6). After re-suspension in RPMI/1% FCS cells at 2×10^5 cells per ml, cells were divided between each of 6 15ml polypropylene tubes, gassed with 5%CO₂/air and incubated at 37°C for 30 minutes before irradiation. Each tube received 0, 0.2, 0.4, 0.6, 0.8, or 1Gy. The cells were then incubated at 37°C for 30 minutes before being fixed. Immunostaining and digital image capture and analysis were performed as previously described. The experiment was repeated 5 times on different days, on each occasion using a fresh blood sample from the same volunteer.

Results:

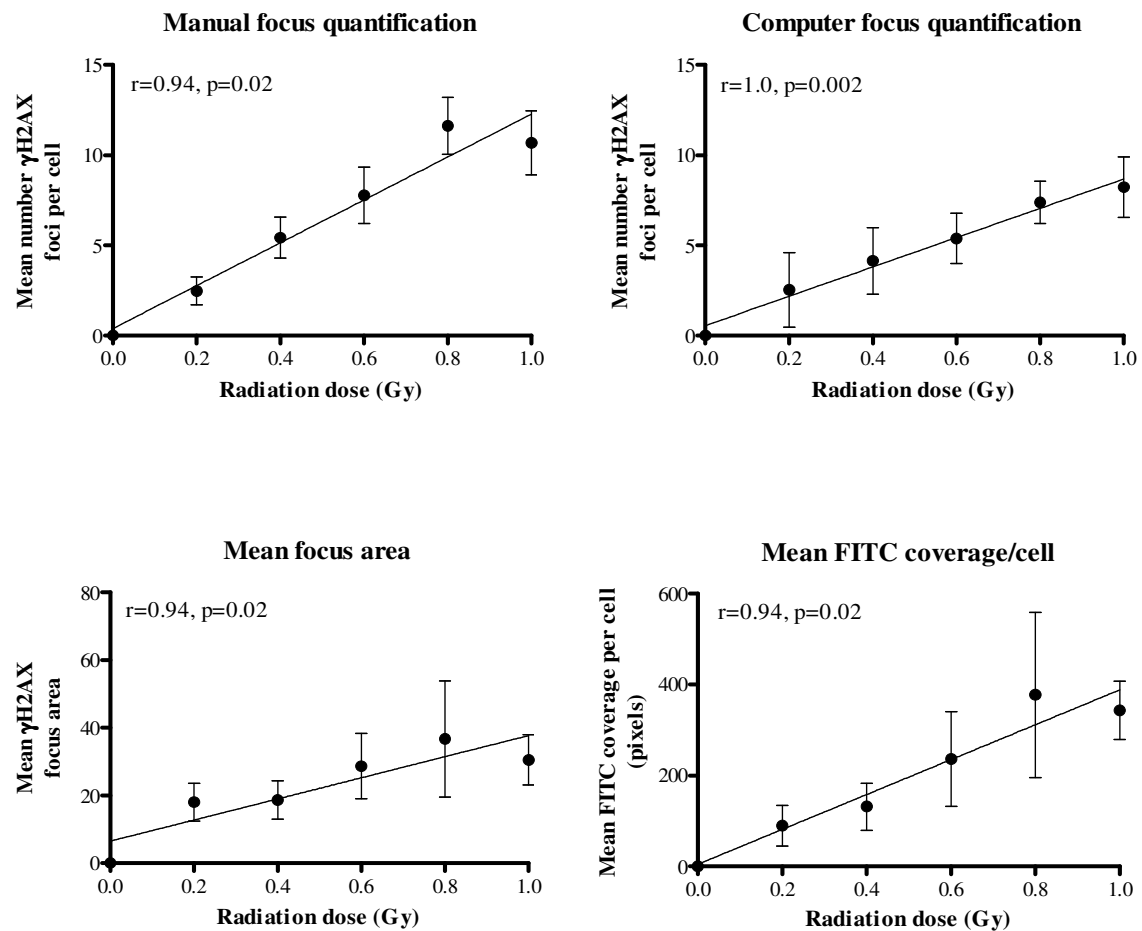


Figure 4.8 Dose-response of γ H2AX focus induction after irradiation (microscopy).

For each data point the mean and standard deviation from 5 separate experiments are shown along with the best fit line (linear regression), correlation coefficient (Spearman) and p-value for each method of γ H2AX focus quantification

The number and size of foci increased linearly with dose up to 0.8Gy. With the exception of the computer count technique there was a suggestion of a “plateauing” effect between 0.8Gy and 1.0Gy most evident in the manual scoring, probably due to significant overlap of foci resulting in a saturation of ability to discriminate visually between foci and masking any increase in focus size. The slope of the linear regression line was shallowest for mean focus size suggesting this parameter has the lowest ability to discriminate between different quantities of γ H2AX. There was considerable inter-experimental

variability in results as demonstrated by the large standard deviation particularly for mean focus area and consequently mean FITC coverage per cell.

A radiation dose of 0.4Gy was chosen as the test dose for further assay development - this resulted in significantly increased levels of γ H2AX compared to baseline when measured by all 4 potential methods of γ H2AX quantification but was still within the linear increase section of the dose response curve, well below any plateau effect.

4.3.2 Optimisation of time between radiation and γ H2AX quantification.

In order to determine the most appropriate time point at which to quantify γ H2AX after test irradiation the kinetics of γ H2AX induction and disappearance after 0.4Gy were examined. PBLs were isolated from a freshly drawn venous blood sample and resuspended at 2×10^5 cells per ml in RPMI/1%FCS, divided into two 15ml Polypropylene tubes. The tubes were gassed with 5%CO₂/air and incubated at 37°C for 30 minutes. A sample was taken from each, centrifuged onto glass slides, fixed and used as a baseline control. The tubes were then gassed again and one tube was then exposed to 0.4Gy gamma irradiation whilst the other was mock irradiated. Both tubes were then returned to the incubator and retained at 37°C. Samples taken from each at 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, and 24 hours. At each sampling point the tubes were re-gassed with 5% CO₂/air. The samples were centrifuged onto glass slides and fixed. Immunocytochemical staining for γ H2AX was performed and foci quantification carried out. The experiment was repeated three times on 3 separate occasions each time using freshly drawn blood from the same healthy volunteer (Subject 2). At each time point the results from the non-irradiated controls were subtracted from the results from irradiated samples to account for any increase in background γ H2AX formation that may have occurred as a result of cell separation and with time in PBLs once isolated from whole blood and stored in vitro.

Results:

The results for all 4 quantification techniques are shown in Figure 4.9. Significant non-specific background staining made quantification of γ H2AX foci difficult both by manual counting and using the modified computer count. In one experiment the non-specific background staining was so severe that image analysis was abandoned and no data was obtained. For the other two experiments, the results from the foci counting both manually (A) and using the computer method (B) were extremely variable. The computer count was especially variable as very small “speckles” of background staining were automatically

counted and varied between different slides. This problem was resolved to some degree by incorporating mean focus area (C) into the modified computer method – the speckled “foci” which were due to non-specific background staining tended to be small whilst the irradiated cells on visualisation clearly contained larger foci presumed to be true γ H2AX foci. By plotting the mean FITC coverage per cell against time a clearer picture of the kinetics of γ H2AX emerges (D) but must still be interpreted with caution due to the paucity of available data points.

The mean FITC coverage per cell increases rapidly after irradiation with 0.4Gy, peaks at 1 hour and falls following this, returning to background level by 6 hours post radiation dose. Non-linear best fit modelling using the method of least squares (GraphPad Prism software) of the kinetics of loss γ H2AX as measured by mean FITC coverage per cell suggests that γ H2AX loss fits with the kinetics of one phase exponential decay (Figure 4.10.) Half life of γ H2AX loss according to this model is 1.74hours (95% CI 1.11 to 3.99 hours).

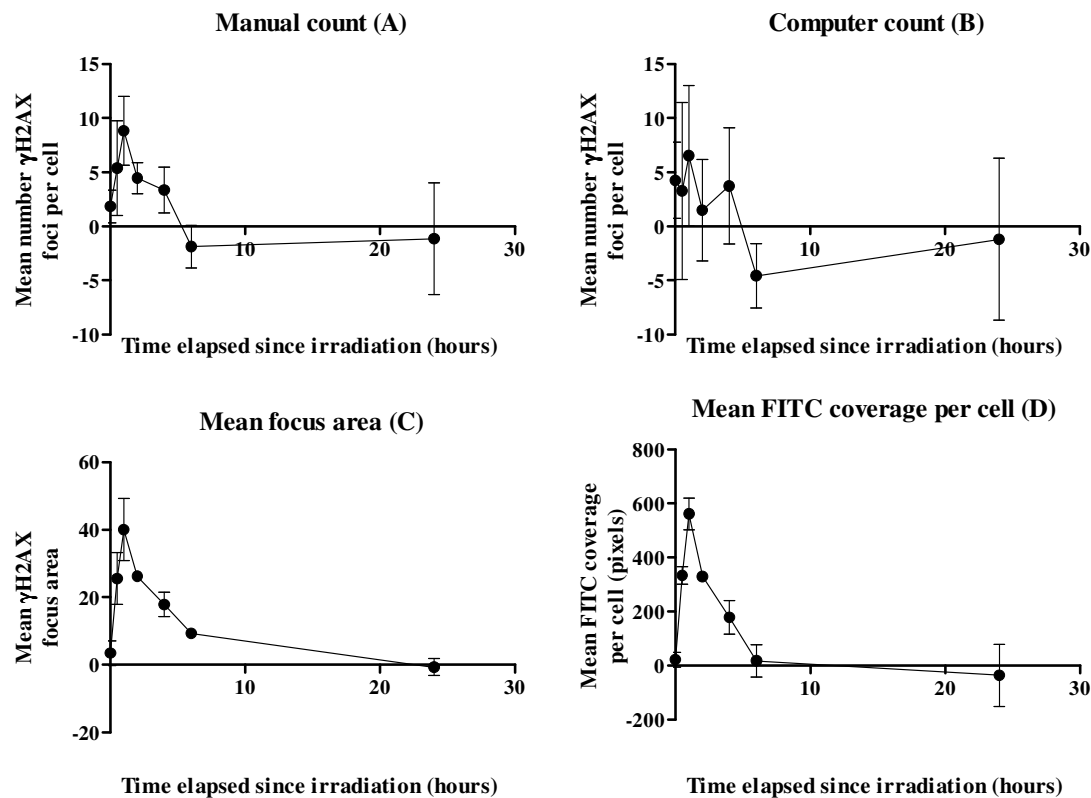


Figure 4.9 Kinetics of γ H2AX focus induction and loss in human PBLs following 0.4Gy γ -irradiation in vitro (microscopy).

Each experiment was repeated three times. No data was available from the third repeat due to extremely inconsistent staining and severe background staining so that analysis not possible. Mean \pm SD from 2 experiments shown in graphs.

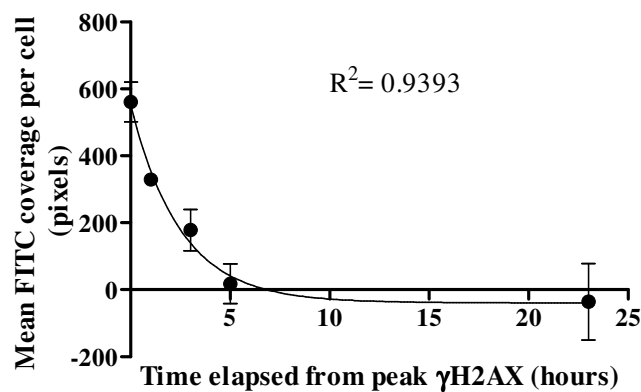


Figure 4.10 Modelling of the kinetics of γ H2AX loss

Non-linear modelling of the kinetics of γ H2AX loss - the loss fits with a model of one phase exponential decay (R^2 =goodness of fit value).

4.3.3 Determination of the most precise method for focus quantification by microscopy - intra-sample precision

A certain amount of variability in results of any assay will naturally occur when a sample is tested repeatedly. Variability is affected by operator technique, environmental conditions, and the performance characteristics of the assay method. The degree of fluctuation in the measurements is indicative of the “precision” of the assay. A high performance assay should be precise with concordance of results from repeated assays on the same blood sample (intra-sample precision) and from repeated assays on different samples from the same individual if the characteristic being measured by the assay is fixed (inter-sample precision).

It was clear that technical difficulties were resulting in inconsistent staining for γ H2AX and high levels of background staining. In order to quantify the variation in assay results due to these technical problems and any other influencing factors, and to determine the most precise method for γ H2AX focus quantification, intra-sample and inter-sample precision was measured for all 4 methods of focus quantification.

The coefficient of variation (CV) is the standard statistical tool used for the comparison of variability in non-identical data sets (e.g. where the assay end points are measured in different units). If data sets are not identical variability must be expressed as a relative rather than an absolute measure in order to allow comparison. This is accomplished by expressing the standard deviation (SD) as a percentage of the mean – i.e. calculating the coefficient of variation (CV).

$$CV = \frac{SD}{\text{mean}} \times 100$$

In order to measure the intra-sample precision for each assay method, PBLs from the same blood sample were divided into 3-4 aliquots and each aliquot analysed in parallel on the same day.

To avoid simultaneous handling of large number of samples, a single time point (30 minutes) post irradiation was chosen as an end point for the measurement of intra-sample precision for all 4 methods of γ H2AX focus quantification rather than a full assessment of γ H2AX kinetics. 30 minutes was chosen because it was clear from the kinetics experiments that there would be a reasonable amount of γ H2AX focus induction at this time after irradiation, but not so much as to result in the coalescence of foci that made manual and computer focus quantification more difficult.

To try to minimise non-specific background staining both the primary and secondary antibodies were titrated to the lowest possible dilution that still resulted in γ H2AX staining. The same batch of both primary and secondary antibody were used throughout. All incubations were performed in a humidified chamber to prevent drying out of samples which may have unpredictably affected staining quality, and all preparation and stages involving the FITC-tagged secondary antibody were performed in a darkroom. The timings for all steps in the procedure were accurately measured. Digital images were captured using same exposure and brightness settings throughout and images thresholded at the same level.

A single fresh peripheral whole blood sample was collected from a volunteer subject. CD4 and CD8 positive PBLs were isolated using immunomagnetic beads and cells resuspended in RPMI/1%FCS at 20,000 cells/ml. 1ml of cell suspension was transferred into each of three pairs of 15ml Polypropylene tubes. Each tube was gassed with 5% CO₂/air and

incubated at 37°C for 30 minutes. One tube in each pair was irradiated with 0.4Gy whilst the other was mock-irradiated. The tubes were then incubated at 37°C for 30 minutes before 0.5ml from each sample was taken for fixing, staining and analysis.

This experiment was repeated 6 times using fresh blood samples from four different volunteers. The coefficient of variation was calculated for each experiment and the mean coefficient of variation for each method of focus quantification calculated (Table 4.1).

Table 4.1 Coefficients of Variation (%) for intra-sample repeats – γ H2AX 30 minutes after 0.4Gy

Quantification technique	Subject 5 (run 1) n=4	Subject 3 (run 1) n=4	Subject 2 (run 2) n=3	Subject 3 (run 3) n=3	Subject 5 (run 3) n=3	Subject 6 (run 3) n=3	Mean CV for each technique
Manual count	16.21	18.49	6.10	4.23	7.72	5.29	9.673%
Computer count	15.28	15.81	7.77	10.51	4.54	6.96	10.15%
Mean focus area	30.24	15.93	9.45	21.34	8.56	7.89	15.57%
Mean FITC area per cell	43.24	26.38	4.86	25.99	13.01	15.05	21.42%

Intra-sample precision was highest (lowest CV) for manual and computer foci counting. There was higher variability in the mean focus area and this variability was compounded in the multiplication required to calculate the mean FITC coverage per cell.

In order to determine the extent to which operator technique may be influencing assay precision the CV for each technique was plotted for each of the dates when the precision testing was performed, operator experience increasing with each run. (Figure 4.11)

Assay precision does appear to improve with time. Given that the same reagents and same techniques were employed throughout it would seem likely that assay precision improves with increasing operator experience.

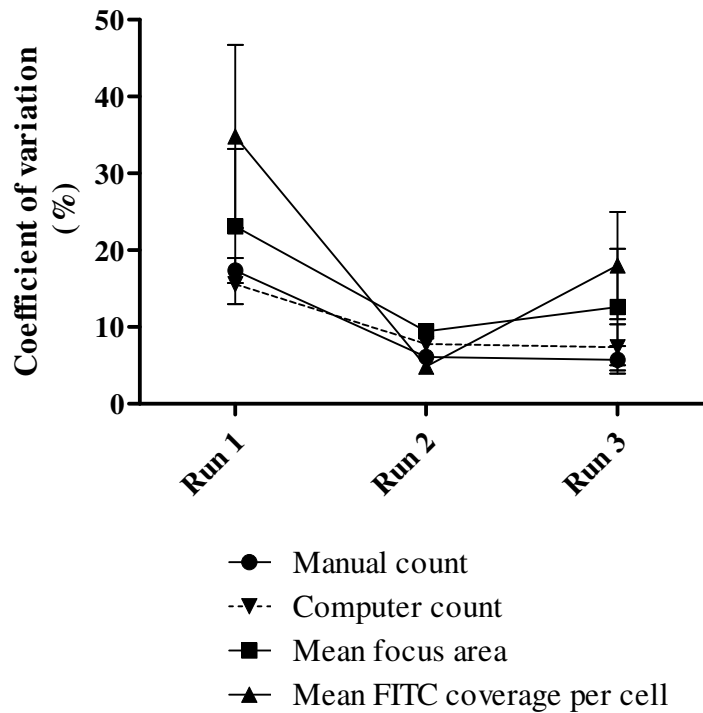


Figure 4.11 Coefficient of variation (mean \pm SD) for intra-sample γ H2AX quantification with increasing operator experience.

4.3.4 Inter-sample precision.

An individual's normal tissue radiosensitivity is thought to be genetically based and therefore should be constant over time. An assay purporting to measure radiosensitivity should therefore give a consistent result when repeated over time in the same individual i.e. there should be inter-sample precision. Lack of inter-sample precision would suggest that the assay is subject to interference by technical or environmental factors during the assay procedure, or confounded by interfering biological factors within the individual which vary over time.

In order to assess inter-sample precision the assay was repeated on 5 occasions over 4 months (i.e. at approximately 3 weekly intervals) using freshly drawn blood samples from the same individual (Subject 2) and assessment of inter-sample variation in assay results performed.

On each occasion blood samples were handled identically. The same batch of reagents and primary and secondary antibody were used each time, and there was strict adherence to the

immunostaining protocol. Images were captured on same microscope and the settings for image capture were the same for each assay.

The individual concerned was a healthy volunteer who admitted to no regular or one off medications at the time of sampling or any concurrent illness over the study period. The subject was not fasted before blood sampling.

Results.

Table 4.2 Inter-sample variability in a single volunteer over 5 experimental runs

	Manual count	Computer count	Mean focus area (pixels)	Mean FITC coverage per cell (pixels)
Run 1	5.09	4.94	18.67	95.76
Run 2	6.56	4.28	22.01	99.02
Run 3	2.29	-2.52	20.13	310.61
Run 4	8.46	8.07	29.54	237.20
Run 5	3.94	1.71	12.52	21.37
CV (%)	45.07%	119.91%	29.88%	77.06%

For all 4 assay parameters assessed there was considerable variability in assay results with the coefficient of variation ranging from 29.88% at best for mean focus area to 119.91% at worst for the computer count.

To try to ascertain whether this variability was due to systematic drift in assay results (e.g. as might occur if any reagent was becoming degraded such as FITC-tagged secondary antibody fading if repeatedly exposed to light), or if variability was secondary to random error or lack of precision or consistency in assay technique, the values for each assay parameter were plotted against time with the mean value for all 5 repeats marked on the same graph. (Figure 4.12)

Interpretation is somewhat limited by the lack of data points available (in clinical laboratories at least 20 data points would be required for Quality Control purposes). However, for each assay parameter there appears to be random dispersion of values around the mean and no clear trend to suggest a systematic drift of assay results upwards or downwards with time.

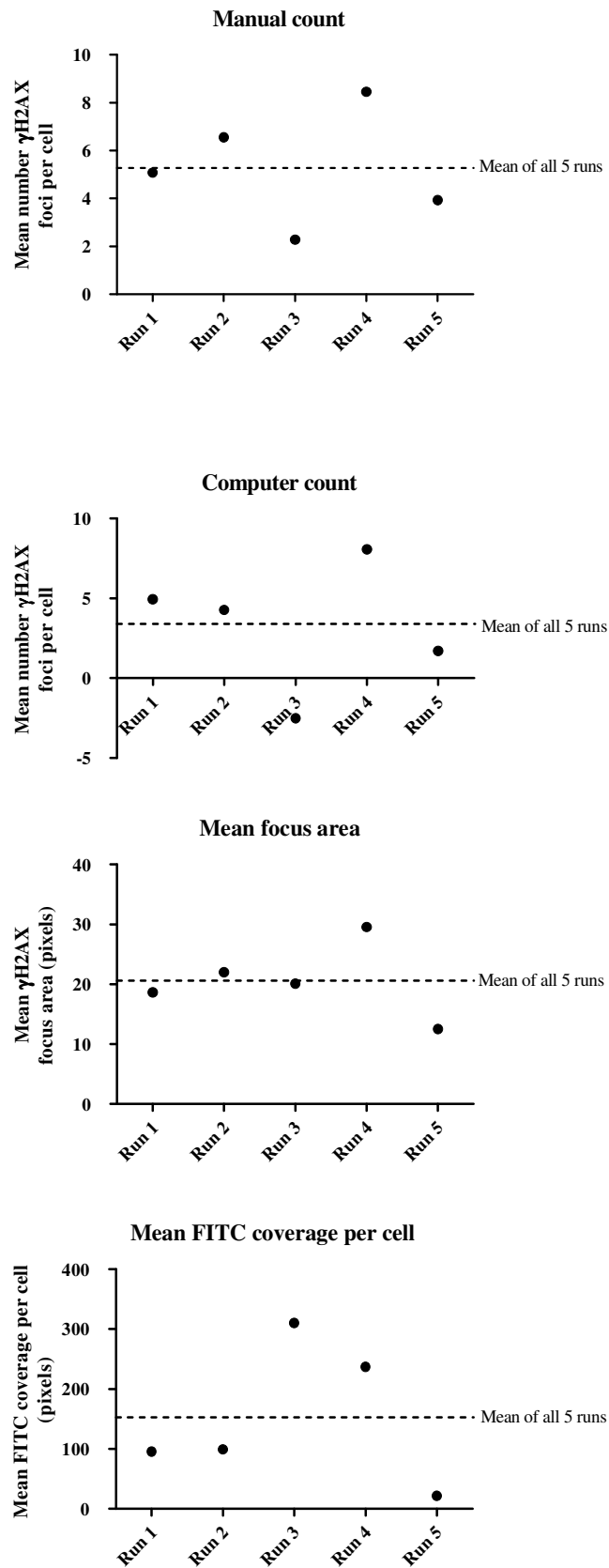


Figure 4.12 Inter-sample variability - assay results plotted against time for 5 assay repeats over 4 months using fresh blood samples from the same individual to assess for systematic drift in results over the study period

4.3.5 Assay failure rate

If any assay is to be useful in influencing clinical practice there must be a high probability of obtaining a result when a sample is sent to the laboratory for analysis. Clinical decisions regarding treatment must be made rapidly and cannot be delayed whilst repeat samples are re-analysed because of previous assay failure.

