In Vitro Chromosomal Radiosensitivity and Cell Cycle Progression in Cancer Survivors

By

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A thesis submitted in partial fulfilment for the requirements for the degree of MSc (by Research) at the University of Central Lancashire in collaboration with Westlakes Research Institute.

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Student Declaration

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I declare that while registered as a candidate for the research degree, I have not been a registered candidate or enrolled student for another award of the University or other academic or professional institution.

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This project formed a subsection of the blood studies carried out as part of the genetic consequences of cancer treatment study www.gcct.org.

Dr Gillian Curwen completed 50% of the chromatid aberration scoring in line with the Westlakes Research Institute procedure for the G_2 chromosomal radiosensitivity assay and I scored the other 50%. I completed all the scoring for the cell cycle delay section.

Signature of Candidate

Type of Award: MSc (by Research)

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ABSTRACT

The *in vitro* G_2 chromosomal radiosensitivity assay is a technique used to investigate variation in the cellular response to radiation. In brief, lymphocytes are irradiated in the G_2 phase of the cell cycle to induce DNA damage, which is exhibited at the subsequent metaphase as chromatid gaps and breaks. Radiation-induced arrest at the end of G_2 is believed to allow time for adequate DNA repair before the onset of mitosis. Therefore, variation in the level of aberrations observed at metaphase is likely to be driven in part by G_2 checkpoint control. This led to an investigation into whether variation in *in vitro* G_2 chromosomal radiosensitivity is related to G_2 checkpoint efficacy.

A modified version of the G_2 chromosomal radiosensitivity assay was validated with samples from staff at Westlakes Research Institute. The standard G_2 assay protocol was altered by the addition of the chemical calyculin A which induces Premature Chromosome Condensation (PCC) in interphase cells enabling visualisation and classification of all cell cycle stages (G_1, S, G_2 and metaphase). Initial attempts at assessing G_2 to metaphase transition by visualising and scoring damage directly in G_2 cells failed. However, by measuring changes in the ratio of PCC-G_2 and metaphase cells before and after irradiation, it was possible to measure G_2 checkpoint delay. Following validation of the PCC technique, both the G_2 assay and the modified assay were applied to a group of 29 cancer survivors and the extent of any individual G_2 checkpoint delay was compared to the radiation-induced chromatid aberration frequency.

No significant relationship between chromatid aberration frequency and G_2 checkpoint delay was observed. Providing that the PCC technique is accurately assessing G_2 delay,

the results suggest that variation in G_2 chromosomal radiosensitivity is more likely to be driven by variation in DNA repair pathways than variation in G_2 checkpoint delay.

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ABBREVIATIONS

AT Ataxia Telangiectasia
ATM Ataxia Telangiectasia Mutated gene
BRCA1 Breast cancer 1 gene
BRCA2 Breast cancer 2 gene
BrDU 5-bromo 2'-deoxyuridine
BS Bloom's syndrome
CDK Cyclin Dependent Kinase
CPR Central Population Register
CV Coefficient of Variation
DSBs Double-Strand Breaks
FA Fanconi's anemia
FACS Fluorescence-activated cell-sorting
G_0 Gap 0
G_1 Gap 1
G_2 Gap 2
ICRP International Commission on Radiological Protection
IKAROS Interactive KARy-Otyping System
KCl Potassium Chloride
MI Mitotic Index
MIn Mitotic Inhibition
NBS Nijmegan breakage syndrome
NCI National Cancer Institute
PCC Premature Chromosome Condensation
PCD Premature Centromere Division
PICR Paterson Institute for Cancer Research
S Synthesis
WRI Westlakes Research Institute

CHAPTER 1

INTRODUCTION

Scope of Study

In vitro assays have demonstrated that cells from cancer prone human genetic syndromes and, indeed, cancer itself exhibit elevated sensitivity to the DNA-damaging agent radiation. One such assay is the *in vitro* chromosomal radiosensitivity technique, in which the amount of radiation-induced chromosome damage observed in metaphase cells is used as a measure of radiosensitivity. In addition to cellular sensitivity, exposure to ionising radiation is known to cause delay in the cell replication cycle. Such checkpoint delay is thought to allow time for genome repair before the onset of replication or mitosis i.e. at G/S borders and G_2/M transition, respectively. Therefore, variation in the level of chromosome damage observed at metaphase is likely to be driven in part by checkpoint control (Scott *et al* 2003; Terzoudi and Pantelias 1997; Terzoudi *et al* 2005; Zampetti-Bosseler and Scott 1981).

This thesis describes the application of a technique called Premature Chromosome Condensation (PCC), which can be used to directly enumerate cell cycle perturbation following radiation exposure, in conjunction with the established *in vitro* chromosomal radiosensitivity assay (Scott *et al*, 1996; Smart *et al*, 2003). The hypothesis tested by this work was that an increase in delay before the onset of mitosis (G_2/M checkpoint) is directly correlated with a visible reduction of chromosome damage in metaphase. The work herein discusses the initial attempts at using the PCC technique in the Westlakes Research Institute (WRI) laboratory and then goes on to describe the application of this methodology to a Danish population of 30 survivors of childhood and young adulthood cancer.

The results failed to provide evidence that checkpoint delay is associated with chromosomal radiosensitivity, at least in this particular cancer survivor cohort. The thesis concludes with a discussion of the possible reasons for these findings, limitations of the assays employed, the importance of intra-individual variation, further work which may be useful and the influence of age, gender and cancer type.

1 INTRODUCTION

1.1 CHROMOSOMAL RADIOSENSITIVITY

1.1.1 Human Genetic Syndromes

A number of human genetic disorders with diverse clinical outcomes have been identified that predispose the individual to a high risk of developing cancer and which exhibit chromosomal instability e.g. Ataxia telangiectasia (AT), Bloom's syndrome (BS) and Fanconi's anemia (FA). Collectively, they have been termed chromosome breakage syndromes (Carney 1999; Futaki and Liu 2001).

AT is an autosomal recessive disorder estimated to occur in approximately 1 in 100,000 live births in the USA (Swift *et al* 1986) and 1 in 300,000 in Great Britain (Woods *et al* 1990). Clinical manifestations of this childhood disease include progressive immunodeficiency, neurological degeneration (ataxia) and dilated blood vessels (telangiectasia) in the corners of the eyes or on the surface of the ears and cheeks (reviewed by Chun and Gatti 2004). Approximately, 25% of those with AT develop cancer, most frequently acute lymphocytic leukaemia or lymphoma; this high cancer predisposition may be linked to a decreased capacity to repair DNA damage. Radiosensitivity in AT was first described in two young individuals treated for cancer by means of radiotherapy (Gotoff *et al* 1967; Morgan *et al* 1968). Two boys aged 9 and 10 years suffered severe adverse reactions to radiation treatment, including dermatitis,

necrosis, dysphagia, and progressive respiratory collapse. The unexpected tissue responses ultimately led to death within four and eight months, respectively. This abnormal sensitivity to radiation leading to enhanced cell killing was confirmed *in vitro* by exposing AT fibroblast cells lines to γ-radiation (Taylor *et al* 1975). Further studies demonstrated an enhanced sensitivity to X-ray irradiation which manifests itself as increased chromosomal damage compared to controls (Bender *et al* 1985; Nagasawa *et al* 1985; Natarajan and Meyers 1979; Taylor 1978). An enhanced sensitivity to radiation, using the endpoint of chromosomal aberrations, has also been observed in BS (Aurias *et al* 1985; Kuhn 1980; Parshad *et al* 1983) and FA (Bigelow *et al* 1979; Higurashi and Conen 1973; Parshad *et al* 1983). However, the results of many investigations into the chromosomal radiosensitivity of chromosome breakage syndromes were inconclusive and difficult to reproduce with only AT patients consistently demonstrating radiosensitivity outside of any control population (reviewed by Murnane and Kapp 1993).

1.1.2 The Cell Cycle Based G_2 Chromosomal Radiosensitivity Assay

In vitro cellular radiosensitivity of cultured cells can be determined using a variety of assays which test for endpoints such as cell death, mutagenicity, cell cycle perturbation, chromosome damage, and DNA damage/repair. The cell cycle based *in vitro* G_2 chromosomal radiosensitivity assay has been one of the most commonly used protocols for the last 30 years and has provided good discrimination in radiation response between individuals. The cell cycle consists of four distinct phases termed gap 1 (G_1), synthesis (S), gap 2 (G_2) and mitosis. In G_1, a high level of protein synthesis occurs and the chromosomes are prepared for S phase, in which duplication of cellular DNA occurs. Following successful DNA replication a short G_2 phase of 4 - 5 hours exists to allow preparation for mitosis, in which cells divide.

The *in vitro* G_2 chromosomal radiosensitivity assay can be performed on any dividing cell population, i.e. either cell lines or on stimulated blood lymphocytes. In brief, the assay involves irradiating PHA-stimulated peripheral blood lymphocytes or fibroblast cell lines *in vitro* to induce DNA damage. A short time for normal repair processes is allowed, before the extent of unrepaired damage, in the form of chromatid gaps and breaks, is measured at metaphase (Figure 1.1). Thus, only cells that were in the G_2 phase at the time of irradiation are sampled by this protocol. The earliest applications of the assay were used to demonstrate that AT cells are abnormally radiosensitive in the G_2 phase of the cell cycle (Rary *et al* 1974). In the late 1970's this cytogenetic assay was further developed and utilised in a number of studies at the National Cancer Institute (NCI), Bethesda, USA by Katherine Sanford and colleagues. Many early studies sampled skin fibroblasts, but difficulties such as bacterial contamination and long pre-culture growth times (Sanford *et al* 1989), led to the G_2 assay being adapted for lymphocytes obtained from a peripheral blood sample (Sanford *et al* 1990). Between 1983 and 1997 the NCI group demonstrated elevated G_2 chromosomal radiosensitivity in a large number of cancer-prone syndromes including FA, familial polyposis coli and BS (Parshad *et al* 1983); chronic ulcerative colitis (Sanford *et al* 1997b); Down's syndrome (Sanford *et al* 1993); familial dysplastic naevus syndrome (Sanford *et al* 1997a); Gardner's syndrome (Parshad *et al* 1983; Takai *et al* 1990); xeroderma pigmentosum (Parshad *et al* 1983; Price *et al* 1991), Li-Fraumeni syndrome (Parshad *et al* 1993) and AT homozygotes (Sanford *et al* 1990).

Many studies have attempted to discriminate between AT heterozygotes, AT patients and normal controls using radiation-induced chromatid aberrations as their endpoint (Bender *et al* 1985; Parshad *et al* 1985; Sanford *et al* 1990; Shiloh *et al* 1986; Shiloh *et*

al 1989; Tchirkov *et al* 1997). These studies produced conflicting results and the present consensus is that radiosensitivity, as measured by induced chromatid aberrations, is an unsuitable endpoint for carrier detection due to considerable overlap between AT heterozygotes and the normal populations. Despite the findings of some groups, the International Commission on Radiological Protection (ICRP 1998) advise that the only cancer-prone syndromes with definitive elevated G_2 radiosensitivity are AT homozygotes and Nijmegan breakage syndrome (NBS) (Weemaes *et al* 1981), which was originally thought to be a variant of AT.

David Scott and colleagues at the Paterson Institute for Cancer Research (PICR) in Manchester applied the NCI assay to control and cancer-prone individuals in an attempt to confirm the clear discrimination previously found at the NCI between the two groups (Scott *et al* 1996). A comparison of control donors at the NCI and PICR uncovered more inter-experiment variability in the PICR control group coupled to clear differences in aberration yields, kinetics of aberration decline and mitotic inhibition. The experimental variability demonstrated by the PICR group when applying the NCI assay was eventually resolved. Scott and colleagues (1996) were able to demonstrate that a centrifugation step prior to irradiation was slowing the progression of some cells into metaphase and the harvesting of cells at 37°C was allowing chromosomal repair throughout the harvesting procedure. By omitting the centrifugation step and harvesting cells at 0°C to stop repair, experimental variability was reduced. Even with these changes, PICR researchers were unable to repeat the results of the NCI group in being able to discriminate between cancer predisposed groups and controls, with complete discrimination only found between controls and AT homozygotes (Scott *et al* 1996). Having established the assay, the PICR laboratory began large-scale investigations into radiosensitivity and predisposition to common cancers.
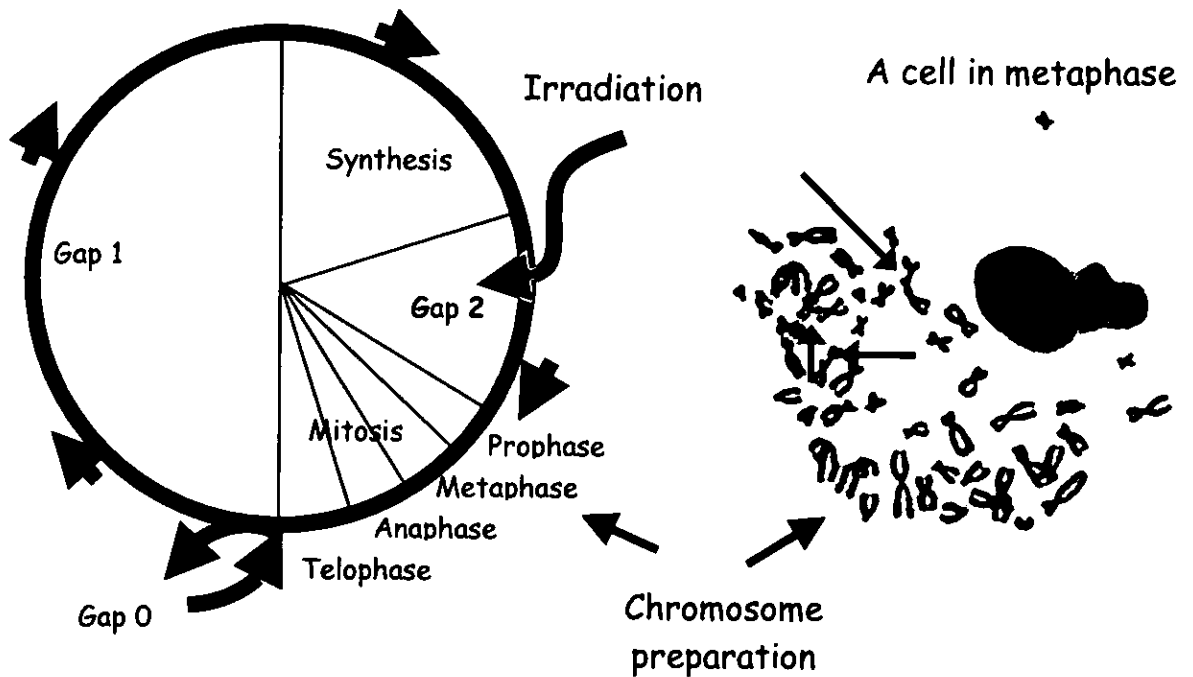
Figure 1.1: The G_2 chromosomal radiosensitivity assay. The cell cycle consists of four distinct phases termed gap 1 (G_1), synthesis (S), gap 2 (G_2) and mitosis. Mitosis is subdivided into prophase, metaphase, anaphase and telephase. Cells not undergoing the four stages of mitosis may also be referred to as interphase cells. Gap 0 cells are quiescent, hence not taking part in the cell cycle. PHA-stimulated peripheral blood lymphocytes are irradiated and after a short interval of 1.5h, to allow G_2 cells to progress to mitosis, the cells are harvested. Chromatid aberrations, indicated here by blue arrows, are viewed in metaphase after cell harvesting and slide preparation. The numbers of chromatid aberrations in 50 or 100 cells are totalled to produce a "G_2" radiosensitivity score.

1.1.3 G_2 Chromosomal Radiosensitivity and Cancer

Although many early G_2 chromosomal radiosensitivity studies concentrated on cancer-prone families, there was a clear interest to research cancer predisposition in conjunction with G_2 chromosomal radiosensitivity in sporadic cancer patients (without a strong family history) with the aim of uncovering genetic markers and evaluating predictive tests. Significantly elevated radiosensitivity has been reported in cells from patients with a diverse range of cancers although results have conflicted between laboratories. A list of the studies undertaken to date is provided in Table 1.1.

To investigate whether individuals with sporadic breast cancer exhibit enhanced G_2 chromosomal radiosensitivity, the G_2 assay was applied to a population of sporadic breast cancer patients in two studies at the PICR (Scott *et al* 1994a; Scott *et al* 1999). A comparison of G_2 scores between a control population of 105 donors and 135 breast cancer patients revealed that approximately 40% (53/135) of breast cancer patients exhibit an elevated chromosomal radiosensitivity compared to 6% of control individuals (Scott *et al* 1999) (Figure 1.2). To discriminate between a sensitive and normal response Scott and colleagues utilised a cut-off value at the 90th percentile in the control distribution and applied this value to the breast cancer patients. Although, this 90th percentile value was, to some extent, arbitrary, it resulted in good discrimination between populations and has since been adopted in the majority of G_2 chromosomal radiosensitivity studies. Earlier studies using fibroblasts utilised a variety of techniques and often sampled only small numbers of individuals. The work of Scott and colleagues was significant in that it was the largest study of its type at the time and the G_2 assay had been standardised for use with peripheral blood lymphocytes to give more reproducible results.

Table 1.1 G_2 chromosomal radiosensitivity in cells from cancer patients.

Cancer Type	Normal sensitivity[1]	Elevated sensitivity[2]
Breast	Docherty *et al* 2007	Baria *et al* 2001; Baeyens *et al* 2002; Howe *et al* 2005b; Parshad *et al* 1996; Patel *et al* 1997; Riches *et al* 2001; Scott *et al* 1994a; Scott *et al* 1999; Terzoudi *et al* 2000
Brain		Terzoudi *et al* 2000
Bladder		Terzoudi *et al* 2000
Head and Neck	Papworth *et al* 2001 (age of diagnosis ≥ 45)	Papworth *et al* 2001 (age of diagnosis ≤ 45) De Ruyck *et al* 2008; Terzoudi *et al* 2000
Colorectal		Baria *et al* 2001; Darroudi *et al* 1995
Cervical	Baria *et al* 2001	Terzoudi *et al* 2000
Lung	Baria *et al* 2001	Terzoudi *et al* 2000
Prostate		Howe *et al* 2005a
Paediatric and Adolescent (treated ≤ 20 years). Includes Hodgkin's disease, non-Hodgkin's lymphoma, osteosarcoma, Wilms' tumour, Rhabdomyosarcoma.	Curwen *et al* 2005[3]	Baria *et al* 2002; Curwen *et al* 2005[3]
Retinoblastoma	Darroudi *et al* 1995	Sanford *et al* 1996
Skin		Terzoudi *et al* 2000
Leukaemia		Terzoudi *et al* 2000
Lymphoma		Darroudi *et al* 1995
Wilms' tumour		Darroudi *et al* 1995

[1, 2] Normal and elevated sensitivity designated on the basis of standards defined within individual studies.
[3] Dependent on control group used as comparison.
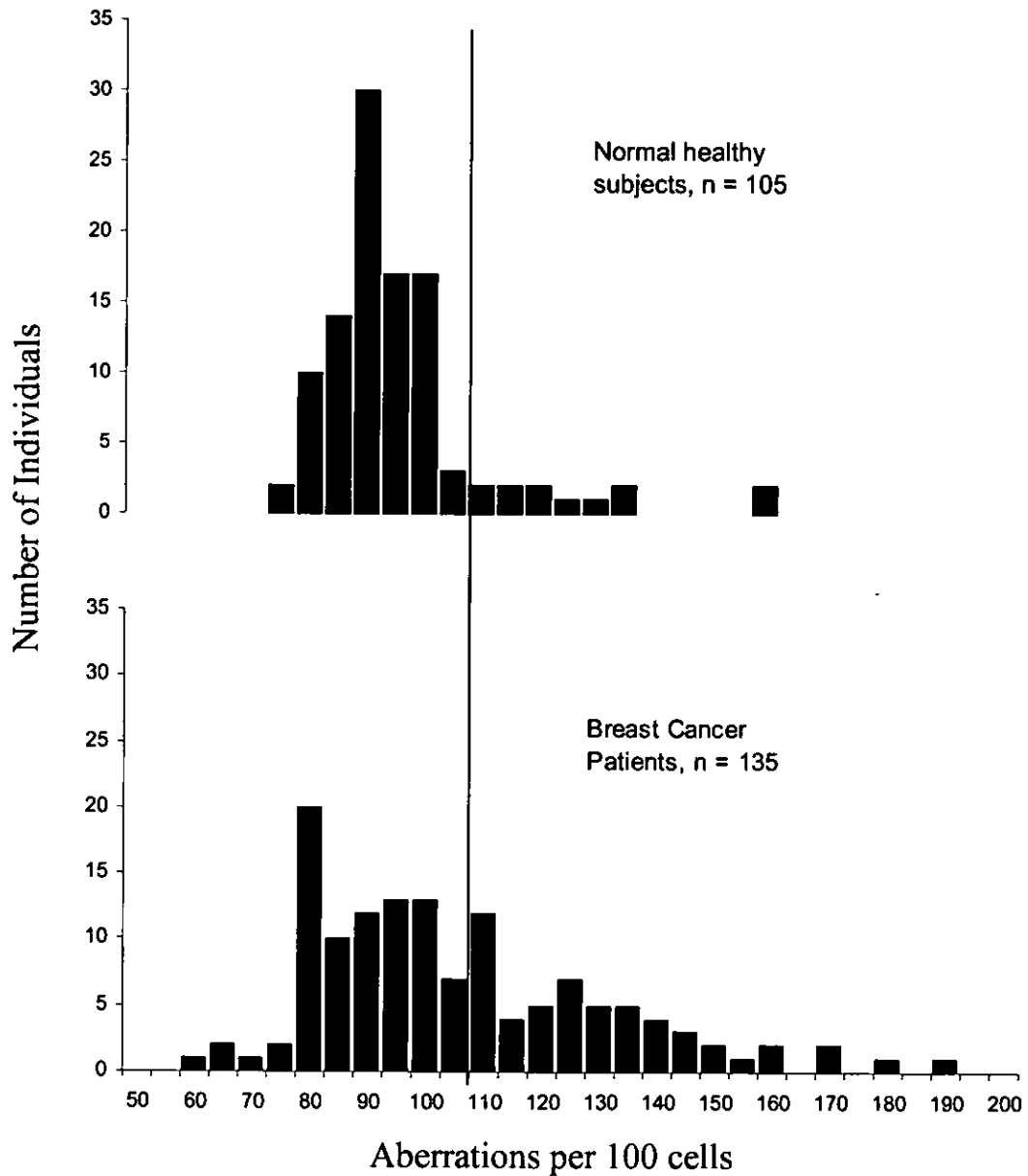
Figure 1.2: G_2 chromosomal radiosensitivity of a group of normal donors (top) and a group of breast cancer patients (bottom). The solid line is at the 90th percentile value of the control group and indicates the cut-off point between sensitive and non-sensitive individuals. Adapted from Scott *et al* (1999) and Scott (2004).

Epidemiological data suggest that 4 – 13% of breast cancer patients could be carriers of the mutated AT gene (Easton, 1994) and this may contribute to the enhanced sensitivity seen in a population profile. However, the enhanced radiosensitivity observed in over 40% of breast cancer patients could not be attributed to the small percentage of AT heterozygotes within the sporadic breast cancer population studied. For this reason, it was postulated that genetic predisposition to breast cancer may be the result of mutations in genes of a low penetrance involved in the processing of DNA damage, and is not confined to those with a strong family history such as carriers of the *ATM* gene and individuals with *BRCA1/ BRCA2* mutations (Scott *et al* 1999; Scott *et al* 2000; Scott 2004). As further evidence, the University of Ghent (Belgium) laboratory failed to demonstrate a role for either *BRCA1* or *BRCA2* (heterozygous carriers) in conferring G_2 chromosomal radiosensitivity (Baeyens *et al* 2004). This suggests that the contribution of *BRCA1/2* towards sporadic breast cancer is perhaps minimal, although a more recent report showed that healthy *BRCA1* carriers had significantly more radiation-induced chromatid aberrations compared to controls matched for age, sex and ethnicity (Barwell *et al* 2007). Epidemiological evidence supporting the hypothesis of Scott includes studies of cancer incidence in twins (Lichtenstein *et al* 2000; Peto and Mack 2000) which indicate that breast cancer, in the majority of cases, arises in genetically predisposed females and cannot be accounted for by relatively rare mutations in *BRCA1* or *BRCA2*. This finding further supports the concept that other low penetrance genes, as yet unidentified, confer an enhanced radiosensitivity. Candidates for low penetrance cancer-predisposition genes include *CHEK2* (Meijers-Heijboer *et al* 2002) and polymorphisms in microsatellites associated with DNA repair genes such as *XRCC1*, *XRCC2* and *XRCC3* (Price *et al* 1997).

