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# Cytogenetic Analysis Of Depleted Uranium Exposure And Age-Dependent Susceptibility To Ionizing Radiation

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**CYTOGENETIC ANALYSIS OF DEPLETED URANIUM EXPOSURE AND AGE-DEPENDENT SUSCEPTIBILITY TO IONIZING RADIATION**

by

**MARINA V. BAKHMUTSKY**

**DISSERTATION**

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

**DOCTOR OF PHILOSOPHY**

2013

MAJOR: BIOLOGICAL SCIENCES

Approved By:

\_\_\_\_\_  
Advisor

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Date

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## DEDICATION

My parents taught me that education is not an optional opportunity, but is an absolutely essential part of a human being. They have sacrificed much to allow me the opportunity to pursue this doctoral degree and I am thankful for all their unconditional love and support, therefore I dedicate this work to them.

## ACKNOWLEDGEMENTS

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## CHAPTER 1

### INTRODUCTION

#### **Ionizing Radiation**

Ionizing radiation is a form of energy that can displace electrons from atoms or molecules, producing ions that can disrupt biological processes and cause damage such as single and double strand DNA breaks. DNA double strand breaks are especially problematic to cells and can lead to cell death, and incorrect DNA repair can result in chromosome aberrations which increase the risk of cancer. Sources of exposure to ionizing radiation include radon, diagnostic procedures such as X-rays, CT scans and mammograms, radiation therapy for cancer treatment, occupational exposures, and radiological accidents.

There are different kinds of ionizing radiation based on the type of particle emitted and the energy and charge of that particle. Directly ionizing radiation can be of two types, alpha particles which are nuclei of helium atoms and beta particles which are electrons. Both carry a charge and can therefore directly interact with atoms in macromolecules. Alpha particles are emitted by the decay of large radioactive nuclei of heavy atoms such as uranium and radon. Indirectly ionizing radiation includes neutrons, gamma rays and X-rays. Neutrons are non-charged particles and when they collide with atomic nuclei they create ions which will in turn cause damage to other molecules. Gamma rays and X-rays are electromagnetic waves of photons emitted either from the nucleus of an atom or from the outer electron shell, respectively. Both gamma rays and

X-rays can ionize atoms through the photoelectric effect by ejecting an electron from an atom they encounter, which in turn will cause damage to other nearby molecules.

## **Radiation Exposures and Associated Risk Factors**

Despite the elevated cancer risks of ionizing radiation exposure, it can be very helpful for diagnostic and therapeutic purposes. Exposure to medical radiation has been rapidly increasing in developed countries over the past 10 years (Shah et al., 2012) and will likely continue to do so. Procedures such as CT scans, radiographs, X-rays, mammograms and fluoroscopy are routine yet very important components of modern medical care. While these procedures use very low doses of radiation, cumulative exposures over time can significantly increase the risk of developing cancer. Age at exposure is an important factor in assessing effects of radiation because it is well known that children experience increased cancer risks after exposure compared to adults (Kleinerman, 2006; Sadetzki and Mandelzweig, 2009; Stephan et al., 2007). Cancer risks are a subsequent consequence of radiation induced DNA damage, yet little is known about the basic cellular radiation susceptibility of cells from children compared to cells from adults. The benefits of medical diagnostic procedures are generally believed to outweigh the risks, but those risks are still important to consider and understand.

Chronic low level exposure in the workplace (Sigurdson et al., 2008a; Yong et al., 2009) and acute high dose exposures from nuclear incidents (Fushiki, 2013; Pierce and Mendelsohn, 1999) are other types of radiation exposures experienced by groups of people. Understanding the effects of ionizing radiation on humans and its implications