In 3 out of 18 assay runs performed no useful data could be obtained due to high background staining on one occasion, and patchy and inconsistent staining on the other. The assay failure rate was therefore 16%.

4.3.6 Time taken to obtain assay results.

Blood sampling to collation of data from image analysis took a minimum of 3 working days. The kinetics experiments took considerably longer due to the larger number of samples and therefore images that required analysis.

Day 1: PBL isolation, irradiation and fixation (6 hours work, intensity dependent on number of samples being handled simultaneously)

Day 2: Immunostaining – (5 hours work, intensity again dependent on number of samples)

Day 3 - 5: Image acquisition and analysis – (image acquisition 3 hours, analysis and collation of data 5-20 hours depending on number of samples)

4.3.7 Inter - individual variation.

Insufficient data was generated to make any meaningful comparisons of γ H2AX focus induction between individuals.

4.4 Discussion

The above evidence demonstrates that it is possible to induce and detect γ H2AX using immunofluorescence staining technique in human PBLs irradiated in vitro.

Foci number and area increased linearly with dose between 0-1 Gy in freshly isolated human PBLs. A linear dose response for γ H2AX induction for dose below 1Gy has previously been reported in other experimental studies. A linear dose response between 0.001 and 2Gy was demonstrated for the human fibroblast cell line MRC-5 when γ H2AX foci were counted by eye (Rothkamm and Lobrich 2003). Olive and co-workers reported a linear dose response in various cell lines irradiated in vitro when γ H2AX induction was

analysed by flow cytometry (MacPhail, Banath et al. 2003; Olive and Banath 2004). Similar findings were reported when examining γ H2AX foci number in human skin irradiated between 0Gy and 1Gy in vivo and then biopsied (Qvarnstrom, Simonsson et al. 2004).

Interpretation of the data regarding the kinetics of γ H2AX focus induction and disappearance is limited due to the fact that data was only available from 2 experiments and there were significant difficulties in accurate focus quantification due to background staining. The peak of γ H2AX induction appears to be at 1 hour post irradiation with rapid loss of γ H2AX following this with a half life of between 1 and 4 hours.

Published data on γ H2AX kinetics after irradiation has shown that the peak level of γ H2AX in cell lines is observed earlier than this, at 10-30 minutes after irradiation (Rogakou, Pilch et al. 1998; Rogakou, Boon et al. 1999; Olive and Banath 2004) although high levels are still seen at 1 hour in some cell cultures, tumour xenografts and normal mouse tissues (Olive and Banath 2004). The half life of γ H2AX loss is reported as between 2-7.6 hours in irradiated mouse tissue depending on tissue type, and the same authors report a half life of 3 hours \pm 0.6 hours for γ H2AX loss in human lymphocytes when analysed by flow cytometry although further information regarding this or experimental details were not included in the relevant paper (Olive and Banath 2004). The limited data available from this study would be consistent with this figure.

Limited data makes modelling of the kinetics of γ H2AX loss difficult – loss may be exponential and computer-modelled curve fitting would fit with this. However, the lack of and unreliability of data points makes firm conclusions about modelling the kinetics of γ H2AX loss impossible based on this data.

Throughout the experiments significant problems were encountered with unpredictable non-specific background staining which made focus quantification difficult and on two occasions impossible. At the other extreme, in one experiment immunofluorescent staining was extremely patchy and only a few cells could be reliably scored and no meaningful data obtained. This was despite careful titration of both primary and secondary antibody to the lowest required concentration and strict adherence to technique. There was no clear evidence that complete experimental failure was more common at the beginning of the

study period whilst the technique was being learned and refined – 2 out of the 3 failures occurred towards the end of the study period. Although the exact causes of assay failure were not determined it is likely that they were due in part at least to operator inexperience and failure rate may have decreased if the study had continued.

The time from tissue sampling to result of 3-5 days with this technique compares favourably with other techniques previously investigated as predictive assays of normal tissue toxicity – if γ H2AX kinetics were demonstrated to predict normal tissue toxicity assay results could be produced within a clinical useful time frame. It should be noted, however, that the actual assay procedure and digital image analysis using these methods are very labour-intensive.

There is no generally accepted definition of an acceptable CV for an assay's intra sample variability but as intra-sample variation is due entirely to experimental error clearly this should be as small as possible. Manual count and computer count result in the lowest intra-sample coefficient of variation but all methods resulted in CVs of approximately 10% or above. Sources of intra-experimental error may be due to failure to mix reagents thoroughly, slight differences in incubation and washing times and possibly differential photo-bleaching during image capture. Only 100 cells per assay are analysed which also increases the chance of random error. There is evidence that for all methods of focus quantification assay precision improved with time presumably due to increasing operator experience.. Whether this is due to improved consistency in the immunostaining procedure or more consistent analysis of foci on digital images is not clear. If microscopic quantification of γ H2AX were to be continued ongoing repeat testing of intra-sample precision would be required to ensure this is a consistent improvement for quality control purposes. The fact that operator experience might influence assay results means that appropriate operator training and an assessment of inter-observer variability in digital image analysis scoring would be required before results from different operators could be comparable if the assay was to be performed by more than one operator in the same laboratory or in different laboratories.

Inter-sample variation was high for all quantification techniques. The CV for the computer count was particularly high (119%) due to difficulty scoring the second experiment due to high background staining which actually led to a negative number once the results from

the control sample had been subtracted. Other cell-based assays of radiosensitivity have reported much lower inter-sample CVs of 2.5-40% (median 15%) as reported in Section 3.1.6.

Inter-sample variation may be due to underlying poor assay precision as well as random technical factors such as unexpected changes in laboratory temperature or lighting, or inconsistencies in laboratory technique. Variability in results between samples may also be due to systematic error such as a sustained seasonal rise or fall in laboratory temperature, or the degradation over time of one of the assay reagents. Examining the plot of assay results against time in Figure 4.12, the assay results seem to be randomly dispersed around the assay mean and there is no clear evidence of a systematic drift in assay results with time. It would be reassuring to have more data points on this graph to confirm this.

Another possible source of inter-sample variability is real biological differences over time in the volunteer giving the blood sample. Biological factors that might affect γ H2AX induction are unknown. They may conceivably include a change in the level of dietary antioxidant intake, infection, medications, alcohol and smoking. The subject in whom inter-sample variation was measured did not smoke or take medications and denied any concomitant viral infections. Dietary intake was not monitored, however. There is evidence that increased dietary antioxidant intake reduces in vitro oxidative DNA damage in isolated human PBLs (Thompson, Heimendinger et al. 1999; Gill, Haldar et al. 2004; Gill, Haldar et al. 2007; Maffei, Tarozzi et al. 2007) and given that ionising radiation generates DNA DSB at least in part via reactive oxygen species it is possible that a fluctuating dietary intake of antioxidants may influence in vitro γ H2AX induction in PBLs in an individual over time. It would be difficult to study biological effects on γ H2AX induction in human PBLs without first improving assay precision and reducing variability due to any technical factors likely to be the source of inter-experimental variation.

Inconsistencies in staining technique and assay throughput could be improved with the use of a fully automated immunostaining facility. Automated systems for immunocytochemistry are commercially available and have an established role in histopathology laboratories resulting in fully automated, rapid and reproducible staining with virtually no background (e.g. DAKO). An automated system therefore may play a

role in improving the precision of any assay of γ H2AX foci and would help speed the throughput of multiple samples if such as assay was being tested in a patient population.

Another major source of imprecision is the analysis of the digital images taken in this study. The sophistication of image analysis was limited by the technological capabilities of available software. To try to maintain consistency images were all taken using the same camera settings and threshold settings. To set a threshold is a way of extracting objects from an image where the threshold is the grey level chosen to separate the objects of interest from the background, resulting in a binary image. Increasing the threshold level when background staining was problematic would have resulted in decreased back ground interference on the image to be analysed, but would have potentially also reduced sensitivity for detecting γ H2AX foci.

Scoring was labour-intensive which meant that it was only realistic to score 100 cells per sample resulting in an increased susceptibility of results to Type 2 error.

Qvarnstrom and colleagues have a more sophisticated method of digital image analysis to allow quantification of γ H2AX foci in irradiated human skin biopsies (Qvarnstrom, Simonsson et al. 2004). The aim of digital image analysis was to transform the γ H2AX and DAPI images each containing approximately 60 cells and obtained manually via a digital camera mounted on a fluorescence microscope, into binary images from which the number of foci could be counted. Qvarnstrom employed mathematical technique used in digital image analysis technique called “a feature-enhancing top hat transformation” before setting the threshold for image analysis. This step results in subtraction of background interference and extracts foci clearly from an uneven background. A common threshold can then be set which detects foci in all of the cells being examined. The top-hat transformation effectively increases sensitivity and specificity of γ H2AX focus detection. The group then scored images using a fully automated system scoring 2000 cells for each sample with each sample being scored in 30 minutes. The group implemented their image analysis methods as Java [™] plug-ins to pre-existing image analysis software.

Bocker and Iliakis (2006) have also recognised the disadvantages of manual focus scoring in γ H2AX focus quantification describing it as tedious, unreliably, subjective and error-prone and requiring substantial training. They have derived a personal computer-based

algorithm for automated focus analysis based on immunofluorescent staining, confocal laser scanning microscopy and computerised image analysis. It too involves a top-hat transformation and operates as a utility on commercially available software can also be extended to images acquired on a digital camera. As well as allowing focus quantification to be performed in large number of cells in a consistent and reproducible manner uncompromised by investigator introduced biases, it can also correct for focus overlap and measure the integrated optical density of each focus. This increases linearly with radiation dose and may be a biologically significant parameter.

In conclusion,

- Quantification of γ H2AX foci by microscopy in irradiated human peripheral blood lymphocytes is technically possible.
- Quantification is imprecise and unreliable using the available laboratory facilities and software
- The intensive labour demands, lack of reliability and imprecision would render this technique impractical for examining γ H2AX kinetics in a large numbers of clinical samples without consideration of automation of staining and image analysis to improve assay reliability, precision and throughput.

5. Quantification of γ H2AX in human peripheral blood lymphocytes by flow cytometry.

Given the poor assay precision and reliability demonstrated for γ H2AX quantification in PBLs using microscopy, an alternative technique for quantification of the signal from cells after immunofluorescent staining for γ H2AX was sought. One of the groups who had shown a relationship between the kinetics of γ H2AX induction and loss and cellular radiosensitivity had measured the signal from FITC-tagged anti- γ H2AX using flow cytometry (MacPhail, Banath et al. 2003; Banath, Macphail et al. 2004; Olive and Banath 2004).

Flow cytometry is a system for measuring and analysing the signals that result as particles flow in a liquid stream through a beam of light (Givan, 2001). A flow cytometer contains:

- 1) A light source (laser) and a means of focussing the light source.
- 2) A fluidics system - Fluid lines and controls to direct a liquid stream containing the particles through the focussed light beam
- 3) An electronic network for detecting the light signals coming from the particles as they pass through the light beam and then converting the signals to numbers that are proportional to light intensity (photodetectors, photomultiplier tubes, and amplifiers)
- 4) A computer for recording the numbers derived from the electronic detectors and then analysing them.

With appropriate permeabilisation procedures flow cytometry can provide a means of analysing intranuclear proteins such as γ H2AX by running immunofluorescent stained cells through the cytometer to measure fluorescence intensity. Under good conditions fluorescence intensity should be related to the amount of intracellular protein present (Givan, 2001). The flow cytometer is useful at comparing intensity of different cells, but not so good at providing absolute value for light intensity it measures. Fluorescence is

therefore best expressed as a ratio of the fluorescence intensity of one cell population compared to another analysed at the same time. McPhail et al express γ H2AX staining as a normalised fluorescence ratio i.e. the ratio of fluorescence in irradiated cells to fluorescence in non-irradiated controls (MacPhail, Banath et al. 2003).

In flow cytometry it is possible to select out a cell population according to scatter characteristics and carry them forward for analysis – this is called "gating". A cell's forward and side scattering of light as it passes through the cytometer is determined by its size and intracellular complexity. Gating employed effectively using light scattering profiles can isolate lymphocytes from within a mixed population of cells from peripheral blood being run through the cytometer. Once experienced gating can be done by eye but confirmed with immunofluorescent phenotyping of the gated cells to confirm that the correct cell type is being analysed. Gating is one of the most powerful aspects of flow cytometry – it needs to be as objective as possible but there is unavoidable subjectivity in selecting the correct population for analysis from a forward and side scatter dot-plot (Givan, 2001).

When compared to immunostaining and foci quantification by microscopy, flow cytometry has immediately apparent advantages and disadvantages:

Advantages –

- 1) Cells are analysed in suspension and therefore obviates need to fix cells to glass slide.
- 2) Gating can select out lymphocytes from any other cell types contaminating samples
- 3) Fluorescence quantification is objective
- 4) Ten thousand cells can be analysed in a few minutes
- 5) After irradiation and fixation cell samples can be stored before immunostaining and analysis

Disadvantages –

- 1) Requires a flow cytometer and relevant expertise in its operation.
- 2) Requires many more cells for analysis than microscopy.
- 3) Cannot give information about individual foci (e.g. area) in individual cells

5.1 Development of basic technique for flow cytometric analysis of γ H2AX staining in human PBLs.

5.1.1 Lymphocyte isolation from whole blood

In order to generate statistically valid results the signal from 10 000 cells per sample analysed is usually measured in flow cytometry. For any radiation dose or time point after irradiation being investigated 4 samples must be prepared – irradiated and non-irradiated cells stained for γ H2AX, along with irradiated and non-irradiated cells stained with the secondary antibody only to correct for non-specific background staining of the secondary antibody and any differences in cellular autofluorescence. Therefore for an experiment examining the kinetics of γ H2AX induction and loss after irradiation a minimum of 40,000 cells are required for final analysis for each time point. To account for approximately 50% cell loss during staining and washing procedures at least 100,000 cells would be required for each time point of γ H2AX kinetics experiments ($7 \times 10^5 - 1 \times 10^6$ for the 7 time point kinetics experiment performed for the microscopy study).

The mean yield of PBLs from whole blood using the immunomagnetic bead isolation technique was 20-40,000 per ml. Using this technique to isolate PBLs for flow cytometry would require 25ml of blood per kinetics assay and a prohibitively large quantity of the immunomagnetic bead separation kit. Using immunomagnetic bead separation for this quantity of blood would also require simultaneous handling of large number of tubes and take a considerable length of time. A more time and cost effective and high yielding method of cell isolation was sought.

A well known technique for mononuclear cell separation from anticoagulated whole peripheral blood is the centrifugation of whole blood through a liquid density gradient medium, the most commonly used medium being Ficoll 400 with sodium metrizonate or sodium diatrizoate (Boyum 1968; Fotino, Merson et al. 1971; Ting and Morris 1971). This technique involves dilution of the blood sample in a buffered solution, careful layering on top of the density gradient medium and then centrifugation, which isolates the mononuclear cells above the medium. The cells are harvested by carefully pipetting them from the above the interface with the medium.

BD Vacutainer Systems have used this technique to develop the BD Vacutainer Cell Preparation Tube (CPT). This combines a blood collection system containing citrate anticoagulant with a Ficoll Hypaque density fluid and a polyester gel barrier which separates the two liquids. Blood is collected directly into the tube during venepuncture and the tube is centrifuged. During centrifugation the mononuclear cells become separated from the denser blood components by virtue of the Ficoll gradient and the gel then moves to form a barrier between the mononuclear cells and other blood components to prevent re-mixing. The tube is a convenient system for the collection of whole blood and separation of mononuclear cells. Samples can be transported after centrifugation without removing from the tube. Each tube has an 8ml draw capacity.

The manufacturer reports recovery of 71% of mononuclear cells in peripheral blood using CPT tubes. Lymphocytes comprise 86% of the recovered cells which are 99.9% viable. The mean absolute mononuclear cell count from a 8ml blood sample is reported as 12.72×10^6 i.e. 1.59×10^6 per ml of whole blood and 1.36×10^6 lymphocytes per ml (BD Vacutainer Systems). Obviously the lymphocyte population will consist of mixed B and T cells rather than the pure CD4 and CD8 positive T cell population used in the focus quantification experiments.

The cost and time advantages of using this system over immunomagnetic bead separation were felt to outweigh any potential disadvantages of using a mixed B and T cell population for γ H2AX analysis.

5.1.2 Immunostaining procedure and flow cytometric analysis

The procedures for cell fixation and immunostaining was adapted from that of McPhail (MacPhail, Banath et al. 2003) and are described in Section 2.2.7.. After cell separation and irradiation cells were fixed in 70% ethanol and stored at -20°C for up to 15 days before the immunostaining procedure.

Following immunostaining cells were analysed by flow cytometry according to the procedure outlined in Section 2.2.8. The mean fluorescence intensity of 10,000 cells was quantified for irradiated cells and their non-irradiated controls and the γ H2AX induced by irradiation was expressed as the normalised fluorescence ratio (NFR) - the ratio of fluorescence intensity in irradiated compared to non-irradiated cells after correction for autofluorescence.

Prior to flow cytometric analysis the flow cytometer was checked using CaliBrite beads according to the procedure described in Section 2.2.10

Immunophenotyping of the gated cell population confirmed that the cells analysed were CD45 positive confirming that they were leucocytes, and CD14 negative confirming that they were not monocytes. The cell population gated for γ H2AX analysis contained a mixture of CD3 or CD19 positive cells confirming a mixed population of T and B lymphocytes although some cells with the FSC and SSC characteristics of lymphocytes did not stain for either CD3 or CD19. This may have been due to low affinity of the antibodies for the cell surface antigen after fixation, or to the presence of a non-B, non-T cell lymphocyte population, possibly natural killer cells (see Appendix 3).

5.1.3 Quantification of the dose response relationship between γ H2AX induction and increasing radiation dose by flow cytometry.

In order to establish that a linear dose response relationship similar to that detected by visual quantification of γ H2AX foci could also be detected by flow cytometry, and to determine a suitable test dose of radiation for examining γ H2AX kinetics, a dose-response experiment was performed. PBLs were isolated from a fresh 8 ml venous blood sample collected from volunteer 6 and resuspended in RPMI/1%FCCS and divided between 8 x 15ml polypropylene tubes. After 30 minutes incubation at 37°C they were irradiated with increasing doses of gamma rays (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0Gy), returned to the incubator for 30 minutes, before centrifugation at 0°C and fixation and storage at -20°C. Samples were immunostained and analysed by flow cytometry. Following staining a sample from each was centrifuged onto glass slides, counterstained with DAPI and visualised by fluorescence microscopy to ensure that the cells had the same γ H2AX foci staining characteristics seen previously during the focus quantification study.

Results

The normalised fluorescence ratio (NFR) increases linearly with increasing dose between 0 and 2 Gy corresponding to an increase in foci seen when the same cells are visualised by microscopy.

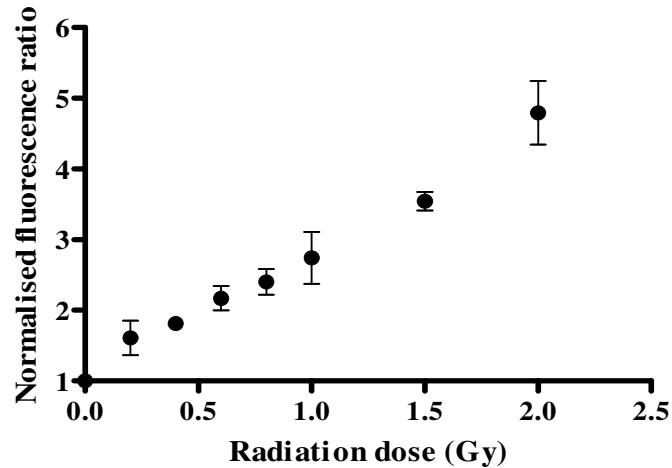


Figure 5.1 Dose response of γ H2AX induction in human PBLs 30 minutes after γ -irradiation in vitro.

Each data point represents the mean \pm SD from 3 separate experiments using lymphocytes from same volunteer. Spearman rank correlation shows a statistically significant relationship between radiation dose and γ H2AX induction as measured by the NFR (Normalised fluorescence ratio = fluorescence in irradiated cells/fluorescence in non-irradiated cells) (Spearman $R = 1.00$, $p < 0.0001$).