Since the breast cancer study of Scott was published in 1994, a number of independent studies have reported significantly elevated G_2 chromosomal radiosensitivity in breast

cancer (Baeyens *et al* 2002; Baria *et al* 2001; Howe *et al* 2005b; Parshad *et al* 1996; Patel *et al* 1997; Riches *et al* 2001; Terzoudi *et al* 2000). However, a more recent study of 211 newly diagnosed breast cancer patients in conjunction with 170 age, sex and ethnically matched controls revealed no significant difference in levels of chromatid breaks between patients and controls (Docherty *et al* 2007). The fact that this study failed to replicate the findings of David Scott's group, as well as other groups, was surprising but may be explained in part by the choice of assay employed. Docherty *et al* (2007) modified the method of Howell and Taylor (1992) which is routinely used at Guy's Hospital to aid the diagnosis of radiosensitivity in patients with phenotypic features of AT and NBS. The Howell and Taylor technique has some differences to the method developed by Scott and colleagues. For example, cell harvesting was carried out at room temperature which may facilitate further rejoining of chromatid gaps and there were minor differences in scoring criteria.

Encouraged by the promising findings of the breast cancer studies, a number of studies investigated whether chromosomal radiosensitivity was associated with other cancer types (Baria *et al* 2001; Baria *et al* 2002; Curwen *et al* 2005; De Ruyck *et al* 2008; Howe *et al* 2005a; Papworth *et al* 2001; Terzoudi *et al* 2000). A large-scale study compared G_2 chromosomal radiosensitivity in 25 normal individuals with a group of 185 cancer patients containing a variety of malignancies including breast, cervix, prostate, larynx, lung, brain, bladder, skin and leukaemia (Terzoudi *et al* 2000). For all cancer types, the mean radiation-induced chromatid aberration yields were higher than in the normal individuals and the average sensitivity of the cancer patients, taken as a whole, was significantly greater than the control group ($P = 0.001$). An examination by the PICR group into colorectal cancer, lung cancer and cancer of the cervix as well as in chronic disease (diabetes mellitus and non-malignant lung disease) revealed that 30%

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(12/37) of colorectal cancer patients exhibited an enhanced sensitivity, which was statistically significant ($P = 0.01$), when compared to the control population (Baria *et al* 2001). Unlike the study of Terzoudi *et al* (2000), elevated G_2 chromosomal radiosensitivity was not found in lung and cervical cancer. Again adopting the 90th percentile cut-off, the proportion of radiosensitive cases in lung cancer was only 23% (8/35) and in cancer of the cervix 11% (3/27) of patients were sensitive, values that were not significantly different. Both lung cancer and cancer of the cervix have a well established environmental aetiology with lung cancer strongly linked to tobacco smoking and cervical cancer linked to infection with human papilloma virus. The lack of a significant elevated radiosensitivity in these malignancies could be explained by the strong environmental aetiology and a far weaker inherited component in comparison with breast cancer. The existence of a genetic predisposition to cancer which is not linked to the repair of radiation induced damage, for example carcinogen metabolism, would not be detectable by the G_2 assay and may provide an alternative explanation. There is some epidemiological evidence of an inherited component in colorectal cancer (Cannon-Albright *et al* 1988; Lichtenstein *et al* 2000) and an elevated chromosomal radiosensitivity of 30%, may well be a marker of low penetrance genes. Another important finding was that patients with chronic disease (diabetes mellitus and non-malignant lung disease) did not exhibit an enhanced radiosensitivity with only 12% sensitive compared to 9% in normals (Baria *et al* 2001). This indicates that elevated radiosensitivity may not be conferred by a diseased state itself.

Continuing their work on cancer patients, the PICR group applied the G_2 assay to a cohort of patients with head and neck cancer (Papworth *et al* 2001). Using the 90th percentile cut-off, 31% (13/42) of patients were sensitive compared to 15% of normals but this was not statistically significant. However, when the patients were divided into

early onset cases (age of diagnosis ≤ 45) and normal onset (age of diagnosis ≥ 45) the difference between the normal and the early-onset group was statistically significant with enhanced radiosensitivity in the early-onset group. The authors suggest that for early-onset cases there is a genetic predisposition which is not present in older patients. A more recent study revealed that 26% of head and neck cancer patients (age range 33 - 91) were significantly radiosensitive compared with only 9% of healthy controls (De Ruyck *et al* 2008). The results of Papworth *et al* (2001), were corroborated by the finding that head and neck cancer patients aged ≤50 years had the highest mean G_2 scores with a mean aberration frequency of 1.32 breaks per cell compared to 1.18 breaks per cell in patients aged >70 (De Ruyck *et al* 2008). Environmental risk factors such as smoking and alcohol consumption are thought to predominate in older patients. Early-onset cases represent less than 5% of all head and neck cancers (Carniol and Fried 1982; Decroix and Ghossein 1981; Son and Kapp 1985), so taken as a whole these studies suggest that head and neck cancer has a smaller genetic component in terms of predisposition, than breast cancer.

1.1.4 Early-Onset Cancer

The early onset of malignancy is thought to be a common feature in cancers that have a high inherited risk. Elevated radiosensitivity has been demonstrated in a mixed group of paediatric cancer patients when compared to age-matched controls (Baria *et al* 2002). When 32 early-onset cases, diagnosed before the age of 20 (age range 0.5 - 19), were compared to 41 young controls (age range 0.25 - 19) and 32 adult normals (age range 20 - 60) the authors found that 44% of patients were sensitive compared to 15% in young controls and 10% in adult controls. The results of this study hinted that a proportion of early-onset cancers may be driven by mutations in genes of low penetrance.

The G_2 assay developed at the PICR was applied, with some minor changes, in our laboratory at WRI to investigate the association of G_2 radiosensitivity with cancer predisposition and the heritability of the trait in a population of Danish survivors of childhood and adolescent cancer and their offspring (Curwen *et al* 2005). In total, four groups were scored for G_2 chromosomal radiosensitivity; 23 survivors of childhood and adolescent cancer, a control group comprising their 23 partners, 38 offspring and an internal control group consisting of 27 volunteers collected at WRI. When the 90th percentile cut-off of the WRI control group was implemented, the proportion of radiosensitive cases was 35% for the partners, 52% for the survivors and 53% for the offspring. There were no significant differences between WRI controls and Danish controls but significant differences between WRI controls and Danish cancer survivors ($P = 0.002$) and WRI controls compared with offspring ($P < 0.001$). However, when the 90th percentile cut-off for the Danish partner control group was applied, no significant differences were observed between the three Danish groups, with only 4% of cancer survivors and 18% of offspring found to be sensitive (Figure 1.3). The higher than expected proportion of radiosensitive individuals seen in the partner control group in comparison with the WRI control group could not be easily explained. Although the authors suggested there was a possibility that partners of cancer survivors may not be an appropriate control group, they concluded it was unlikely that the partners would form a distinct group with elevated radiosensitivity. The inability to distinguish between cancer survivors and their partner controls suggests that any association between elevated G_2 chromosomal radiosensitivity and childhood cancer predisposition should be regarded with caution. Moreover, the WRI controls may not be an appropriate group for comparison with childhood and adolescent cancer. That being the case, the inability of the study to distinguish between cancer survivors and cancer partners seems to contradict the earlier findings by Baria *et al* (2002).
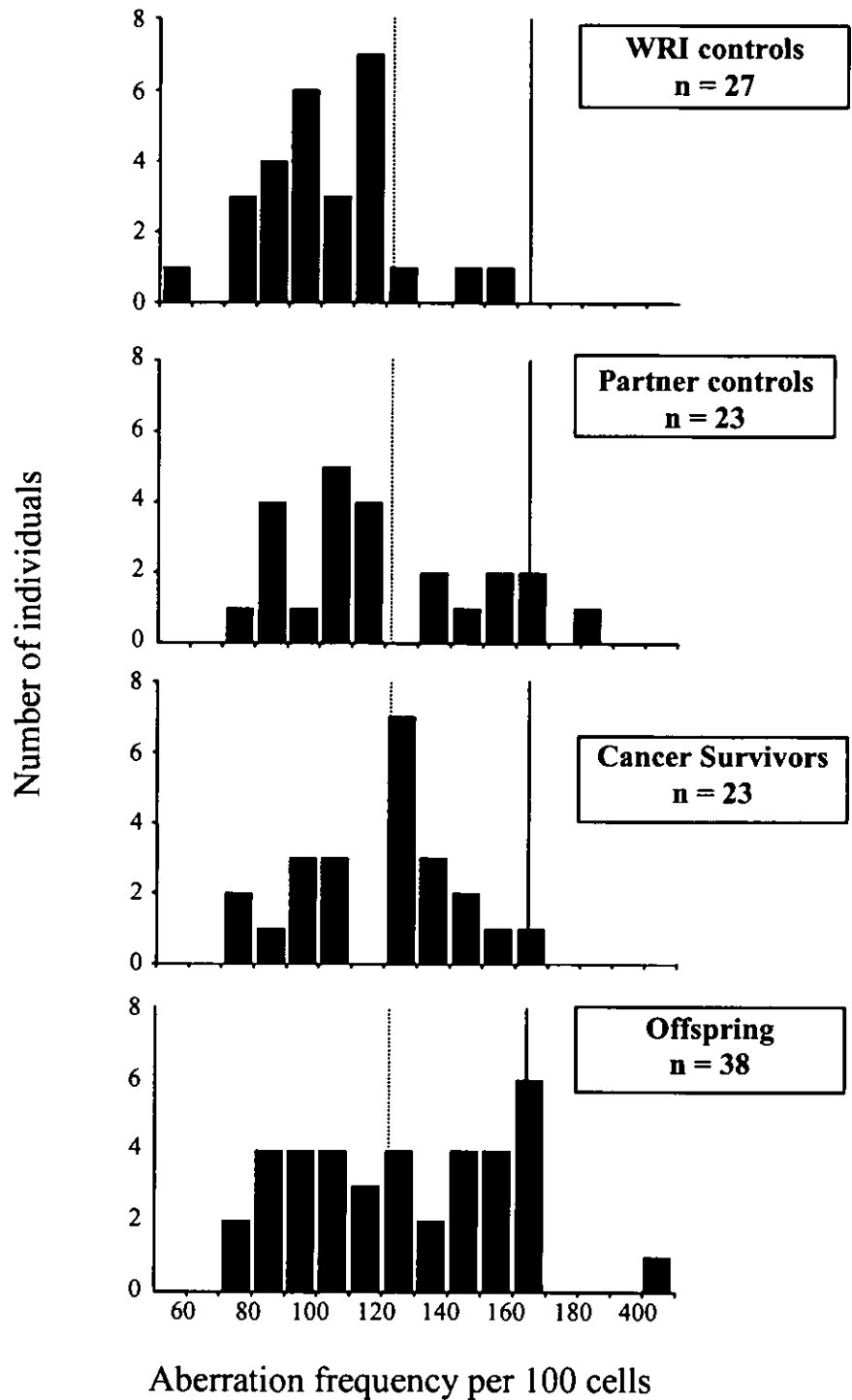
Figure 1.3: Distributions of G_2 chromatid aberration frequencies in WRI controls, partner controls, cancer survivors and offspring of cancer survivors. The vertical lines represent the cut-off points for a normal and radiosensitive response, based on the 90th percentile of the WRI control (red-dotted line) and partner control (solid black line) groups. Figure adapted from Curwen *et al* (2005) and reproduced with kind permission.

1.2 THE INFLUENCE OF RADIATION ON CELL CYCLE KINETICS

1.2.1 Cell Cycle Control

Cell cycle control is maintained by checkpoints at G_1/S transition and $G_2/Mitosis$ transition and is regulated by key proteins such as p53, ATM, BRCA1 and various Cyclin Dependent Kinase (CDK) molecules. The G_1 checkpoint exists to prevent cells from entering DNA synthesis with DNA damage which can then become 'fixed' in the genome. At this stage cells may be temporarily stopped from dividing and enter a state of quiescence called G_0 phase. The G_2 checkpoint prevents the proliferation of damaged cells and allows time for DNA repair before transition to metaphase. Efficient cell cycle control is crucial for maintaining genomic integrity and stability, thereby preventing unregulated cell proliferation which leads to cancer.

1.2.2 The Effect of Radiation upon the Cell Cycle

Since the 1920's it has been recognised that radiation can affect cellular growth (Mottram *et al* 1926). By 1953 an accurate representation of cell cycle progression was established using radiolabelling of S phase cells with ^{32}P (Howard and Pelc 1953). Howard and Pelc discovered that X-ray irradiation prolonged both the G_1 and G_2 phases and later work utilising HeLa cells revealed this delay to be dose-dependent (Yamada and Puck 1961). Such cell cycle delays are now thought to represent a co-ordinated cellular response to radiation in order to prevent damaged cells from progressing through the cell cycle. Investigations into G_2 checkpoint delay utilising mutant cells of *Saccharomyces cerevisiae* that are unable to arrest in response to irradiation revealed that the observed cell cycle defect was also coupled to an increased radiosensitivity (Weinert and Hartwell 1988; Weinert 1992; Weinert *et al* 1994). The authors postulated that cells contain checkpoints which arrest in response to DNA damage and that these checkpoints exist to allow time for DNA repair.

1.2.3 ATM Function in Cell Cycle Checkpoints

ATM kinases are vital components of the pathway which controls DNA repair (Jeggo *et al* 1998) and the length of the G_2 phase (Shackelford *et al* 1999). Due to the lack of functional ATM kinase in cells from AT patients, this group is a vital source for enabling a thorough exploration of the role of ATM kinase in DNA repair and cell cycle checkpoint processes. Investigations into the role of ATM in checkpoint function have produced a range of apparently conflicting results. For example, some studies have shown that AT cells fail to arrest at the G_2 checkpoint after irradiation and progress immediately into metaphase (Beamish *et al* 1996; Scott *et al* 1994b; Zampetti-Bosseler and Scott 1981), whilst other studies suggest a prolonged G_2 arrest compared to normal cells (Beamish *et al* 1994; Beamish and Lavin 1994; Scott *et al* 1994b). These apparently opposing viewpoints may be explained by the existence of two distinct G_2 arrest mechanisms (Xu *et al* 2002). Utilising a variety of cell cycle assays the authors demonstrated that a transient ATM-dependent checkpoint is activated shortly after irradiation to prevent damaged cells, irradiated in the G_2 stage of the cell cycle, from progressing to metaphase. The second mechanism is measurable several hours after irradiation and is represented by the accumulation of cells in G_2 phase that were irradiated in the S or G_1 phase of the cell cycle. Crucially, this mechanism appears to be ATM independent, hence the accumulation of both AT and normal cells irradiated in the earlier stages of the cell cycle.

1.2.4 Measuring G_2 Arrest

The total length of the G_2 phase in irradiated lymphocytes and controls can be estimated using a number of techniques such as [^{3}H]TdR labelling (Pincheira *et al* 1994; Pincheira *et al* 2001), fluorescence-activated cell-sorting (FACS) (Bates and Lavin 1989; Herzenberg *et al* 2002; Hong *et al* 1994; Hu *et al* 2001; Hu *et al* 2002) and 5-bromo 2'-

deoxyuridine (BrDU) incorporation (Palitti *et al* 1999). G_2 checkpoint delay has been considered from a chromosomal radiosensitivity perspective using the mitotic index (MI) as a measure of the proportion of cells reaching mitosis. Mitotic inhibition (MIn) is calculated as the percentage reduction in the MI in irradiated cell cultures compared to non-irradiated cultures. It is postulated that MIn could be used as a reliable indicator of G_2 checkpoint efficacy providing that MIn values are truly representative of mitotic delay. Lymphocytes from 20 donors were used to investigate the presence of an X-ray induced adaptive response, sensitivity to X-ray irradiation in G_2 phase and G_2 checkpoint response (Pretazzoli *et al* 2000). Checkpoint activation was tested at both 0.02Gy and 0.3Gy and was measured by MI (as a % of control) and labelling with [^{3}H]TdR. One donor in particular consistently exhibited a strong reduction in MI in combination with low breakage frequency. The reduction in MI may represent a longer period of G_2 delay allowing more time for the repair of damage and thus, fewer aberrations are observed at metaphase. When the data for all twenty donors was analysed an increase in chromatid breaks was associated with a decrease in mitotic delay induced at 0.02Gy but not at 0.3Gy.

To evaluate the G_2 checkpoint efficacy of cells with a known checkpoint defect, MIn was used to determine the extent of cell cycle delay induced by X-ray irradiation in G_2 phase in a selection of AT homozygotes, AT heterozygotes and a control population (Scott *et al* 1994b). The mean inhibition for control samples was calculated at 88.1% compared to 44.2% in AT homozygotes whilst heterozygotes demonstrated similar levels of inhibition (88.5%) to controls. These results suggest that AT cells, on average, have lower levels of G_2 checkpoint delay compared to normal healthy individuals following radiation exposure in the G_2 phase of the cell cycle. These findings were consistent with earlier studies based upon MI measurements, all of which demonstrate

that irradiation in G_2 results in less delay in AT cells than controls (Hansson *et al* 1984; Mozdarani and Bryant 1989; Scott and Zampetti-Bosseler 1982). The group of Scott *et al* (2003) also calculated MIn in 129 breast cancer patients and 105 normal controls, which were originally processed for the G_2 assay (chromatid aberrations reported in Scott *et al* 1999). Inhibition in the breast cancer patients was significantly lower compared to female controls ($P = 0.009$) suggesting decreased G_2 checkpoint efficacy in patients compared to female controls. The authors suggest that this reduction in MIn may contribute to the enhanced chromosomal radiosensitivity of these patients, by allowing less time for the repair of chromatid damage before it is fixed and viewed in metaphase.

1.2.5 PCC (Premature Chromosome Condensation)

Chromatin condenses during the mitotic phase of the cell cycle in a highly ordered pre-determined fashion. However, using molecular techniques, chromosome condensation can be uncoupled from mitotic events and be induced prematurely in cells in the interphase stage of the cell cycle. Originally, this was achieved by the deliberate fusion of interphase cells to mitotic cells using Sendai virus (Johnson and Rao 1970), later improved using polyethylene glycol (PEG)-mediated fusion (Pantelias and Maillie 1983), and can now be achieved by the addition of the phosphatase inhibitors calyculin A or okadaic acid (Gotoh *et al* 1995). PCC enables categorisation of each cell cycle phase due to the visualisation of distinct morphologies: G_1 phase chromosomes are univalent, S phase cells are pulverised in appearance and G_2 phase chromosomes are of similar appearance to those in metaphase in that they contain bivalent condensed chromosomes but can be distinguished due to the absence of a visible centromeric region (Gotoh *et al* 1995; Hatzi *et al* 2007; Hatzi *et al* 2008; Terzoudi *et al* 2005).

Early application of the PCC technique revealed that arrested G_2 cells repair many of their DNA breaks before mitosis (Hittelman and Rao 1974) indicating that one of the purposes of G_2 delay is to allow time for the repair of DNA damage. Therefore, the efficacy of the G_2 to metaphase checkpoint could influence the G_2 radiosensitivity score measured at metaphase. The PCC technique has recently been combined with a version of the G_2 radiosensitivity assay to investigate the role of the G_2 checkpoint in the repair of DNA double-strand breaks (DSBs), in normal and AT cells (Terzoudi *et al* 2005). In this protocol the effect of complete checkpoint abrogation upon chromatid aberration burden was directly measured by comparing aberration levels in both normal and AT lymphocytes before and after G_2 to mitosis transition. The key finding of this work was that there was no discernable difference in the number of chromatid breaks scored directly in artificially condensed G_2 phase AT and normal cells prompting the authors to suggest that DNA DSBs are repaired in AT and normal cells with similar kinetics, and that the differences in frequencies of chromatid breaks in normal and AT cells is primarily due to the G_2 checkpoint difference. Analysis of normal cells at metaphase revealed a two- to three-fold reduction in the number of breaks in comparison to G_2 phase whilst AT cells did not exhibit any strong reduction in chromatid aberration level. To confirm that normal cells exhibit a two- to three-fold reduction in chromatid damage following checkpoint transition, the G_2 checkpoint was artificially abolished using caffeine, which acts as an ATM inhibitor. Following caffeine addition the number of chromatid aberrations in metaphase in normal cells was similar to that observed in AT cells. These investigations provided direct evidence that activation of the ATM-dependent G_2 checkpoint following irradiation is a key event in the reduction of chromatid damage observed at metaphase. In addition to analysing chromatid damage, this group calculated the ratio of cells in G_2 to cells in G_2 and metaphase in an attempt to measure the level of G_2 delay following irradiation. An increase in this ratio was

observed in normal and AT heterozygote cells whereas there was no change in this ratio for AT homozygotes following irradiation. This was further proof that AT cells are unable to undergo checkpoint activation in response to irradiation in G_2 phase. This laboratory has also used PCC methodology to evaluate the combined effects of radiation and the potential mutagens hydroquinone (Hatzi *et al* 2007) and glutaraldehyde (Hatzi *et al* 2008) upon cell cycle progression and chromosomal radiosensitivity. These studies suggest that the direct enumeration of each cell cycle phase is a promising indicator of G_2 checkpoint delay.

1.3 SCOPE AND AIMS OF THIS PROJECT

The Genetic Consequences of Cancer Treatment study is a multi-national collaboration between research groups in the U.S.A, U.K., Denmark and Finland which utilise epidemiology, molecular genetic techniques and cytogenetics. The objective is to investigate whether preconception radiotherapy and chemotherapy received by children and young adults contribute to adverse pregnancy outcomes (Boice *et al* 2003) (http://www.gcct.org/). Pilot studies using blood of Danish trios (cancer survivor, partner and offspring) attempted to elucidate whether minisatellite mutations are indicative of transmissible radiation-induced damage (Rees *et al* 2006) and if chromosomal radiosensitivity is a marker of cancer predisposition (Curwen *et al* 2005). The initial pilot study using blood has now been extended to further samples received from Danish families. This provided an opportunity to explore G_2 chromosomal radiosensitivity in relation to G_2 checkpoint function.

In the first instance, development work to investigate the project viability using the PCC technique was undertaken employing samples from WRI staff. Once the methodology was fully developed, the technique was applied to a Danish population of 30 survivors of childhood and young adulthood cancer. The aim of this study was to apply PCC methodology in combination with the G_2 radiosensitivity assay and to use this technique to investigate cell cycle perturbation following irradiation in relation to the frequency of chromatid aberrations observed at metaphase. Samples were cultured for the G_2 and the G_2 + PCC assay to determine the G_2 radiosensitivity score and G_2 checkpoint delay, respectively. Any correlations between the two sets of data were investigated in the hope of illuminating the relationship between G_2 checkpoint control and G_2 chromosomal radiosensitivity.

CHAPTER 2

VALIDATION OF THE PREMATURE

CHROMOSOME CONDENSATION

(PCC) TECHNIQUE

2.1 INTRODUCTION

Initial experiments were performed employing a group of healthy volunteers to ensure that the technique described in the literature could be performed in the WRI laboratory before commencing a study of cell cycle perturbation in cancer survivors (see Section 3). The initial goal was to observe chemically-induced PCC in peripheral blood lymphocytes, to study chromosome morphology, assign cell cycle stage and to score chromatid aberrations directly in G_2 phase as achieved by Terzoudi *et al* (2005) and Febrer *et al* (2008).

2.2 METHODS

2.2.1 Validation Study Population

Samples were taken from WRI staff willing to volunteer blood. One individual donated blood on more than one occasion. All volunteers gave written informed consent before a blood sample was taken (see Appendix A for copy of consent form) and blood samples were coded to ensure anonymity. Slides made from these blood cultures were further coded by a member of staff not directly involved in the study to prevent scorer bias. As the majority of the volunteers also gave blood as part of the WRI G_2 assay validation study (Smart *et al* 2003) or the Danish Trio Pilot study (Curwen *et al* 2005) the same coding system was adopted. In total seven donors participated, comprising of four males and three females.