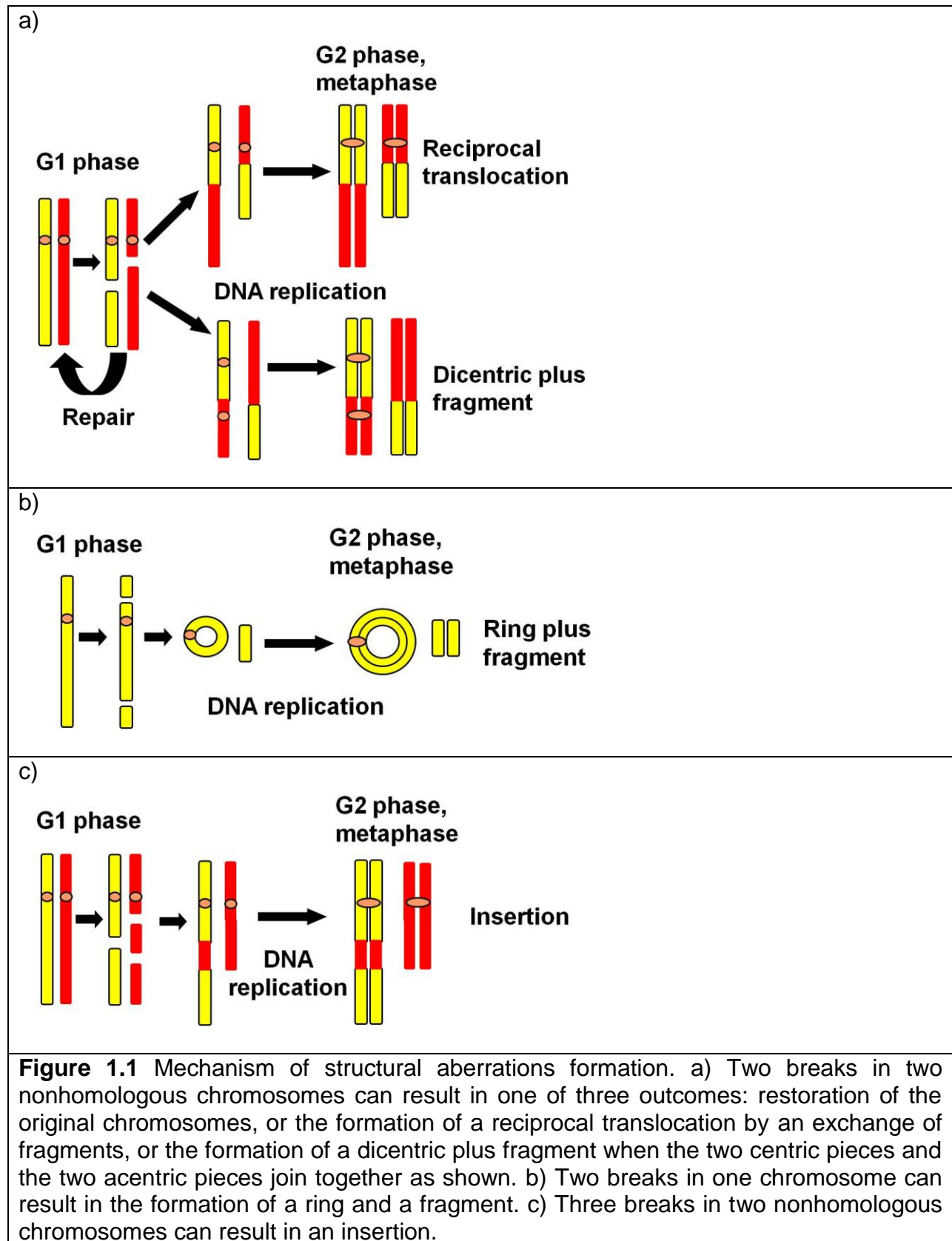
for health are important because while radiation can be a powerful tool, its use requires care and diligence to avoid adverse consequences.