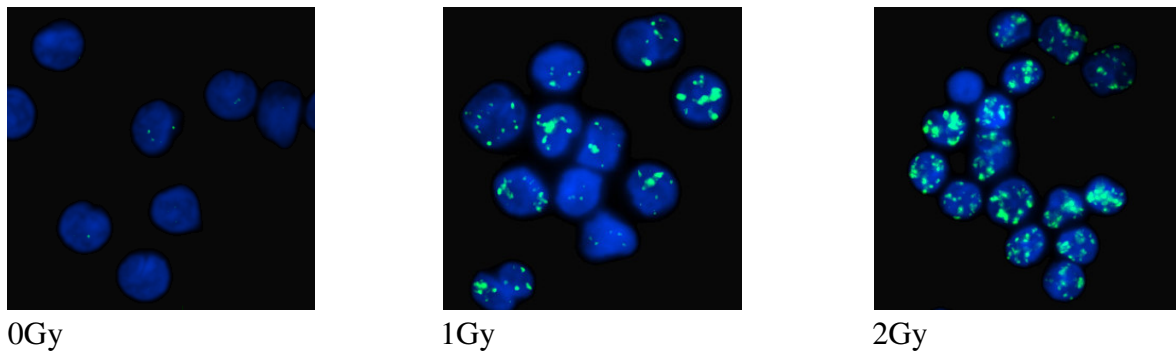


Figure 5.2 Visual confirmation H2AX focus induction in samples analysed by flow cytometry

The corresponding digital images of the same samples, cytopspun onto glass slides after the immunostaining procedure, counterstained with DAPI and visualised by fluorescence microscopy (x100) confirm that discrete foci have been induced and detected after irradiation which increase in number with increasing radiation dose

The plateau effect above 0.8Gy observed during quantification by microscopy is not present suggesting that quantification by flow cytometry is not saturable in the dose range examined. The slope of the dose response curve is shallower than that seen with direct

visual focus quantification suggesting that this technique is not as sensitive at detecting γ H2AX induction after low doses of radiation e.g. <1Gy. There is a statistically significant difference overall in γ H2AX levels observed following different radiation doses (Kruskall-Wallis, $p=0.0023$) but direct comparison between different pairs of dose levels could only detect a statistically significant difference between non-irradiated cells and cells which had received 2Gy ($p<0.05$, Dunn's multiple comparisons test). 2Gy was therefore used as the test dose of radiation for examining the kinetics of γ H2AX induction and loss in subsequent experiments. This dose is also the usual dose per fraction used in clinical radiotherapy schedules and there is therefore the potential to use a clinically relevant test dose of radiation in this assay.

5.1.4 Kinetics of γ H2AX induction and loss after 2Gy

The kinetics of γ H2AX induction and loss in irradiated human PBLs after 2Gy as measured by flow cytometry were examined. Potential endpoints for measuring γ H2AX induction and loss which could be used in a predictive assay were assessed for reproducibility.

A fresh peripheral blood sample (4x8ml CPT tubes) was taken from subject 2. The sample was immediately processed and isolated PBLs resuspended in RPMI/1% FCS at 1.5×10^6 /ml, divided into 13x15ml polypropylene tubes, gassed with 5% CO₂/air and incubated at 37°C for 30 minutes. The cells in one tube were fixed to measure baseline γ H2AX levels. 6 of the remaining tubes were irradiated (2Gy) and the other 6 were mock irradiated. All were then returned to the incubator and at each pre-specified time point irradiated and non-irradiated samples were fixed. All samples were stored at -20°C until immunostaining. Immunostaining of all samples from the same experiment was performed simultaneously and analysed by flow cytometry. The normalised fluorescence ratio was calculated for each time point.

Kinetics of H2AX induction and loss were measured 11 times in subject 2. The pre-specified time points for quantification of γ H2AX were 0, 0.5, 1, 2, 4, 6 and 24 hours.

Results

After the first 3 repeats of this experiment it became clear that 24 hours after irradiation it was not possible to gate a population of lymphocytes during flow cytometry. The FSC/SSC plot of the 24 hour sample consistently showed particles with a wide variability

in forward and side scatter characteristics and it was not possible to define a population for gating that gave a clearly discernable peak in green fluorescence. For the remaining kinetics experiments the 24 hour time point was omitted.

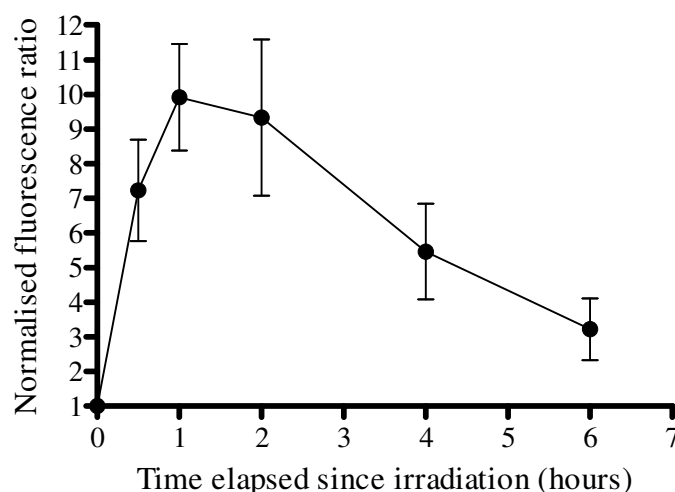


Figure 5.3 Kinetics of γ H2AX induction and loss in human peripheral blood lymphocytes after 2Gy

The mean and standard deviation for each time point are shown (n=11)

γ H2AX levels peak at 1 hour post irradiation, appear to plateau between 1 and 2 hours and then fall between 2 and 6 hours. Levels are still approximately 3 times greater than baseline at 6 hours after irradiation. Insufficient number of time points makes it very difficult to ascertain the precise kinetics of γ H2AX loss. The decrease in γ H2AX between 2 and 6 hours may either be occurring in a linear fashion (linear regression analysis, $R^2=0.7128$, non-significant departure from linearity on runs test) or be the early part of an exponential decay curve (non-linear best fit of one phase exponential decay curve, $R^2=0.7295$). Assuming that γ H2AX is lost in a linear fashion the half life for loss is 3.26 hours (95% CI 2.944 to 3.582 hours). Using the exponential decay model, the half-life of γ H2AX loss is 2.55 hours (95% CI 0.9836 to +infinity).

5.1.5 Potential assay end-points for measuring γ H2AX kinetics

A diagnostic test needs a defined end-point to allow comparisons between individuals. Possible assay end points for describing the kinetics of γ H2AX include:

- Area under the Curve (AUC) – this would give an integrated measurement of the effect of time on γ H2AX levels after irradiation using data from all time points.
- Half-life of γ H2AX loss – Given the lack of time points for analysis calculating the half-life for γ H2AX loss assuming exponential loss will be inaccurate if not impossible on data from individual experiments. Therefore, as a compromise, half life for γ H2AX loss assuming a linear relationship between time and γ H2AX levels between 2 and 6 hours and using the slope of the best fit line calculated by linear regression was investigated as a possible assay endpoint. The fact that this method of assessing γ H2AX kinetics assumes a linear relationship which may not actually be present, and that the slope of a linear regression line is an mathematical estimate rather than a direct observation are both potential disadvantages of this method.
- A more pragmatic endpoint based on direct observations and making no assumptions about the kinetics of γ H2AX loss is the ratio of γ H2AX peak levels compared to those at 6 hours.

The half-life of γ H2AX loss, peak:6 hour level of γ H2AX and AUC of γ H2AX kinetics, as well as the measured levels of γ H2AX at each specified time point were assessed as potential assay endpoints, firstly by estimating their precision.

Intra-sample precision

Because cells isolated from a single blood sample can be stored after fixation it is possible to measure assay precision both within a single run and between runs of the immunostaining procedure for the same blood sample.

It was inappropriate to take sufficient blood at one time from one individual to allow repeated measurement of γ H2AX kinetics on a single blood sample. Therefore estimates of intra-sample precision were based on the ability of the assay to predictably and reliably quantify γ H2AX levels at a single time point after radiation. The time point chosen was 1 hour post irradiation when peak levels of γ H2AX were demonstrated in subject 2. Intra-run and between-run precision were measured for PBLs isolated from the same blood sample.

a) Intra- sample precision (intra-run)

A peripheral blood sample was taken from subject 2 (5X8ml CPT tubes). PBLs were isolated and resuspended in RPMI/1% FCS at 1 million/ml. 4 million cells were transferred into each of 12 pairs of 15ml polypropylene tubes, gassed with 5%CO₂/air and incubated at 37 °C for 30 minutes. One tube of each pair was irradiated with 2 Gy whilst the other was mock irradiated and all were incubated at 37°C for 1 hour before being cooled and fixed. The samples were stained for γ H2AX and analysed by flow cytometry on the same day. The coefficient of variation was calculated as an estimate of within run intra- sample precision.

b) Intra- sample precision (inter-run)

A peripheral blood sample was taken from subject 2 – cells were separated and resuspended at a concentration of 1 million per ml in two separate 15ml polypropylene tubes gassed with 5%CO₂/air and incubated at 37 °C for 30 minutes. One of each pair was irradiated and the other mock-irradiated and all were incubated at 37°C for 1 hour before being cooled and fixed. Fixed cells divided into 12 pairs of storage tubes (one irradiated and one control per pair). Three pairs of samples were analysed separately on 4 consecutive days.

Table 5.1 Intra-sample precision – mean results, standard deviation and coefficient of variation for flow cytometric quantification of γ H2AX in human PBLs at 1 hour post 2Gy

	Mean level γ H2AX 1 hour post 2 Gy (NFR)	Standard deviation	Coefficient of variation
Intra- run precision (n =12)	8.13	0.69	8.41%
Inter run precision. (n=4)	5.872	0.66	8.74%

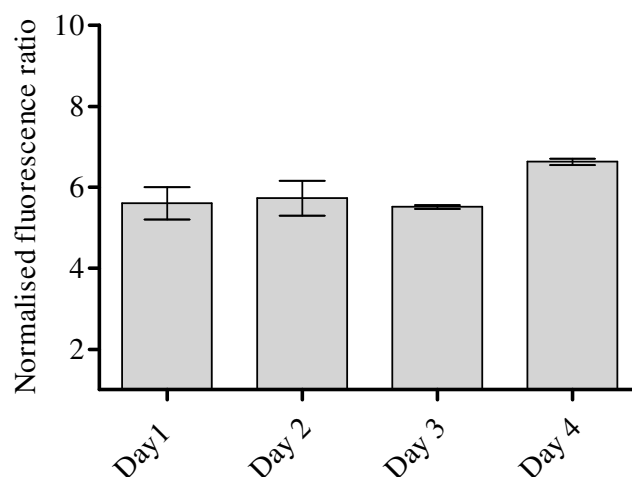


Figure 5.4 Intra-sample/inter run precision – mean and standard deviation for flow cytometric quantification of γ H2AX in human PBLs at 1 hour post 2Gy from a single blood sample.

Cells were isolated, irradiated and fixed on the same day but immunostained and analysed over 4 consecutive days. There was no statistically significant difference in results over the 4 days ($p = 0.0752$, Kruskal-Wallis test)

Inter-sample precision

In order to measure inter-sample precision for all potential assay end points γ H2AX kinetics after 2Gy were measured in freshly collected human peripheral blood lymphocytes from the same volunteer on repeated occasions ($n=11$) over 12 months. The coefficient of variation for each potential assay endpoint was calculated. PBLs were isolated immediately from each blood sample with strict adherence to the protocols for irradiation, fixation, immunofluorescent staining and flow cytometric analysis throughout. After irradiation and fixation samples were stored for at -20°C for differing durations before analysis up to a maximum of 15 days. Subject 2 did not admit to any concurrent illness or taking any medication at any time over the study period. Diet was not assessed.

Table 5.2 Inter-sample precision for potential end-points of an assay of the kinetics of γ H2AX induction and loss after in vitro irradiation in human PBLs.

The mean NFR, 95% confidence intervals and coefficient of variation for 11 repeated measurements on the same individual over a 12 month period for each assay endpoint are given.

	Mean	95% CI	Coefficient of Variation
γH2AX at 0.5 hour	7.23	6.23-8.21	20.26%
γH2AX at 1 hour	9.91	8.88-10.95	15.55%
γH2AX at 2 hours	9.34	7.82-10.84	24.14%
γH2AX at 4 hours	5.46	4.53-6.39	25.32%
γH2AX at 6 hours	3.22	2.62-3.82	27.83%
Area Under Curve	33.44	28.41-38.47	22.39%
Half-life of γH2AX loss (h)	3.26	2.94-3.58	14.55%
Ratio peak:6 hour γH2AX	3.32	2.96-3.68	16.10%

There was considerable inter-sample variability in results - the lowest variability, suggesting the best precision is for γ H2AX levels at 1 hour, half-life of γ H2AX loss and the ratio of peak levels to γ H2AX at 6 hours. Inter-sample variation is higher than intra-sample-variation measured for γ H2AX levels at 1 hour (15.55% vs. 8%).

To ascertain if the inter sample imprecision of each assay end point was due to random error or a systematic drift in results over time, the results from each end point were plotted against the experiment repeat number and results examined for any evidence of a correlation between assay result and time.

There was a statistically significant positive correlation between γ H2AX levels at 2 hours after irradiation and the time in the study period (Spearman R 0.66, $p=0.03$, shown in Figure 5.5). For the other assay end points there appeared to be a trend towards increasing assay result over the sampling period, but the increase was not statistically significant. (Figure 5.5 and Table 5.3)

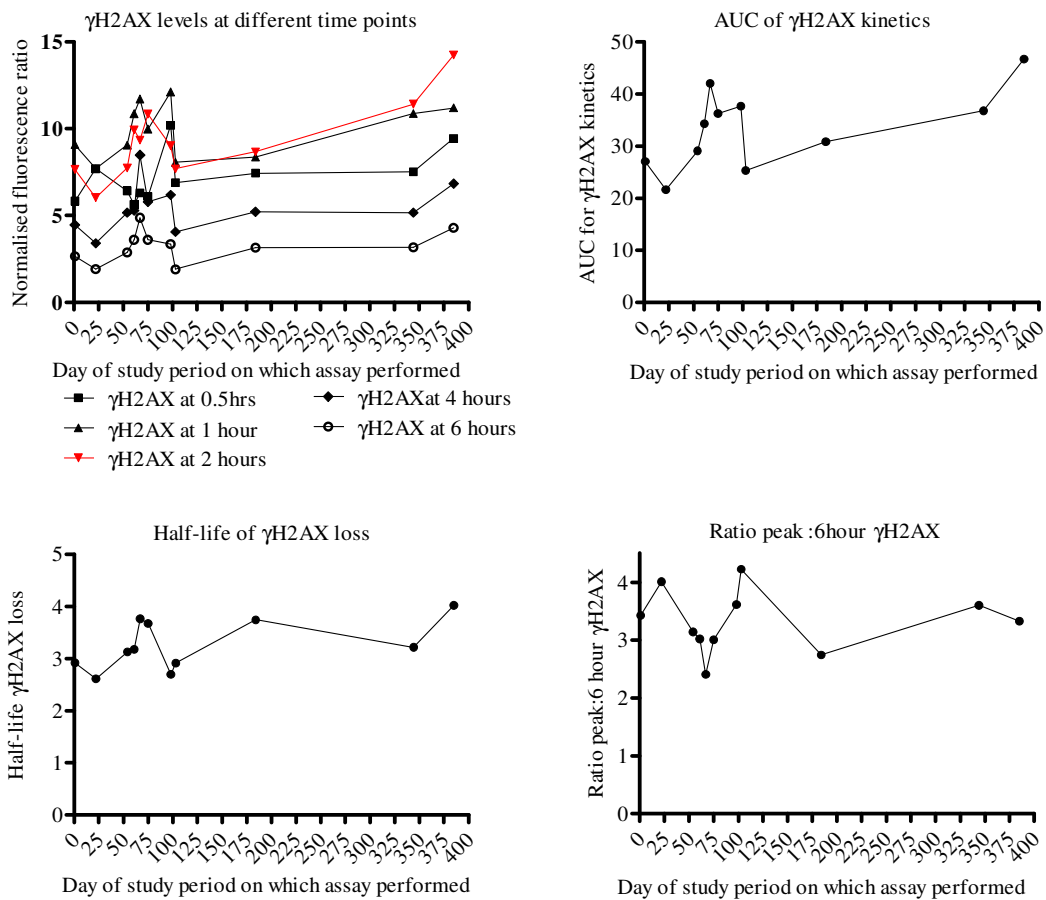


Figure 5.5 Change in γH2AX assay results in a single individual over the study period. Assay results are shown plotted against the day of the study period on which the assay was performed (first experimental run was performed on day 1)

Table 5.3 Relationship between assay results and time in a single individual over the study period (corresponds to Figure 5.5)

Spearman rank correlation was used to test for any relationship between the assay results and timing of the experimental run within the study period for different end-points over 11 repeats in a single individual over 12 months.

	Assay end-point							
	γ H2AX at fixed time point after irradiation (hours)					AUC	Half-life γ H2AX loss	Ratio peak:6 hour γ H2AX
	0.5	1	2	4	6			
Spearman R	0.54	0.33	0.66	0.35	0.33	0.56	0.52	0.00
p-value (two-tailed)	0.09	0.33	0.03	0.30	0.33	0.07	0.10	1.00

After cell fixation, samples for assessment of inter-sample precision were stored at -20°C before immunostaining and analysis. Duration of storage varied from 1-15 days. As all other parameters of the assay technique were standardised throughout the study period the possibility that duration of sample storage after fixation and before analysis may have affected assay results was examined. Duration of storage was plotted against assay result for each of the potential assay endpoints (Figure 5.6). There was no correlation between duration of storage and assay results for any of the end-points (Table 5.4)

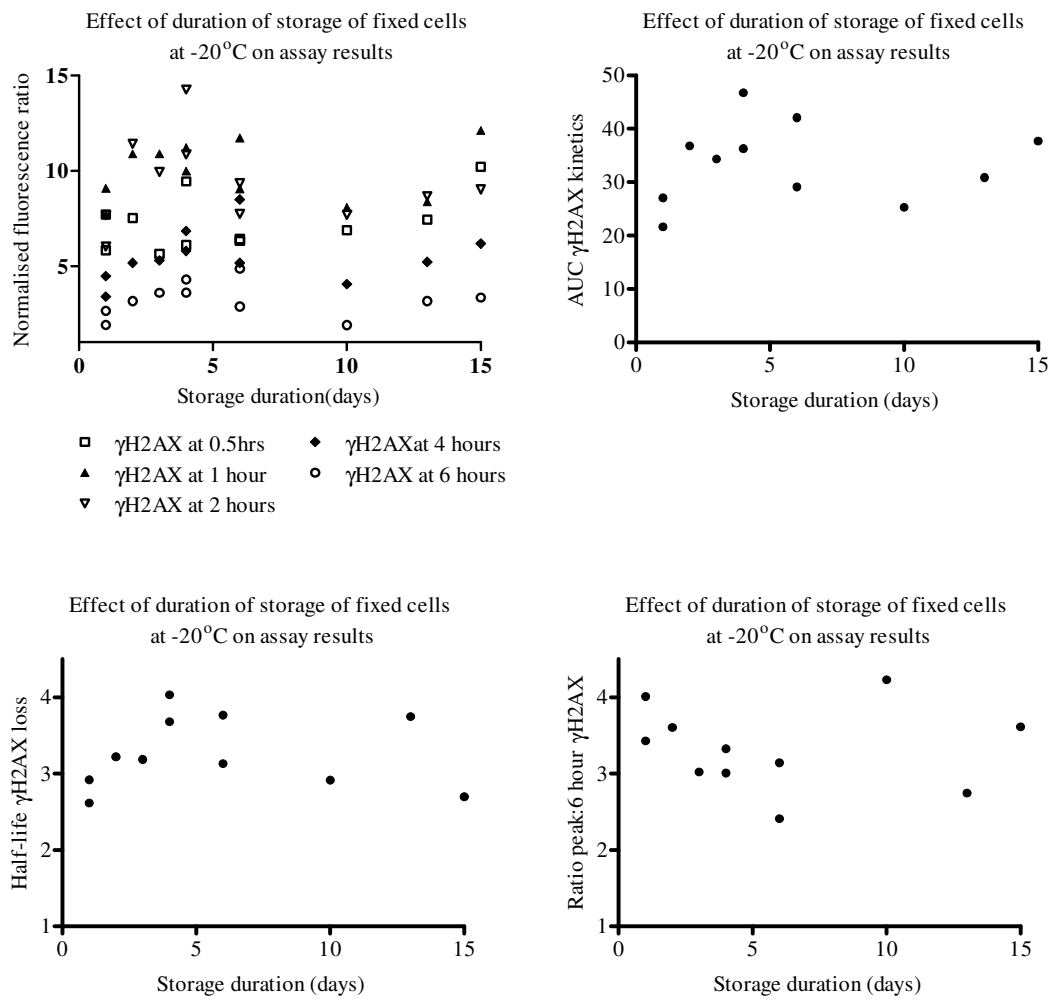


Figure 5.6 Relationship between duration of storage of fixed samples and assay results

Table 5.4 Correlation (Spearman rank) between duration of sample storage at -20°C and assay results for potential assay end-points.(Corresponds to Figure 5.6)

	Assay end-point							
	γ H2AX at fixed time point after irradiation (hours)					AUC	Half-life γ H2AX loss	Ratio peak:6 hour γ H2AX
	0.5	1	2	4	6			
Spearman R	0.30	0.20	0.05	0.42	0.13	0.26	-0.16	0.11
p-value (two-tailed)	0.37	0.56	0.87	0.20	0.70	0.43	0.63	0.74

5.1.6 Assay practicality:

The number of times it was not possible to determine a γ H2AX level for any given pair of irradiated and non-irradiated samples was calculated to give an estimate of assay reliability. 1/86 pairs of irradiated vs. control samples over the course of the assay precision experiments failed, giving an assay-failure rate of 1.16%

Assay results were available 2 working days following blood sample collection. Freezing of samples after irradiation and fixation meant that staining and flow cytometric analysis could be performed at convenient time.

5.1.7 Kinetics of γ H2AX induction and loss in human PBLs after different radiation doses

In order to determine if the kinetics of γ H2AX induction and loss in isolated G0 human PBLs varied with applied radiation dose, γ H2AX kinetics after 5, 10 and 20Gy were measured in freshly collected peripheral blood samples from the same volunteer (Volunteer 2). Each experiment was performed independently on different days and for each dose level was repeated between 2 and 4 times (Figure 5.7). The AUC, peak: 6 hour ratio and half-life were measured for each independent experiment and the presence of any relationship between resulting assay end-points and radiation dose was examined by Spearman rank correlation. The results from the previous experiments in the same volunteer examining the kinetics after 2 Gy were included in the analysis.