2.2.2 Sample Collection

All samples were collected at WRI by a principal genetic counsellor. Blood was drawn into 5 ml lithium heparin vacutainers (BD Vacutainer Systems, Ref. 367684) and allowed to stand overnight at room temperature.

2.2.3 Cell Culture

For each blood sample two T25 cm^3 culture flasks (VWR International Ltd, Catalogue No. 734-0031) were set up. The day before culturing, the volume of RPMI-1640 medium (Sigma$^®$, Catalogue No. R8758) which was required for the particular sample size was supplemented with 15% foetal calf serum (Invitrogen Corporation, Catalogue No. 10099-133), 1% phytohaemagglutinin (M-form) (Gibco™, supplied by Invitrogen Corporation, Catalogue No. 10576-015) and 1% L-glutamine (Invitrogen Corporation, Catalogue No. 25030-032). A single foetal calf serum batch (Lot. 4955944s) was used for all samples throughout the validation work and the Danish cancer survivor study. The culture medium was placed in a 37°C, 5% CO_2/95% air incubator and left overnight to pre-warm and undergo gaseous exchange. For each culture flask, 1 ml of blood was added to 9 ml of complete culture medium in a T25 cm^3 culture flask. All culture flasks were mixed gently and then placed upright in the incubator with the caps loose. The time of culture set up was then noted to keep to the strict timings required for this procedure. After exactly 48 hours of culturing 7 ml of the spent medium was removed using pre-warmed pipettes, taking care not to disrupt the cell layer. This medium was replaced with 7 ml of fresh pre-warmed, pre-gassed medium and the flasks were mixed by gentle inversion before been placed back into the incubator with the caps loose.

2.2.4 X-ray Irradiation

At 15 min prior to irradiation, flasks were gently mixed and placed in a 37°C portable incubator and transported by car to the X-ray facility (Siefert), located on the Westlakes Science Park in the Geoffrey Schofield Laboratories a short distance away (approximately ¼ mile). The X-ray set was maintained by regular warm-up operations and tested to ensure safety and the correct dose delivery. Before sample irradiation the

X-ray room was pre-warmed using a radiator and the set itself was warmed-up using a pre-programmed procedure. After exactly 72 hours total culture time, the flasks were either irradiated with 0.5 Gy 300kV X-rays or 'mock-irradiated' i.e. treated in an identical manner to the irradiated culture flasks apart from receiving X-rays. The dose received varied marginally between irradiated culture flasks with all exposures in the range 0.49 – 0.51 Gy. The exact dose was recorded for each sample. 'Mock-irradiated' control flasks were simultaneously removed and returned to the incubator with the corresponding irradiated flasks, but were not irradiated. This 'mock-irradiation' ensured identical treatment of both control and irradiated cultures. Each culture flask was outside the portable incubator for the shortest period possible to minimise any drop in temperature. Following irradiation, flasks were transported back to the laboratory and placed back in the incubator. After a recovery period of exactly 30 min, 100µl of pre-warmed KaryoMax colcemid® (10 µgml⁻¹) (Invitrogen Corporation, Catalogue No. 15210-057) was added to the culture flasks, which were then mixed gently by inversion and returned to the incubator. Colcemid enabled the collection and visualisation of chromosome spreads at metaphase by blocking mitosis via inhibition of spindle formation.

2.2.5 PCC Induction

The G_2 assay was combined with PCC methodology in a protocol based on the study by Terzoudi *et al* (2005) (see Figure 2.1). The protocol adopted for PCC induction followed the methodology of the G_2 assay with the exception that calyculin A (Sigma®, Catalogue No. C5552-10UG) was added in addition to colcemid. Three time points for the addition of calyculin A were tested to establish optimum conditions for PCC induction, visualisation of chromatid damage and good discrimination between G_2 and metaphase spreads. At either 30 min, 60 min or 75 min post-irradiation, 5µl of

calyculin A (0.1 mM) was added to the culture flasks, which were then mixed and returned to the incubator as before.

| Blood Culture Set up | | Media Change (48hr) | 0.5Gy | Add colcemid | Add calyculin A | Harvest Cells |

72 hrs · · · · · · · · · 30 min · · · ? · · · ?
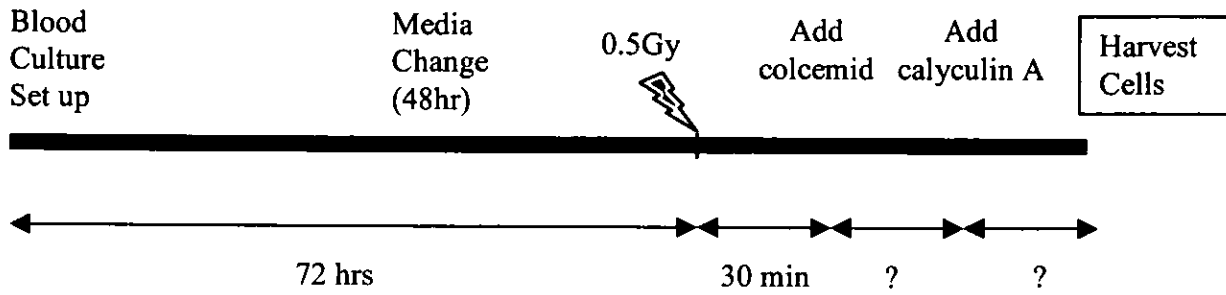
Figure 2.1: The protocol for evaluating PCC induction. PHA-stimulated peripheral blood lymphocytes were cultured for 72 hours using standard techniques with a media change at 48 hours. At 72 hours cultures were irradiated with 0.5Gy X-rays, colcemid was added at 30 min post-irradiation which was 1 hour prior to cell harvesting. The time point of calyculin A addition was attempted at 3 time-points: 30 min, 60 min and 75 min post-irradiation.

2.2.6 Cell Harvesting

Almost 90 min after irradiation, the contents of each culture flask were transferred to centrifuge tubes (Barloworld Scientific Limited, Catalogue No. 144AS) before being plunged into ice chippings at exactly 90 min post-irradiation. The tubes were left for 2-3 min to facilitate rapid cooling to approximately 0°C to prevent further DNA repair. Tubes were then spun at 400 g in a pre-cooled centrifuge (0°C-4°C) for 5 min. Following centrifugation, the supernatant was aspirated within 1.5 ml of the pellet and the cells were then vortexed before treatment with cold potassium chloride (KCl) solution (VWR International Ltd, Catalogue No. 101984L) for 20 min with regular inversion of tubes. After 5 min of centrifugation at 400 g cells were fixed slowly with a mixture of methanol (VWR International Ltd, Catalogue No. 10158 6B) and acetic acid (VWR International Ltd, Catalogue No. 10001CU) in the ratio 3:1, respectively. After a further centrifugation and fix, cells were stored at -20°C. The following week, these cell pellets were washed and fixed a further four times (six in total) and stored for a minimum period of 24 hours before making slides.

2.2.7 Slide Preparation and Staining

SuperFrost® Slides (Scientific Laboratory Supplies, Catalogue No. MIC3024 and MIC3022) were cleaned with methanol, washed briefly under tap water and plunged into ice chippings for 30-60 min prior to preparing cell suspension. Meanwhile, centrifuge tubes containing cell pellets and fixative were removed from the -20°C freezer and left on the laboratory bench to equilibrate to room temperature for 30-60 min. To prepare the cell suspension, fixed cells were centrifuged at 400 g for 5 min. After centrifugation, cells were re-fixed once as described in Section 2.2.6. The supernatant was completely aspirated making sure not to remove any cellular material and a further 0.5-1 ml of fresh fixative was added to create a milky suspension. Next, 40 μl of cell suspension was dropped from a height of approximately 30-50 cm onto

cold, wet slides which were immediately passed through a flame. This technique of edge flaming was vital in producing evenly distributed chromosome spreads throughout the slide, which also had good quality morphology. Low humidity had been shown previously to adversely effect chromosome spreading and thus slides were often made over a sink of steaming water, no slide making was attempted when the humidity of the laboratory was below 40%, and the air conditioning was switched off. When dry, slides were arranged in glass troughs and stained with Giemsa stain solution (improved R66), (VWR International Ltd, Catalogue No. 350864X) diluted 1:19 with Gurr® buffer (1 tablet supplied by BDH Limited dissolved in 1l H₂O) and air-dried. Once completely dry, slides were mounted by applying DPX mountant (VWR International Ltd, Catalogue No. 360294H) onto coverslips (20 x 50mm) (VWR International Ltd, Catalogue No. 631/0137) and firmly placing the slides on top ensuring air bubbles were eradicated.

2.2.8 Microscopy

Prior to scoring, all slides were scanned using the Metasystems Metafer4 scanning system which comprises a Zeiss Axioplan 2 imaging microscope with a Marzhauser motorized scanning stage connected to Metafer 4.MSearch software (Metasystems, Germany. www.metasystems.de), (see Appendix B for photograph). This software and microscope package enabled the user to search an entire slide, to record and subsequently capture images of any cells which appeared to have 'metaphase-like' morphology. For automated pinpointing of each metaphase, mounted and coded slides were fixed into the microscope bays to allow for scanning at x10 magnification. This automated system had a few key advantages over standard light microscopy. Slides with low numbers of chromosome spreads could be identified immediately following scanning and discarded in favour of superior slides or more slides could be made if

required. The speed of cytogenetic analysis was increased approximately two-fold because thumbnail images of poor quality spreads could be discarded prior to scoring and the user could move from cell to cell immediately with a mouse click instead of manually scrolling through an entire slide.

To greatly improve the correct identification of cells that have visible chromosome spreads, as opposed to intact cells or non-nuclear material, the image capturing mechanism was trained using built-in software. This classifier training was used to set parameters for future scans and make the scanning process more efficient. In brief, the Metafer4 scanning system was used to capture a large number of images, which were then used to define objects, in this case metaphase spreads. A number of slides were scanned using a default classifier and a number of image fields were captured. At this stage, the computer was not used to do any automated analysis to recognise metaphase spreads. Instead, these training fields were reviewed manually. If a metaphase was present, the field was marked as 'Positive' and a green circle was drawn around the metaphase; everything not marked was recorded as 'Negative'. Objects that showed some characteristics of metaphase but were incomplete metaphases or non-cellular material such as 'dirt' were rejected by drawing a red circle around them. More than 600 metaphases from several slides were required to fully train the software and create a fully functional classifier. A new classifier called 'G$_2$ metaphases' was created and the command 'Compute Classifier' was initiated. The computer was left overnight to compute the classifier to complete the training. This new classifier was selected when scanning all the Danish trio slides. Following training, the number and quality of spreads identified increased greatly.

2.3 RESULTS

2.3.1 The Effect of Calyculin A upon Chromosome Morphology

The number, morphology and distribution of chromosome spreads varied substantially between samples for a variety of reasons, which may include intrinsic cellular characteristics, thickness of cell suspension used and slide making technique. Using the definitions and photos provided by others (Febrer *et al* 2008; Gotoh *et al* 1995; Terzoudi *et al* 2005; Hatzi *et al* 2007; Hatzi *et al* 2008) an attempt was made to distinguish between cells in G_1, S, G_2 and metaphase. Examples of the types of cell morphology visualised are shown in Figures 2.2, 2.3, 2.4, 2.5 and 2.6. G_1 phase cells often take the form of a condensed metaphase-like shape containing univalent chromosomes, whilst S phase cells take a 'pulverised' form and the chromosomes have thick and thin sections to them (Gotoh *et al*, 1995). G_2 phase PCC cells contain bivalent condensed chromosomes which are similar in shape to metaphase chromosomes. However, the key difference is that the two sister chromatids have no visible centromeric region conferring a distinctive morphology, easily distinguished from metaphase spreads (Hatzi *et al* 2007; Hatzi *et al* 2008; Terzoudi *et al* 2005).
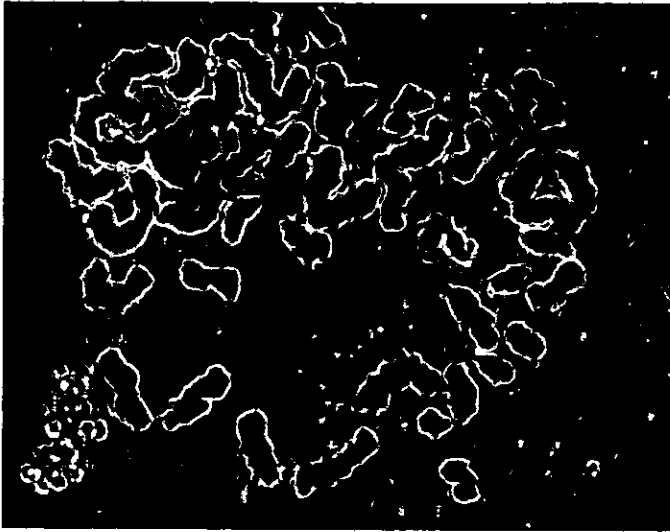
Figure 2.2: Chromosome spread with characteristics of PCC-G_1 phase. These cells often take the form of a condensed metaphase-like shape containing 46 univalent chromosomes. In this and subsequent figures in Chapter 2, the photographs are of cells from the 7 different subjects used in the validation study population (n = 7).
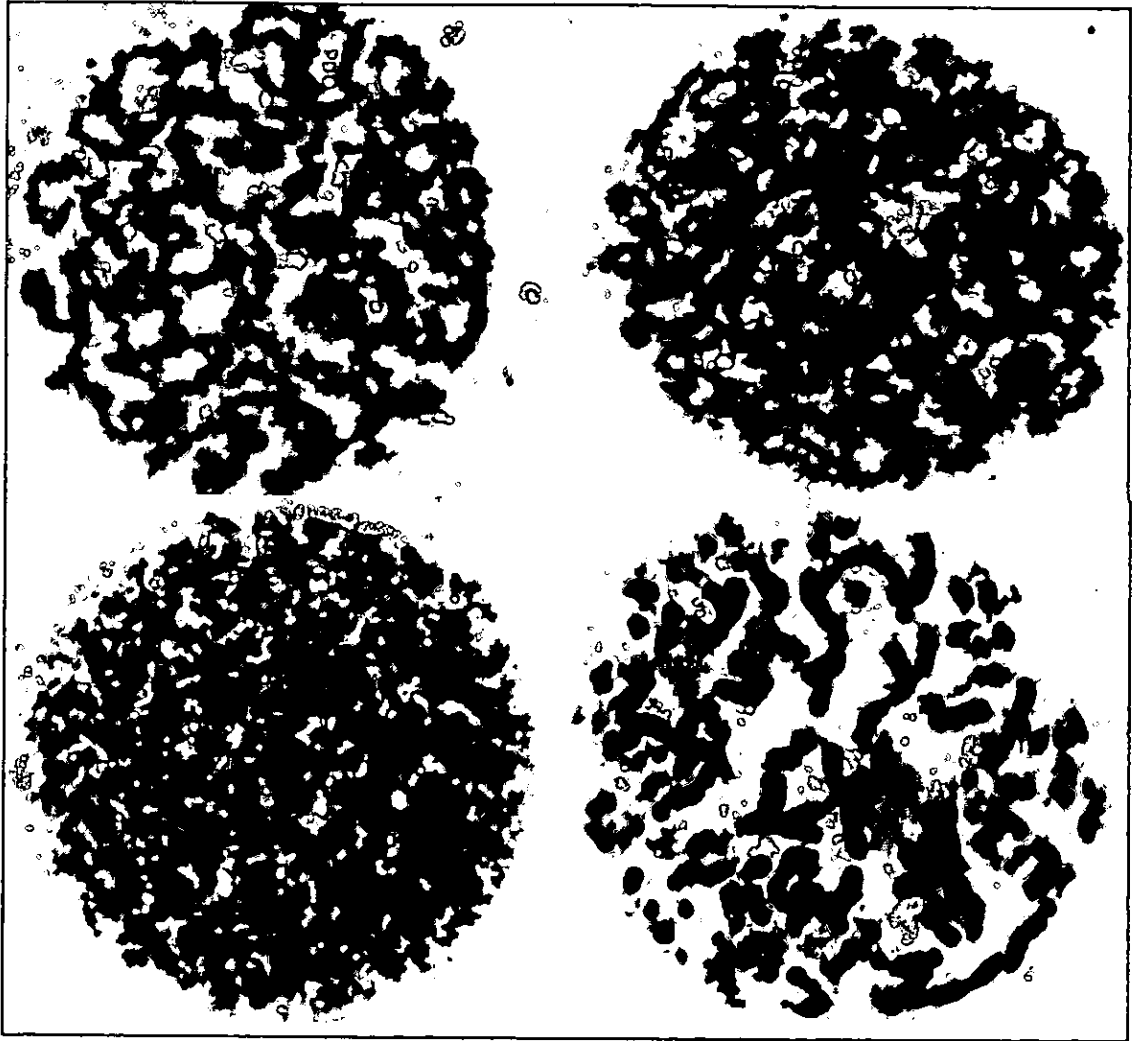
Figure 2.3: Chromosome spreads with characteristics of PCC-S phase. These cells take a 'pulverised' form and the chromosomes have thick and thin sections to them. Typical of 7 such experiments using the validation study population (n = 7).
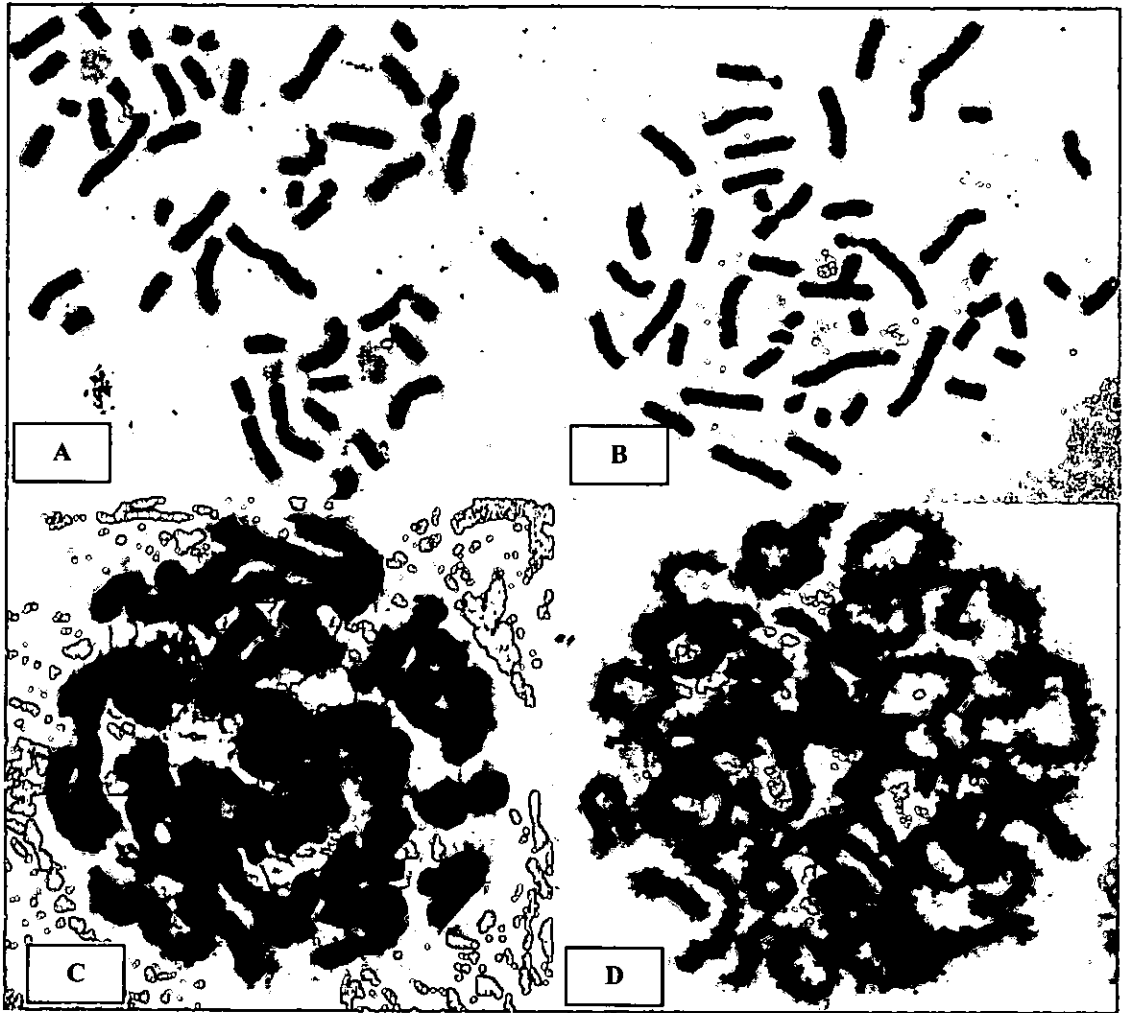
Figure 2.4: Panels A-D are cells containing chromosome spreads with characteristics of

PCC-G_2 phase. These cells contain bivalent condensed chromosomes. The two sister

chromatids have no visible centromeric region conferring a distinctive morphology,

easily distinguished from metaphase spreads. Panels A and B have clearly visible sister

chromatids. Panels C and D are PCC-G_2 cells containing tangled and overlapping

chromosomes. Panels A-D are typical of 7 such experiments using the validation study

population (n = 7).

Figure 2.5: Chromosome spreads with characteristics of metaphase. These cells contain bivalent condensed chromosomes with a visible centromeric region conferring a distinctive morphology. Typical of 7 such experiments using the validation study population (n = 7).
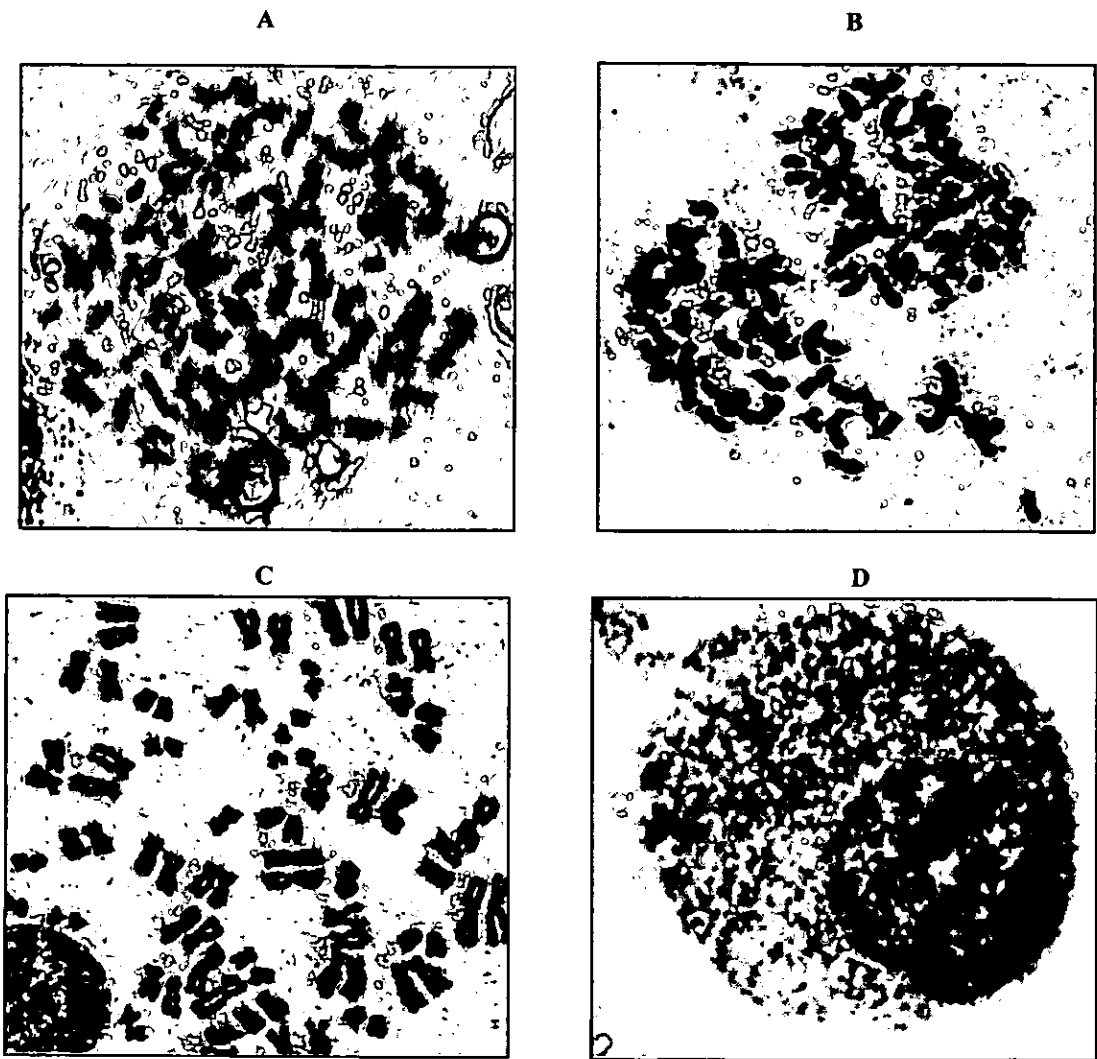
Figure 2.6: Miscellaneous chromosome spreads. Typical of 7 such experiments using the validation study population (n = 7).