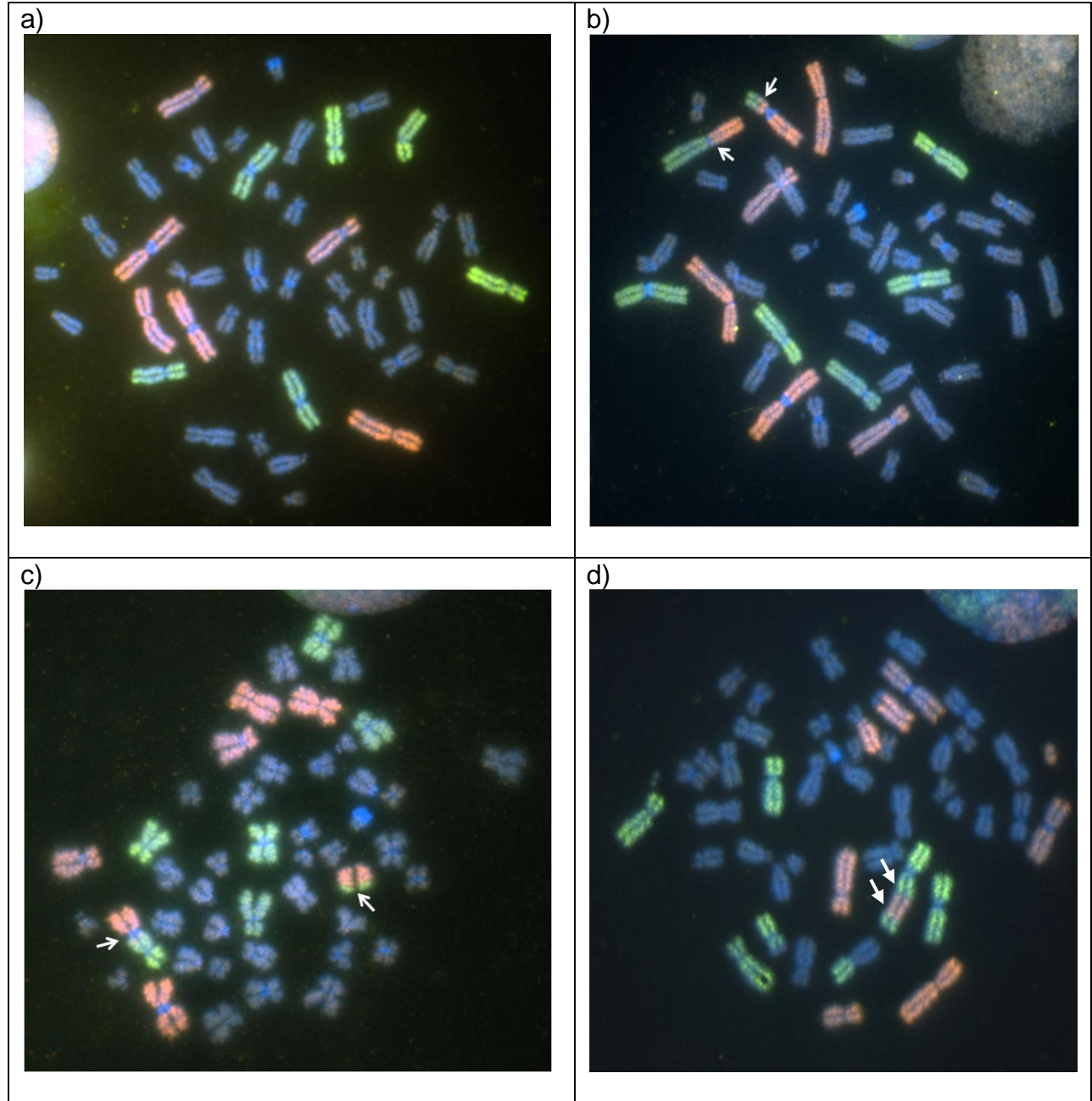
## **Structural Chromosome Aberrations**

Structural chromosome aberrations are a highly relevant biological endpoint because they are both a biomarker of exposure and a biomarker of effect. Structural aberration frequencies are known to be closely associated with cancer formation and to have some predictive ability for determining cancer risks (Bonassi et al., 2008; Bonassi et al., 2004; Hagmar et al., 1994; Hagmar et al., 2004). Nearly all cancer cells contain some form of chromosomal rearrangements (Frohling and Dohner, 2008; Gasparini et al., 2007), some of which have been characterized at the molecular level (Edwards, 2009; Kaye, 2009).