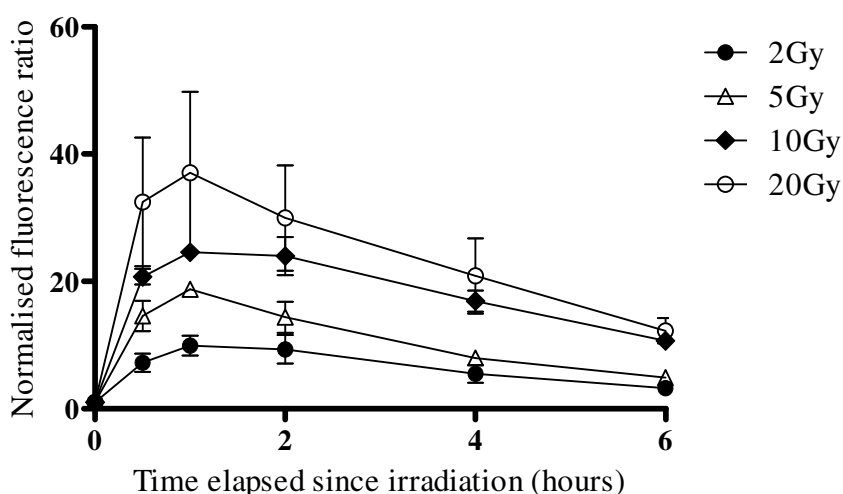


Figure 5.7 Kinetics of γ H2AX induction and loss in isolated PBLs from a single volunteer following different test doses of in vitro irradiation

Each data point represents the mean and standard deviation from between two (5Gy) and eleven (2Gy) independent repeats.

At all of the time points examined there was a trend to an increase in induced γ H2AX levels with increasing radiation dose, but at no time point did this trend become statistically significant (Figure 5.8A and Table 5.5). A similar relationship was seen for AUC measurements (Figure 5.8B and Table 5.5). There was a suggestion that the dose-response curves for these end-points was beginning to plateau at 20Gy. The end-points examining the kinetics of γ H2AX loss (half-life and peak: 6 hour ratio) did not appear to correlate with increasing radiation dose (Figure 5.8A and Table 5.5). There was, however,

a statistically significant difference between peak: 6 hour ratio and half-life of γ H2AX loss for different radiation doses ($p=0.03$, Kruskal-Wallis) with Dunn's multiple comparisons test showing a significant difference between 5Gy and 10Gy but not between other radiation doses for both endpoints ($p<0.05$ for both),

Table 5.5 Correlation between radiation test-dose and assay end-points. Spearman R and p-values suggest that there is no relationship between radiation test dose and assay results for any of the assay end-points.

	Assay end-point							
	γ H2AX at fixed time point after irradiation (hours)					AUC	Half-life γ H2AX loss	Ratio peak:6 hour γ H2AX
	0.5	1	2	4	6			
Spearman R	1.00	1.00	1.00	1.00	1.00	1.00	0.60	-0.60
p-value (two-tailed)	0.08	0.08	0.08	0.08	0.08	0.08	0.42	0.42

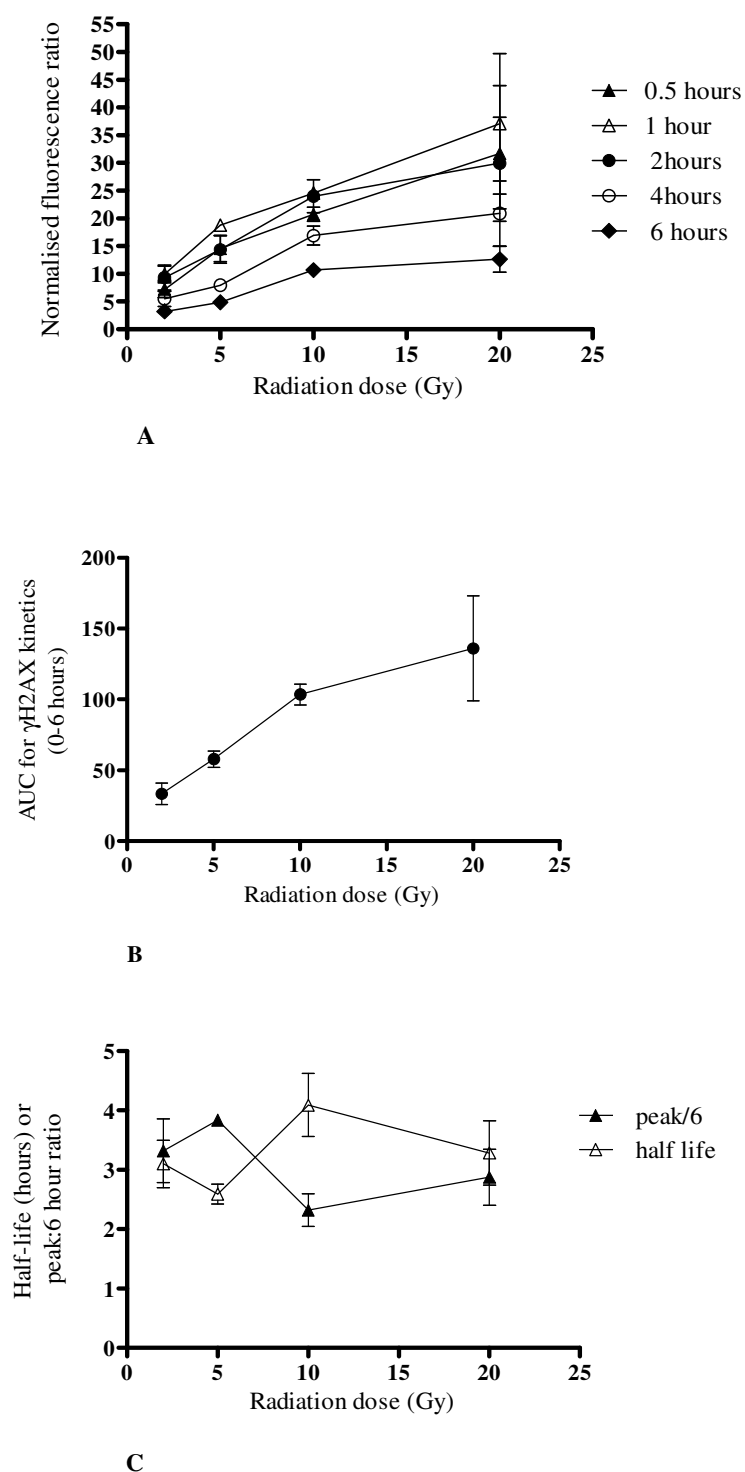


Figure 5.8 Correlation of end-points of γ H2AX kinetics experiments with in vitro test dose of radiation

There was a non-significant trend for an increase in γ H2AX levels at all time points (A) and the AUC of the kinetics curve (B). There was no clear relationship between measures of γ H2AX loss and applied radiation dose (C)

5.1.8 Comparison of the kinetics of radiation-induced DNA double strand break repair and γ H2AX induction and loss in human peripheral blood lymphocytes.

The data presented in Section 5.1.7 illustrates that it is possible to use flow cytometry to measure γ H2AX kinetics following a radiation dose of 20Gy. Quantification of DNA double-strand breaks (DSBs) by constant field electrophoresis is a relatively insensitive technique compared to γ H2AX quantification and requires doses in excess of 10Gy to allow detection of DNA DSBs. Using flow cytometry to quantify γ H2AX rather than foci therefore allows simultaneous comparison of the kinetics of γ H2AX and DSBs in same cell population after the same radiation dose to test the assertion that γ H2AX foci are equivalent to DNA DSBs and that loss of γ H2AX foci equates to DNA DSB repair.

Using the technique described in Section 2.2.11 DNA DSBs in isolated human peripheral blood lymphocytes (from Subject 2) were measured immediately after exposure to increasing doses of gamma irradiation (0, 10, 20 and 30Gy). This confirmed a linear dose-response relationship with an increase in induction of DNA DSBs with rising radiation dose. (see Appendix 5).

Peripheral blood was collected from volunteer 2 (8x8ml CPT tubes). The mononuclear cell fraction was isolated, washed and resuspended in RPMI/1%FCS. The cell suspension was transferred to a tissue culture flask and incubated at 37°C for 1 hour to remove the monocyte fraction. The cells were then counted and resuspended at a concentration of 1×10^6 cell/ml in RPMI/1%FCS. 4 ml was transferred to each of 18 x 15ml Polypropylene tubes for the H2AX analysis (Set 1). 150 microlitres was transferred to another set of 18 tubes for the DSB assay (Set 2), RPMI/1% FCS was added to all tubes to bring the volume up to 7ml. Each tube was gassed with 5% CO₂ in air and incubated at 37°C for 30 minutes. The tubes were then cooled on ice. 9 tubes from each set were irradiated on ice (20Gy) whilst the non-irradiated controls were kept on ice. After irradiation one irradiated and one control tube from each set was kept on ice whilst the others were returned to the incubator. At the pre-specified time points one irradiated and one control sample from each set were cooled rapidly on ice and held on ice at 0°C until all time points had been reached. The tubes were then processed according to the usual protocols for each assay. The time points chosen were 0, 0.25, 0.5, 1, 2, 6, 18 and 24 hours following irradiation. The number of DNA DSBs and γ H2AX as quantified by the normalised fluorescence ratio were plotted against time. The experiment was repeated 4 times.

Results

As would be expected peak levels of DSBs were detected at 0 hours, immediately following irradiation and before any repair had occurred (Figure 5.9 A). The number of DSBs fell rapidly in the first hour after irradiation before plateauing between 2 and 6 hours and then rising again steadily at 18 and 24 hours after irradiation. At 24 hours the number of DSBs was higher than originally induced by irradiation.

γ H2AX levels rose rapidly following irradiation, peaking at 1 hour and then falling steadily in an apparent linear fashion between 1 and 6 hours. As previously experienced in the examination of γ H2AX kinetics after 2 Gy, no clear cell population could be gated for analysis of irradiated cells at 24 hours. The same was also true at the 18 hour time-point. This was despite there being a definite cell pellet visible after centrifugation of sample tubes at all stages of sample preparation.

In order to facilitate comparison of the kinetics of DSBs and γ H2AX, both were plotted as proportions of their peak levels against time for the first 6 hours after irradiation (Figure 5.10). 37% and 42% of DSBs had disappeared by 30 minutes and 1 hour after irradiation, with the proportion of DSBs repaired reaching a maximum of 57% at 2 hours after irradiation. In contrast the levels of γ H2AX were rising rapidly over the first hour after irradiation whilst the numbers of DNA DSBs were falling. At 2 hours when the number of DSBs had reached its nadir, only 15% of the induced γ H2AX had been lost. The proportions of DSBs and γ H2AX remaining after irradiation only reached equivalence at 4-6 hours after irradiation.

Annexin-V staining and flow cytometric analysis of unfixed cells 24 hour after irradiation suggested that the proportion of cells undergoing apoptosis was significantly increased in irradiated compared to non-irradiated cells (Figure 5.11).

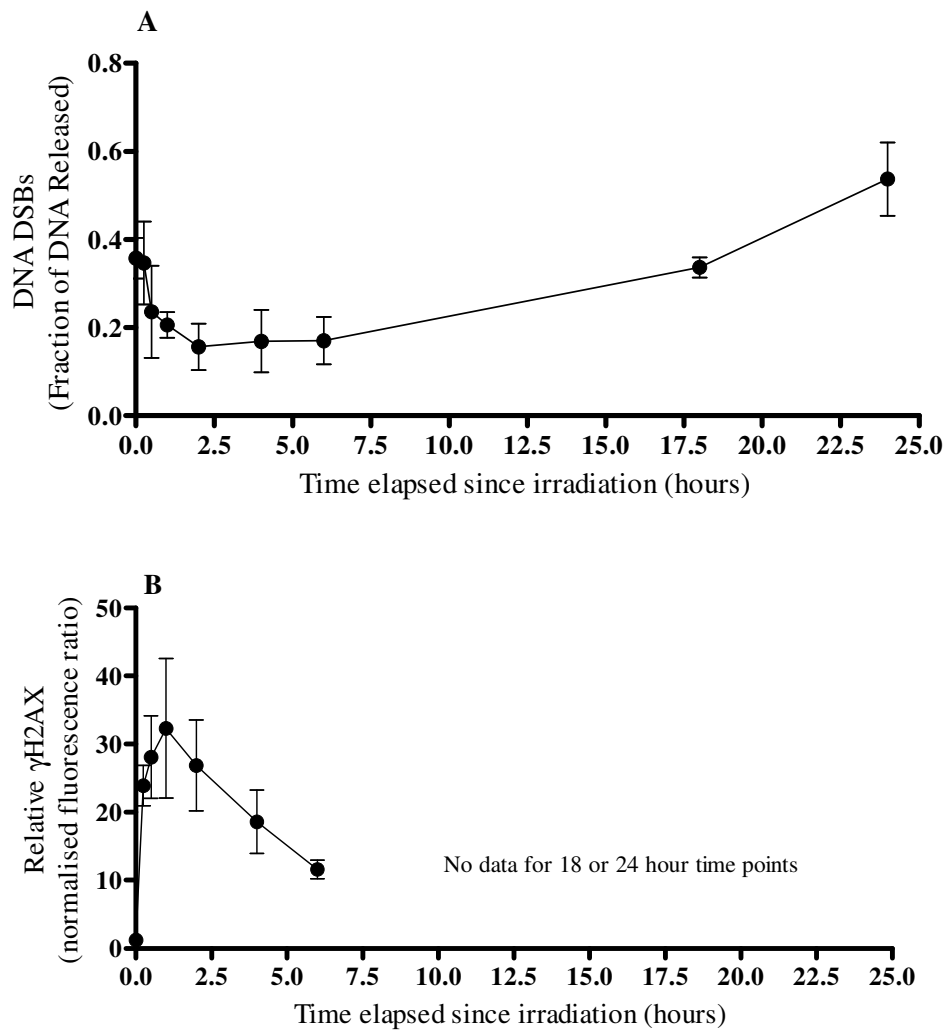


Figure 5.9 Kinetics of DNA double strand break formation and repair and γ H2AX induction and loss in PBLs after 20Gy

DNA DSBs as measured by constant field gel electrophoresis (A) with simultaneous measurement of γ H2AX induction and loss by flow cytometry (B) in isolated human peripheral blood lymphocytes after in vitro irradiation (20 Gy). Each data point represents mean \pm standard deviation from 4 experiments.

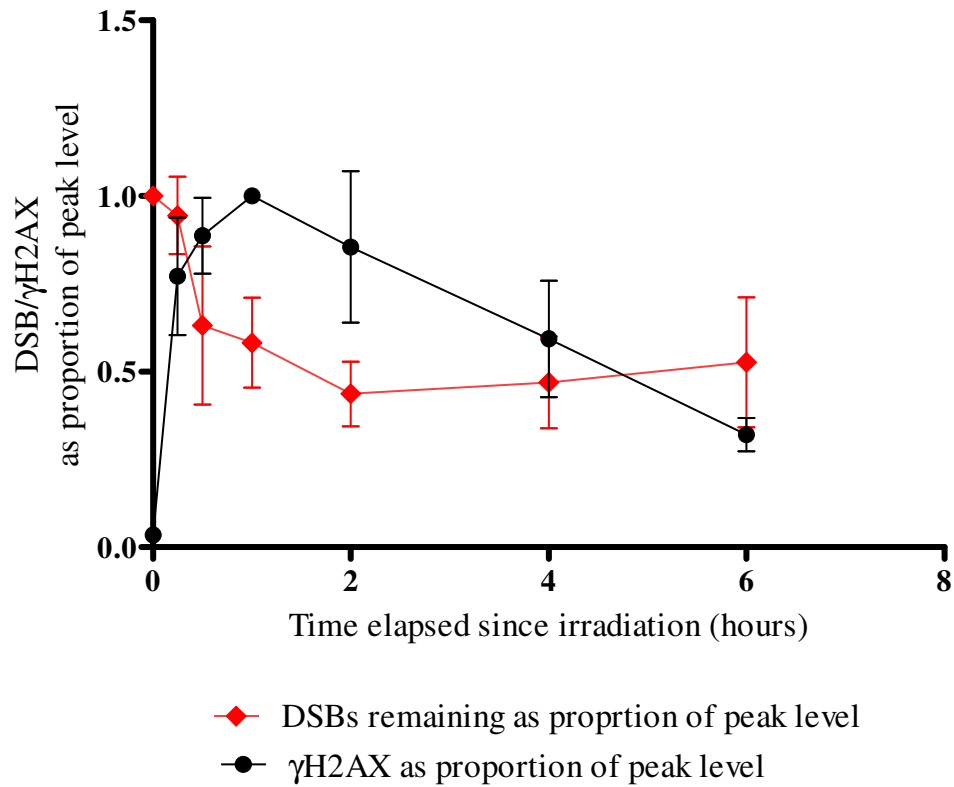


Figure 5.10 Kinetics of DSB induction and repair and γ H2AX induction and loss for the 6 hours immediately post irradiation (20Gy) in isolated human peripheral blood lymphocytes.

The values for each time point are expressed as the proportion of DSBs or γ H2AX present compared to the peak level of each. The peak level was at 0 hours for DSBs and 1 hour for γ H2AX. DSBs are falling as γ H2AX is rising in the first hour following irradiation (see text).

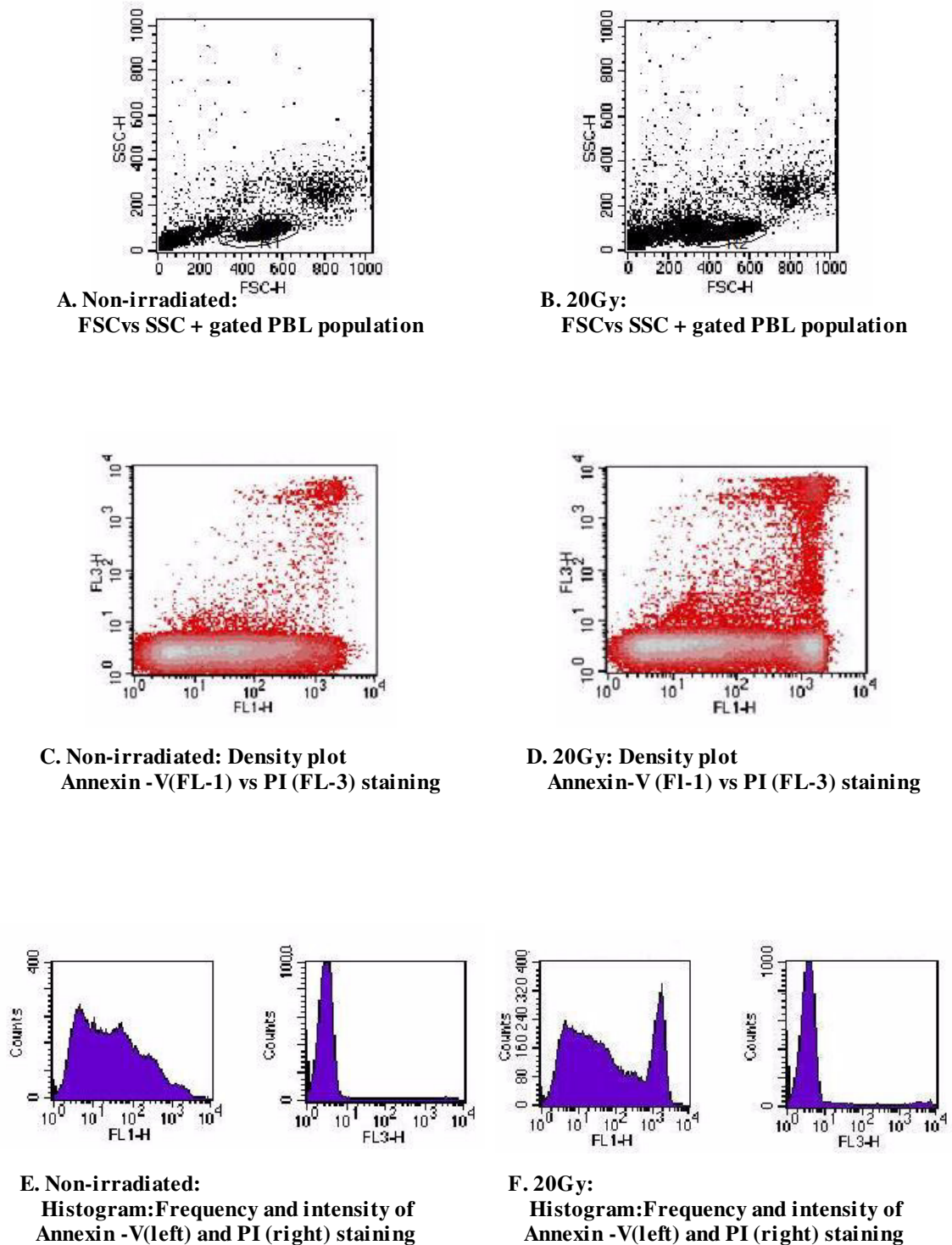


Figure 5.11 Apoptosis of isolated G0 human peripheral blood lymphocytes held in culture 24 hours post irradiation (20Gy)

Compared with the non-irradiated control (A), there is more small volume cell debris present in the irradiated sample but it is still just possible to gate a lymphocyte population

on the FSC/SSC dot plot (B). Density plots show that after 24 hours there is a discrete subpopulation of irradiated cells staining with FITC-labelled Annexin-V detected on the FL-1 channel (D) compared with the non-irradiated control (C). There is also increased staining with propidium iodide (PI) after irradiation (FL-2 channel) suggesting an increased number of non-viable cells. Frequency histograms for FITC-labelled Annexin-V (FL-1) show a discrete peak at a fluorescence intensity of 1×10^3 (F) which is not present in non-irradiated controls (E), confirming the presence of an Annexin-V positive and probably apoptotic cell population induced by irradiation. Frequency histograms of PI staining (FL-2) show a very small population of strongly PI positive and therefore non-viable cells after irradiation only.

5.1.9 Discussion

Using immunofluorescent staining and analysis by flow cytometry, it is possible to measure the kinetics of γ H2AX in PBLs after in vitro irradiation with a clinically relevant radiation dose. The assay is quick and the ability to store samples between irradiation and immunostaining means that it is more convenient than γ H2AX focus quantification by direct visualisation by microscopy. Assay failure rate is low at 1.16%.