Panel A and B: Spreads contain more than 46 chromosomes, which are often smaller than seen in other spreads. **Panel C**: Chromosome spread showing the typical features of endoreduplication, a cell cycle defect found in cells released from G_2 arrest in order to undergo mitotic catastrophe. Chromosome duplication without mitotic cell division results in multiple chromosomes. **Panel D**: Non-dividing G_0 cell.

The classification of cells into either PCC-G_1, PCC-S, PCC-G_2 or metaphase is not always clear-cut, as some spreads appear to have characteristics of more than one phase. Late PCC-S phase cells, which have completed their DNA replication apart from a few chromosomal areas, often look like PCC-G_2 cells but contain more than 46 chromosomes, have attenuated areas and many small breaks (email correspondence with Dr Gabriel Pantelias), (Figure 2.7). Upon the addition of calyculin A, these incomplete areas of replication condense and lead to breakage, explaining the high number of chromosome pieces observed. In contrast, PCC-G_2 cells have fully completed DNA replication and form sister chromatids without any visible discontinuity or areas of attenuation.

Some of the cells visualised contained chromosomes with premature centromere division (PCD), (Figure 2.8). Although they share the key feature of PCC-G_2 cells in that they contain no visible centromere they appear morphologically distinct. One of the effects of calyculin A addition seems to be an increase in PCD with reported rates of 16-17% in amniotic fluid cultures and 10% in lymphocyte cultures (Srebniak *et al* 2005). Although high levels of PCD in calyculin A treated cultures were not seen, these cells were more common than in colcemid only cultures.
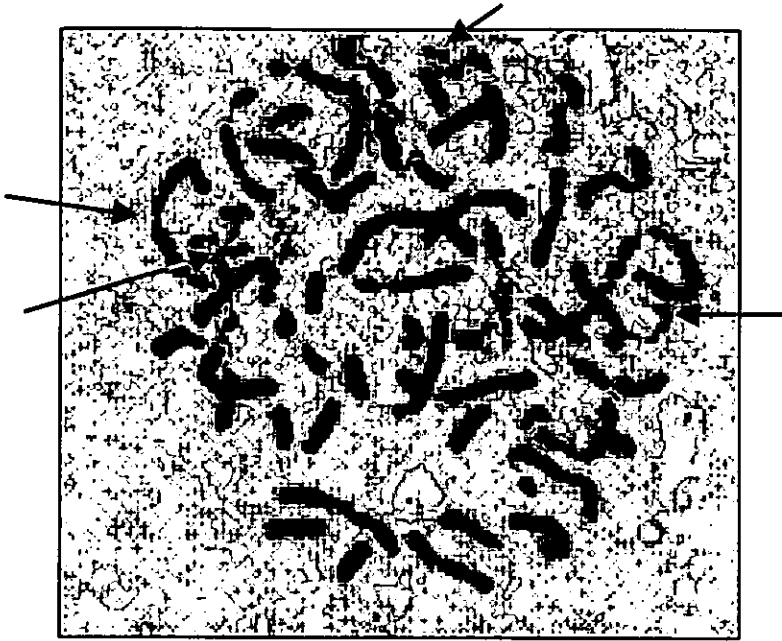
Figure 2.7: Late PCC-S phase cells. Chromosome number is higher than 46. Arrows mark possible areas of incomplete DNA replication. Typical of 7 such experiments using the validation study population (n = 7).

Figure 2.8: Premature Centromere Division (PCD). Typical of 7 such experiments using the validation study population (n = 7).

2.3.2 Differentiation of PCC-G_2 and Metaphase Cells

In initial attempts at differentiating between PCC-G_2 and metaphase, only cells with well spread chromosomes were included to maintain integrity in the scoring procedure (Figure 2.9). However, by leaving out many tight, unclear spreads which were most likely PCC-G_2 cells there may have been a danger of underestimating the number of cells in G_2 phase in comparison to metaphase cells, which have, on the whole, an unambiguous morphology. The cell cycle is a continuous process and some cells, which are likely to be close to transition points, display characteristics of both S and G_2 phase or both G_2 and metaphase. Due to the presence of such cells in combination with tight overlapping chromosomes, the classification of cell cycle stage was more difficult than at first anticipated. Crucially, the definition of what comprised a PCC-G_2 phase cell was decided upon before embarking on the Danish cancer survivor samples and strict criteria were applied throughout that part of the study to both control and irradiated cultures. Examples of cells included and excluded in analysis are shown in Figures 2.10, 2.11 and 2.12.

Figure 2.9: PCC-G$_2$ cell from an unirradiated sample with good spreading, two clearly visible sister chromatids and no visible centromeric region. Typical of 7 such experiments using the validation study population (n = 7).
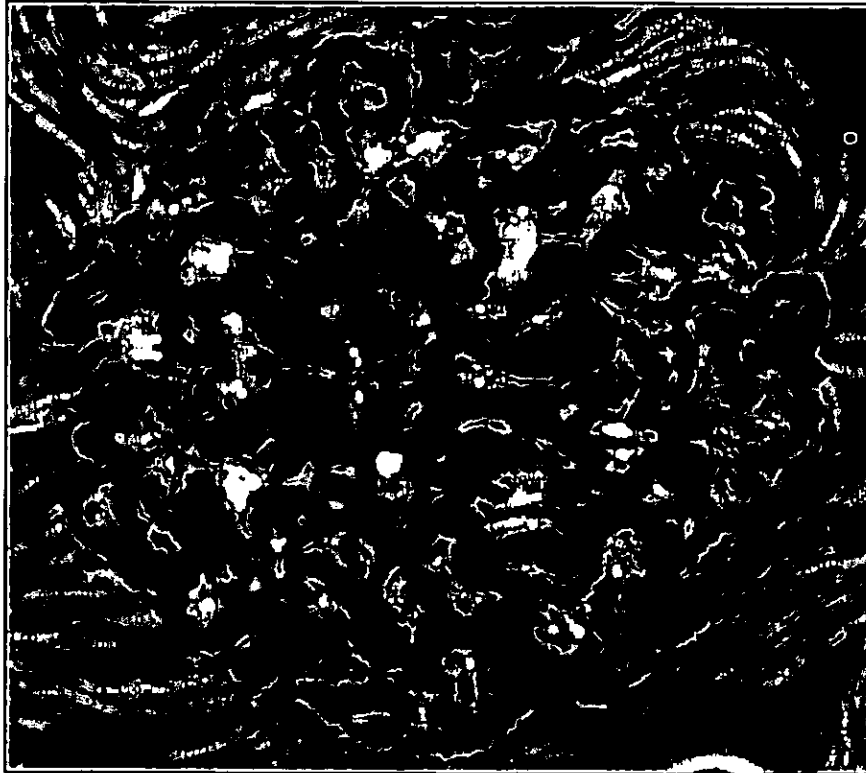
Figure 2.10: Cells with characteristics of both PCC-S and PCC-G_2 phase. In the

scoring criteria chosen such cells would be excluded and not classed as a PCC-G_2 cell.

Typical of 7 such experiments using the validation study population (n = 7).

Figure 2.11: PCC-G_2 cell. Despite overlapping and difficulty in differentiating sister chromatids the cell contains bivalent chromosomes with no visible centromeric region. In the scoring criteria chosen such cells would be classed as PCC-G_2 cells. Typical of 7 such experiments using the validation study population (n = 7).
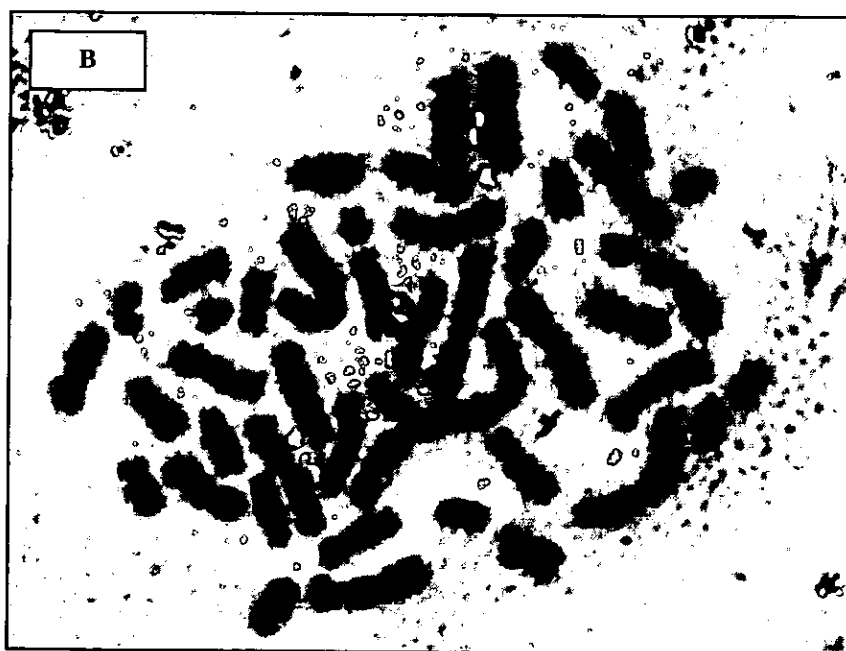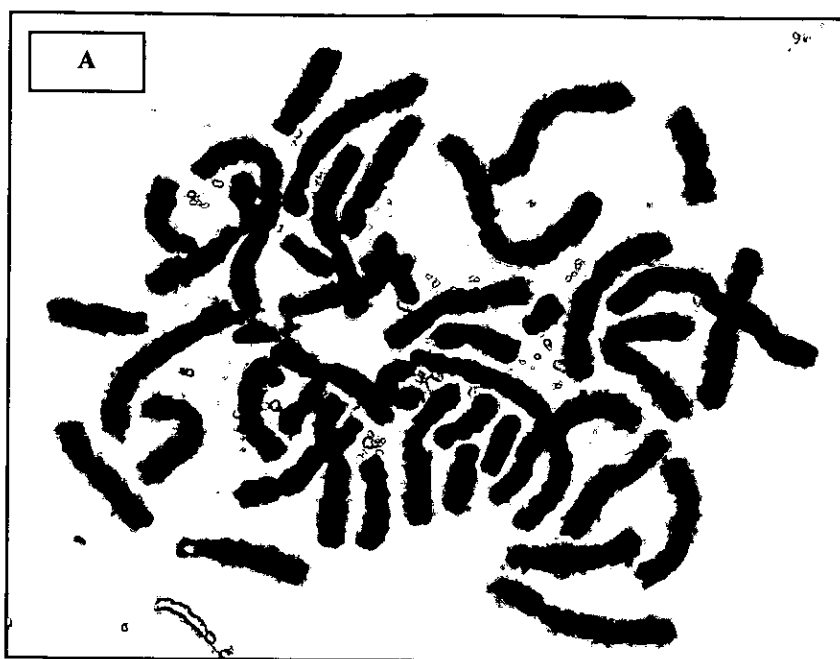
Figure 2.12: Cells with characteristics of both PCC-G$_2$ and metaphase. Due to the presence of centromeric constriction in many of the chromosomes these cells would be scored as metaphase cells in the scoring criteria chosen. Typical of 7 such experiments using the validation study population (n = 7).

2.4 DISCUSSION

2.4.1 Timing of Calyculin A Incubation

Studies which have used the chemically-induced PCC technique in conjunction with the G_2 chromosomal radiosensitivity assay have added calyculin A at either 60 min (Febrer *et al* 2008; Terzoudi *et al* 2005) or at 75 min (Shovman *et al* 2008) post-irradiation. This current study undertook a small number of experiments to assess three prospective time points at 30 min, 60 min and 75 min post-irradiation. Addition of calyculin A at 75 min post-irradiation failed to produce many discernible PCC-G_2 cells in the slides examined. The addition of calyculin A at 75 min post-irradiation i.e. 15 min pre-harvest, has recently been combined with G_2 assay methodology in an attempt to improve the traditional colcemid-only assay (Shovman *et al* 2008). The authors describe a substantial decrease in cells with split centromeres in comparison with longer calyculin A incubation times. In addition, the mitotic index was higher and thus, an increase in scorable condensed chromosome figures was observed. However, to assess the G_2 checkpoint in the first few hours after irradiation it is vital that the assay employed can distinguish mitotic cells from G_2 cells (Xu *et al* 2002). For this study, differences in centromeric constriction, as applied by Terzoudi *et al* (2005), were used to distinguish between metaphase and G_2 cells. Based on the limited data, the 75 min post-irradiation time did not allow visualisation of such morphological differences and therefore was not suitable for this specific project.

The addition of colcemid and calyculin A together at 30 min post-irradiation resulted in the vast majority of cells resembling PCC-G_2 spreads making a comparison of PCC-G_2 to metaphase ratio difficult. By delaying the addition of calyculin A for another 30 min and instead adding at 60 min post-irradiation, more G_2 cells are allowed to pass into metaphase before artificial condensation of the entire cell population. Therefore, the 60

min post-irradiation time enables visualisation of a substantial number of both PCC-G_2 and metaphase cells and thus, an accurate evaluation of any change in the ratio of PCC-G_2 to metaphase cells can be calculated. In addition, the 60 minute post-irradiation timing enabled visualisation of chromatid damage within a proportion of these irradiated cells. In line with the protocol of other groups (Febrer *et al* 2008; Terzoudi *et al* 2005) the 60 min post-irradiation timepoint was adopted.

2.4.2 Scoring Chromatid Aberrations in the G_2 Phase of the Cell Cycle

One of the original aims was to score chromatid aberrations in PCC-G_2 cells. Unfortunately, there was limited success. Few PCC-G_2 cells with good spreading in combination with clear sister chromatids were observed which made scoring gaps and breaks far more difficult than in cells routinely seen in metaphase. When scoring was attempted extra care was taken when analysing PCC-G_2 cells. Only PCC-G_2 cells which had good quality morphology comparable to metaphase cells were analysed (Figure 2.13). Chromatid aberrations were only recorded if visible as sharp breaks which were almost certainly caused by X-irradiation rather than unclear areas of attenuation, faded bands or scratches produced by coverslip damage, incomplete DNA replication or any other disruption to cell morphology. By obtaining a digital image of individual cells using a microscope mounted camera in conjunction with an image analysis software package called Interactive **KARy-O**typing System (IKAROS), (Metasystems, Germany) and applying sharpening filters, it is possible to improve the visualisation of damage. However, this is a time consuming process and the morphology must still be of a reasonable standard. To maintain integrity, the analysis of manipulated images, as opposed to the scoring of actual cell damage visualised using microscopy, should be undertaken with caution.

A number of laboratories employing the PCC technique for the study of chromatid gaps and breaks before the onset of mitosis have utilised cell lines or isolated lymphocytes rather than peripheral blood cultures (Bryant *et al* 2008; Gotoh *et al* 1995; Gotoh *et al* 1999; Hittelman and Rao 1974; Terzoudi *et al* 2000; Terzoudi and Pantelias 1997; Wang *et al* 2006). However, there have been some recent successes in scoring chromatid damage directly in G_2 phase by adding calyculin A to peripheral blood cultures (Febrer *et al* 2008; Terzoudi *et al* 2005). Further work would be useful to assess the visualisation of damage in both cell lines and in blood cultured lymphocytes to confirm which cell type allows accurate analysis.

Figure 2.13: Aberrations observed in a PCC-G_2 cell following 0.5Gy X-ray irradiation. Red arrows show chromatid gaps and breaks. Typical of 7 such experiments using the validation study population (n = 7).

2.5 CONCLUSIONS

The results have shown that the methodology implemented enabled chemically-induced PCC to be observed in peripheral blood lymphocytes. PCC was investigated as a technique for studying perturbation in the G_2 cell cycle checkpoint in a group of healthy volunteers. Unfortunately, direct analysis of chromatid aberrations in the G_2 phase proved unreliable. Following a number of failed attempts to visualise and accurately score damage directly in PCC-G_2 cells the decision was taken to instead score the ratio of cells in each cell cycle stage before and after irradiation. By calculating the ratio of PCC-G_2 cells versus PCC-G_2 + metaphase cells before and after the G_2 to mitosis transition point, it was possible to measure the extent of any radiation-induced G_2 checkpoint delay. The next stage was to apply the PCC technique to a group of Danish cancer survivors to assay radiation-induced G_2/mitosis cell cycle perturbation and make direct comparisons to G_2 chromosomal radiosensitivity measured in metaphase.

CHAPTER 3

EXAMINING G_2 CHROMOSOMAL RADIOSENSITIVITY AND CELL CYCLE PROGRESSION IN CHILDHOOD AND YOUNG ADULTHOOD CANCER SURVIVORS

3.1 INTRODUCTION

Following the establishment of optimum conditions for PCC induction in the WRI laboratory and the determination of practical scoring criteria, the G_2 assay and the $G_2 +$ PCC assay were applied to survivors of childhood and young adulthood cancer. The relationship between chromatid aberration frequency, as determined by the G_2 assay, and cell cycle perturbation, as determined by the $G_2 +$ PCC assay, was investigated.

The UCLan Faculty Ethics Committee was able to register as an approved Institutional Review Board who reviewed and approved the overall project. In addition, ethical permission was obtained in Denmark from the Danish Scientific Ethical Committee and the Danish Data Protection Agency, as well as, the Westlakes Ethics Committee.

3.2 METHODS

3.2.1 The Cancer Survivor Group

Dr Jeanette Falck-Winther (Institute of Cancer Epidemiology, Danish Cancer Society, Copenhagen, Denmark. http://www.cancer.dk/epi%20research/) was the co-ordinator for family selection, sample collection and transport for the Danish blood studies section of the Genetic Consequences of Cancer Treatment project (www.gcct.org).

In Denmark, a national Central Population Register (CPR) was established in 1968 based upon a personal identification number for each citizen. This information can be linked to population-based health registries including the Danish Cancer Registry, the Danish Central Cytogenetic Registry, the Danish Medical Birth Registry and the Abortion Registry. Dr Jeanette Falck-Winther used these databases to target a suitable cohort of eligible survivors, spouses and offspring. Inclusion criteria required that patients were alive on, or born after, 1st April 1968 when the national Central Population

Register (CPR) was established, were diagnosed with cancer at age <35 years between 1943 and 2002, had survived until a fertile age of 15, had received moderate to high doses of scattered radiation to the gonads, had live offspring and were treated at either the Rigshospitalet (State Hospital) in Copenhagen or the Aarhus Kommunehospital (Community Hospital) in Jutland. Dr Falck-Winther contacted eligible survivors by letter to determine willing participants which produced a final study group of 30 Danish survivors of cancer. Information on cancer in relatives, cancer type, medical treatment, radiation exposure and aspects of lifestyle was obtained from a questionnaire and family health portrait completed by each survivor (see Appendix C for copy of questionnaire). To ensure anonymity each family was assigned a study number (T29 – T59) and the blood samples were labelled accordingly before being sent to WRI. This study continued the numbering system adopted for the pilot study of 28 Danish cancer trios (cancer survivor, partner and offspring) labelled T1 to T28 (Curwen *et al* 2005). Blood samples from the partners and the offspring of the cancer survivors were also transported to WRI along with the survivors, as part of the over-arching study into the Genetic Consequences of Cancer Treatment (www.gcct.org) but were not used in this project.

3.2.2 Transport and Internal Assay Controls

To monitor any intra-sample variability and provide data on any transportation effect two volunteers were sampled in Denmark on the same day as the family blood samples were drawn and set-up in culture for the G_2 chromosomal radiosensitivity assay. The two volunteers were not related to the participating families and had no previous incidence of cancer or radiation exposure. In addition, one volunteer acted as an internal assay control and was sampled at WRI and cultured in parallel to the Danish transport controls and the Danish trios for both the G_2 chromosomal radiosensitivity

assay and the G_2 + PCC assay. Details of sample collection for all three control samples are provided in Table 3.1.

3.2.3 Sampling and Transport

The WRI internal assay control, the two Danish transport controls, and the 30 cancer survivors together with their families provided written informed consent before a blood sample was taken (see Appendix A for copy of internal consent form and Appendix D for copies of Danish consent form and information leaflet).

All Danish families and the two transport controls had peripheral blood drawn into lithium-heparin vacutainers at the State Hospital, Rigshospitalet, Copenhagen or the Skejby Hospital, Aarhus during the Monday of the sampling week. Blood was kept at room temperature prior to being shipped to WRI via courier. The internal WRI control was also sampled on the Monday of the sampling week and allowed to stand overnight at room temperature. The inclusion of a piece of dental X-ray film with each shipment, subsequently analysed by the Dosimetry Department at the Sellafield Nuclear Reprocessing Plant, Cumbria, U.K., revealed no evidence of radiation exposure during flight. All shipments were received by 8 am on Tuesday and cultures were set up in family groups at two or three time-points (depending on shipment volume) throughout the day to allow for manageable sample processing. Where possible, blood samples were set up in culture within 24 hours, with some samples set up between 24 and 28 hours of being drawn. In total, 8 shipments containing 122 blood samples were transported to WRI between June and December 2006. Due to one of the survivor blood samples failing to culture the final analysed study group comprised a total of 29 cancer survivors. Details of the cancer survivor group are provided in Table 3.2.

Table 3.1: Information on transport and internal assay controls.

Donor	Code	Sex	Date of sample collection								Total samples	
			19/06/08	26/06/08	03/07/08	30/10/06	06/11/06	13/11/06	04/12/06	11/12/06	G_2 assay	G_2 + PCC assay
Internal	G_2NN	F	✓	✓	✓	✓	X	✓	✓	✓	7	4
Transport 1	G_2AA	F	✓	✓	✓	✓	✓	✓	✓	✓	8	0
Transport 2	G_2ZZ	F	✓	✓	X	✓	X	X	✓	✓	5	0

Table 3.2: Details of the cancer survivor group.

Cancer Survivor ID	Shipment Date	Sex	Age at sampling (years)	Age at diagnosis (years)	Cancer diagnosis
2901	19/06/06	M	41	26	Hodgkin's disease
3001	19/06/06	M	45	13	Hodgkin's disease
3101	26/06/06	F	47	17	Hodgkin's disease
3201	26/06/06	F	32	1	Neuroblastoma
3301	11/12/06	F	62	9	Non-Hodgkin's lymphoma
3401	03/07/06	F	41	8	Non-Hodgkin's lymphoma
3501	03/07/06	F	44	9	Non-Hodgkin's lymphoma
3601	03/07/06	M	46	17	Non-Hodgkin's lymphoma
3701	03/07/06	F	34	13	Hodgkin's disease
3801	03/07/06	F	41	15	Hodgkin's disease
3901	30/10/06	F	61	9	Non-Hodgkin's lymphoma
4001	30/10/06	M	43	9	Non-Hodgkin's lymphoma
4101	06/11/06	F	61	15	Hodgkin's disease
4201	06/11/06	M	48	27	Hodgkin's disease
4301	13/11/06	M	41	28	Hodgkin's disease
4401	06/11/06	M	56	17	Non-Hodgkin's lymphoma
4501	06/11/06	F	55	16	Non-Hodgkin's lymphoma
4601	06/11/06	F	32	3	Wilms' tumour
4701	13/11/06	M	43	30	Hodgkin's disease
4801	13/11/06	M	52	19	Hodgkin's disease
4901	13/11/06	M	47	28	Hodgkin's disease
5001	13/11/06	M	38	3	Hodgkin's disease
5101	13/11/06	M	60	10	Non-Hodgkin's lymphoma
5201	04/12/06	M	50	14	Hodgkin's disease
5301	04/12/06	M	68	32	Testis (seminoma)
5401	04/12/06	F	54	16	Hodgkin's disease
5501	04/12/06	M	61	24	Testis (teratoma)
5601	04/12/06	M	55	28	Testis (seminoma)
5701	11/12/06	M	58	30	Testis (teratoma)
5801	11/12/06	M	52	24	Testis (seminoma)
Mean			48.9 ± 1.74	17.0 ± 1.62	

3.2.4 The G_2 Chromosomal Radiosensitivity Assay

The assay described herein, was based upon the method described by Scott *et al* (1996). Cell culture was carried out as described in Section 2.2.3 but with the following changes: For each cancer survivor two culture flasks were set up and labelled as 'G_2 assay irradiated' and 'G_2 assay control'. For each culture flask 2 ml of blood was added to 18 ml of culture medium and the flasks were placed in the 37°C CO_2 incubator for 72 hours culture. After exactly 48 hours of culturing 15 ml of the spent medium was removed using pre-warmed pipettes, and this medium was replaced with 15 ml of fresh pre-warmed, pre-gassed medium. The flasks were mixed by gentle inversion before been placed back into the incubator with the caps loose.