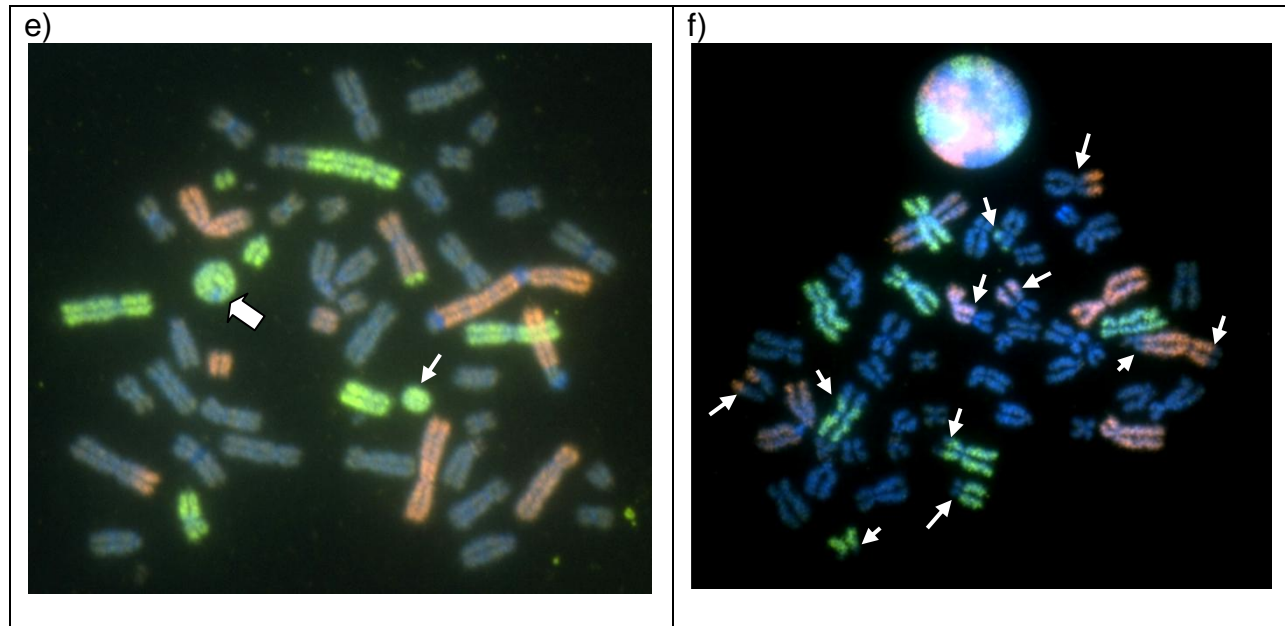
Translocations are one of the aberration types often used as a biomarker for detection of exposures from genotoxic agents and ionizing radiation (Tucker, 2002, 2008b). Translocations are the most stable aberration type because cells with these exchanges generally do not undergo much negative selection pressure during mitosis. For this reason translocations accumulate with exposures that are chronic (Tucker et al., 1997), or highly fractionated (Spruill et al., 1996), and they can persist for years in an individual. Translocations are ideal for use in radiation biodosimetry where acute as well as chronic exposures to individuals (Tucker and Luckinbill, 2011) and populations (Sigurdson et al., 2008a; Yong et al., 2009) can be detected. Baseline translocations in unexposed individuals increase steadily with age (Sigurdson et al., 2008b).

Figure 1.1 shows the mechanism of formation for reciprocal translocations, dicentrics, fragments, rings and insertions. Each of these aberration types is the result of multiple double strand DNA breaks induced in one or more chromosomes that subsequently are mis-repaired. Exchanges that are even more complex are possible, and their number and complexity will depend on how many double strand DNA breaks are induced in a cell and how they are mis-repaired (Natarajan and Boei, 2003). Figure 1.2 shows an example of a normal metaphase cell as well as cells with different types of aberrations that are commonly seen following radiation exposure.