It was disappointing that it was not possible to obtain data for γ H2AX levels at 24 hours after irradiation. The FSC/SSC characteristics of the particles present in the irradiated immunostained samples after 24 hours suggests a population of varying sizes and likely represents cellular debris. Lymphocytes are susceptible to apoptosis so it is possible that cells were undergoing radiation apoptosis by 24 hours post radiation - this would result in a mixed population of cells of varying sizes as cells shrink and break up into apoptotic bodies. The subsequent examination of the kinetics of DNA DSBs and γ H2AX after 20Gy in section 5.1.8 again found it was difficult to consistently gate a lymphocyte population in fixed irradiated cells which corresponded to an increase in DNA DSBs and Annexin-V positivity suggesting that at least after 20Gy cells were undergoing apoptosis at 24 hours.

The kinetics of γ H2AX induction and loss between 0-6 hours after 2Gy including estimation of half life does seem to concur with published data from McPhail et al relating to cultured cells and murine normal tissues and the reported half-life of γ H2AX loss of 3 hours in human PBLs (MacPhail, Banath et al. 2003; Olive and Banath 2004). It also corresponds with the limited data that was derived from γ H2AX focus quantification by

microscopy where peak levels were seen at 1 hour. The estimated half-life of γ H2AX loss was longer in PBLs after 2 Gy analysed by flow cytometry (3.26 hours, 95% CI 2.94-3.58, Figure 5.3) than after 0.4Gy when analysed by microscopy (1.74 hours, 95% CI 1.11 to 3.99 hours, Figure 4.10). The half-life estimates from microscopy were based on only 2 experimental repeats - the 95% confidence intervals are large and overlap with those obtained from measurement of half-life of γ H2AX loss by flow cytometry so it is unlikely that a true significant difference in half-life exists.

Intra- sample-intra- run precision is measured is 8.4%. Intra- sample-inter-run imprecision is similar at 8.74%. This suggests that this method of γ H2AX quantification is more precise than by foci quantification by microscopy. However, these coefficients of variation would also suggest that despite following a strict protocol and using the same reagents on throughout there are still technical sources of variation in staining or analysis during an experimental run and between different experimental runs which lead to inconsistencies in results from different sample tubes containing the same sample. The cells in each of the different sample tubes were irradiated and fixed in the same batch before separation demonstrating that source of variability must be inherent in the storage and/or staining and/or flow cytometry analysis. As all samples in the intra-run experiments were treated with reagents (including primary and secondary antibody) from the same solutions, variation in results must come from small variations in the timings of antibody incubations and washings which result from having to handle several tubes at once (although all tubes were handled in the same order at each step), or variability at the analysis step, such as possible FITC fading in some samples whilst waiting for analysis. There is no clear way of accounting for these technical variations and improving intra- sample-intra--run precision.

Intra- sample-inter run variation may have come from the same sources, with possible additional confounding by differing environmental conditions within the laboratory on different days for the immunofluorescent staining procedure (such as change in temperature or light intensity) or day-to-day inaccuracies in antibody dilution. The other possible source of variation is the increasing duration of storage at -20°C over the 4 days of the experiment. Data points are limited but there is no clear evidence of a systematic drift in results to suggest that samples were degrading in the freezer during storage (Figure 5.4).

There is significant inter-sample variation in results for all assay end-points when measured in a single individual over a 12 month period. The lowest inter sample imprecision occurs for assay endpoints which examine rate of γ H2AX loss (half-life and peak: 6 hour ratio). Assays of γ H2AX loss seem likely to be most important as potential predictive assays of normal tissue radiosensitivity given the correlations between H2AX loss and cell survival reported by Olive and MacPhail (MacPhail, Banath et al. 2003; Olive and Banath 2004) so the finding that these end-points appear to be the most precise is encouraging. Imprecision in measurements at the specific time points is high and this is reflected in high inter sample variability in AUC measurements which integrate results from all time points.

There is evidence that there is a systematic upwards drift in measured γ H2AX levels after irradiation as the sampling period progressed which was statistically significant for the 2 hour time point (Table 5.3 and Figure 5.5). This apparent systematic drift over time may reflect a systematic change in sample handling, irradiation, fixation, storage, immunostaining or flow cytometry analysis.

The duration of exposure to radiation was constant throughout the 12 month period - the upward drift in results is not likely to be due to radioactive decay of the gamma-irradiation source which if anything would have decreased γ H2AX induction over the sampling period.

Two separate batches of both primary and secondary antibody were used over the sampling period – it is possible that differing binding affinities may have affected results but if this were the case this would be expected to result in a sudden step wise change in results when the batch was changed rather than the slow drift observed.

Increasing operator experience resulting in faster processing of sample or decreased background staining should have been compensated for by the corrections derived from simultaneous analysis of non-irradiated and non-stained controls. The CPT tubes used throughout were from the same batch number and all were utilised within their expiry date.

It is possible that there was a systematic drift in the performance of the flow cytometer. The optimal settings for fluorescence compensation may have changed in time resulting in “leaching” of any red fluorescence signal generated by the cells being mistakenly

measured as part of the green FITC fluorescence signal. Fluorescent bead calibration and compensation correction was performed when the cytometer was serviced. Recalibration and setting of fluorescence compensation parameters should perhaps have been performed more regularly perhaps even at the beginning of each experimental run, as would be standard with any machine being used in a clinical laboratory.

Finally, there may have been a systematic biological change in the susceptibility to radiation DNA damage and response to DNA damage over time in Subject 2. Apart from the passage of time no other easily appreciated biological changes occurred in subject 2 over the 12 month study period with no admission of new medication, illness, or change in diet or lifestyle. Background DNA damage as measured by the micronucleus assay does increase with increasing donor age (Ganguly 1993; Bolognesi, Abbondandolo et al. 1997) but no relationship between donor age and response to in vitro irradiation has been found (Bishay, Ory et al. 2001). Whilst a biological reason for the systematic drift in assay results is possible, it seems less likely than a technical cause.

As well as the systematic drift in assay results there is clearly random scattering of assay values over the sampling period which accounts for most of the assay imprecision between samples. As discussed in Chapter 4, this random variability may be due to inconsistencies in experimental technique or environmental conditions, or random biological variations in subject 2 that may influence assay results in an unpredictable fashion. The only obvious technical variation between handling of the different samples over the study period was the varied duration of storage of fixed samples at -20°C before immunostaining. However, there was no evidence of a correlation between length of sample storage and assay result for any end point examined (Table 5.4 and Figure 5.6).

Comparison of the kinetics of γ H2AX induction and loss after different radiation doses shows an increase in detected γ H2AX with increased dose for all time points and a corresponding increase in AUC (Figure 5.8 A and B). Given that increased radiation dose results in an increased number of induced DNA DSBs (Appendix 5) this is not unexpected. The fact that there is no statistically significant correlation between dose and γ H2AX at the measured time-points or the AUC of the kinetics experiments as measured by Spearman rank correlation (Table 5.5) probably reflects the small number of data points on which the analysis was based. There is a suggestion that the dose response relationship for all time-

points, and consequently the AUC, becomes less steep between 10 and 20Gy, possibly implying that the immunofluorescent quantification of γ H2AX by flow cytometry is saturable at high radiation doses using this technique and these antibody concentrations.

There is no clear relationship between radiation dose and the rate of γ H2AX loss measured by half-life or the peak: 6 hour ratio (Figure 5.8 C), reflected in the Spearman R values of 0.6 and -0.6 respectively (Table 5.5). The statistically significant difference between 5 and 10 Gy for both end-points of γ H2AX loss as detected by the Kruskal-Wallis and Dunn's multiple comparisons test is probably due to the lack of data points for the 5 Gy data set which has generated a type 1 error. It seems unlikely that the half-life of γ H2AX loss would significantly increase between 5 and 10Gy only to decrease again for 20Gy. It would therefore seem probable that the process of γ H2AX removal proceeds at same rate irrespective of the initial "concentration" of γ H2AX suggesting that the process of γ H2AX removal had not become saturated at the doses examined.

It is therefore possible to use flow cytometry to measure γ H2AX kinetics at 20Gy, which is a dose sufficiently high to also allow simultaneous DSB quantification by constant-field gel electrophoresis. Simultaneous comparison of the kinetics of γ H2AX and DSB induction and loss in same cell population after the same radiation dose is therefore feasible and can test the assertion that γ H2AX foci are equivalent to unrepaired DNA DSBs (Rothkamm, Kruger et al. 2003; Rothkamm and Lobrich 2003; Lobrich, Rief et al. 2005; Takahashi and Ohnishi 2005; Sedelnikova, Nakamura et al. 2007). From Figures 5.9 and 5.10 it is clear that whilst the number of DSBs in irradiated cells is falling steeply between 0 and 1 hour after irradiation, γ H2AX levels are rising. When the number of DSBs has reached a nadir at 2 hours, 85% of induced γ H2AX remains. Equivalence in remaining proportions of DSBs and H2AX is only reached between 4 and 6 hours. This data would suggest that between 0 and 6 hours following irradiation, in G0 human peripheral blood lymphocytes at least, γ H2AX levels do not equate to DSBs. This would be consistent with evidence suggesting that 53BP1/ γ H2AX foci do not correspond with DSBs in the first 24 hours following irradiation in human fibroblasts (Markova, Schultz et al. 2007) and that dephosphorylation of γ H2AX and DNA DSB repair can be independent phenomena (Antonelli, Belli et al. 2005) or at least correlate only at very low radiation doses (Bouquet, F., C. Muller, et al. 2006).

In this study it was not possible to measure γ H2AX in irradiated PBLs 24 hours after irradiation due to lack of a clear population in irradiated samples to gate during flow cytometry. This disappearance of an analysable cell population for γ H2AX quantification corresponds to a marked rise in DNA DSBs between 6 and 24 hours in irradiated cells. At 24 hours there is an increase in Annexin-V staining in irradiated PBLs compared to non-irradiated cells suggesting that irradiated PBLs are undergoing apoptosis. This would explain the rising number of DSBs as DNA is cleaved during the apoptotic process. One would expect this to correspond to an increase in γ H2AX (Rogakou, Nieves-Neira et al. 2000) and it was disappointing not to be able to detect this. In non-fixed cells it was possible to gate a population for PBLs for analysis in the Annexin-V assay, albeit with more difficulty than in non-irradiated cells because of an increased amount of cell debris (Figure 5.11 B) The lack of a similar “gate-able” cell population in samples containing apoptotic cells prepared for γ H2AX quantification and therefore fixed in ethanol may reflect a change in cell morphology induced by the fixation process which is pronounced in apoptotic cells and makes cells indistinguishable from debris on the FSC/SSC dot plot.

If γ H2AX is formed at the sites of DNA DSBs, but the break is actually already repaired by the time the phosphorylation of H2AX has occurred H2AX phosphorylation must have another role in DNA repair other than simply signalling an unrepaired break. γ H2AX may act as a signal marking the site of a repaired DSB that may require “checking” to determine the fidelity of the repair process. γ H2AX is then dephosphorylated once the checking process is over. The process by which γ H2AX is removed from a cell after DNA repair is not completely understood. It does not appear to be due to removal of the whole molecule and is probably by in-situ dephosphorylation (Nazarov, Smirnova et al. 2003). The trigger for dephosphorylation is unknown. A residual γ H2AX focus after irradiation may represent a non checked DSB rather than un-repaired DSB. H2AX null cells and mice can still repair DNA DSBs but are more prone to genomic instability and tumour susceptibility suggesting that H2AX may have a role in maintaining the integrity of the genome and reducing mutations resulting from inaccurate repair of DNA damage (Bassing, Chua et al. 2002; Bassing, Suh et al. 2003).

How might this relate to the ability of the kinetics of γ H2AX loss to predict radiation sensitivity of cell lines and normal tissues as previously reported? The ability of a cell to label a DSB and mark it for checking and the subsequent rate of fidelity checking and then

γ H2AX dephosphorylation may determine how many unchecked DSBs are present in a cell at time of cell division. If a certain number of γ H2AX foci marking non-checked DSBs are present the cell may undergo apoptosis rather than divide and produce potentially genetically damaged progeny. This is very much speculation - the molecular mechanisms of DNA DSB repair are increasingly understood (Kobayashi, Iwabuchi et al. 2008), but detailed information about the pathways and interactions of the molecular components of the process has not yet been elucidated.

In conclusion:

- Using flow cytometry it is possible to measure the kinetics of γ H2AX induction and loss over the 6 hours following in vitro irradiation of isolated human PBLs. The intra- sample precision of one off measurements of γ H2AX levels at 1 hour post irradiation is acceptable.
- It is not possible to measure γ H2AX in human PBLs 24 hours following irradiation due to loss of a discernable cell population for gating at flow cytometry.
- A variety of possible endpoints for a predictive assay of normal tissue radiosensitivity can be derived from the kinetics data. Inter –sample precision is best for γ H2AX at 1 hour (corresponding to peak γ H2AX levels), half-life of γ H2AX loss and the ratio of peak: 6hour γ H2AX levels, but the coefficient of variation is moderately high for all end-points
- The assay produces results in a clinically relevant timescale and has a low failure rate.
- If utilised in a clinical study a strict programme of flow cytometer instrument calibration would be required.
- γ H2AX foci do not appear to equate to DNA DSBs in human PBLs at 0-6 hours following irradiation.

6. Inter-individual comparison of kinetics of γ H2AX induction and loss in irradiated human peripheral blood lymphocytes.

6.1 Introduction

Further information on the reproducibility of assay endpoints can be derived by measuring and comparing intra-individual and inter-individual variation in assay results in a cohort of volunteers. Ideally inter-individual differences in assay results should exceed intra-individual differences suggesting that the assay has sufficient precision and power to discriminate between individuals.

A study of intra-individual and inter-individual variation in the kinetics of γ H2AX induction and loss in irradiated human peripheral blood lymphocytes from healthy volunteers was therefore undertaken.

6.2 Methods: a study of 8 volunteers.

Eight volunteer subjects were recruited from within the department (Table 6.1.). The sex, smoking status and age of each participant was noted as smoking and increasing age have both been reported to affect background levels of DNA damage and repair capacity in peripheral blood lymphocytes and could represent potential confounding factors in assay results (Dhawan, Mathur et al. 2001; Bajpayee, Dhawan et al. 2002; Diem, Ivancsits et al. 2002; Marcon, Andreoli et al. 2003; Fracasso, Doria et al. 2006; Hofer, Karlsson et al. 2006). Peripheral blood samples were taken from each on three separate occasions. All blood samples were taken in morning within the department and were processed immediately. The kinetics of γ H2AX induction and loss were measured for each sample after 2Gy according to the protocols described in Sections 2.2.6-2.2.9.

The mean value and standard deviation for each potential assay end-point was calculated for each individual. One way ANOVA was performed for all potential assay end points to determine if there were any statistically significant inter-individual differences in assay results. Statistical comparison of results from males vs. females and smokers vs. non-smokers were performed using an unpaired t-test, and results from volunteers aged <30, 31-40 and >40 years old were compared by one way ANOVA. Intra- and inter-individual variations in assay results were compared by examining the mean squared values for the

ANOVA table for each assay end-point, and calculating the mean intra-individual and inter-individual coefficient of variation for each assay end-point

Table 6.1 Basic demographics of the eight volunteer blood donors who participated in the study of inter-individual variation in γ H2AX kinetics

Subject	1	2	3	4	5	6	7	8
Age	65	35	32	44	33	28	28	22
Sex	Male	Male	Female	Female	Female	Female	Female	Male
Smoker?	No	No	No	Yes	No	No	No	No

6.3 Results: Individual results for 8 volunteers and intra- and inter-individual variation for each assay end-point.

Table 6.2 Summary of results (mean and standard deviation from 3 repeats) for each assay end-point examined in 8 individuals

A. γ H2AX levels at 30 minutes post 2Gy (normalised fluorescence ratio) (n=3)

Subject	1	2	3	4	5	6	7	8
Mean	7.00	6.52	6.71	6.38	8.07	7.23	8.01	9.31
SD	1.68	0.95	0.18	0.47	1.51	2.10	1.86	1.02

B. γ H2AX levels at 1 hour post 2Gy (normalised fluorescence ratio) (n=3)

Subject	1	2	3	4	5	6	7	8
Mean	9.86	10.26	8.60	9.41	11.53	9.71	11.57	12.00
SD	3.27	1.05	0.58	2.16	4.30	2.72	1.47	3.26

C. γ H2AX levels at 2 hours post 2Gy (normalised fluorescence ratio) (n=3)

Subject	1	2	3	4	5	6	7	8
Mean	9.25	9.70	7.62	10.07	9.88	10.28	10.26	8.96
SD	2.61	1.85	0.18	1.72	2.36	0.96	3.19	2.28

D. γ H2AX levels at 4 hours post 2Gy (normalised fluorescence ratio) (n=3)

Subject	1	2	3	4	5	6	7	8
Mean	5.30	5.21	4.81	5.72	6.37	6.87	5.84	5.69
SD	0.65	0.07	0.57	0.50	1.35	1.89	1.76	1.92

E. γ H2AX levels at 6 hours post 2Gy (normalised fluorescence ratio) (n=3)

Subject	1	2	3	4	5	6	7	8
Mean	3.61	3.21	2.67	2.96	3.64	3.33	3.79	3.35
SD	0.46	0.36	0.88	0.05	0.71	1.24	0.55	0.53

F. AUC of γ H2AX kinetics post 2Gy (normalised fluorescence ratio) (n=3)

Subject	1	2	3	4	5	6	7	8
Mean	33.24	33.4	27.37	34	38.14	37.63	37.80	36.08
SD	7.82	3.93	1.60	4.08	10.81	6.31	7.33	9.97

G. Half-life of γ H2AX loss post 2Gy (hours) (n=3)

Subject	1	2	3	4	5	6	7	8
Mean	2.78	3.25	4.37	3.66	3.20	3.59	3.19	3.55
SD	0.88	0.31	2.25	0.69	0.45	1.14	0.20	0.45

H. Ratio of peak to 6 hour levels of γ H2AX post 2Gy (n=3)

Subject	1	2	3	4	5	6	7	8
Mean	3.54	3.03	2.90	2.87	3.22	2.99	3.31	3.25
SD	0.84	0.23	0.44	0.22	0.30	0.50	0.50	0.26

No statistically significant difference could be detected between male and female volunteers, or smoker vs. non-smokers (unpaired t test $p > 0.05$ for all end-points) or when comparing age below 30 vs. aged 31-40 vs. age > 40 years ($p > 0.05$, one way ANOVA) for any of the end-points examined

Table 6.3 Summary of one-way ANOVA results for each assay end-point.

The intra- and inter-individual variance is taken as within-subject and between subject mean square values from the ANOVA table.

	Assay end-point							
	γ H2AX at fixed time point after irradiation (hours)					AUC	Half-life γ H2AX loss	Ratio peak:6 hour γ H2AX
	0.5	1	2	4	6			
Intra-individual variance	1.67	6.94	4.45	1.65	0.47	50.70	1.02	0.21
Inter-individual variance	3.07	4.38	2.39	1.29	0.70	38.53	0.65	0.16
Is inter- > intra-individual variance?	Yes	No	No	No	Yes	No	No	No
p- value	0.15	0.72	0.79	0.61	0.24	0.63	0.72	0.61

Within-individual variance is greater than between-individual variance for all end-points examined except for time points 30 minutes and 6 hours. For no endpoint is the inter-individual variance statistically different from the intra-individual variance, and no statistically significant difference between individuals could be detected for any assay endpoints ($p > 0.05$ for all).

Table 6.4 Intra- versus inter-individual variation - coefficients of variation

To further illustrate that intra-individual variation exceeded inter-individual variation the mean intra-individual coefficient of variation and the inter-individual coefficient of variation were calculated for each assay endpoint. For all end-points intra-individual exceeded inter-individual variation.

Assay end-point	Intra-individual variation	Inter-individual variation
0.5 hours	17.63%	12.48%
1 hour	22.95%	11.80%
2 hours	20.33%	9.43%
4 hours	21.38%	11.0%
6 hours	21.06%	14.79%
AUC	19.49%	10.32%
Peak:6 hour ratio	22.83%	13.4%
Half life	13.53%	7.26%

6.4 Discussion

When the kinetics of γ H2AX induction and loss were measured on three separate occasions in 8 healthy volunteers no statistically significant differences could be detected between individuals for any of the assay end-points examined. There appeared to be no statistically significant influence of age, sex or smoking status on results although the data sets examined were very small so a possible confounding influence by these factors cannot be entirely excluded.

Using ANOVA intra-individual variance exceeded inter-individual variance for all endpoints except γ H2AX levels at 30minutes and 2 hours post-irradiation but no statistically difference in inter- and intra -individual variance could be detected for any assay end-point. This suggests that between-sample variation in assay results within the same individual whether caused by technical inconsistencies in assay technique or confounding biological factors may mask differences between individuals and raises considerable doubt as to whether the measurement of γ H2AX kinetics using this technique will be sufficiently precise to allow discrimination between individuals of differing normal tissue radiosensitivity.

Of course, the volunteer study reported here sampled blood from individuals of unknown intrinsic radiosensitivity. Given that the estimated proportion of highly radiosensitive individuals in the population is 5% it is unlikely that this small sample of 8 volunteers contained an individual with high normal tissue radiosensitivity. If all the volunteers were of average normal tissue radiosensitivity then perhaps no significant difference between assay results would be expected.

Ismail et al (Ismail, Wadhra et al. 2007) recently reported a study of γ H2AX quantification after 8Gy by flow cytometry in cryopreserved human peripheral blood lymphocytes in 20 volunteer patients and described a two-fold inter-individual difference in the γ H2AX signal at 0.5 hours and a 0.3-fold difference in DNA repair capacity as measured by the ratio of γ H2AX signal at 0.5 hours to that at 5 hours after irradiation. This inter-individual variation is considerably higher than demonstrated here for similar estimates of γ H2AX induction and loss. Ismail et al have not attempted to measure intra-individual variation in assay results. Inspection of the crude data from the γ H2AX kinetics in the 8 volunteers in the current study shows that within an individual the γ H2AX levels at fixed time points after irradiation and peak: 6 hour ratio can vary between 1.5 and 2-fold over time, and without a similar estimate of intra-individual variation it is not possible to be certain that that Ismail et al were detecting a true inter-individual variation in γ H2AX assay results.