Samples were irradiated as described in Section 2.2.4. Following irradiation flasks were transported back to the laboratory and placed back in the incubator. After a recovery period of exactly 30 min, 200 µl of pre-warmed KaryoMax colcemid® was added to the culture flasks, which were then mixed gently by inversion and returned to the incubator.

Cell harvesting and slide preparation and staining were carried out as detailed in sections 2.2.6 and 2.2.7, respectively.

3.2.5 Scoring Metaphase Cells

Prior to any actual sample analysis, each microscope user scored the same 50 cells from a sample collected for an earlier study. This scoring check ensured that the same scoring criteria were applied throughout the study and eliminated any scorer bias. Two Cytogeneticists, using either the Zeiss Axioplan 2 imaging microscope linked to image analysis equipment or a conventional Nikon© halogen microscope, scored 50 cells per irradiated sample using different slides, giving a total of 100 scored cells. A Student t-

test was utilised to measure variation in the number of aberrations for each set of 50 cells scored per sample. This monitoring method revealed that any fluctuation between analysts was non-significant ($P = 0.86$).

Upon identifying a metaphase, an assessment was made on whether the cell was suitable for scoring. Cells were checked for reasonably well spread morphology and the absence of scratches. Cells that were discarded contained obviously fewer than 46 chromosomes, had extremely compact morphology or contained many overlapping chromosomes. For the remaining cells, all chromosome pieces were counted and checked for one centromere per chromosome. If 46 chromosome pieces with only one centromere per chromosome were present, these cells were marked as normal and assessed for chromatid damage. Metafer 4.MSearch software was used to improve the efficiency of the manual microscope analysis by calculating the co-ordinates of each cell relevant to the user's microscope. Therefore, there was no need for the user to manually scroll through the whole slide for good quality chromosome spreads, which can be a time-consuming process.

3.2.6 Assessment of Chromatid Damage

Chromatid aberrations were scored using previously outlined criteria (ISCN, 1995) that have been applied in a number of studies (Curwen *et al* 2005; Scott *et al* 1996; Scott *et al* 1999; Smart *et al* 2003). Chromatid gaps were defined as single aligned discontinuities larger than the width of a chromatid and chromatid breaks were defined as distinct dislocation and mis-alignment of the broken segments (Figure 3.1). For each sample, the number of gaps and breaks were combined to produce a total chromatid aberration yield. The other type of aberrations noted but not used to determine the G_2 radiosensitivity score were chromosome gaps and breaks defined as a break through

both chromatid arms (Figure 3.2). Gaps which were smaller than the width of a chromatid were also recorded but did not contribute to the overall aberration score.

There has was some confusion in the classification of 'gaps' and 'breaks' with different laboratories using slightly different criteria. The scoring of chromatid aberrations was discussed in detail at a G_2 assay workshop in 2001 (Bryant *et al* 2002). Although some groups did score aberrations smaller than the width of a chromatid, it was used as a measure of radiosensitivity (Vral *et al* 2002). It is likely that all types of discontinuities in a single chromatid arm were derived from DNA DSBs (Bryant 1984) and evidence obtained from correlating chromatid aberrations with the comet assay suggests that 'small gaps' are indicative of DNA damage (Paz-y-Mino *et al* 2002). For this reason it was likely that these 'small gaps' were biologically significant and some laboratories believed that all visible discontinuities should compose the final G_2 score (Bryant *et al* 2002). It was demonstrated that the results obtained when scoring with and without small gaps were comparable although the variability was increased when gaps smaller than the width of a chromatid were included (Adema *et al* 2003). The authors speculated that the inclusion of small gaps might be less suitable for discriminating between individuals with small differences in chromosomal radiosensitivity (Adema *et al* 2003). The standard procedure at the WRI laboratory was to record data on 'small gaps' but to only publish G_2 scores which comprised of clearly defined breaks and gaps larger than the width of a chromatid.

It was common practice for induced aberration yields to be calculated by subtracting the number of chromatid breaks and gaps in control samples from those in the corresponding irradiated sample. Following a review of current data on spontaneous yields and data cited in many other studies, that laboratory at WRI stopped scoring

unirradiated samples. In control samples the number of chromatid aberrations was usually low (0 - 4 per 100 cells) and did not correspond to the sensitivity of an individual. This decision had substantially decreased the amount of time taken to analyse the cohort. Control cultures were still processed and are available for scoring if necessary.

All results were recorded on the 'G$_2$ Radiosensitivity Score Sheet' (Appendix E) either by hand or using the image analysis electronic form. On completion of sample analysis, all score sheets were audited to ensure that all additions were correct.
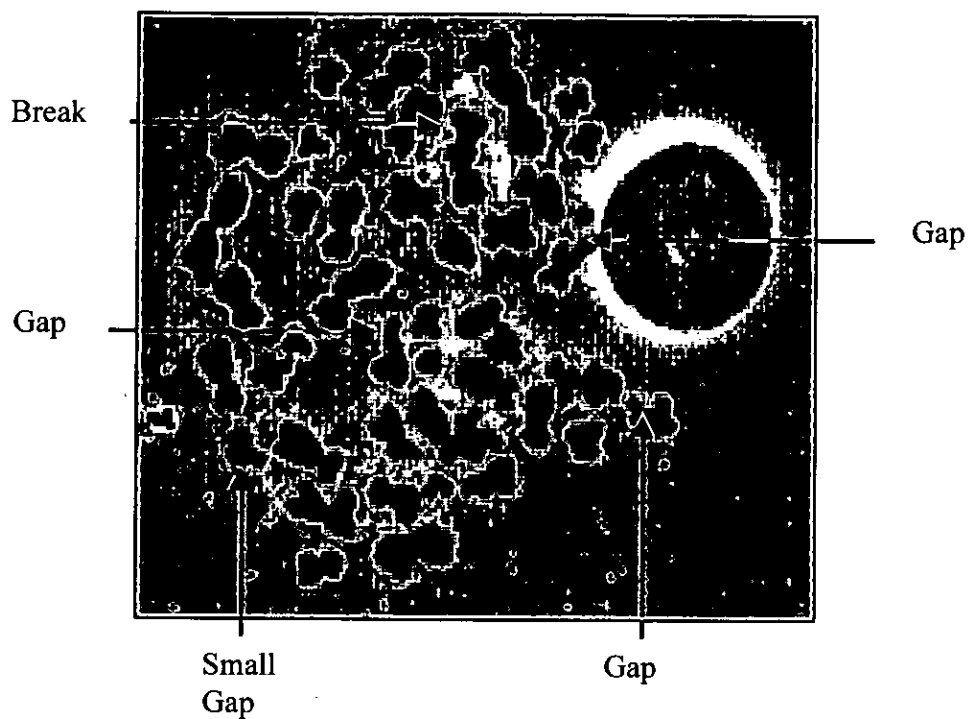
Figure 3.1: Chromatid aberrations observed in metaphase following 0.5Gy X-ray irradiation. The total aberration yield for this cell is four. (The small gap did not contribute to the G_2 radiosensitivity score). Typical of 29 such experiments using the cancer survivor population (n = 29).
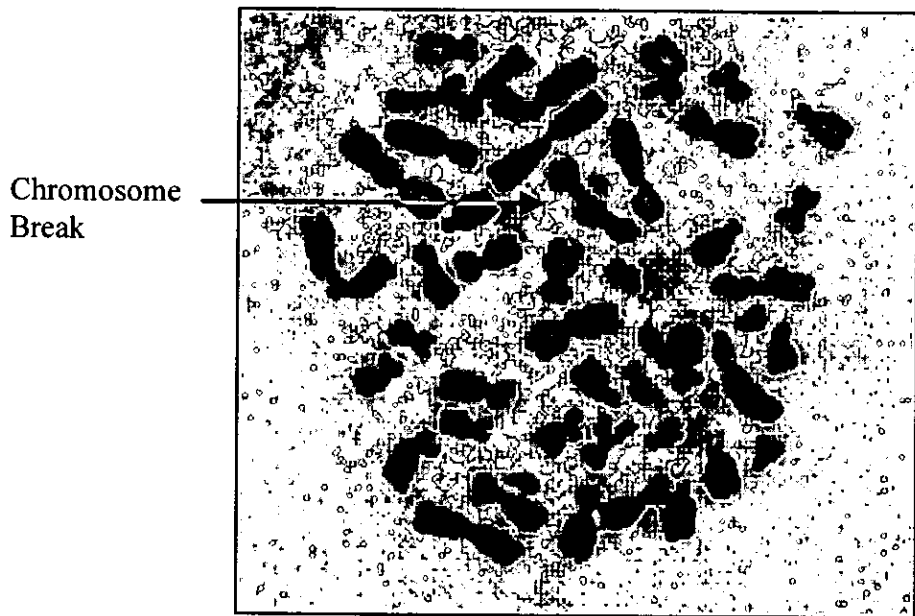
Chromosome
Break

Figure 3.2: Metaphase from an irradiated peripheral blood culture containing a chromosome aberration. Both chromatid arms are broken and mis-aligned. Typical of 29 such experiments using the cancer survivor population (n = 29).

3.2.7 The G_2 + PCC Assay

The protocol adopted was a minor modification of the G_2 chromosomal radiosensitivity assay which differed only by the addition of calyculin A. For this reason it was referred to as the G_2 + PCC assay. For each of the 29 cancer survivors two culture flasks were set up and labelled as 'G_2 + PCC assay irradiated' and 'G_2 + PCC assay control'. The protocol followed was detailed in sections 2.2.3, 2.2.4, 2.2.5, 2.2.6 and 2.2.7. The chosen time point for calyculin A addition was at 60 minutes post-irradiation (see Section 2.3.3). A flow diagram in Figure 3.3 summarised the protocols for the G_2 assay and the G_2 + PCC assay.
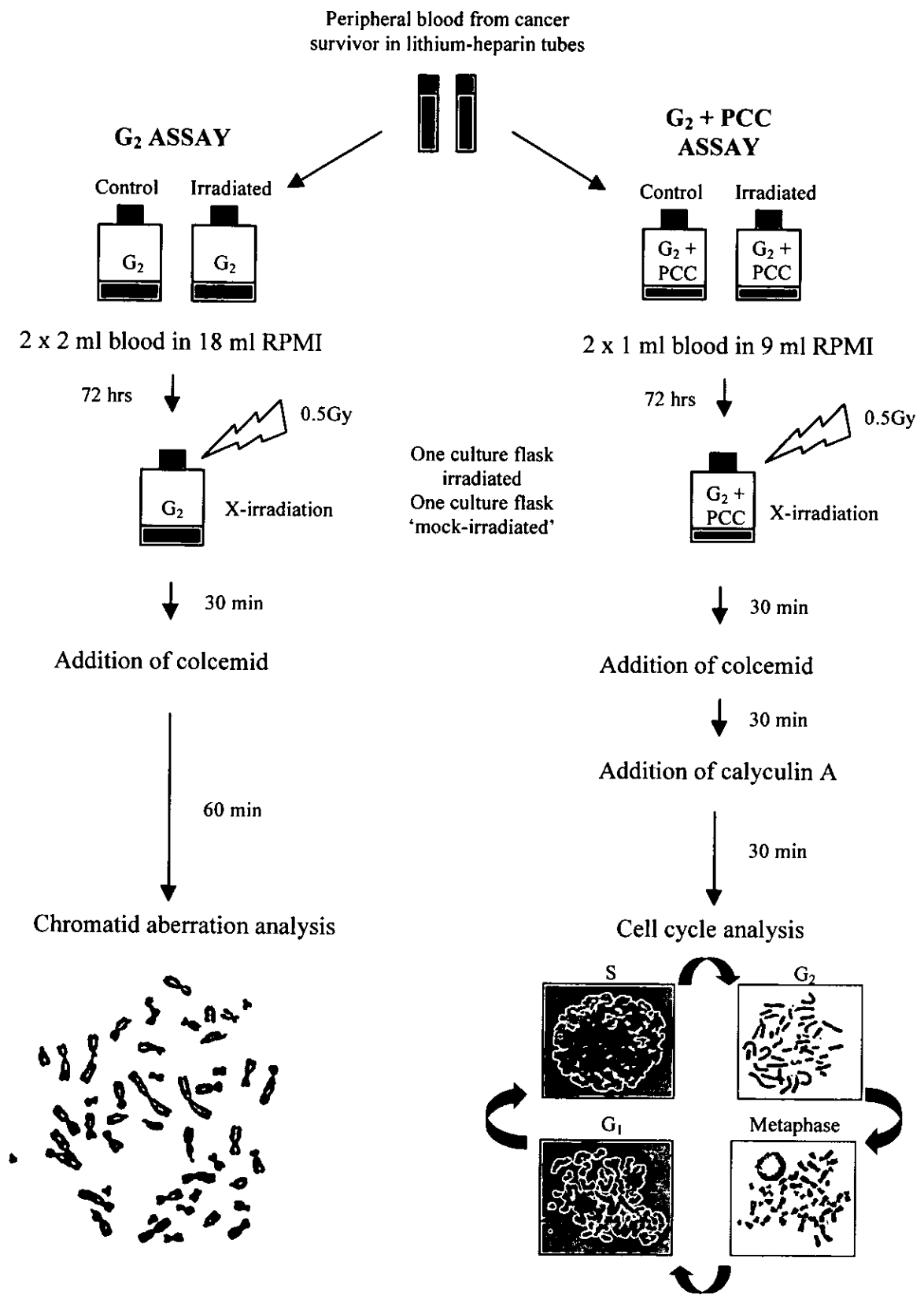
Figure 3.3: The procedure for the G_2 assay and the G_2 + PCC assay. n = 29 for cancer patients and n = 3 for healthy controls.

3.2.8 Measuring G₂ Checkpoint Delay

The automated image analysis machine was used to scan slides and pinpoint chromosome spreads. All detected spreads were analysed sequentially under a x100 lens. PCC-G_2 cells, metaphase cells, PCC-G_1 phase cells, PCC-S phase cells and cells of an unknown origin were marked on the score sheet but only the PCC-G_2 and metaphase cells were used in the ratio calculation. For each sample, a combined total of at least 500 PCC-G_2 and metaphase cells were recorded and used to calculate the ratio of PCC-G_2 to metaphase cells before and after irradiation.

The effect of irradiation on G_2 checkpoint delay (A) was assessed by calculating the proportion of cells in G_2 phase in irradiated cultures *vs* unirradiated cultures:

$$A = \left(\frac{G_2}{G_2 + M} \right)^{Ir} - \left(\frac{G_2}{G_2 + M} \right)^{Un}$$

Where G_2 is the number of PCC-G_2 cells, M is the number of metaphase cells, *Ir* is the proportion of PCC-G_2 cells relative to metaphase cells in the irradiated culture and *Un* is the proportion of PCC-G_2 cells relative to metaphase cells in the unirradiated culture.

In contrast to the scoring of chromatid aberrations, all chromosome pieces were not counted, although it was still vital to check whether the cell appeared intact and had no obvious loss of chromosomes. For each sample, the ratio was recorded and calculated on an electronic form generated by the image analysis machine (see Appendix F). The extent of G_2 checkpoint delay was compared to the G_2 chromosomal radiosensitivity scores to examine any correlation. A strong correlation would suggest that cell cycle

delay, as measured by this technique, directly affects the level of chromatid aberrations at metaphase.

3.2.9 Statistical Methods

The distributions of chromatid gaps and breaks amongst metaphase cells were analysed for approximation to the Poisson distribution and standard errors were calculated taking into account overdispersion as described by Savage (1970) and applied previously at WRI (Smart *et al* 2003). The mean numbers of aberrations, the standard deviation, variance and the ratio of variance to mean were calculated for each donor. A ratio of variance to mean of one would be expected for a Poisson distribution which indicated that every cell had an equal chance of developing an aberration. A value of greater than one indicated that the distribution of aberrations in all samples was overdispersed. Inter-individual and intra-individual variation in aberration frequencies were examined by chi-squared (χ^2) analysis using the formula ($\chi^2 = \Sigma(O-E)^2/EZ$) where O was the observed value of aberrations, E was the expected value of aberrations and Z was the overdispersion factor calculated as the average value of ratio of variance to mean. This analysis was carried out using Microsoft® Excel and a *P* value was obtained using the CHIDIST command =CHIDIST(SUM, DF), where SUM is the sum total of all chi-squared values for the population and DF was the total degrees of freedom. Percentage coefficients of variation (CV) were calculated by dividing the standard deviation by the mean.

Standard errors were calculated by adjusting for overdispersion of chromatid-type aberrations using the appropriate overdispersion factor. The standard error for the cancer survivors was calculated according to the formula $\sqrt{}$(Total number of aberrations x Z)/Total number of samples taken. For internal and transport controls, where repeat

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sampling had occurred, any additional intra-individual variation introduced was also compensated for. Standard errors for control samples were calculated according to the formula √(Mean no. of aberrations per sample x Z x Y)/ Total number of samples taken. Y was the sum of all the values of chi-squared divided by the total degrees of freedom.

The Mann-Whitney U test was used to examine whether the probability distributions were equal in the two sets of data. The null hypothesis that observations in one group tend to be larger than observations in the other group was tested and a *P* value generated. In this project, the Mann-Whitney U test was used to compare the data sets for males and females and to examine differences in cancer types. Spearman's rank correlation analysis was used to test the null hypothesis that there were no relationships between data sets. Two columns of data were inputted into columns and ranked before analysis. A correlation coefficient (R) which falls between +1 and -1 was calculated which indicates the direction of correlation and its strength. An R value of -1 would indicate a strong negative correlation and an R value of +1 would indicate a strong positive correlation. A *P* value was then calculated to determine the significance level. For this project, Spearman's rank correlation analysis was used to examine the relationship between G_2 chromosomal radiosensitivity and G_2 checkpoint delay as measured by the G_2 + PCC assay, as well as any influence of age. All analyses were performed using the Minitab statistical software package (www.minitab.com) and/or Microsoft® Excel.

3.3 RESULTS

3.3.1 G_2 Chromosomal Radiosensitivity in Internal Assay and Transport Controls

Table 3.3 displays the frequencies of radiation-induced chromatid aberrations for all collected samples from the internal assay control and the two transport controls, in addition to their corresponding coefficient of variation. The average ratio of variance to mean for each control was 1.55 for the internal assay control, 1.78 for transport control 1 and 1.45 for transport control 2. These results indicate that the distribution of aberrations in all the control samples is overdispersed. This result is consistent with the pilot Danish trio study carried out at the WRI which gave a ratio of variance to mean which was, on average, 1.5 (Smart *et al* 2003). To take into account any overdispersion the expected values for the yields of chromatid gaps and breaks per 100 metaphases were adjusted by a factor of 1.55, 1.78 and 1.45 for the internal assay control, transport control 1 and transport control 2, respectively. The mean radiation-induced chromatid aberration frequencies per 100 cells $\pm$ standard error were 113.57 $\pm$ 3.35, 131.29 $\pm$ 5.85 and 124.80 $\pm$ 7.47 for the internal assay control, transport control 1 and transport control 2, respectively. The coefficients of variation (CV) were calculated as 20.65%, 31.17% and 29.94% for the internal assay control, transport control 1 and transport control 2, respectively. Chi-squared analysis revealed statistically significant intra-individual variation for the internal assay control ($\chi^2_6 = 18.74$, $P = 0.005$), transport control 1 ($\chi^2_6 = 42.95$, $P < 0.001$), and transport control 2 ($\chi^2_4 = 30.85$, $P < 0.001$).

Table 3.3: Radiation-induced chromatid aberration frequencies in internal assay and transport control donors. n = 29 for cancer patients and n = 3 for healthy controls.

Donor	Chromatid aberrations per 100 cells at sampling shipment								Mean aberration yield ± S.E. per 100 cells	CV (%)
	1	2	3	4	5	6	7	8		
Internal assay control	132	126	150	85	-	97	111	94	113.57 ± 3.35	20.65
Transport control 1	149	123	*	75	160	143	82	187	131.29 ± 5.85	31.17
Transport control 2	148	152	-	61	-	-	123	140	124.80 ± 7.47	29.94

S.E. = standard error, CV = coefficient of variation, - = sample not collected. * = sample not analysed due to infection of culture.

3.3.2 The Relationship between G_2 Checkpoint Delay and G_2 Chromosomal Radiosensitivity in the Internal Assay Control

This individual was sampled on seven occasions for the G_2 assay and blood was cultured for both the G_2 assay and the G_2 + PCC assay on four of those occasions. Even though significant intra-individual variation was found for all seven samples, as shown in Section 3.3.1, when the four samples cultured for both the G_2 assay and the G_2 + PCC assay were analysed in isolation no significant intra-individual variation for G_2 chromosomal radiosensitivity was revealed (χ^2_3 = 4.70, P = 0.195), although a CV of 14.67 was calculated. A scatter plot of the radiation-induced chromatid aberration frequencies and the corresponding G_2 checkpoint delay for the four samples is illustrated in Figure 3.4. Although only four samples were taken a trend is suggested. However, Spearman's rank correlation analysis revealed there was no significant relationship between G_2 checkpoint delay and radiation-induced chromatid aberrations in this individual (r = -0.800, P = 0.200).

Table 3.4: The radiation-induced chromatid aberration frequencies and the corresponding value of G_2 checkpoint delay for the internal assay control.

Sample	Aberration yield per 100 cells	CV^1 (%)	G_2 delay^2 (A)	Spearman's rank correlation	
				r^3	P^4
G2NN-1	132		-0.02		
G2NN-2	126		0.16		
		14.67		-0.80	0.20
G2NN-7	111		0.14		
G2NN-8	94		0.40		

^{1}Coefficient of variation for G_2 radiosensitivity, $^2G_2$ delay (A) was determined by subtracting the proportion of PCC-G_2 cells relative to metaphase cells in the unirradiated culture from the proportion in the irradiated culture, ^{3}Correlation coefficient for G_2 delay, ^{4}Significance level achieved when using Spearman's rank correlation analysis.

Figure 3.4: Correlation between G_2 checkpoint delay (A), as measured by the G_2 +

PCC assay, and chromatid aberration frequencies for the internal assay control. A is

calculated by subtracting the proportion of PCC-G_2 cells relative to metaphase cells in

the unirradiated culture from the proportion in the irradiated culture.

3.3.3 The Relationship between G_2 Chromosomal Radiosensitivity and G_2 Checkpoint Delay in the Cancer Survivor Group.

Table 3.5 illustrates the radiation-induced chromatid aberration frequencies and the corresponding level of G_2 checkpoint delay for the cancer survivor group. The average ratio of variance to mean for the cancer survivor samples was 1.73 indicating that the distribution of aberrations in all the cancer survivors is overdispersed. The mean aberration frequency was 137.21 ± 2.86 per 100 cells and a CV of 25.3% was determined for inter-individual variability. In addition, chi-squared analysis revealed a statistically significant difference between the samples at the 0.05 significance level ($\chi^2_{28} = 142.09$, $P < 0.001$). The distribution of the radiation-induced chromatid aberration frequencies is in Figure 3.5.

A scatter plot of the radiation-induced chromatid aberration frequencies and the corresponding G_2 checkpoint delay for each sample is illustrated in Figure 3.6. No significant relationship was observed between G_2 checkpoint delay and chromatid aberration frequency ($r = -0.206$, $P = 0.284$).