**Figure 1.2** Examples of cells in metaphase labeled with FISH whole chromosome paints and counterstained with DAPI showing structural chromosome aberrations. a) Normal cell in metaphase with chromosomes 1, 2 and 4 painted red and chromosomes 3, 5 and 6 labeled simultaneously in green; all other chromosomes are counterstained with DAPI (blue). b) Reciprocal translocation (arrows) between a red and a green chromosome. c) Dicentric and an acentric fragment with a color junction (arrows). d) Insertion of a red segment into a green chromosome (arrows). e) Centric ring chromosome (thick arrow) and an acentric ring (thin arrow). f) Complex damage in a cell with at least 11 different color junctions (arrows). Several other types of structural aberrations are also present in panels (d) and (e) which are not marked.

There are many ways to visualize and count chromosome aberrations. Giemsa stained unbanded chromosomes (Mateuca et al., 2006) is a classic assay that allows detection of unstable events such as dicentrics, fragments and rings. It is inexpensive but compared to some other methods, scoring cells stained in this manner is time consuming and somewhat challenging. A major disadvantage is that stable rearrangements such as translocations and insertions cannot easily be detected. Giemsa banding (Bayani and Squire, 2004), with or without karyotyping, is a very thorough way to detect stable and unstable events in every chromosome in a cell. However analysis of G-banded cells is very time consuming and technically challenging, and is better suited when only small numbers of cells need to be analyzed. A more current technique is multicolor banding (mBAND) which produces bands in an array of colors, enabling the analysis of intra-chromosomal exchanges (Hada et al., 2011). Spectral karyotyping (SKY) (Belaud-Rotureau et al., 2003; MacLeod and Drexler, 2013) and multicolor-FISH (mFISH) (Liehr et al., 2004) are techniques where each homologous chromosome pair is painted in a single unique color using fluorescent *in situ* hybridization (FISH) whole chromosome paints. SKY and mFISH permit visualization and detection of all inter-chromosomal exchanges in a cell. However, these methods are very expensive, and technically demanding and time consuming to perform, much like karyotyping. Their major strength lies in analyzing the composition of tumor cells. However, for radiation exposure assessments, scoring a sufficient number of cells with banded chromosomes, SKY, or mFISH can be problematic because of the time required for analysis. At high doses of radiation an additional problem arises with these assays because the large number of aberrations makes the analyses even