The influence of the high intra-individual variability and poor assay precision on the discriminatory ability of γ H2AX kinetics in the identification of individuals of high normal tissue radiosensitivity depends on the magnitude of any difference in assay results that might exist between an individual of high and an individual of “average” normal tissue radiosensitivity. If the difference is very large, the poor assay precision” will be less important and the assay may well possess discriminatory ability. If, however, the difference in assay results is small, then poor assay precision will be of major importance as the high intra-individual variation in results may well reduce or negate any discriminatory power the assay may possess. In a crude estimate of how large the difference in assay results between non-radiosensitive and radiosensitive individuals would need to be for the assay to achieve some discriminatory power, a ROC analysis was performed using hypothetical data. Individual results from the three repeats of γ H2AX kinetics in the 8 volunteers in this study were taken as the results from “non-radiosensitive” controls with the spread of results giving a crude indication of the

variability in assay results or “noise”. Hypothetical values of assay results were generated for a “radiosensitive population” with hypothetical assay results being 5, 10, 25, 50 and 100% greater than those for the “non-radiosensitive” controls. ROC analysis was then performed and the AUC of the ROC curve calculated (Table 6.5). As discussed in Chapter 3, if the AUC of the ROC curve is 0.5 the assay possesses no discriminatory power, whereas an AUC of 1.0 represents an assay with perfect discriminatory ability. As the AUC rises from 0.5 to 1.0 the discriminatory values of the assay improves. As seen in the table the assay results for all potential end-points in radiosensitive individuals would need to be at least 50% greater than that of controls to result in an AUC consistently greater than 0.8. This hypothetical model is very crude and underestimates the negative affect confounding “noise” from intra-individual variation in assay results will have on assay discriminatory power. It also makes the assumption that the volunteers in this study were all individuals of normal intrinsic radiosensitivity which may not be true, and which again may have led to an overestimate of assay discriminatory power in this hypothetical data set. This hypothetical data does suggest however that the true difference between non-radiosensitive and radiosensitive individual will have to be at least 50%, and probably higher to overcome the “noise” generated by high intra-individual variation in assay results. Given the results from other studies of functional cell-based assays reported in Chapter 3 the existence of a consistent difference of this magnitude would seem unlikely.

The only way of truly determining the magnitude of any difference in assay results in individuals of high and average normal tissue radiosensitivity and to properly assess its discriminatory power is to perform a clinical study comparing assay results from individuals previously treated with radiotherapy (and who therefore have known normal tissue sensitivities) and looking for differences in assay results in patients with high versus normal levels of toxicity after apparently identical radiotherapy regimens and correction for other confounders that may influence normal tissue response. ROC analysis of the results should then give a true idea of whether this assay has any clinical utility.

The most efficient way to achieve this goal would be to perform a retrospective case-control study which would require relatively small numbers of patients and could give answers quickly. If ROC analysis yielded a value for AUC close to 0.5 then it would be clear that either there is no relationship between γ H2AX kinetics and normal tissue toxicity or that the poor assay precision was masking such a relationship, and it would then be

inappropriate to spend further time and resources on its investigation as a predictive assay of normal tissue radiosensitivity

Table 6.5 ROC analysis for hypothetical differences in assay results between radiosensitive and non-radiosensitive individuals.

Table shows the AUC of the ROC curve and 95% confidence intervals (in brackets) for the different assay endpoints for different hypothetical percentage differences in assay results between radiosensitive and non-radiosensitive individuals

Assay end-point	Hypothetical % difference in assay results between radiosensitive and non-radiosensitive individuals				
	5%	10%	25%	50%	100%
0.5 hours	0.57 (0.45-0.71)	0.65 (0.52-0.79)	0.79 (0.67-0.90)	0.93 (0.67-0.99)	1.00 (0.99-1.00)
1 hour	0.57 (0.43-0.71)	0.63 (0.49-0.76)	0.76 (0.64-0.88)	0.92 (0.85-0.98)	0.99 (0.97-1.01)
2 hours	0.58 (0.43-0.72)	0.61 (0.47-0.75)	0.76 (0.64-0.88)	0.90 (0.83-0.97)	0.99 (0.98-1.01)
4 hours	0.59 (0.45-0.73)	0.63 (0.50-0.77)	0.76 (0.64-0.88)	0.89 (0.81-0.97)	0.99 (0.96-1.01)
6 hours	0.57 (0.43-0.72)	0.63 (0.49-0.70)	0.75 (0.63-0.88)	0.87 (0.78-0.97)	0.96 (0.90-1.01)
AUC	0.57 (0.43-0.72)	0.65 (0.51-0.78)	0.77 (0.65-0.88)	0.92 (0.85-0.98)	0.99 (0.98-1.01)
Peak:6 hour ratio	0.59 (0.45-0.73)	0.65 (0.51-0.78)	0.79 (0.68-0.90)	0.98 (0.83-1.00)	0.97 (0.93-1.01)
Half-life (h)	0.63 (0.49-0.79)	0.71 (0.58-0.84)	0.90 (0.81-0.98)	0.98 (0.96-1.01)	1.00 (1.00-1.00)

7. Effect of blood sample storage duration and conditions on γ H2AX induction in vitro.

7.1 Rationale for investigation of effects of sample storage on assay results.

The high intra-individual variation of assay results demonstrated in the volunteer study raises concern about the potential clinical utility of the examination of γ H2AX kinetics in PBLs after in vitro irradiation as a predictive assay of normal tissue sensitivity. Before firm conclusions about the clinical utility of assay can be made, the discriminatory ability of the assay must be tested in a population of individuals of known normal tissue radiosensitivity. A study population of known radiosensitivity would consist of patients who had already been treated with radiotherapy and whose normal tissue toxicity had been documented.

All measurements of γ H2AX kinetics up to this point have been performed using fresh blood samples, with PBLs separated, irradiated and fixed immediately following blood sample collection. Realistically immediate processing of blood samples would not be a feasible option when collecting samples from patients. Blood samples would be collected at hospital outpatient clinics when patients were attending for follow-up and would need to be transported to the laboratory which is situated 15 miles from the nearest oncology outpatients department. As samples would not always be taken in the morning they will likely need to be stored overnight prior to cell preparation. For any study comparing results from different samples it is important to assess whether blood sample storage and storage conditions could potentially affect assay results and confound the study outcome. If storage does affect results it is also important to determine if it does so in a predictable fashion which would mean that sample handling could be standardised to minimise differences in assay results between individuals caused by differences in sample handling rather than a difference in normal tissue radiosensitivity.

7.2 Method

As in the studies of assay precision, γ H2AX induction in PBLs at 1 hour after 2Gy was taken as the end-point for experiments examining the effects of sample storage and storage conditions on assay results. Although ideally the kinetics of γ H2AX induction would have been measured for different storage durations and conditions it was not feasible to collect

the large quantity of blood that would have been required for such an experiment from one individual at one time. It would also have required simultaneous handling of an excessively large number of sample tubes during subsequent analysis.

In order to determine if blood sample storage affected assay results γ H2AX induction in PBLs at 1 hour after 2Gy in blood taken from a single individual and stored at room temperature or at 4°C for up to 24 hours. Peripheral blood (13 x8ml CPT tubes) was collected from a single volunteer (Subject 2). PBLs from 1 tube were separated, irradiated and fixed immediately as per the usual protocol outlined in Chapter 2. Six tubes were centrifuged and the resulting monocyte layer re-suspended in plasma by inversion of the tubes. The remaining six tubes were not centrifuged before storage. Three pre-separated tubes and three tubes containing whole blood were refrigerated (4°C) and three pre-separated tubes and three tubes containing whole blood were stored at room temperature (21°C), all with light excluded. At pre-specified time points (2, 6 and 24 hours) PBLs were isolated, irradiated and fixed from one pre-separated and one tube of whole blood from each storage temperature.. All samples once fixed were then stored at -20°C for up to 14 days. All were immunostained and analysed simultaneously. The experiment was repeated 7 times.

The measured radiation-induced γ H2AX level for each storage condition was plotted against storage time. The Area Under the Curve (AUC) for each was calculated. The Kruskal-Wallis test followed by Dunn's multiple comparison tests were performed to determine if storage conditions and duration had a statistically significant effect on the levels of γ H2AX induced 1 hour after 2 Gy. Spearman rank correlation was performed to examine if there was a relationship between duration of storage and assay results for the four different storage conditions.

7.3 Results

For all seven repeated experiments it was not possible to obtain results for the whole blood sample stored at 21°C for 24 hours. Although a normal cell yield was obtained following separation from whole blood, only cell debris was evident at flow cytometry following irradiation, fixation and immunostaining and no consistent lymphocyte population could be gated. Lymphocytes could be consistently gated in the 24 hour samples for whole blood samples stored at 4°C and for the pre-separated samples stored at both temperatures.

Table 7.1 Mean/standard deviation γ H2AX levels 1 hour after 2Gy in PBLs from blood stored between 0 and 24 hours under different conditions before analysis

Also shown are the AUC and Spearman correlation coefficients for γ H2AX levels with increasing storage duration (n=7)

	Whole blood 21°C	Pre- separated 21°C	Whole blood 4°C	Pre- separated 4°C
Fresh sample	12.01/2.32	12.01/2.32	12.01/2.32	12.01/2.32
Stored for 2 hours	14.82/2.44	13.60/2.72	12.22/1.67	12.77/1.61
Stored for 6 hours	16.12/3.99	13.18/2.24	11.49/3.14	12.83/1.68
Stored for 24 hours	Not available	15.93/1.11	9.99/3.53	8.49/2.6
AUC 0-24 hours	Not available	342.2/39.5	267.1/52.45	268.8/22.54
AUC 0-6 hours	85.13/16.74	80.19/12.56	72.46/12.48	75.48/12.92
Correlation (0-24 hours) Spearman r and p-value	Not available	0.8, p=0.33	-0.8, p=0.33	-0.8, p=0.33
Correlation (0-6 hours) Spearman r and p-value	0.5, p=1.0	0.5, p=1.0	-0.5, p=1.0	-0.5, p=1.0

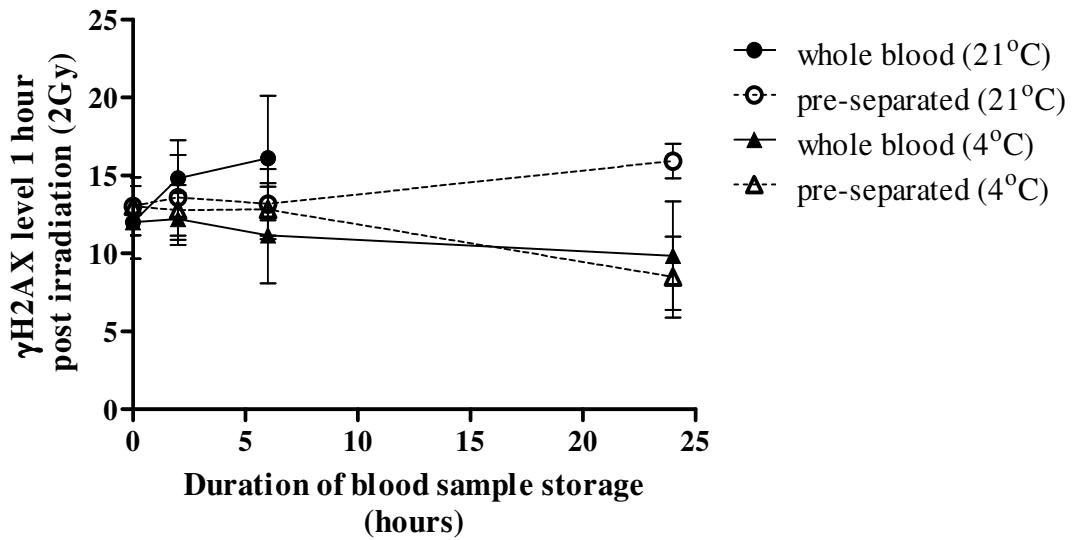


Figure 7.1 Effect of storage temperature and duration on γ H2AX levels in isolated human PBLs 1 hour after 2Gy in whole and pre-separated blood samples

There is a statistically significant difference in γ H2AX levels 1 hour after 2Gy in blood samples stored for different durations under different storage conditions ($p=0.0057$, Kruskal-Wallis test). Dunns multiple comparison tests show that radiation-induced γ H2AX levels are significantly higher in pre-separated blood samples stored at room temperature for 24 hours compared with pre-separated samples refrigerated for the same duration and this is confirmed by Mann-Whitney test on the same data ($p=0.0286$). There were no other significant differences between γ H2AX levels induced following irradiation in samples stored under different conditions. There was no significant correlation between assay results and duration of storage for any of the storage conditions ($p>0.05$ for all) and no significant difference between assay result at any time point for any storage condition and the results obtained from fresh samples. There was, however, a trend for γ H2AX levels 1 hour after irradiation to increase with duration of storage in samples stored at room temperature and to decrease with duration of storage in refrigerated samples.

7.4 Discussion.

Blood sample storage conditions and duration of storage do appear to influence the levels of γ H2AX induced in isolated human peripheral blood lymphocytes after in vitro irradiation.

It is not possible to obtain results for samples stored as whole blood at room temperature for 24 hours due to the absence of a clearly defined lymphocyte population on flow cytometric analysis despite an apparent normal yield of mononuclear cells from the CPT tubes after storage. Storing whole blood at 4°C or separating the mononuclear cells from whole blood by centrifugation and re-suspension in plasma before storage at room temperature seems to protect against this effect. The main effect of centrifugation within the CPT tubes is to separate the monocyte population and plasma from erythrocytes and granulocytes which are trapped below the gel layer within the tubes and prevented from re-mixing with the monocytes layer. The protective effect of separating the granulocytes and erythrocytes before blood storage at room temperature suggest that these cells are exerting an influence on the lymphocyte population during storage that results in their loss during the subsequent irradiation, fixation and staining procedure. The fact that storage of whole blood at 4°C also protects against loss of analysable cells after irradiation suggest that the effect of granulocytes and erythrocytes is a temperature-dependent process possibly enzyme-mediated. It has been reported that lysis of erythrocytes can exert oxidative damage on lymphocytes with lymphocytes analysed by the alkaline comet assay manifesting a 10-fold increase in DNA damage if analysed in the presence of red blood cells compared to isolated lymphocytes (Narayanan, O'Donovan et al. 2001). This DNA damage increases with increased duration of blood storage, is more pronounced if samples stored at room temperature compared to refrigerated samples but does still occur even if whole blood is stored at 4°C.

Radiation-induced γ H2AX levels increased with increased duration of storage up to 6 hours in whole blood samples stored at room temperature suggesting that during storage lymphocytes became more prone to radiation DNA damage or that the enzymes involved in H2AX phosphorylation became more rapidly able phosphorylate H2AX. Cells stored in whole blood at room temperature under oxidative stress with ongoing DNA damage such as that due to lysis of granulocytes or erythrocytes will perhaps have increased induced

levels of ATM/ATR and hence increased ability to form γ H2AX after irradiation. It is possible that with longer duration of oxidative stress these cells will also be more susceptible to apoptosis after irradiation. The lack of cells for analysis in the irradiated lymphocytes stored at room temperature for 24 hours may have been due to rapid cell death after in vitro irradiation in pre-stressed cells. Exploration of this hypothesis would require analysis of apoptotic profiles of irradiated and non-irradiated lymphocytes following different storage conditions and duration.

The apparent decrease in γ H2AX induction in refrigerated samples either stored whole or pre-separated implies that cells stored at low temperatures are either less susceptible to DNA damage or that they are less able to respond by enzymatic phosphorylation of H2AX than cells stored at room temperature. Storage at low temperatures does lead to decreased enzyme activity. Although cells were incubated at 37°C for 30 minutes prior to irradiation it is possible that this time was insufficient to allow full recovery of enzyme activity. An alternative explanation is that the extreme cold may induce stress response genes whose products then act to protect the cell against subsequent radiation-induced DNA damage. Cold-induced proteins such as HSP-70 and HSP-90 have been shown to be upregulated in mammalian cells when returned to 37°C after transient cold shock at 4°C (Cox, Moseley et al. 1993; Liu, Bian et al. 1994). There is some evidence that HSP 70 protects against DNA damage caused by ionising radiation (Calini, Urani et al. 2003; Hunt, Dix et al. 2004).

It does seem clear that if patient blood samples are not to be analysed immediately after blood collection, storage must be standardised for any clinical study as storage conditions do seem to affect γ H2AX levels. Given that storage of whole blood at room temperature for 24 hours yields no results and that there is a possibility that granulocyte and erythrocyte lysis may exert oxidative stress on lymphocytes which may occur even at low storage temperatures it would seem sensible to separate the mononuclear cells before storage by centrifugation of the CPT tubes immediately after blood collection. A major advantage of using the CPT tubes system is that separation can be performed quickly and safely without opening of tubes and exposure of staff to blood biohazard or potential bacterial contamination of blood samples prior to storage.

As storage temperature seems to affect results the samples must then be stored at a standardised temperature prior to analysis. After 24 hours there was a significant

difference between induced γ H2AX in separated samples stored at room temperature or refrigerated but no difference between either and fresh samples. It would therefore be reasonable to store samples at either temperature as long as the temperature was standardised and controlled. Refrigeration of blood samples is probably the easiest way to do this as room temperatures within different hospital departments or laboratories can vary significantly and may even vary overnight depending on hospital heating policy (!).

In conclusion:

- for the purposes of a clinical study requiring blood samples to be taken from patients and stored before preparation and analysis, peripheral blood samples should be immediately centrifuged to separate out the erythrocyte and granulocyte population and then stored at 4°C for up to 24 hours until ready for further preparation and analysis..

8. Final discussion

Despite over a decade of research, mostly involving assays testing the response of a cell to a test dose of radiation in vitro, no predictive assay of normal tissue radiosensitivity has been developed and incorporated into clinical practice.

The majority of studies of functional cell-based assay identified in this systematic review are of poor methodological design, or have been reported with insufficient detail to convince clinicians that possible confounding factors and sources of bias have been sufficiently taken into account and therefore that the conclusion of the study, whether it be positive or negative, is valid. Heterogeneity of assay techniques, the patients recruited, the radiotherapy delivered, assessment of toxicity and statistical analysis makes it difficult to compare studies of different assays or combine the results from studies which are examining the same assay. This heterogeneity may also account for the inconsistency of study results and conclusions. Overall, it is impossible to come to any firm conclusions about the discriminatory ability of functional cell based assays in the prediction of normal tissue radiosensitivity on the current evidence base and the increasingly held perception that they have no potential clinical utility cannot be justified on this evidence alone.

By analysing individual patient data extracted from the published studies and calculation of diagnostic odds ratios (DOR) it is possible to remove at least the heterogeneity of statistical analysis in those studies which report individual patient data. Although for most the DOR suggest that the assays perform poorly, a small number were shown to possess a DOR and 95% CI which suggest possible discriminatory power, at least within the context of the study from which the data was extracted. Perhaps the most convincing DOR was for a study of DNA DSB induction in peripheral blood lymphocytes and the prediction of acute normal tissue toxicity in radiotherapy for head and neck cancer (Wang, Chen et al. 2005). It should be noted, however, that this result has not been validated in subsequent studies.

Nevertheless this observation does justify the attempt to develop another cell-based assay based on the detection of γ H2AX foci and their subsequent loss in irradiated human peripheral blood lymphocytes. Clearly any such assay needed to be developed in a

methodical fashion with sufficient attention to and reporting of the technical parameters, such as reproducibility, important in Level 1 assay development.

The data presented here show that it is possible to measure and follow the kinetics of γ H2AX after a clinically relevant test dose of in vitro radiation in isolated human peripheral blood lymphocytes using immunofluorescent staining and analysis by flow cytometry. Assay failure rate is low and results are produced within a clinically useful time frame. The assay clearly measures a response to radiation but, at least in the first 6 hours after irradiation, it may not simply reflect DNA DSB rejoining as discussed in Section 5.1.10.

The high intra-individual variation in γ H2AX induction and loss exceeds inter-individual variation and immediately raises concerns that the power of the assay to discriminate between individuals of differing normal tissue radiosensitivities may be low. As the individuals studied in Chapter 6 were of unknown radiosensitivity the discriminatory power of the assay must be tested in a population of known radiosensitivities with appropriate attention to confounding factors to be certain that the high intra-individual variation in assay results really does limit its clinical utility. As blood sample storage duration and conditions seem to affect assay results any such study must include standardised sample storage conditions in its protocol and ensure to report this subsequently.

Unless there is a significant difference (at least 1.5-fold) in assay results between individuals of high and normal intrinsic radiosensitivity, it seems likely that the intra-individual variability will mask any differences in assay results between individuals of differing radiosensitivities. This intra-individual variation between samples, whether caused by day to day variations in technical performance, such as subtly varying laboratory conditions, or day to day biological variation within an individual potentially caused by diet, concurrent illness or stress, will be very difficult to reduce. Intra-individual variation is also evident in other studies of cell-based assays which (when documented) report an inter-sample coefficient of variation of 15%. It must be likely that even if future studies of cell-based assays of normal tissue radiosensitivity are designed and conducted with flawless attention to study design and confounding factors that the inherent and difficult to control for biological and technical day-to-day variations may render cell-based

assays insufficiently reproducible and therefore precise to be of any clinical value. Proven lack of reproducibility resulting in low diagnostic accuracy in otherwise well designed studies may ultimately be the justification for the abandonment of functional cell-based assays in the prediction of normal tissue radiosensitivity.

A problem with assay reproducibility notwithstanding, it is unclear as to whether the principle of measuring the response of a surrogate solitary cell type (e.g. a fibroblast or lymphocyte) to a single test dose of radiation to predict tissue response is a valid one. Clinical fractionated radiotherapy schedules involve repeated exposure of normal tissues to radiation on a daily basis often for 4-6 weeks and measuring cellular response to a single test dose of radiation in vitro does not reflect this process of repeated radiation insult.