Table 3.5: Details of the cancer survivor group including radiation-induced G_2 aberration frequencies and the corresponding level of G_2 checkpoint delay.

Cancer Survivor ID	Sex	Age at sampling (years)	Cancer diagnosis	Aberration Yield per 100 cells	G_2 delay[1] (A)
2901	M	41	Hodgkin's disease	147	-0.20
3001	M	45	Hodgkin's disease	102	-0.29
3101	F	47	Hodgkin's disease	153	-0.10
3201	F	32	Neuroblastoma	123	0.09
3301	F	62	Non-Hodgkin's lymphoma	123	0.33
3401	F	41	Non-Hodgkin's lymphoma	74	0.20
3501	F	44	Non-Hodgkin's lymphoma	110	0.32
3701	F	34	Hodgkin's disease	170	0.12
3801	F	41	Hodgkin's disease	142	0.06
3901	F	61	Non-Hodgkin's lymphoma	95	0.16
4001	M	43	Non-Hodgkin's lymphoma	172	-0.25
4101	F	61	Hodgkin's disease	114	-0.16
4201	M	48	Hodgkin's disease	135	-0.19
4301	M	41	Hodgkin's disease	127	0.08
4401	M	56	Non-Hodgkin's lymphoma	107	0.10
4501	F	55	Non-Hodgkin's lymphoma	105	-0.20
4601	F	32	Wilms' tumour	183	0.27
4701	M	43	Hodgkin's disease	98	0.32
4801	M	52	Hodgkin's disease	204	-0.05
4901	M	47	Hodgkin's disease	187	-0.08
5001	M	38	Hodgkin's disease	124	0.05
5101	M	60	Non-Hodgkin's lymphoma	180	-0.12
5201	M	50	Hodgkin's disease	206	0.32
5301	M	68	Testis (seminoma)	101	0.39
5401	F	54	Hodgkin's disease	133	-0.18
5501	M	61	Testis (teratoma)	113	0.06
5601	M	55	Testis (seminoma)	134	0.20
5701	M	58	Testis (teratoma)	165	0.04
5801	M	52	Testis (seminoma)	152	0.26
Median	-	**48**	-	**133**	**0.05**

[1]G_2 delay (A) was determined by subtracting the proportion of PCC-G_2 cells relative to metaphase cells in the unirradiated culture from the proportion in the irradiated culture.
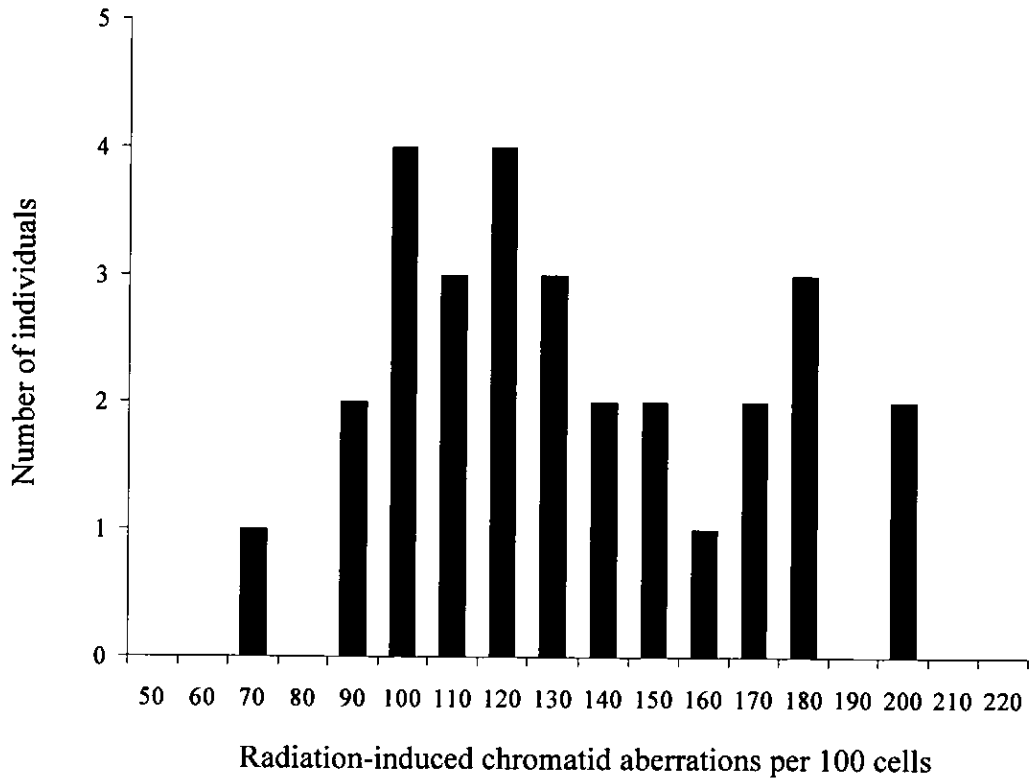
Figure 3.5: Radiation-induced chromatid aberration frequencies in the cancer survivor group. Mean level of chromatid aberrations = 137.21 ± 2.86 per 100 cells, n = 29.
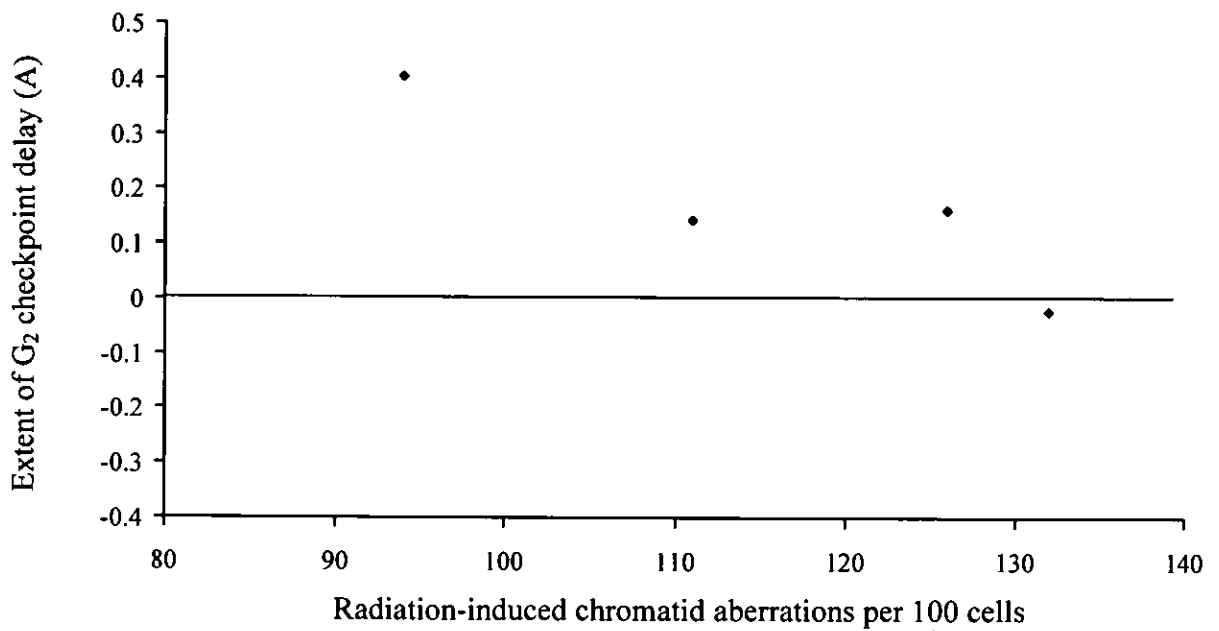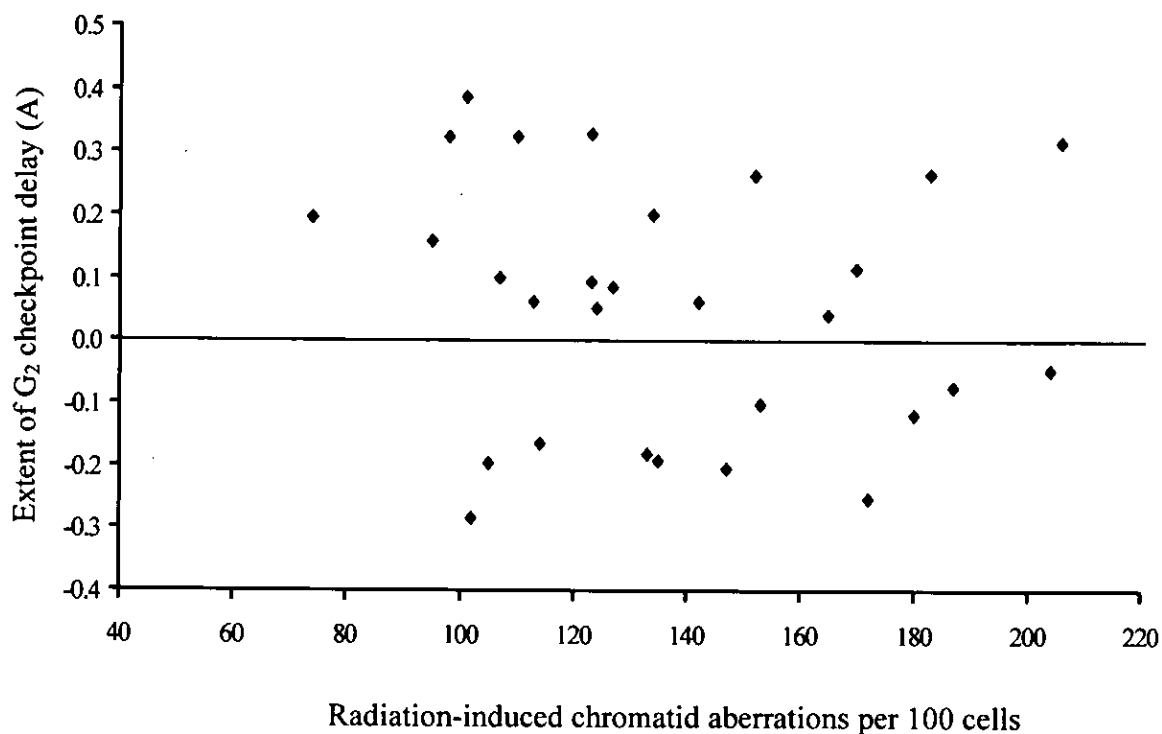
Figure 3.6: Correlation between G_2 checkpoint delay (A), as measured by the G_2 + PCC assay, and chromatid aberration frequencies in the cancer survivor group. A is calculated by subtracting the proportion of PCC-G_2 cells relative to metaphase cells in the unirradiated culture from the proportion in the irradiated culture, n = 29.

3.3.4 The Influence of Age, Gender and Cancer Type upon G_2 Chromosomal Radiosensitivity and G_2 Checkpoint Delay

Spearman's rank correlation analysis revealed that there was no significant correlation between G_2 chromosomal radiosensitivity and age (r = -0.207, P = 0.282) and no significant correlation between G_2 checkpoint delay and age (r = 0.057, P = 0.767). A scatter plot of the age of each survivor at sampling and the corresponding chromatid aberration score is illustrated in Figure 3.7 and a scatter plot of the age of each survivor and the corresponding G_2 delay value is shown in Figure 3.8.

Comparison of data sets using Mann-Whitney U test revealed that there were no significant differences between genders for either G_2 chromosomal radiosensitivity (P = 0.241) or G_2 checkpoint delay (P = 0.479) (Table 3.6). The distribution of radiation-induced chromatid aberrations according to gender is illustrated in Figure 3.9. Figure 3.10 shows a scatter plot of the relationship between G_2 chromosomal radiosensitivity and G_2 checkpoint delay according to gender.

Dividing the cancer type into two groups as follows: haematological (Hodgkin's/non-Hodgkin's) versus other cancers (testis/wilms'/neuroblastoma) resulted in no significant differences between the two groups for either G_2 chromosomal radiosensitivity (P = 0.879) or G_2 checkpoint delay (P = 0.067) when using the Mann-Whitney U test (Table 3.6). The distribution of radiation-induced chromatid aberrations according to cancer type is illustrated in Figure 3.11. Figure 3.12 shows a scatter plot of the relationship between G_2 chromosomal radiosensitivity and G_2 checkpoint delay according to cancer type.
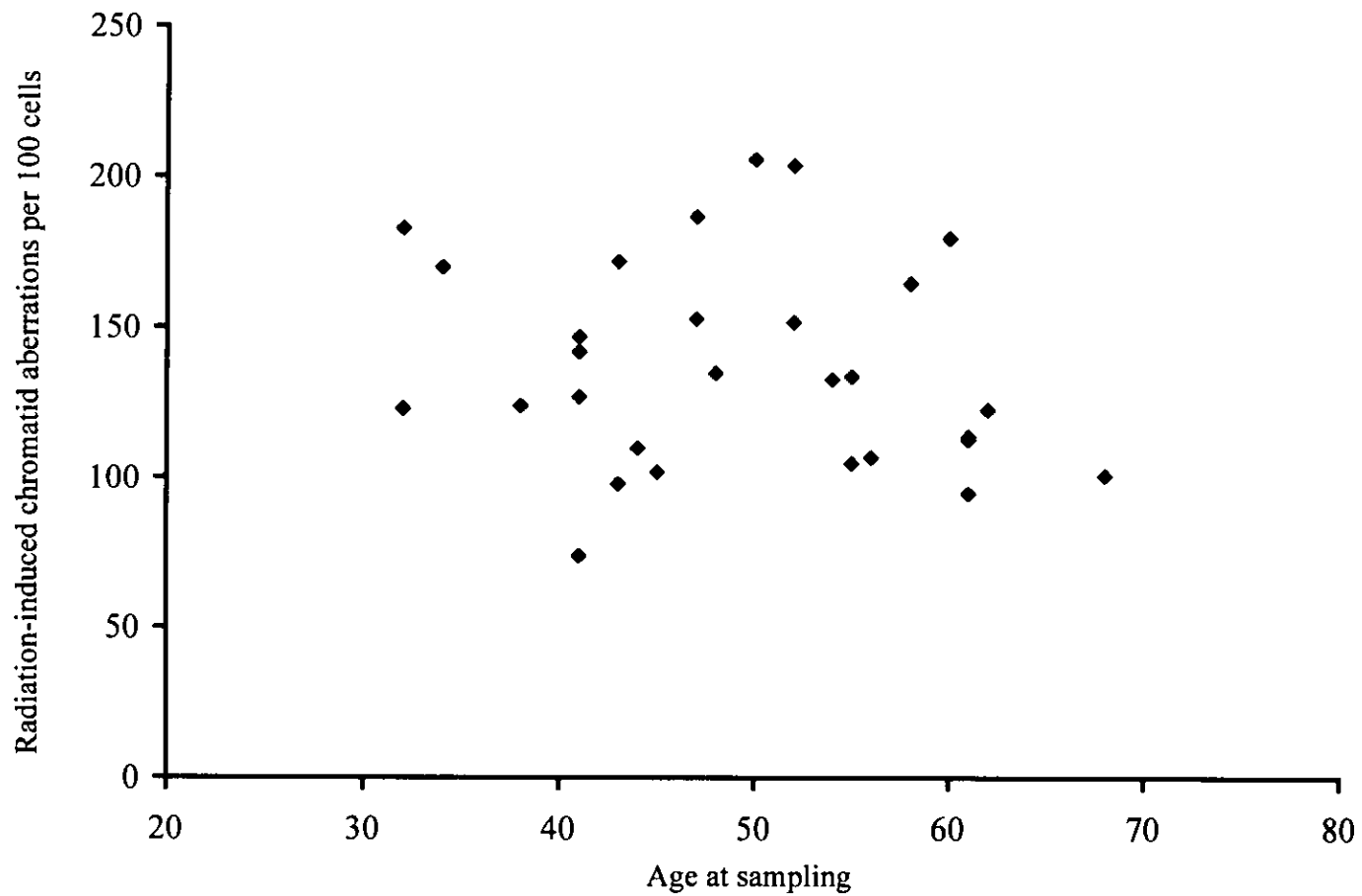
Figure 3.7: Correlation between age at sampling and radiation-induced chromatid aberration frequencies for the cancer survivor group, n = 29
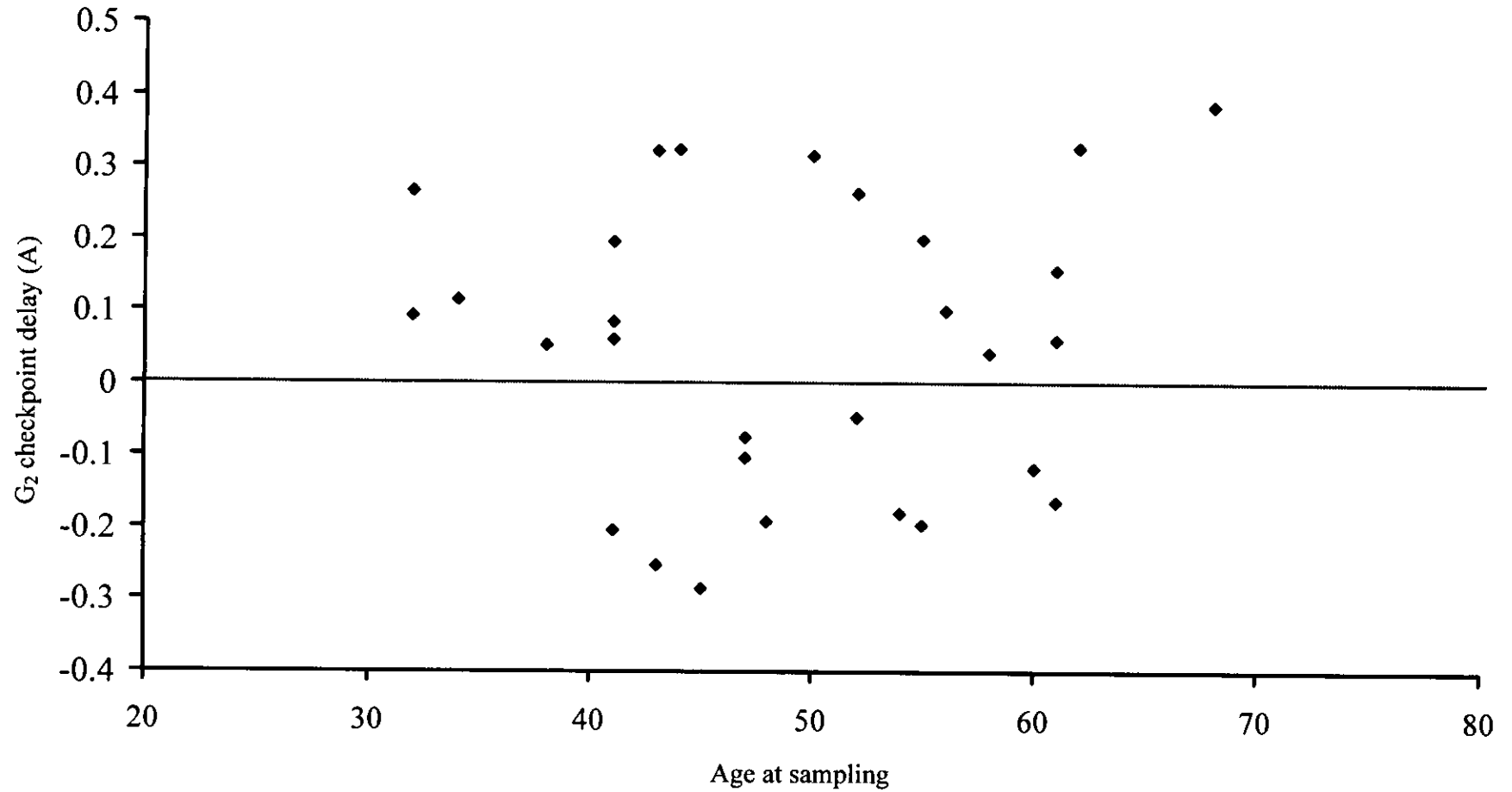
Figure 3.8: Correlation between age at sampling and G_2 checkpoint delay (A) for the cancer survivor group. A is calculated by subtracting the proportion of PCC-G_2 cells relative to metaphase cells in the unirradiated culture from the proportion in the irradiated culture, n = 29.

Table 3.6: G_2 chromosomal radiosensitivity and G_2 checkpoint delay according to gender and cancer type, n = 29.