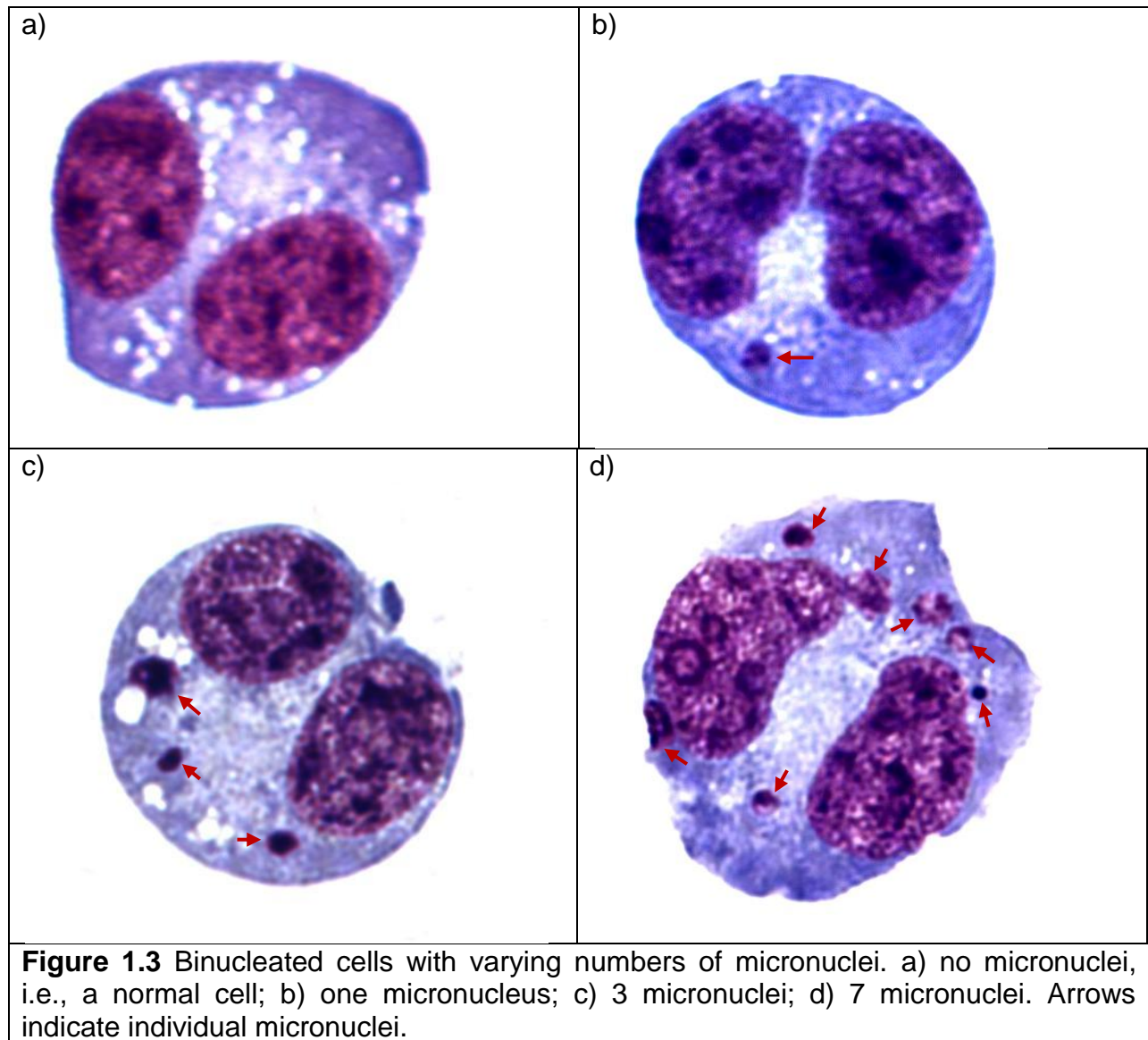
slower. For these reasons, a different type of assay is needed in studies where large numbers of cells must be scored quickly and efficiently, particularly where obtaining sufficient statistical power is a concern.

An approach that solves the twin problems of labor intensiveness and high cost involves FISH whole chromosome painting where the 6 largest chromosome pairs are painted in 2 contrasting colors (Tucker, 2010b). This method is advantageous because it enables large numbers of cells to be scored relatively quickly and easily, making it possible to collect data from many events in order to have enough statistical power to detect biologically relevant differences in response to radiation. Training people to be accurate slide readers is usually easy, and once trained, they can screen many cells quickly. Another advantage is that this method allows detection of both stable and unstable events such as translocations, dicentrics, fragments, rings and insertions. However, only exchanges between genetic material labeled in different colors can be seen, therefore only a portion of all exchanges can be quantified. To compensate for this issue, more metaphase cells can be scored.

### **Cytokinesis Block Micronucleus Assay**

Another cytogenetic method used to assess chromosome damage is the cytokinesis block micronucleus assay (CBMN) (Fenech and Morley, 1985), which has become extensively used for evaluating genotoxic exposures in mammalian cells (Bonassi et al., 2007; Mateuca et al., 2006). Micronuclei are now a well-accepted cytogenetic biomarker of exposure and effect (Bonassi et al., 2005; Garcia-Sagredo,

2008; Vral et al., 2011). DNA damage is visualized and measured simply by counting the number of micronuclei (MN) in binucleated cells. Any one micronucleus can contain whole chromosome(s) as the result of a non-disjunction event, and / or acentric fragment(s) that did not segregate to one of the daughter nuclei (Eastmond and Tucker, 1989). As cells complete mitosis, cytokinesis is blocked by the addition of cytochalasin B and all the products of mitosis are retained in the cell membrane; any chromosomes or fragments that were not successfully segregated to either nucleus are visualized as MN. This method is inexpensive, it is easy to train personnel for scoring, and large numbers of cells can be analyzed very quickly. Figure 1.3 illustrates examples of binucleated cells with different numbers of micronuclei.