Although the pathophysiology underlying the normal tissue response to radiation is not completely understood, radiation normal tissue injury is thought to behave like a complex wound with the severity of expression of injury related to damage to a large number of cell types (Denham and Hauer-Jensen 2002). Radiation injury occurs in organised tissues comprising a large number of interactive mutually dependent cell lineages that, along with the extracellular stroma, all contribute individually to the welfare of the tissue as a whole. Whilst ionising radiation can cause the specific cellular responses as tested for in cell-based predictive assays, it has been increasingly recognised that it is the differing but interacting responses of all of the cell types within tissue and modification of extracellular stroma that dictates the severity of normal tissue injury after radiotherapy (Denham and Hauer-Jensen, 2002).

Acute radiation toxicity has classically been attributed to loss of functional cells due to the death or proliferative arrest of stem cell populations (Stone et al, 2003). However, the acute effects of radiation do not appear to depend entirely on the lethal effects of radiation. Radiation causes an immediate increase in tissue blood flow and vascular permeability via indirect radiation effects on mast cells and endothelial cells, resulting in generation of thrombin and release of histamine and prostaglandin I₂ and E₂ and neutrophil adhesion to the endothelial cell surface in the hours following radiation exposure (Potten et al, 1978; Dunn et al, 1986; Panes et al, 1995; Park et al, 2000). Histologically the features are of an acute inflammatory response. As treatment continues, the acute inflammatory response builds due to a combination of direct and indirect cell injury and death, with each fraction

of treatment adding another insult to an already damaged tissue. Microscopically acutely irradiated tissues demonstrate margination of neutrophils and perivascular infiltration (Slauson et al, 1976; Narayan et al, 1982; Reinhold et al, 1990). Radiation endothelial cell apoptosis may cause increased vascular permeability and micro vascular thrombosis (Fajardo and Stewart, 1971; Rubin and Greim, 1998). Leucocytes drawn to the site of injury adhere to the endothelium, migrate into the extracellular matrix and release proteolytic enzymes and reactive oxygen species. Necrosis of other parenchymal and inflammatory cells will exacerbate the acute inflammatory response. Increased levels of cytokines such as TGF- β may delay re-epithelialisation (Denham and Hauer-Jensen, 2002). Loss of barrier function may also predispose to further injury, both physical and microbial which if persistent may lead to consequential late damage. The clinical phenotype of acute radiation injury in normal tissue is therefore due to a combination of cell loss due to radiation cell killing of stem cells, and the acute inflammatory response, which is triggered and sustained by radiation cell killing and vascular changes.

The mechanisms that lead to late normal tissue injury are likely to involve death of functional parenchymal cells as classically described by the radiation target theory. There is increasing evidence, however, that non-lethal damage to endothelial cells and fibroblasts within the irradiated volume play a significant role. In many respects the tissue response to radiation has similarities to the wound healing response to traumatic injury (Denham and Hauer-Jensen 2002). The genetic and molecular events triggered by the initial radiation injury may be sustained for many months if not years with ongoing production of pro-inflammatory cytokines, and chemokines produced by macrophages, epithelial cells and fibroblasts. The cytokines lead to an adaptive response within the tissue and cellular infiltration. There is strong evidence that TGF- β 1 is over expressed in irradiated tissues and this induces fibroblast proliferation – these fibroblasts may be post-mitotic but retain ability to produce collagen and so lead to fibrosis (Rodemann and Bamberg, 1995). Vascular sclerosis is a recognised feature of late radiation injury, and may have a major role in its pathogenesis. The capillary network is particularly vulnerable to injury. Obstruction of the lumen follows swelling of the endothelial cell cytoplasm (Reinhold, 1972; Reinhold and Buisman, 1973) , followed by detachment of proliferating endothelial cells (Hopewell et al, 1986; Kwock et al, 1998), thrombosis, rupture of the capillary wall and loss of entire capillary segments (Fajardo, 1997). Arterioles show subendothelial and

adventitial fibrosis (Sams, 1965). In addition to apoptosis, radiation is thought to induce long-term phenotypic changes in endothelium up regulating expression of cell adhesion molecules, chemokines and cytokines, which can then promote further thrombosis, recruitment of inflammatory cells and fibrosis (Law, 1981). Hypoxia may result from progressive endothelial and vascular damage and ischaemia – this hypoxia may result in the continuous overproduction of ROS which can lead to further molecular injury, effectively causing a vicious circle of ongoing injury for months if not years (Vujaskovic et al, 2001). Hypoxia itself may promote inflammation and activate the pro-fibrotic cytokine TGF β . Late normal tissue damage may become clinically apparent when a critical threshold of normal tissue dysfunction is reached after a latent period during which tissue injury is ongoing. Alternatively, further injury to the irradiated tissue, perhaps in the form of surgery may trigger another cascade of inflammation and cytokine production resulting in sudden exacerbation of normal tissue damage and its clinical manifestation.

In theory there could be inter-individual variation in any of the molecular, cellular and tissue responses to ionisation radiation that contribute towards acute and late normal tissue toxicity and it seems unlikely that a single cellular response in a single cell type will in isolation predict normal tissue radiosensitivity.

Assays of gene expression profiling go some way to measuring the complex nature of cellular radiation responses, aiming to recognise differences in patterns of radiation-induced gene expression in cells derived from radiosensitive and non-radiosensitive individuals. Their advantage is that the expression pattern of thousands of genes can be measured simultaneously without even having to know the function of the genes being examined, which is useful given that the molecular pathophysiology of radiation toxicity is not completely understood. There is increasing evidence that gene expression profiling is reproducible within and between laboratories (Canales, Luo et al. 2006; Patterson, Lobenhofer et al. 2006; Shippey, Fulmer-Smentek et al. 2006; Tong, Lucas et al. 2006; Chen, Hsueh et al. 2007; Fuscoe, Tong et al. 2007). However, these gene expression profiling assays are still susceptible to the criticism that they are analysing radiation response in a solitary surrogate cell type and that this cellular response may not reflect the tissue response (Begg 2006).

The move to radiogenomics avoids the problems associated with lack of assay reproducibility due to biological variation and the issues of surrogate tissue analysis since the genetic code being analysed should be the same in all cell types and not susceptible to the influences of biological or environmental conditions. By detecting single nucleotide polymorphisms in candidate genes likely to be involved in the tissue response to radiation it is hoped to develop a genetic profile associated with increased intrinsic normal tissue radiosensitivity (Andreassen, Alsner et al. 2002). This profile can then be measured in any easily accessible cell type including peripheral blood lymphocytes and with the advent of high throughput technology results should be available within a clinically useful time frame. One of the current limitations of this technique is choosing the appropriate genes for analysis given the lack of understanding of the molecular, cellular and tissue response to radiation. Gene expression studies may help guide researchers to appropriate targets.

The radiogenomics approach has had limited success so far with some studies reporting an association between genetic polymorphisms and radiosensitivity (Andreassen, Alsner et al. 2003; Andreassen, Alsner et al. 2005; Cesaretti, Stock et al. 2005; Chang-Claude, Popanda et al. 2005; De Ruyck, Van Eijkeren et al. 2005; Alsner, Andreassen et al. 2008). Hypothesis generating studies have not been validated in independent subjects so far (Andreassen, Alsner et al. 2006).

The establishment of tissue banks with associated detailed information regarding tumour type, radiotherapy details, standardised and accurate normal tissue toxicity reporting and recording of patient and therapy related confounding factors means that studies based on genotyping assays can proceed quickly now with positive or negative results being likely to convince clinicians of assay utility due to improved study design assuming these confounding factors are dealt with appropriately.

The development of predictive assays of normal tissue radiosensitivity illustrates the need for translational research in radiotherapy and radiobiology and illustrates how interested parties (clinicians, biologists and statisticians) must cooperate to generate useful results quickly and efficiently. The purpose of the research strategy is to produce a diagnostic test which can influence clinical decision making and patient management.

The clinician must specify the maximum degree of invasiveness of tissue sampling before a test becomes unacceptable, e.g. an assay requiring a brain biopsy be unlikely to be taken

up in routine clinical practice unless samples could be taken at the time of surgical tumour resection. The clinician must also specify the maximum acceptable period between sampling and results before the assay becomes of no clinical use. The collection of robust and reliable data regarding radiotherapy dosimetry, patient characteristics, toxicity, and potential confounders is incumbent on the clinician. The clinician must also define the potential use of the assay result in the clinical decision making process, as this will influence the assay performance requirements – if the results are being used to screen out radiosensitive individuals for treatment with a lower radiation dose or alternative non-radiotherapy treatment without making any change to the treatment for the remainder, the assay specificity may be more important than sensitivity to avoid erroneous screening out of an individual of normal radiosensitivity and perhaps subjecting them to radical surgery when an organ-preservation approach may have been feasible. On the other hand if individuals identified as non-radiosensitive are to be treated with dose-escalated radiotherapy then sensitivity may be more important than specificity in order to ensure that all sensitive individuals have been identified and screened out of dose escalation, which for them would potentially be extremely toxic if not life-endangering.

The biologist's pivotal role is to determine which biological parameters are likely to be useful predictors of normal tissue radiosensitivity and to determine how to measure these parameters reliably and reproducibly. The drive to develop a predictive assay will hopefully generate more knowledge about the biological processes underlying the normal tissue radiation response.

As is considered routine in the development of clinical randomised controlled trials a statistician must be involved in developing study design including power calculations for adequate sample size and analysis of results to maximise the potential to generate useful and convincing results and therefore maximise research efficiency.

This cooperation and thorough attention to study design is vital – the consequence to an individual patient of changing therapy based on the result of a predictive assay can be profound if the assay does not perform well. Therefore clinicians and ethics committees will require robust and convincing evidence of the diagnostic performance of a predictive assay before patients will be recruited into any studies of assay-directed therapy and this evidence will only be generated by well conducted adequately powered research studies.

Equally, if an assay really has no potential clinical utility it is important to provide convincing evidence of this quickly to prevent on-going and wasted devotion of time and financial resource.

As the technical and physical individualisation of radiotherapy treatment reaches its maximum capabilities with the advent of intensity-modulated and image-guided radiotherapy, the pursuit of biological individualisation of treatment to try to further improve the therapeutic index of radiotherapy is clearly a worthwhile goal. The current literature on cell-based predictive assays of normal tissue radiosensitivity is of insufficient quality to make any firm conclusions about their potential utility but the inherent susceptibility to biological and environmental influences, as evident in the γ H2AX assay, is likely to limit their usefulness. Whilst the establishment of tissue bank data-bases such as those associated with the GENEPI project (West, McKay et al. 2005) will hopefully mean that progress in the field of radiogenomics and the prediction of normal tissue radiosensitivity will be made, the advent of assay-directed therapy and attainment of levels 5 and 6 on Fryback and Thornbury's hierarchy with improved patient outcomes and societal gains in population tumour control rates and reduced rates of toxicity seems some way off.

8.1 Future work

As discussed earlier final conclusions about the potential utility of measuring γ H2AX induction and loss in peripheral blood lymphocytes can only be made after testing for a difference in assay results between individuals of known high normal tissue radiosensitivity and normal or low radiosensitivity.

The ROSES study, which recruited patients from radiotherapy centres in Dundee and Edinburgh between 2000-2002 (Wells, Macmillan et al. 2004) examined the role of skin care in the management of acute skin toxicity during and following radiotherapy for breast, head and neck and some anal cancers. During this study skin toxicity data was collected prospectively using reflectance spectrography and RTOG/EORTC scoring. Radiotherapy details were recorded as were details regarding BMI, smoking, blood pressure, haemoglobin, breast size, and chemotherapy, and concurrent medications.

It should be possible to perform a case-control study examining the role of γ H2AX in the prediction of acute skin toxicity by identifying living patients who experienced severe acute skin toxicity (RTOG/EORTC grade 3 or 4) and matching then to one or ideally two individuals who experienced RTOG 0-2 toxicity. Matching criteria would be for tumour site, stage, radiotherapy schedule, chemotherapy, smoking status, haemoglobin, skin care regimen, BMI and (for breast radiotherapy) breast size. Sufficient time has now elapsed for late toxicity to have developed. Collection of late toxicity data then matching of cases with severe late toxicity with controls without toxicity using similar matching criteria as for acute toxicity as well as for duration of follow up may help determine if γ H2AX induction and kinetics in PBLs can predict late skin/subcutaneous toxicity. Clearly this study would be susceptible to the potential bias introduced by the attrition of patients from the original ROSES study due to progression of their disease or other factors, but may give sufficient information to either confirm the lack of utility of the γ H2AX assay or justify the establishment of a larger prospective study if any signs of discriminatory power were evident.

As a clinician, my most important contribution to the process of predictive assay development is likely to be the prospective collection of high quality information regarding radiotherapy, confounding therapies and patient related factors and high quality acute and late toxicity data from patients receiving treatment under my care, which then has the potential to be used in future studies of predictive assays, along with the establishment and maintenance of useful working relationships with biology colleagues as part of a translational research team to help guide assay development from the perspective of the clinician who might one day use it in day to day practice.

9. References

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10. Appendices

Appendix 1 : Materials

1.1 Laboratory equipment

Centrifuge Heraeus Labofuge 400R (Heraeus, Thermo Scientific)

CO₂ cell incubator (Heraeus, Thermo Scientific)

Coulter counter - Z2™ COULTER COUNTER® Cell and Particle Counter (Beckman-Coulter, Fullerton, USA)

Cytocentrifuge - Shandon Cytospin 2 (Thermo Inc, USA)

Dynal Magnetic Particle Concentrator (MPC) MPC –L (Dynal, Oslo, Norway).

Flow cytometer - Becton Dickinson FACScan Analytic Flow Cytometer (Becton, Dickinson and Company, Franklin Lakes, USA)

Gamma irradiator (IBL437C Cis Bio International, High Wycombe, UK).

Gel documentation and analysis system (SynGene, Synoptics, Cambridge, UK)

Gel mould (Bio-Rad Laboratories Ltd, Hemel Hemstead, UK)

General glass wares (Schott)

Horizontal Electrophoresis apparatus - Bio-Rad Sub-Cell 96 (Bio-Rad Laboratories Ltd, Hemel Hemstead, UK)

Humidified chamber (made in-house)

Laminar Flow Hood Class 2 - Herasafe HSP 12 (Thermo Kendro)

Motorized Pipette Controller – Pipetboy (IBS Integra)

One-channel Air-Displacement Pipette, various sizes (Pipetman, Gilson, Wisconsin, USA)

Power Pack (Bio-Rad)

UV Spectrophotometer

Weigh machine (Sartorius)

Zeiss Axioplan fluorescence microscope (Carl Zeiss Ltd)

1.2 Chemicals and enzymes

EDTA (0.5M for molecular biology, Sigma-Aldrich)

Ethanol (BDH Laboratory Supplies, UK)

Ethidium Bromide, 1% solution (Sigma)

Low melting point agarose (Sigma-Aldrich)
MOWIOL-DAPI (1 microgram/ml) (prepared in-house by biochemistry department)
Paraformaldehyde (Sigma-Aldrich)
Proteinase K (Sigma-Aldrich)
Sodium N-lauryl sarcosine (Sigma-Aldrich)
Triton X-100 (Sigma-Aldrich)
Ultrapure agarose (Life Technology LTD, Paisley, UK)
Vectashield (Vector Laboratories, Burlingame, USA)

1.3 Kits

CD4 positive isolation kit (Dynal Biotech, Oslo, Norway)
CD8 positive isolation kit (Dynal Biotech, Oslo, Norway)
CaliBrite two-colour kit (containing 3 individual vials of polymethylmethacrylate microspheres - unlabelled, FITC labelled and PE-labelled) (BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, USA)
Annexin V-FITC apoptosis detection kit (BD Pharmingen, BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, USA)

1.4 Consumables

BD Vacutainer 5ml draw lithium heparin blood collection tubes, BD (Becton, Dickinson and Company, Franklin Lakes, USA)
BD Vacutainer 8ml draw sodium citrate CPT tubes, BD (Becton, Dickinson and Company, Franklin Lakes, USA)
BD Vacutainer one-use holders, BD (Becton, Dickinson and Company, Franklin Lakes, USA)
BD Vacutainer one use sterile needles, BD (Becton, Dickinson and Company, Franklin Lakes, USA)
1.5 ml safe lock tubes (Eppendorf)
Cell culture flasks, filter capped, 80cm² (Nunc)
Disposable cytofunnels with filter cards (Shandon, Thermo Inc, USA)
Disposable pipette tips, various sizes (VWR)
Disposable sterile pipettes, 1ml, 5ml and 10ml, (Sterilin)
Glass slides and coverslips
Laboratory film (Parafilm)

Sterile polypropylene 15 and 50 ml tubes (Falcon), (BD Bioscience, Becton, Dickinson and Company, Franklin Lakes, USA)

5ml polystyrene tubes for flow cytometry sample injection (BD Bioscience, Becton, Dickinson and Company, Franklin Lakes, USA)

1.5 Cells and antibodies

Muntjac fibroblast cell line (kindly supplied by Dr Peter Bryant)

Anti-phosphohistone H2AX (Ser 139) clone JBW301 mouse monoclonal IgG1, (Upstate, Lake Placid, USA)

Fluorescein (FITC)-conjugated Affinipure Donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories Inc, Westgrove, PA, USA))

FITC-labelled Mouse anti-human CD3 IgG (BD Bioscience, Becton, Dickinson and Company, Franklin Lakes, USA)

PE-labelled Mouse anti-human CD19 IgG (BD Bioscience, Becton, Dickinson and Company, Franklin Lakes, USA)

Becton Dickinson Simultest™ LeucoGATE™ (CD45/CD14) (BD Bioscience, Becton, Dickinson and Company, Franklin Lakes, USA)

FITC-labelled Mouse IgG₁ Isotype Control (BD Bioscience, Becton, Dickinson and Company, Franklin Lakes, USA)

1.6 Solutions, sera, buffers and media

Minimal Essential Medium (For Muntjac cell culture):

Minimal Essential Medium x1 (Gibco, UK)

+ 0.3g/litre L-glutamine (Gibco, UK)

+ 10% foetal calf serum (Gibco, UK)

RPMI 1640 (for re-suspension of peripheral blood lymphocytes after isolation from whole blood):

RPMI 1640 Media x1 without L-glutamine (Gibco, UK)

+ 0.3g/litre L-glutamine (Gibco, UK)

+ 1% foetal calf serum (Gibco, UK)

Sera:

Foetal calf serum (Gibco, UK)

Donkey serum (Sigma, St Louis, USA)

Flow cytometry sheath fluid:

FACSFlow, BD (Becton, Dickinson and Company, Franklin Lakes, USA)

Phosphate Buffered Saline:

Phosphate Buffered Saline 10x w/o Mg^{2+} , Ca^{2+} (Gibco, UK) diluted to 1x in sterile deionised H_2O , pH 7.4

50x TAE buffer:

242 g Tris base

57.1 ml Glacial acetic acid

100 ml 0.5 M EDTA pH 8.0

In 1 litre deionised H_2O

Fricke stock solution:

0.9804 g ammonium ferrous sulphate ($Fe(NH_4)_2(SO_4)_2$)

0.1460g sodium chloride

3.1668g caesium sulphate (Cs_2SO_4)

6.93ml concentrated sulphuric acid (H_2SO_4) "Superpure"

in 250ml deionised H_2O

1.7 Software

Laboratory systems

Cellquest Version 3, (Becton, Dickinson and Company, Franklin Lakes, USA)

Cytovision System Version 3(Applied Imaging, Sunderland, Tyne & Wear, UK)

FACSComp (Becton, Dickinson and Company, Franklin Lakes, USA)

Syngene Genetools software (Syngene, Synoptics, Cambridge, UK)

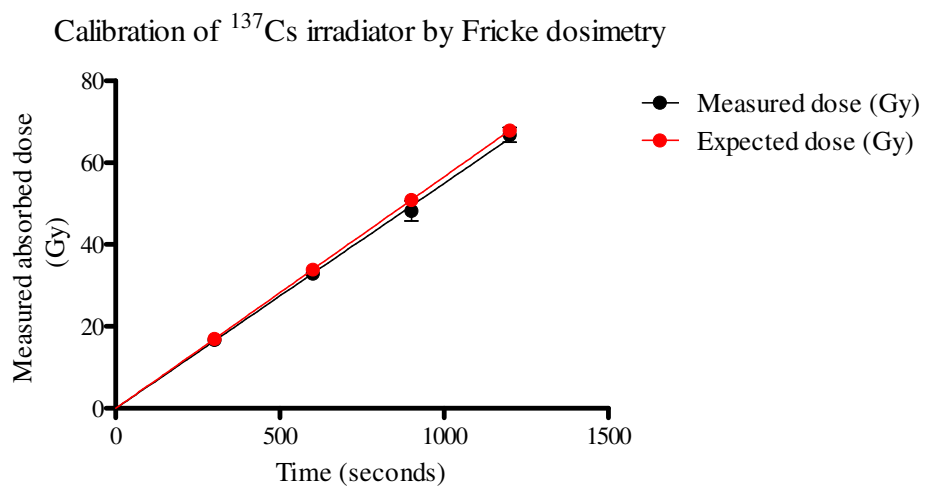
Statistical analysis:

GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com

Stats Direct Version 2.6.6. (www.statsdirect.com)

Appendix 2 : Gamma Irradiator Dosimetry.