Endpoint	Subjects	Median (range)	*P* value, Mann-Whitney U-test
G_2 chromosomal radiosensitivity	Male survivors	135 (98 – 206)	0.241
	Female survivors	123 (74 – 183)	
	Haematological cancer (Hodgkin's/non-Hodgkin's)	130 (74 – 206)	0.879
	Other cancer (testis/wilms'/neuroblastoma)	134 (101 – 183)	
G_2 checkpoint delay	Male survivors	0.052 (-0.285 – 0.386)	0.479
	Female survivors	0.061 (-0.197 – 0.329)	
	Haematological cancer (Hodgkin's/non-Hodgkin's)	0.011 (-0.285 – 0.329)	0.067
	Other cancer (testis/wilms'/neuroblastoma)	0.200 (0.041 – 0.386)	
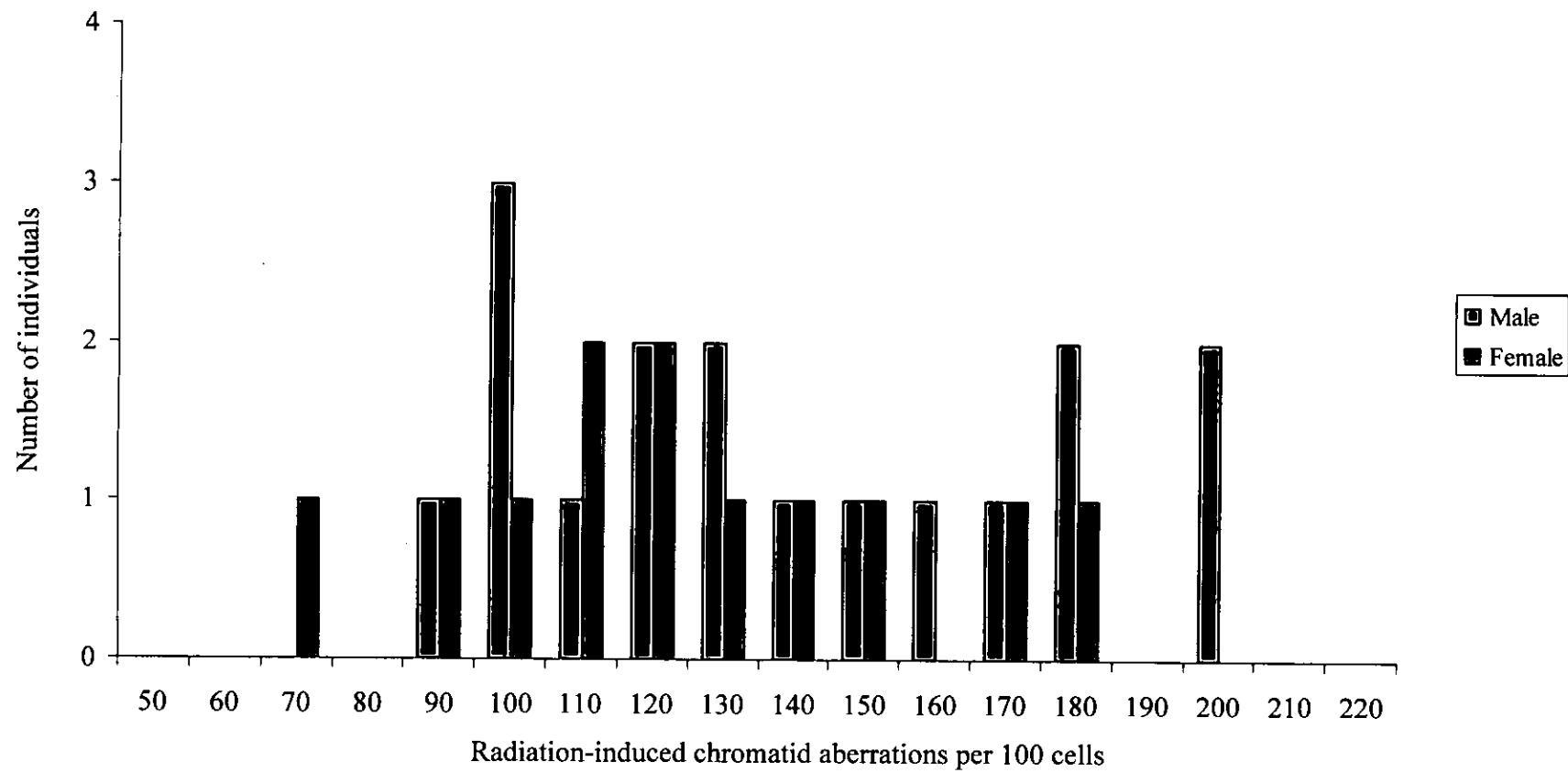
Figure 3.9: Distribution of radiation-induced chromatid aberrations according to gender in the cancer survivor group, n = 29.
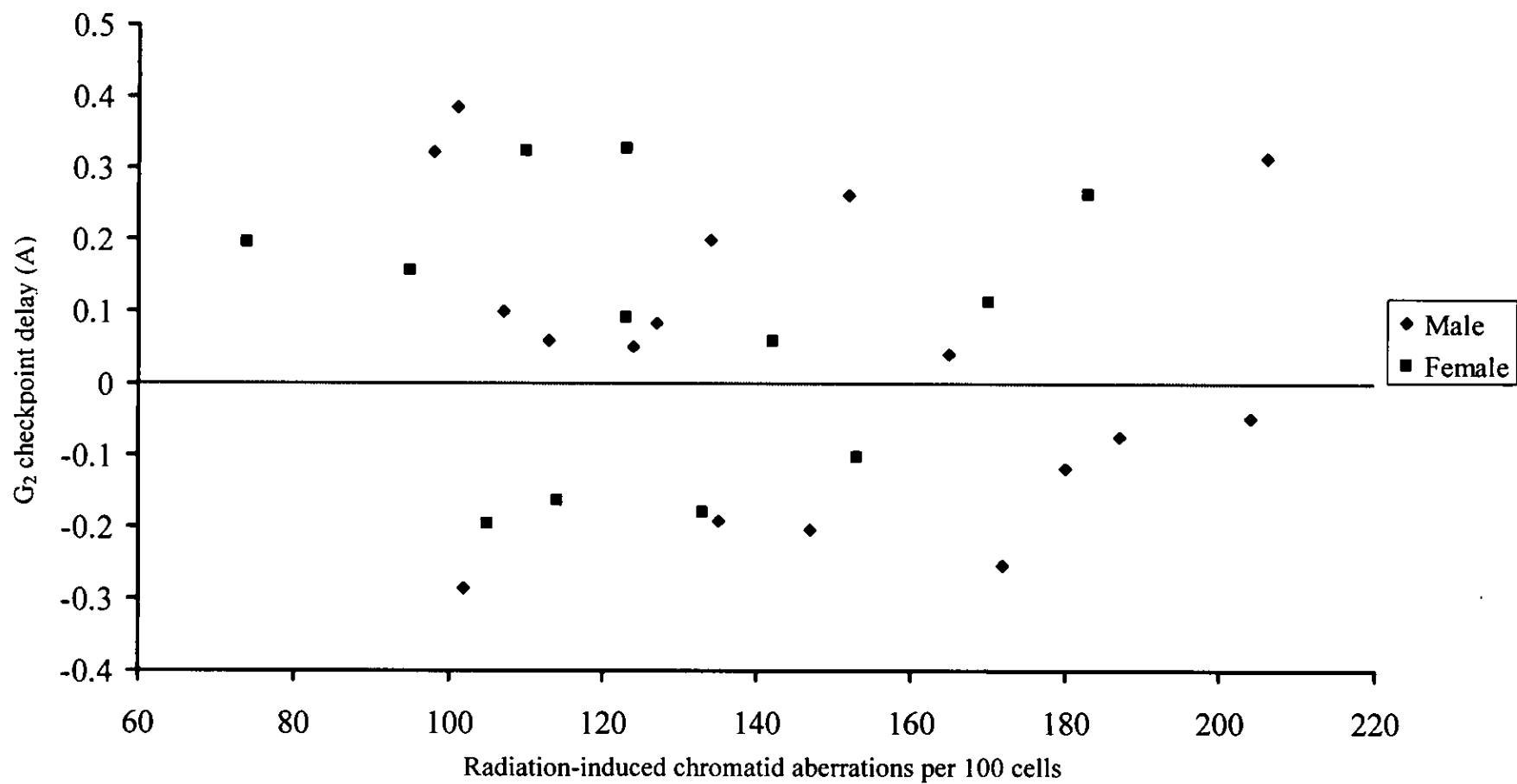
Figure 3.10: The relationship between G_2 chromosomal radiosensitivity and G_2 checkpoint delay according to gender in the cancer survivor group, n = 29.
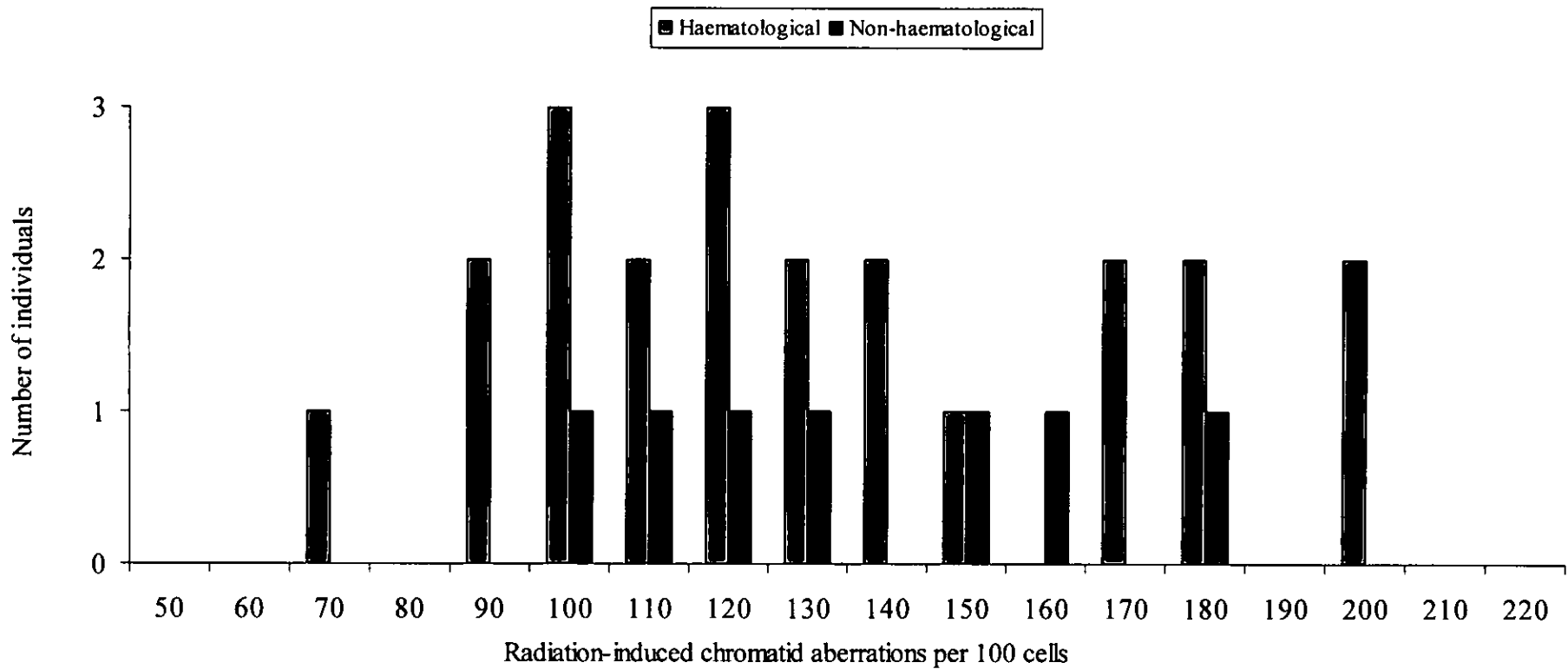
Figure 3.11: Distribution of radiation-induced chromatid aberrations according to cancer type in the cancer survivor group, n = 29.
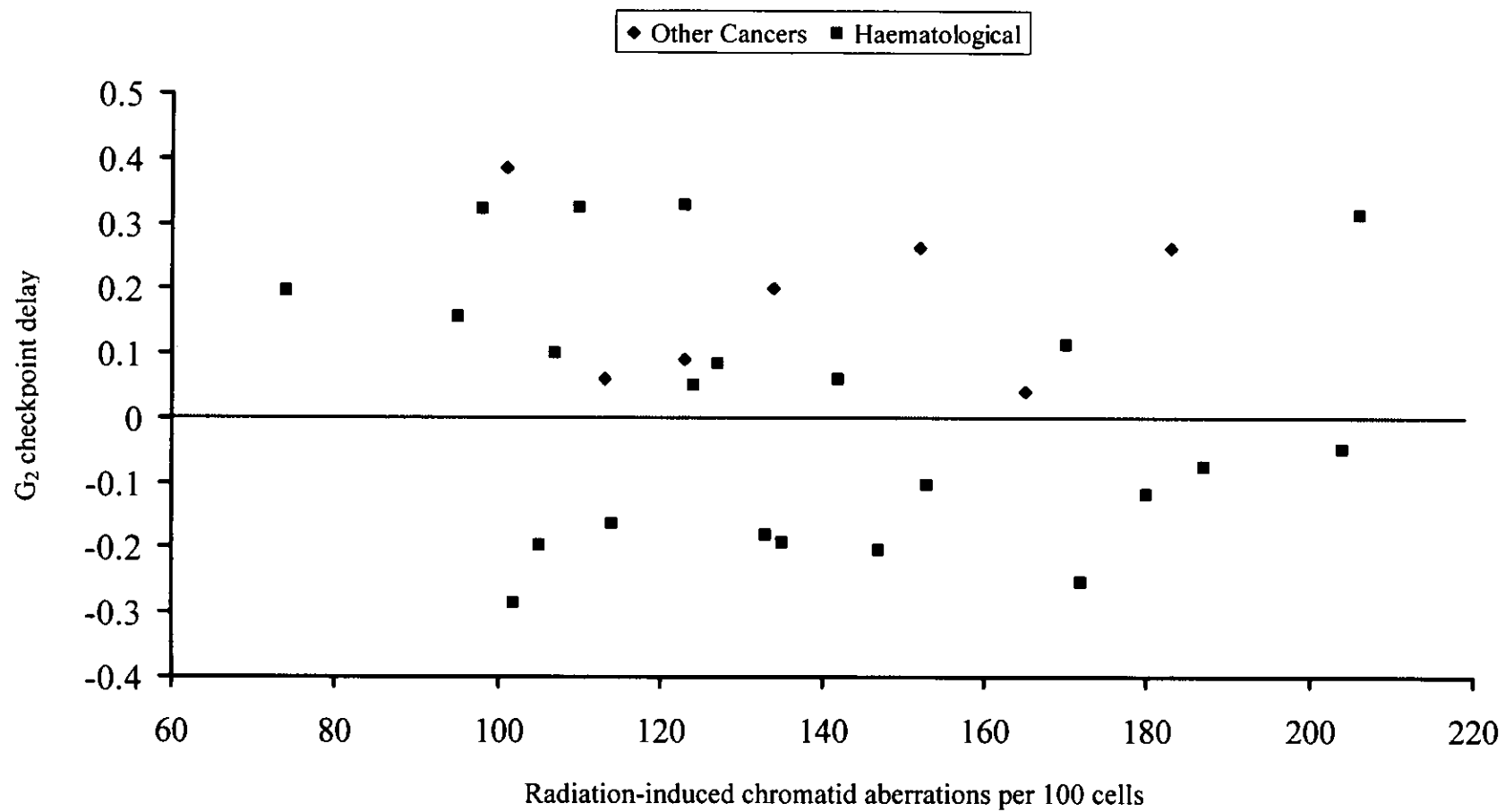
Figure 3.12: The relationship between G_2 chromosomal radiosensitivity and G_2 checkpoint delay according to cancer type in the cancer survivor group, n = 29.

3.4 DISCUSSION

3.4.1 G_2 Chromosomal Radiosensitivity in Internal Assay and Transport Controls

Investigations into the effect of *in vitro* radiation exposure on cells from patients with cancer prone syndromes suggested that elevated G_2 chromosomal radiosensitivity is associated with cancer predisposition (Bender *et al* 1985; Parshad *et al* 1983; Parshad *et al* 1993; Rary *et al* 1974; Sanford *et al* 1987; Sanford *et al* 1989; Sanford *et al* 1990; Shiloh *et al* 1989; Taylor *et al* 1975; Taylor 1978). More recent studies have revealed elevated levels of chromatid damage in a variety of cancer types in comparison to healthy control groups (Baeyens *et al* 2002; Baria *et al* 2001; Baria *et al* 2002; De Ruyck *et al* 2008; Howe *et al* 2005b; Papworth *et al* 2001; Parshad *et al* 1996; Riches *et al* 2001; Scott *et al* 1994a; Scott *et al* 1999; Terzoudi *et al* 2000). Regarding assessment of cancer risk, the G_2 chromosomal radiosensitivity assay has proved less useful in the clinical setting due to considerable overlap between patients and normal individuals in the vast majority of studies. This overlap, coupled to doubts regarding reproducibility of repeat samples, signifies that it may not be useful in determining risk at the individual level especially after only one blood sample (Vral *et al* 2002; Vral *et al* 2004). However, providing that the inter-individual variation exceeds the intra–individual variation, as shown by Scott *et al* (1999), the G_2 chromosomal radiosensitivity assay is still useful for providing quantification of risk in population based studies.

It is widely recognised that the G_2 chromosomal radiosensitivity assay is technically exacting and requires validating before setting up in the laboratory. In the WRI laboratory, G_2 assay reproducibility of separate samples from the same donor was confirmed and revealed that intra-individual variation was non-significant for seven out of the nine healthy donors sampled (Smart *et al* 2003). However, χ^2 analysis revealed statistically significant variation in two of the donors, although removal of the highest

G_2 score sampling point for each of these donors resulted in non-significant variation (Smart *et al* 2003). Similarly, in the 2005 Danish trio study that was undertaken at the WRI laboratory, the intra-individual variation in the internal assay control was found to be non-significant and a CV of 13.56% was reported (Curwen *et al* 2005). However, when this individual was sampled a further seven times, as part of this project, an increased CV of 20.65% was calculated for these seven samples which was confirmed as statistically significant using χ^2 squared analysis. Moreover, results for the two transport controls also showed statistically significant intra-individual variation.

Relatively stable intra-individual variation, with CVs in the range of 7 –10%, has been reported by the PICR group in four separate studies (Baria *et al* 2001; Papworth *et al* 2001; Roberts *et al* 1999; Scott *et al* 1999), as well as in other laboratories (Riches *et al* 2001). Validation of the technique at the Dublin Institute of Technology also revealed reproducible G_2 assay scores in three out of four healthy donors producing CVs between 4.61% and 5.1%. However, one donor had a CV of 22.9%, which was statistically significant (Howe *et al* 2005b). An investigation at Ghent University, Belgium, in which two individuals gave blood on nine separate occasions over a period of one year revealed that intra-individual variability was not significantly different from the inter-individual variability (Vral *et al* 2002) corroborating the findings of other studies (Baeyens *et al* 2002; Baria *et al* 2002). In addition, an individual previously determined to be radiosensitive using the 90th percentile cut-off gave radiosensitivity scores in the normal range at two subsequent repeat sampling points. A follow-up study conducted over a period of three years in which 14 donors were repeatedly sampled, revealed non-significant variation in three out of the four donors that had multiple sampling (5 – 15 repeat samples) (Vral *et al* 2004). This suggests that there is good reproducibility for three out of four of these individuals.

Many laboratories have now reached the consensus that a single sample is insufficient to ascertain the G_2 chromosomal radiosensitivity of an individual and that multiple blood sampling of the same individual may be required to make definitive conclusions (Bryant *et al* 2002; Vral *et al* 2002; Vral *et al* 2004). Vral *et al* (2004) speculate that a blood sample taken on a single occasion may not be reproducible because the ratio of lymphocyte subsets may change with time and blood composition is influenced by hormone levels, diet and immune status. Support for this comes from a number of *in vivo* and *in vitro* studies which demonstrate that hormone levels influence radiosensitivity (Kanda and Hayata 1999; Ricoul and Dutrillaux 1991; Ricoul *et al* 1997; Roberts *et al* 1997). In the WRI 2005 Danish trio study, it was suggested that the significant intra-individual variation observed in the transport control was caused by hormonal changes due to the donor becoming pregnant during the study (Curwen *et al* 2005). Interestingly, analysis of the first five samples received pre-pregnancy revealed no significant variation hinting at a hormonal effect due to pregnancy (Curwen *et al* 2005). This individual acted as transport control 1 in this study. Subsequent re-sampling of transport control 1 for this current project, who was not pregnant at any stage, revealed statistically significant variation between the seven samples indicating that intra-individual variation is more likely to be an intrinsic characteristic of transport control 1 and not linked to pregnancy.

There have been some suggestions that G_2 score may be influenced by a transport effect (Bryant *et al* 2002; Roberts *et al* 1999; Scott *et al* 1999). Although a transportation effect cannot be completely discounted, the significant variability of the WRI internal assay control, in which blood did not leave the laboratory, suggests that variability may be an intrinsic characteristic of all three of the control donors used for this project. Further support for the intrinsic nature of intra-individual variation in aberration yields

comes from studies which have investigated inter-experimental parameters. When multiple cultures are set up from the same blood sample, high levels of assay reproducibility have been observed at WRI (Smart *et al* 2003) and in other laboratories (Vral *et al* 2002). Moreover, multiple sampling of an individual throughout a single day has not revealed significant variation (Docherty *et al* 2007). Thus, it seems unlikely that experimental factors such as irradiation conditions, medium and minor timing differences influence variability in chromatid aberration yields observed when an individual is sampled on separate occasions. Although there is a paucity of available data on individuals with multiple sampling, it is not always the case that when more samples are taken the more likely it is that variation becomes significant. For example, good reproducibility has been demonstrated in two separate donors following 13 (Smart *et al* 2003) and 15 samples (Vral *et al* 2004).

As demonstrated in the majority of studies, significant intra-individual variability only occurs in a proportion of donors (Smart *et al* 2003; Vral *et al* 2002; Vral *et al* 2004). Thus, providing the cohort consists of sufficient numbers then population-based assays can still provide valuable information on the relationship between G_2 radiosensitivity, cancer predisposition and heritability of the G_2 radiosensitivity phenotype. In addition, G_2 radiosensitivity scores have been shown to correlate with gene expression level (Sims *et al* 2007) and adverse radiotherapy response (De Ruyck *et al* 2005) which demonstrates that the G_2 radiosensitivity assay is reliable enough for comparison with other endpoints. However, due to the high intra-individual variability observed in all three control samples used in this study, the reproducibility of the G_2 radiosensitivity scores for each of the cancer survivors is open to conjecture.

3.4.2 The Relationship between G_2 Checkpoint Delay and G_2 Chromosomal Radiosensitivity in the Internal Assay Control

Although only four samples were taken from the internal assay control there is a hint that G_2 to metaphase progression in response to radiation varies within an individual. The suggested trend, although not statistically significant, indicates that an increase in G_2 checkpoint delay correlates with a decrease in chromatid aberrations at metaphase. This is an interesting finding and multiple sampling of a single individual could confirm whether this was a statistical anomaly. It has been postulated that heterogeneity of cell cycle progression rather than DNA repair capacity is responsible for the variation in G_2 chromosomal radiosensitivity observed in normal individuals (Palitti et al 1999). These limited data set of four samples provide some evidence for this hypothesis.

3.4.3 The Relationship between G_2 Chromosomal Radiosensitivity and G_2 Checkpoint Delay in the Cancer Survivor Group

It is known that cells have checkpoints which arrest in response to DNA damage and it has been postulated that these checkpoints exist to allow time for DNA repair (Weinert et al 1994). Ionising radiation delivered in the G_2 phase of the cell cycle can cause a transient ATM-dependent cell cycle arrest which allows time for repair and prevents the progression of damaged cells from G_2 phase into mitosis (Xu et al 2002). Studies employing radiation-induced MIn have revealed that the arrest in G_2 is much less pronounced in cells from patients with AT than in normal cells (Scott et al 1994b; Scott and Zampetti-Bosseler 1982; Zampetti-Bosseler and Scott 1981). The hypothesis was that a low MIn value represents a deficient checkpoint in which less time is allowed for the repair of chromosome damage before the onset of mitosis and thus, higher aberration yields would be observed at metaphase (Scott et al 2003; Terzoudi and Pantelias 1997; Zampetti-Bosseler and Scott 1981). More recent studies utilising PCC

methodology support this idea. The enumeration and classification of G_2 and metaphase cells following irradiation have revealed that a less efficient G_2 checkpoint is responsible for the enhanced G_2 chromosomal radiosensitivity observed in AT cells (Terzoudi *et al* 2005) and in normal lymphocytes pre-treated with the benzene metabolite hydroquinone (Hatzi *et al* 2007). However, investigations of prostate cancer (Howe *et al* 2005a) and *BCRA1* heterozygotes (Febrer *et al* 2008) have revealed that an increase in G_2 checkpoint delay is related to increased chromatid gaps and breaks at metaphase. Heterozygous females (*BRCA1$^{+/-}$*) underwent significantly more delay in G_2 than control females and yet had more chromatid damage at metaphase (Febrer *et al* 2008). The authors suggest that the increased levels of chromatid aberrations observed in *BRCA1$^{+/-}$* females may be a result of reduced repair capability but they do not rule out the possibility that the G_2 checkpoint is less proficient despite the increase in G_2 delay observed. For example, a key finding of this work was that the number of chromatid breaks observed directly in G_2 did not differ between *BRCA$^{+/+}$* and *BRCA$^{+/-}$* females but the reduction of chromatid damage following G_2 to metaphase transition was 32-63% in *BRCA$^{+/+}$* females compared to only 13-28% in *BRCA$^{+/-}$* females. The authors propose that the reduction in damage from G_2 to metaphase is a better endpoint for differentiating between radiosensitive and non-radiosensitive groups rather than the conventional G_2 assay method of observing chromatid aberrations in metaphase which show considerable overlap between patient and control populations in most studies.

Investigations into well characterised mutations in genes such as *ATM* and *BRCA* enable a fully controlled examination of the role of G_2 to metaphase transition in the reduction of chromatid damage at metaphase. Although a G_2 checkpoint defect has been clearly detected in AT patients and linked to their inherent elevated radiosensitivity, less substantial evidence exists for a relationship between G_2 checkpoint delay and G_2

chromosomal radiosensitivity in sporadic cancer patients. An increase in the extent of MIn has been associated with a decrease in the amount of chromatid damage in a mixed population of breast cancer patients and normal females following an acute dose of 0.5 Gy (Scott *et al* 2003). Moreover, this study revealed less G_2 arrest in breast cancer patients in comparison to healthy female controls suggesting a putative G_2 checkpoint defect which may contribute to the enhanced G_2 chromosomal radiosensitivity seen in approximately 40% of cases. However, the authors proposed that only a very small proportion of radiosensitive patients may have a G_2 checkpoint deficiency and conclude that chromatid aberration frequency and the extent of mitotic inhibition may not be causally related.

Prior to using PCC, an earlier study at WRI investigated the relationship between G_2 checkpoint delay and G_2 chromosomal radiosensitivity in survivors of early-onset cancer, their spouses and offspring using mitotic inhibition (Curwen, PhD thesis 2007). There was no significant correlation between G_2 checkpoint delay and chromatid aberration frequency. In the present project, chemically-induced PCC was applied to a group of 29 childhood and young-adulthood cancer survivors in an attempt to investigate any relationship between G_2 checkpoint delay and G_2 chromosomal radiosensitivity. Again, there was no significant correlation between G_2 checkpoint delay and chromatid aberration frequency. Examination of alternative radiosensitivity endpoints such as clonogenic survival have also demonstrated that defective G_2 checkpoints, such as those found in *BRCA1* mutated cell lines, are not linked to radiosensitivity (Xu *et al* 2002). The exact causes of the large inter-individual variation observed when using the G_2 chromosomal radiosensitivity assay have not yet been elucidated. Such variation may be a consequence of disparity between individuals in the initial yield of chromatid aberrations, differences in DNA repair capacity, and

variation in cell cycle control during G_2 to M transition, although other explanations and influencing factors cannot be ruled out. For example, a recent study at the University of St. Andrews provides evidence that inter-individual variation in chromatid break frequency may result from differences in the level of topoisomerase IIα expression (Terry *et al* 2008).

An unexpected finding in the Danish cancer survivor cohort, which was not observed in the initial attempts at applying the PCC technique, was that a number of samples displayed negative G_2 checkpoint delay values indicating a greater proportion of 'metaphase-like' cells in the irradiated sample than in the control sample. A possible explanation is that differentiating between G_2 and metaphase cells based upon a lack of centromeric constriction as seen in other studies (Febrer *et al* 2008; Gotoh *et al* 1995; Hatzi *et al* 2007; Hatzi *et al* 2008; Terzoudi *et al* 2005) is an imperfect technique. A shorter calyculin A incubation of 15 minutes has recently been used to improve the G_2 assay by increasing the mitotic index of blood cultures whilst still resulting in a majority of cells with clear centromeric constriction (Shovman *et al* 2008). The authors suggested that a proportion of cells with 'metaphase-like' morphology and centromeric constriction are in fact cells in G_2 phase which have been artificially condensed.

3.4.4 The Influence of Cancer Type on G_2 Chromosomal Radiosensitivity and G_2 Checkpoint Delay.

The effect of cancer type on G_2 chromosomal radiosensitivity and G_2 checkpoint delay was not a key aim of this MSc project. Moreover, early-onset cancer accounts for less than 2% of all cancers diagnosed in the U.K (Baria *et al* 2002) and thus, comparisons between groups of patients with a specific type of early-onset cancer would have, in practice, proved difficult. Nonetheless, for the purpose of analysis, cancer type was

divided into haematological (Hodgkin's/non-Hodgkin's) and other cancers (testis/wilms'/neuroblastoma). As perhaps might be expected, no significant differences between the two groups were observed for either G_2 Chromosomal Radiosensitivity or G_2 Checkpoint Delay.

The aim of the over-arching study into the genetic consequences of cancer treatment was to investigate the contribution of radiotherapy and/or chemotherapy towards adverse health outcomes in the offspring of survivors of cancer (www.gcct.org). The cancer survivors were primarily recruited based on the likelihood of high doses received to the gonads, hence, the large proportion of Hodgkin's disease and Non-Hodgkin's lymphoma patients in this study. These two malignancies both have a different aetiology to breast, colorectal and lung cancer and crucially, are more likely to be caused by defects other than DNA damage/repair or cell cycle checkpoint deficiency. For example, Hodgkin's lymphoma is caused by a combination of infection with Epstein-Barr Virus (Kapatai and Murray 2007), re-arrangement defects in the immunological system (Mathas 2007) and genomic alterations (Weniger *et al* 2006). Thus, it is possible that low-penetrance cancer predisposition genes, putatively manifest in breast cancer patients as elevated G_2 chromosomal radiosensitivity (Scott *et al* 1999) or to a lesser extent decreased G_2 delay (Scott *et al* 2003), are not discernible in the Danish cancer survivor cohort.

3.4.5 The Influence of Age and Gender upon G_2 Chromosomal Radiosensitivity and G_2 Checkpoint Delay.

Radiation-induced MIn studies by the PICR group have revealed significant age and gender influences. For example, MIn was shown to be significantly greater in female than in male controls (Scott *et al* 2003) and MIn has been shown to decrease with age

(Scott *et al* 1994b; Scott *et al* 2003). Despite MIn declining with age, no relationship between age and chromatid aberration frequency has been uncovered (Scott *et al* 1999). Moreover, sex and/or age differences have not been observed when considering G_2 chromosomal radiosensitivity in breast cancer in other laboratories (Baeyens *et al* 2005; Riches *et al* 2001; Scott *et al* 1999), other cancers (Baria *et al* 2001; De Ruyck *et al* 2008; Sanford *et al* 1996), common variable immune deficiency (Aghamohammadi *et al* 2008) or in clinically normal donors (Borgmann *et al* 2007; Cadwell *et al* 2008; Papworth *et al* 2001). Interestingly, Docherty *et al* (2007) found that G_2 chromosomal radiosensitivity decreased with age but only when chromatid gaps smaller than the width of a chromatid were included in the analysis. When breaks (discontinuities larger than the width of a chromatid) were considered alone, as is the case in many laboratories, no significant correlation was observed (Docherty *et al* 2007). The influence of age has become apparent in head and neck cancer studies with patients in the youngest age groups showing enhanced sensitivity over control groups (De Ruyck *et al* 2008; Papworth *et al* 2001), although no significant correlation between age and chromatid aberration frequency has been established (De Ruyck *et al* 2008). Environmental influences, such as smoking and alcohol, predominate in the older group indicating a lower genetic component compared to the youngest patients. Hence, late-onset cases had similar G_2 scores to controls within the 45 and over age group (Papworth *et al* 2001). In this study of childhood and young-adulthood cancer survivors no significant age or gender effects were found when comparing G_2 checkpoint delay between different sub-groups or when investigating the relationship between radiation-induced G_2 checkpoint delay and G_2 chromatid aberration frequency.

3.4.6 Conclusion

In conclusion, the results of this study have shown that inter-individual variation in G_2 chromosomal radiosensitivity is not driven by variation in G_2 checkpoint delay, at least in this group of cancer survivors. In addition, the results have demonstrated that age, gender and cancer type have no significant influence upon either cell cycle delay or G_2 chromosomal radiosensitivity.

3.4.7 Limitations

As applied in this study, the PCC technique appears to have a number of limitations including difficulty in scoring damage directly in PCC-G_2 cells, doubts over the accuracy and validity of cell cycle categorisation and limitations due to intra-individual variability in both G_2 chromosomal radiosensitivity and G_2 checkpoint delay. For these reasons it was difficult to establish whether the PCC technique has provided useful information on the impact of G_2 to metaphase transition upon the levels of chromatid damage observed in metaphase. However, other studies have shown that the PCC technique can add considerable value to the G_2 radiosensitivity assay with only a minor change to the already established protocol (Febrer *et al* 2008; Shovman *et al* 2008; Terzoudi *et al* 2005).

In normal donors, an increased chromatid aberration frequency was associated with a decrease in mitotic delay induced by 0.02 Gy but not when a dose of 0.3 Gy was used to induce delay (Pretazzoli *et al* 2000). The authors speculate that a saturation effect exists at higher doses. Further evidence for a saturation effect at even higher doses is that the extent of G_2 delay is independent of dose in the range 1 – 10 Gy (Xu *et al* 2002). One possible explanation for the negative results of this study is that the X-ray dose

employed in the G_2 chromosomal assay is too high to uncover subtle differences in G_2 checkpoint delay.

3.4.8 Scope for Future Work

Further experiments could be used to obtain a dose response curve to pinpoint the dose that provides best discrimination for uncovering a possible relationship between G_2 chromosomal radiosensitivity and G_2 checkpoint delay. Results from this study make it difficult to ascertain whether the PCC technique has any advantages over MIn in measuring the extent of G_2 checkpoint delay. The technique of MIn can be carried out quickly without any modification to the G_2 assay and uses the same slides which are used for chromatid aberration analysis making it less expensive and more efficient than PCC analysis. In addition, it is possible to train the Metafer software to automatically scan and score mitotic indices. It would be of interest to compare G_2 checkpoint delay values obtained using PCC with the conventional method of MIn. In this cohort, the intra-individual variation was too variable to assign a definitive score of G_2 chromosomal radiosensitivity on an individual basis and the same level of caution should be applied when assigning a score of G_2 checkpoint delay, especially after a single sample. A well-controlled study on a small number of normal individuals with repeated sampling for both G_2 checkpoint delay and G_2 chromosomal radiosensitivity may be needed to tease out any causative relationship.

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APPENDICES

APPENDIX A: WRI CONSENT FORM

CONSENT FORM FOR BLOOD SAMPLES FOR IN VITRO STUDIES OF EXPOSURE TO RADIATION

I am willing to provide a blood sample.

I understand that the sample will be used for research studies associated with in vitro exposure to radiation. I understand any information pertaining to the sample will be protected by the principles of confidentiality and will conform to the Data Protection Act (1998) and the Human Tissue Act (2004).

Signed: ....Date: ....

Name: ....(first name) .... (family name)
(please print)

Date of Birth: .... Gender: ....

Are you a smoker?   Yes   No   Ex-smoker

Have you ever had any radiotherapy/chemotherapy?   Yes   No

Comments

APPENDIX B: ZEISS AXIOPLAN 2 IMAGING MICROSCOPE WITH A MARZHAUSER MOTORIZED SCANNING STAGE
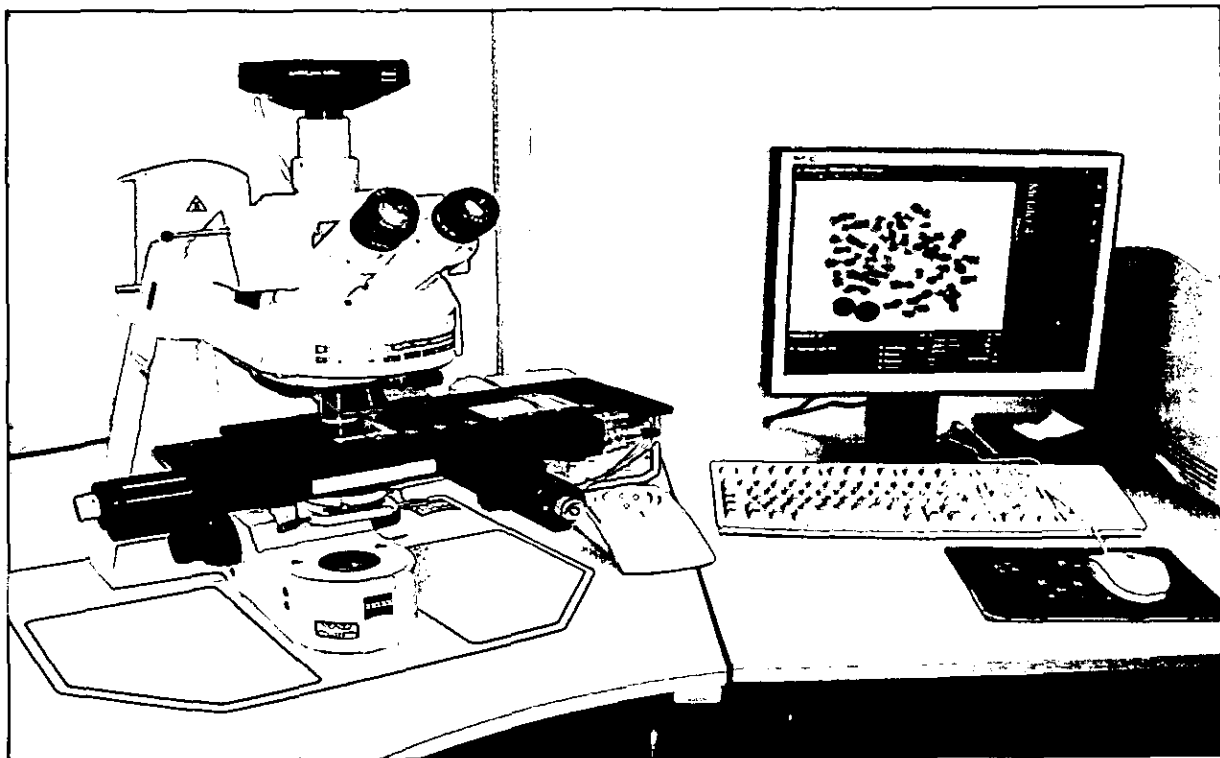
QUESTIONNAIRE

Indication of Genetic Damage Transmitted to Children of Danish Survivors of Childhood Cancer - A Feasibility Blood Collection Study

Institute of Cancer Epidemiology
Danish Cancer Society
Strandboulevarden 49
DK-2100 Copenhagen Ø

Study no.: _____   Date of interview: _____

1. Basic information

1.1 Sex

M F

|__|__|

1.2 Age

Year

|__|__|

2. Cancer in the family

Yes No Not
sure

2.1 Has anyone in your nearest biological family had cancer?

|__|__|__|

(Parents, grandparents, siblings, children, parent's siblings; i.e. aunts and uncles, but not adopted children, stepfamily or family in-laws)

2.2 If yes, please specify:

Family member	Type of cancer/Not sure	ICD-8

3. Smoking habits

3.1 **Are you a current smoker?**
(at least 1 cigarette per day in 6 months or cigar/pipe)

Yes No
|__|__|

3.2 **Have you previously been a current smoker? (defined as above)**

Yes No
|__|__|

3.3 **How much do/did you smoke in average per day/week/month?**

Number of cigarettes
|__|__|

Per day
|__|

Per week
|__|

Per month
|__|

3.4 **Age at start of smoking?**

Age
|__|__|

3.5 **Age at quitting, if former smoker?**

Age
|__|__|

3.6 **Total years of daily smoking?**

Year
|__|__|

4. Medications

4.1 Do you currently use any form of medication?

We ask you about prescription and over-the counter drugs as well as alternative
medicine.

Yes No

|＿|＿|

If yes, please specify the name of the drug(s), duration of use as well as the indication

Drug name	Duration of use in months (m)/years (y)	Indication

4.2 Have you previously received large doses of chemotherapy or similar drugs due to serious illness?

Yes No

|＿|＿|

Drug name	Duration of use in months (m)/years (y)	Indication

5. Use of Hormones (women only)

5.1 Do you use oral contraceptives?

Yes No

|__|__|

If yes, please specify the name of the drug(s) and duration of use

Drug name	Duration of use in months (m)/years (y)

5.2 Do you use any other type of hormones, such as estrogens and/or progesterones?

Yes No

|__|__|

If yes, please specify the name of the drug(s), duration of use and type of hormones

Drug name	Duration of use in months (m)/years (y)	Type of hormones*

* Estrogen only, progesterone only, combination pills, others (please specify)

APPENDIX D: CONSENT FORM AND INFORMATION FOR DANISH FAMILIES

Informed Consent

Indication of Genetic Damage Transmitted to the Children of Danish Survivors of Childhood Cancer - A Feasibility Blood Collection Study

I have read the information brochure, and I hereby confirm that I agree to participate in the study.

Furthermore, I give permission to having my and my child/children's blood drawn.

I understand that participation in the study is entirely voluntary and that I can withdraw my and my child/children's commitment without giving any explanation.

Do you allow your blood sample to be at stored at the Institute of Cancer Biology, Danish Cancer Society, Copenhagen, and to be used in future studies on childhood cancer after renewed approval from the Danish Ethical Committee?

☐ Yes
☐ No

Do you allow your child/children's blood sample to be at stored at the Institute of Cancer Biology, Danish Cancer Society, Copenhagen, and to be used in future studies on childhood cancer after renewed approval from the Danish Ethical Committee?

☐ Yes
☐ No

Date: _____

Name: _____

Signature: _____

∞

Institute of Cancer Epidemiology
Danish Cancer Society
Strandboulevarden 49
DK-2100 Copenhagen, Denmark

Approved by the Danish Scientific Ethical Committee
If a scientific study of a certain disease or disorder includes human beings the purpose of the study must be to prevent or treat the disease. This is included in the regulations of the Scientific Ethical Committee consisting of both medical doctors and laymen. These regulations are confirmed by law. This scientific investigation is approved by the Scientific Ethical Committee and therefore satisfies the ethical requirements of the committee.

Project investigators
Responsible project investigators are Ms. Jeanette Falck Winther, MD and senior researcher and Mr. Jørgen H. Olsen, DMSc and Director, both from the Institute of Cancer Epidemiology, in collaboration with Catherine Rechnitzer, DMSc and in charge of the Late Effect Clinic, Juliane Marie Center, Rigshospitalet, and senior researcher Per Guldberg, Institute of Cancer Biology, Danish Cancer Society. These Danish investigators are part of an international collaboration with researchers from England and the US.

Identity safety
On all blood samples and questionnaires study numbers will replace personal identity numbers. All information gained will be used for tabulations of statistics only where no identification of data at the personal level can be found.

Institute of Cancer Epidemiology
Danish Cancer Society
Strandboulevarden 49
2100 København Ø
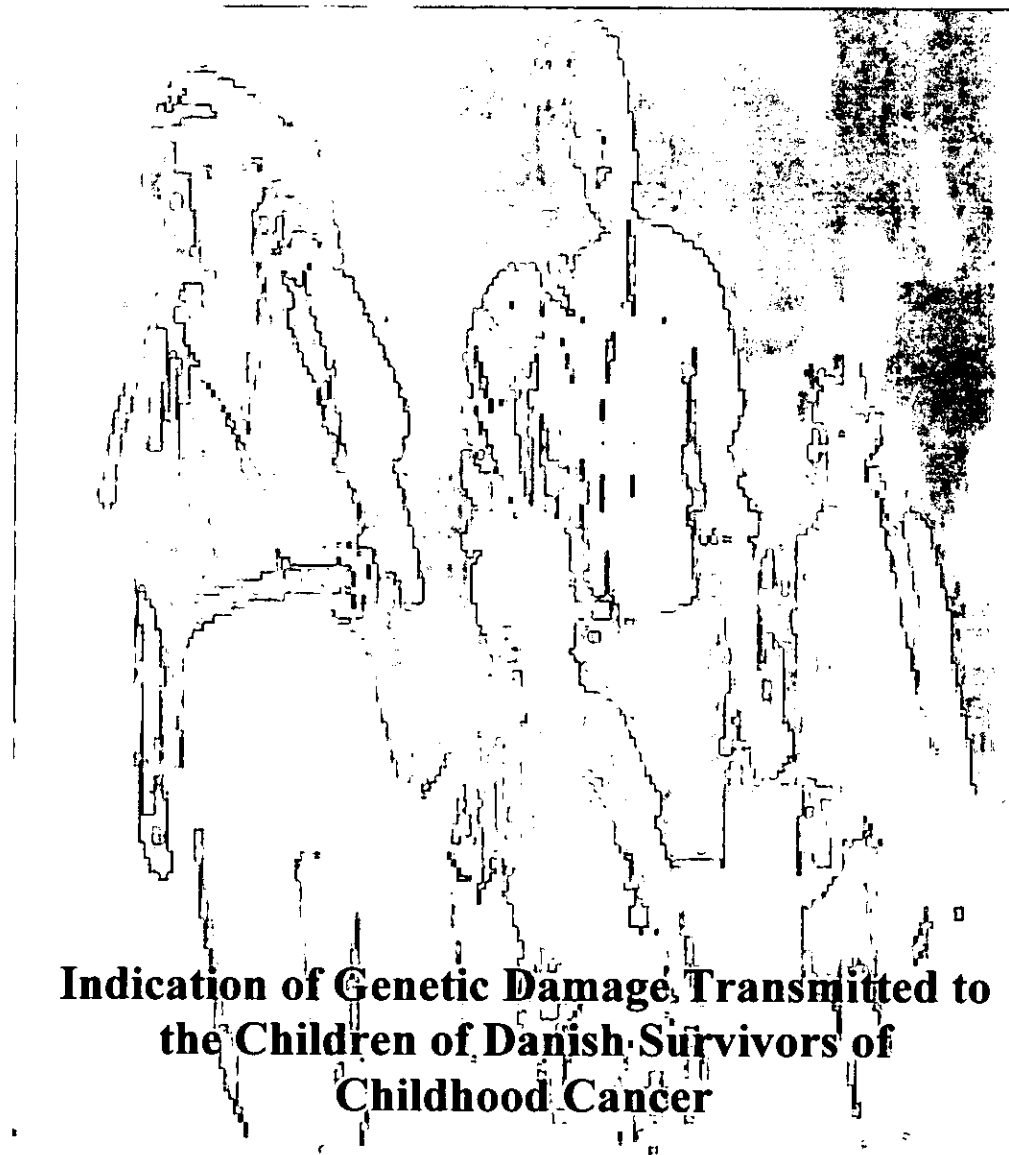
Indication of Genetic Damage Transmitted to the Children of Danish Survivors of Childhood Cancer

Background

It is still not evident whether radiotherapy frequently used to treat childhood and adolescent cancer can damage the germ cells (the sperm or the eggs) and whether such alterations at the genetic level, if introduced, are passed onto the next generation.

Fortunately, it is well documented that offspring of patients treated for childhood cancer bear no increased risk for cancer. This also seems to be the case for other serious disorders as e.g. congenital malformations. Nonetheless, it cannot be ruled out that radiotherapy might introduce alterations in the germ cells at the molecular level (mutations) affecting health of the next generation to a minor degree or leading to no clinical observable manifestation at all.

Aim

This is what we would like to investigate – in a laboratory study at first - enrolling families consisting of the parents, one treated for cancer in childhood or adolescence, and all - or at least one - child.

By thoroughly investigating and comparing variations in the genomic material of blood cells from the mother, father and children it is possible to find changes, if any, in the child's genes, which are introduced by radiation treatment of one of the parents being the cancer survivor.

How you and your family can contribute

If your reply letter indicates that you and your family wish to participate in our investigation the project coordinator and medical doctor Jeanette Falck Winther will call you to get an appointment for a meeting at one of the Pediatric Oncology Outpatient Clinics in Denmark (Monday afternoons). When you arrive at the clinic the doctor will help you fill out a short questionnaire. Afterwards you, your partner and child(ren) will be asked to have a blood sample drawn. Reimbursement of transport costs will of course be provided.

Questionnaire

The questionnaire, which will be filled out by you and your partner, includes information on family history of cancer, smoking habits, medication and hormone use (oral contraceptives). With your permission, information of treatment for your childhood cancer will be abstracted from your medical record and included in the study.

Blood sample

Before the meeting, we mail some analgetic plasters for your child(ren) to minimize pain (instruction included). The blood sample from your partner will be used as a control to ensure that any alterations in the genes of your children, if hereditary, are not associated with radiation treatment.

The blood samples will be analyzed at laboratories at Westlakes Research Institute in England. With the permission of you and your partner, the remaining blood will be frozen down and stored in a biobank at the Institute of Cancer Biology situated at the Danish Cancer Society so that the blood can be used for future studies of childhood cancer.

We would like to stress that the result of the analyses based on bloods from you and your family can only reveal a pattern if combined with results of other families participating in the study. Therefore, you and your family will not receive a letter with the result of the analyses based on your own blood samples. Instead you will receive a newsletter in 18 to 24 months when we have finalized the investigation.

Voluntary and confidential

Participation in the investigation is entirely voluntary and you can at any time withdraw your commitment without giving any explanation. All information will be treated confidentially and will not be available for anyone else but the project investigators.

APPENDIX E: THE WRI G$_2$ RADIOSENSITIVITY SCORE SHEET

Code:-   Irradiated [   ] Control [   ]   Microscope no.:-   Scorer:-
Date:-

Slide	No.	CO-ORD	2N	N	chsg	chsb	chtg	chtb	COMMENTS.
	1								
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	3								
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APPENDIX F: THE WRI PCC SCORE SHEET

PCC cell proportions

Evaluated
24

SlideName	SrchBy	ChngBy	ChngDate	NCells	Undefined	Rejected
KC TEST G2 & P	Adm	Adm	23/09/05	460	432	4

No	CellID	G2	Met	G1	S	Int	Unkn
1	1	1	0	0	0	0	0
2	2	0	1	0	0	0	0
3	3	0	1	0	0	0	0
4	4	0	1	0	0	0	0
5	5	0	1	0	0	0	0
6	6	0	1	0	0	0	0
7	7	0	1	0	0	0	0
8	8	1	0	0	0	0	0
9	9	0	1	0	0	0	0
10	10	0	1	0	0	0	0
11	13	1	0	0	0	0	0
12	14	1	0	0	0	0	0
13	15	0	1	0	0	0	0
14	16	0	1	0	0	0	0
15	17	0	1	0	0	0	0
16	18	0	1	0	0	0	0
17	19	0	1	0	0	0	0
18	21	0	1	0	0	0	0
19	22	0	1	0	0	0	0
20	23	0	1	0	0	0	0
21	24	0	1	0	0	0	0
22	26	0	1	0	0	0	0
23	27	0	1	0	0	0	0
24	28	0	1	0	0	0	0
		4	20	0	0	0	0

Signature of Scorer .... Date ....