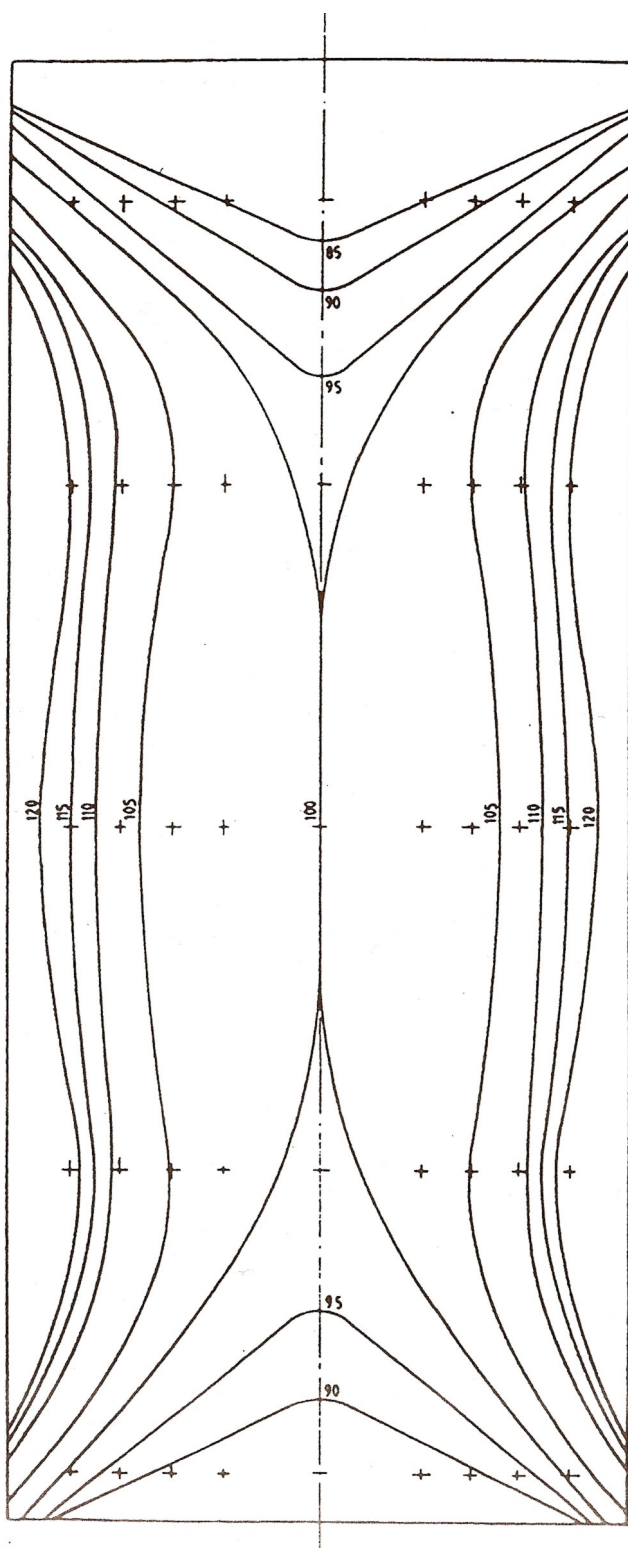
2.1 Results from Fricke dosimetry of gamma irradiator.



Time (seconds)	Measured dose (Gy)			
	Y1	Y2	Y3	Y4
300	16.512370	17.091750	17.671140	15.643300
600	33.604120	32.445360	33.024740	32.445360
900	48.957730	47.219590	45.481440	51.275260
1200	66.049480	68.367010	68.077320	64.601030

Measured dose rate = 3.297Gy per minute on 25/1/2005

2.2 Theoretical isodose distribution within the irradiation chamber (taken from manufacturer's information – Cis Bio International)

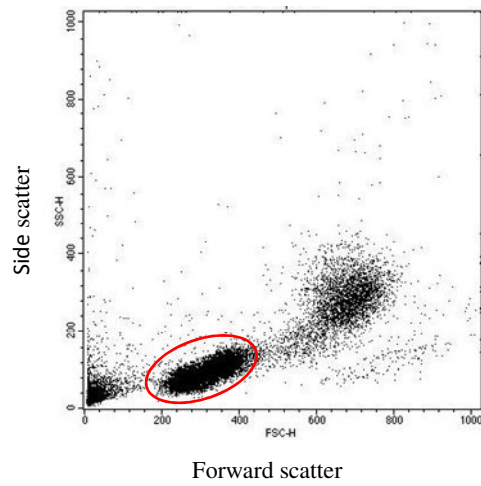


Appendix 3 : Immunophenotyping of cell population under investigation

Immunophenotyping of fixed mononuclear cell preparations was performed as described in Section 2.2.8.

Analysis as shown below confirmed that the gated population that was analysed in the γ H2AX quantification experiments was CD45 positive i.e. made up of leucocytes, and CD14 negative (i.e. did not contain monocytes) (see Figure C below). Given that centrifugation in CPT tubes had removed the granulocyte population it is reasonable to assume that this non-monocyte population consists of lymphocytes. From Figure D it can be seen that the majority of the gated cells were CD3 positive (i.e. were T-lymphocytes), and a clear population of CD19 positive cells (B-lymphocytes) was also present. Some of the gated cells did not stain for either CD3 or CD19 despite all staining for CD45 and not for CD14. This may be because of decreased antibody/receptor affinity in fixed samples (the antibodies to cell surface antigens for leucocyte immunophenotyping are recommended for use in fresh unfixed blood samples for this reason), or because of the presence of a population of non-B and non-T lymphocytes or other white cells with the same FSC and SSC characteristics as lymphocytes. Natural killer cells are lymphocytes which lack the characteristic B and T cell surface antigens – and may make up some of this non-stained population.

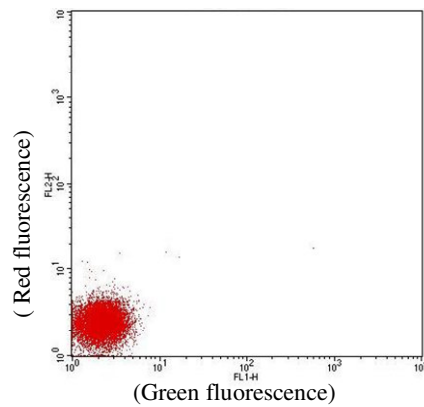
Analysis of the sample stained with the FITC-conjugated mouse antihuman isotype control showed a minor degree of non-specific binding only.



A.

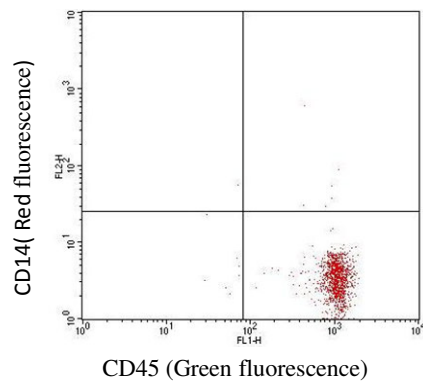
Forward and side scatter characteristics of isolated monocytes after fixation and immunostaining

The red oval surrounds the gated population taken forward for analysis.



B.

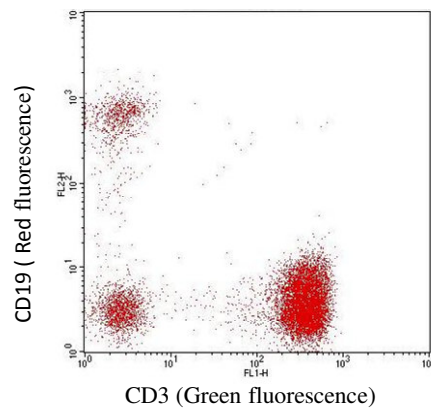
Green vs red fluorescence of gated population of non-stained sample confirming no background red or green fluorescence



C.

Gated population stained with FITC-conjugated anti CD45 and PE-conjugated anti CD14.

Cells within the gated region are CD45+ve confirming that they are leucocytes, and CD14 -ve, confirming that they are not monocytes



D.

Gated population stained with FITC-conjugated anti CD3 and PE-conjugated anti CD19.

Cells within the gated region are CD3+ve (bottom right quadrant) confirming that they are T-lymphocytes (and representing the majority), and CD19 +ve, confirming that they are B-lymphocytes (top left quadrant). A number of cells remain unstained (bottom left). These must represent either B or T lymphocytes to which antibody has not bound or a separate white cell population without CD3 or CD19 antigens, possibly natural killer cells.

Appendix 4 : Papers and protocols for Systematic review.

4.1 List of identified studies fulfilling entry criteria for systematic review of the literature on functional cell-based predictive assays of normal tissue radiosensitivity

- Alsbeih, G., S. Malone, et al. (2000). "Correlation between normal tissue complications and in vitro radiosensitivity of skin fibroblasts derived from radiotherapy patients treated for variety of tumors." Int J Radiat Oncol Biol Phys **46**(1): 143-52.
- Barber, J. B., W. Burrill, et al. (2000). "Relationship between in vitro chromosomal radiosensitivity of peripheral blood lymphocytes and the expression of normal tissue damage following radiotherapy for breast cancer." Radiother Oncol **55**(2): 179-86.
- Barber, J. B., C. M. West, et al. (2000). "Detection of individual differences in radiation-induced apoptosis of peripheral blood lymphocytes in normal individuals, ataxia telangiectasia homozygotes and heterozygotes, and breast cancer patients after radiotherapy." Radiat Res **153**(5 Pt 1): 570-8.
- Begg, A. C., N. S. Russell, et al. (1993). "Lack of correlation of human fibroblast radiosensitivity in vitro with early skin reactions in patients undergoing radiotherapy." Int J Radiat Biol **64**(4): 393-405.
- Borgmann, K., B. Roper, et al. (2002). "Indicators of late normal tissue response after radiotherapy for head and neck cancer: fibroblasts, lymphocytes, genetics, DNA repair, and chromosome aberrations." Radiother Oncol **64**(2): 141-52.
- Brock, W. A., S. L. Tucker, et al. (1995). "Fibroblast radiosensitivity versus acute and late normal skin responses in patients treated for breast cancer." Int J Radiat Oncol Biol Phys **32**(5): 1371-9.
- Burnet, N. G., J. Nyman, et al. (1994). "The relationship between cellular radiation sensitivity and tissue response may provide the basis for individualising radiotherapy schedules." Radiother Oncol **33**(3): 228-38.
- Crompton, N. E., R. Miralbell, et al. (1999). "Altered apoptotic profiles in irradiated patients with increased toxicity." Int J Radiat Oncol Biol Phys **45**(3): 707-14.
- Crompton, N. E., Y. Q. Shi, et al. (2001). "Sources of variation in patient response to radiation treatment." Int J Radiat Oncol Biol Phys **49**(2): 547-54.
- De Ruyck, K., M. Van Eijkeren, et al. (2005). "Radiation-induced damage to normal tissues after radiotherapy in patients treated for gynecologic tumors: association with single nucleotide polymorphisms in XRCC1, XRCC3, and OGG1 genes and in vitro chromosomal radiosensitivity in lymphocytes." Int J Radiat Oncol Biol Phys **62**(4): 1140-9.

- Dickson, J., B. Magee, et al. (2002). "Relationship between residual radiation-induced DNA double-strand breaks in cultured fibroblasts and late radiation reactions: a comparison of training and validation cohorts of breast cancer patients." *62*(3): 321.
- Dikomey, E., I. Brammer, et al. (2000). "Relationship between DNA double-strand breaks, cell killing, and fibrosis studied in confluent skin fibroblasts derived from breast cancer patients." *Int J Radiat Oncol Biol Phys* **46**(2): 481-90.
- Djuzenova, C., B. Muhl, et al. (2004). "Normal expression of DNA repair proteins, hMre11, Rad50 and Rad51 but protracted formation of Rad50 containing foci in X-irradiated skin fibroblasts from radiosensitive cancer patients." *Br J Cancer* **90**(12): 2356-63.
- El-Awady, R. A., M. Mahmoud, et al. (2005). "No correlation between radiosensitivity or double-strand break repair capacity of normal fibroblasts and acute normal tissue reaction after radiotherapy of breast cancer patients." *Int J Radiat Biol* **81**(7): 501-8.
- Geara, F. B., L. J. Peters, et al. (1993). "Prospective comparison of in vitro normal cell radiosensitivity and normal tissue reactions in radiotherapy patients." *Int J Radiat Oncol Biol Phys* **27**(5): 1173-9.
- Herskind, C., S. M. Bentzen, et al. (1998). "Differentiation state of skin fibroblast cultures versus risk of subcutaneous fibrosis after radiotherapy." *Radiother Oncol* **47**(3): 263-9.
- Hoeller, U., K. Borgmann, et al. (2003). "Individual radiosensitivity measured with lymphocytes may be used to predict the risk of fibrosis after radiotherapy for breast cancer." *Radiother Oncol* **69**(2): 137-44.
- Hummerich, J., G. Werle-Schneider, et al. (2006). "Constitutive mRNA expression of DNA repair-related genes as a biomarker for clinical radio-resistance: A pilot study in prostate cancer patients receiving radiotherapy." *Int J Radiat Biol* **82**(8): 593-604.
- Johansen, J., S. M. Bentzen, et al. (1994). "Evidence for a positive correlation between in vitro radiosensitivity of normal human skin fibroblasts and the occurrence of subcutaneous fibrosis after radiotherapy." *Int J Radiat Biol* **66**(4): 407-12.
- Johansen, J., S. M. Bentzen, et al. (1996). "Relationship between the in vitro radiosensitivity of skin fibroblasts and the expression of subcutaneous fibrosis, telangiectasia, and skin erythema after radiotherapy." **40**(2): 101.
- Kearsley, J. H., Z. M. Fang, et al. (1998). "Clinical radiohypersensitivity screening using radiation-induced chromosomal aberrations." *Australas Radiol* **42**(3): 219-21.
- Kiltie, A. E., J. B. Barber, et al. (1999). "Lack of correlation between residual radiation-induced DNA damage, in keratinocytes assayed directly from skin, and late radiotherapy reactions in breast cancer patients." *Int J Radiat Oncol Biol Phys* **43**(3): 481-7.

- Kiltie, A. E., A. J. Ryan, et al. (1999). "A correlation between residual radiation-induced DNA double-strand breaks in cultured fibroblasts and late radiotherapy reactions in breast cancer patients." Radiother Oncol **51**(1): 55-65.
- Kizilian-Martel, N., R. C. Wilkins, et al. (2003). "Prediction of radiosensitivity by measurement of X-ray induced apoptosis in human blood using the comet assay." Anticancer Res **23**(5A): 3847-54.
- Lavin, M. F., I. Bennett, et al. (1994). "Identification of a potentially radiosensitive subgroup among patients with breast cancer." J Natl Cancer Inst **86**(21): 1627-34.
- Lee, T. K., R. R. Allison, et al. (2003). "Lymphocyte radiosensitivity correlated with pelvic radiotherapy morbidity." Int J Radiat Oncol Biol Phys **57**(1): 222-9.
- Leong, T., M. Chao, et al. (2003). "Radiation-hypersensitive cancer patients do not manifest protein expression abnormalities in components of the nonhomologous end-joining (NHEJ) pathway." Br J Cancer **88**(8): 1251-5.
- Loeffler, J. S., J. R. Harris, et al. (1990). "In vitro radiosensitivity of human diploid fibroblasts derived from women with unusually sensitive clinical responses to definitive radiation therapy for breast cancer." Radiat Res **121**(2): 227-31.
- Lopez, E., R. Guerrero, et al. (2005). "Early and late skin reactions to radiotherapy for breast cancer and their correlation with radiation-induced DNA damage in lymphocytes." Breast Cancer Res **7**(5): R690-8.
- Mariano Ruiz de Almodovar, J., D. Guirado, et al. (2002). "Individualization of radiotherapy in breast cancer patients: possible usefulness of a DNA damage assay to measure normal cell radiosensitivity." Radiother Oncol **62**(3): 327-33.
- Nachtrab, U., U. Oppitz, et al. (1998). "Radiation-induced micronucleus formation in human skin fibroblasts of patients showing severe and normal tissue damage after radiotherapy." Int J Radiat Biol **73**(3): 279-87.
- Neubauer, S., J. Dunst, et al. (1997). "The impact of complex chromosomal rearrangements on the detection of radiosensitivity in cancer patients." Radiother Oncol **43**(2): 189-95.
- Oppitz, U., K. Baier, et al. (2001). "The in vitro colony assay: a predictor of clinical outcome." Int J Radiat Biol **77**(1): 105-10.
- Oppitz, U., S. Denzinger, et al. (1999). "Radiation-induced comet-formation in human skin fibroblasts from radiotherapy patients with different normal tissue reactions." Strahlenther Onkol **175**(7): 341-6.
- Oppitz, U., S. Schulte, et al. (2002). "In vitro radiosensitivity measured in lymphocytes and fibroblasts by colony formation and comet assay: comparison with clinical acute reactions to radiotherapy in breast cancer patients." Int J Radiat Biol **78**(7): 611-6.

- Ozsahin, M., N. E. Crompton, et al. (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.
- Peacock, J., A. Ashton, et al. (2000). "Cellular radiosensitivity and complication risk after curative radiotherapy." Radiother Oncol **55**(2): 173-8.
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4.2 DATA EXTRACTION PROTOCOL FOR SYSTEMATIC REVIEW

Reference:

Predictive Assay under investigation:

Stated study objectives:

MATERIALS AND METHODS SECTION

Patients

Source

Inclusion/exclusion criteria:

Disease sites TNM or AJC stage:

Radiotherapy details – site, dose, fractionation, energy, treatment centre/s, field sizes and planning techniques

Toxicity scoring details:

System used e.g. RTOG/EORTC:

Endpoint assessed: early, late:

Median time from radiotherapy to toxicity scoring.

Who is the person recording toxicity? Have they been trained?

Scorer blinded to assay result? - if so, how was this achieved?

Inter-scorer variability assessed? – if so how was this done?

Specimen characteristics:

Biological material used

Methods of sampling/ transport/storage. Is this the same for both cases and controls?

Assay methods:

Is a detailed protocol or reference for protocol included?

Brief summary of technique

How long is the interval between taking the sample and obtaining a reliable result?

For the investigating laboratory are:

quality control procedures reported?

reproducibility assessments reported?

quantification methods reported?

scoring and reporting protocols reported? (give details)

Has technique been reported before by other groups?

Have effects of blood/tissue sampling conditions and sample storage been assessed?

For subjective assays – has inter-scorer variability been assessed and reported?

Are assays performed blinded to patient outcome? If so- how was this achieved?

Study design:

Prospective or retrospective

Method of case selection – e.g. definition of case vs. control

Case matching/stratification employed?

Period over which cases taken/recruited

Sample size

Rationale for sample size (including power calculations)

Hypothesis generating set/ test set (or other method for avoiding recursive reasoning)

Statistical analysis:

Specify all statistical methods used

Is appropriate statistical analysis used?

Is a statistician involved?

Has ROC analysis been performed?

Is sensitivity/specificity/PPV reported?

RESULTS SECTION

Data Reporting

How many patients are included at each stage of analysis and what are the reasons for drop out (e.g. failure to get assay result, patients died before late toxicity could be assessed)

Basic demographic characteristics reported for cases and controls?

Potential confounding factors reported for cases and controls?

e.g. concurrent chemotherapy, gaps in treatment, bolus, comorbidity, smoking

Are cases and controls well matched for basic demographic characteristics and potential confounding factors?

How are the assay results reported? (e.g. table, histogram etc)

Is individual patient data reported?

Are the number of missing values reported?

Analysis

Is there a difference in assay result between individuals with “normal radiation toxicity and individuals with “increased” radiation toxicity?

What is the size of the difference?

Is the difference statistically significant?

Give p values and 95% CI

Does the analysis account for all potential confounding factors?

Has ROC analysis been performed? If so what is the AUC?

Have optimum cut off values been determined?

4.3 SCORING SYSTEM FOR QUALITY OF PREDICTIVE ASSAY STUDY DESIGN AND REPORTING

Radiotherapy homogeneity

15: Radiotherapy delivered to the same single site in all patients.

Patients treated prospectively with dose, fractionation, beam energy, field size, boost, bolus, planning technique, concurrent or subsequent chemotherapy listed for all and clearly homogenous OR case-control study with matching of cases and controls for the above features

5: Radiotherapy delivered to the same single site in all patients.

No matching or listing of features to confirm but homogeneity likely

0: Multiple sites treated, or same site treated with multiple different radiotherapy schedules with no subsequent adjustment in analysis or no description of radiotherapy or chemotherapy

Patient homogeneity

15: Patients recruited prospectively into study fulfilling clear entry and exclusion criteria to reduce presence of confounders with all remaining potential confounders (e.g. smoking status) listed and accounted for in subsequent analysis OR matched case control study with careful matching of confounding factors in cases and controls

5: Apparent homogeneity of patient population but insufficient documentation of demographics, inclusion/exclusion criteria to be certain

0: Clear heterogeneity of patient population or no description on any patient demographics or potential confounders

Toxicity Scoring

10: Toxicity measured as a single end-point in a single tissue with matching or subsequent adjustment for follow up duration for late toxicity

5: Toxicity measured as multiple end points in a single tissue

0: Toxicity measured in multiple tissues, no adjustment for follow up time between cases and controls or no definition of “case” or “control” with respect to toxicity

Inter-scorer reproducibility assessment

3: only one scorer for toxicity, or if more than one inter-scorer reproducibility conformed

0: no reporting of scorer number or reproducibility

Assay intra-sample reproducibility assessment

5: Assay technique optimised and intra-sample reproducibility assessments performed by same group before study commenced

2: Inter-sample reproducibility assessed during the clinical study

Assay inter-sample reproducibility assessment

5: Assay technique optimised and inter-sample reproducibility assessments performed by same group before study commenced

2: Intra-sample reproducibility assessed during the clinical study

Sample handling

5: clear description of sample handling prior to analysis, whether it is stored, whether storage affects results and if handling is the same for all specimens

0: no description of sample handling

Blinding

2: Scorer of toxicity blinded to results of assay and scorer of assay blinded to results of toxicity

1: one of the above criteria fulfilled

0: No report of scorer blinding

Treatment for possible confounding factors

10: possible confounding factors accounted for in study design or analysis

0: no attempt to account for confounding factors

Sample size

10: >50

5: >20

1 : < 20

Statistical analysis

20: construction of ROC curve, selection of cut offs and calculation of sensitivity and specificity

10: appropriate analysis e.g. correlation, comparison of means, Kaplan-Meier with adequate reporting of results

0: no reporting of statistical methods or results

Appendix 5 : DNA DSB induction by ionising radiation in isolated human peripheral blood lymphocytes

Human peripheral blood lymphocytes were isolated from fresh peripheral blood samples, irradiated with increasing doses of ionising radiation (^{137}Cs gamma source) and then analysed for the presence of DNA double strand breaks as per the method described in Section 2.2.11. The number of DNA DSBs was calculated as the Fraction of DNA Released (FDR) from each well with the subtraction of results from non-irradiated control samples– the mean of results and standard deviation from 3 independent experiments using PBLs from the same volunteer (subject 6) are plotted against radiation dose and is shown below. This confirms a linear dose response in DNA DSB induction between 0 and 30Gy (r^2 0.9752, linear regression analysis). Examples of the images of the gel after electrophoresis used for image analysis are also shown)