## **Repair of Ionizing Radiation Induced DNA Damage**

Maintaining an intact and error free genome throughout the life of a cell is important for avoiding cancer. Mutations in the DNA can lead to cell death, loss of cell cycle control and uncontrollable growth indicative of cancer. To protect themselves against these undesirable events, mammalian cells have evolved a complex and diverse mechanism to oversee the integrity and repair of its genome. Double strand DNA breaks (DSBs) can wreak havoc on genomes by promoting the loss of genetic material through degradation of broken ends by exonucleases, by failing to replicate the broken ends faithfully, or by the loss of DNA fragments during mitosis. To avoid these dangers, DSBs are quickly repaired via two major pathways of non-homologous end joining (NHEJ) and homologous recombination repair (HRR). NHEJ is the major pathway to repair ionizing radiation-induced DSBs; HRR also plays a smaller role in DSB repair mainly in S and G2 phases of the cell cycle. The repair machinery does not recognize which broken end belongs to which chromosome so there is a significant chance that a cell with two double strand breaks will incorrectly repair the loose ends and end up with a chromosome rearrangement. A detailed account of DNA repair is described in a recent comprehensive review (Thompson, 2012).

## **Uranium, Depleted Uranium and Heavy Metal Toxicity**

Many heavy metals are known to be human carcinogens (Hayes, 1997; Jarup, 2003) and clastogens (Vainio and Sorsa, 1981) that have been linked to cancer formation as a result of chronic exposures in the workplace. Uranium is a naturally

occurring, extremely dense, heavy metal found in the soil and seawater that is mined and extracted for use in military as well as civilian applications. It is weakly radioactive due to the unstable nature of its isotopes  $^{234}\text{U}$ ,  $^{235}\text{U}$  and  $^{238}\text{U}$ , which emit alpha particles upon decay. The  $^{235}\text{U}$  isotope has fissile properties and can maintain a nuclear chain reaction to generate heat for production of electricity and can be used to generate nuclear explosions. Natural Uranium must be enriched in the  $^{235}\text{U}$  isotope before it can be used as nuclear fuel; the byproduct of this process is Depleted Uranium (DU) which has less  $^{235}\text{U}$  and more  $^{234}\text{U}$  and  $^{238}\text{U}$  than natural Uranium. DU is much more stable than natural U and has a very low probability of fission. DU is most commonly used by the military in the manufacture of munitions and tank armor plating. However, whether DU exposure has long term consequences for human health has been debated (Bleise et al., 2003; Briner, 2010; Sztajnkrzyer and Otten, 2004). Many studies have shown carcinogenic effects of DU in cell lines and animal models (Berradi et al., 2008; Darolles et al., 2010; Hao et al., 2009, 2012; LaCerte et al., 2010; Shaki et al., 2012; Xie et al., 2010) but there is little evidence of adverse human health effects (Bakhmutsky et al., 2011; Squibb et al., 2012).

## **Future Directions**

### **a) Long-term Depleted Uranium Exposure on Gulf War Veterans**

Chapters 2 and 3 describe the long term genotoxic effects of Depleted Uranium exposure through inhalation of DU dust and wound contamination on Gulf War 1 Veterans as measured by MN formation and FISH whole chromosome painting. Even though no association was found between DU exposure and the cytogenetic biomarkers, for the 80 Veterans enrolled in this health surveillance program it will be important to monitor them for possible long term health effects for the rest of their lives. Since some of these Veterans are as young as 40 years of age, most likely another 40 years of monitoring would be necessary, since it could be detrimental to miss any possible long term health effects related to DU. Of greatest concern are the individuals with embedded DU fragments because they continue to excrete high levels of Uranium in their urine, and because some individuals show tissue changes around the fragment site. These tissue changes may present future problems such as pain and discomfort, irritation and possibly benign or even cancerous growths.

### **b) Susceptibility to Ionizing Radiation Increases with Age in Humans**

Chapter 4 presents important findings that newborns are more susceptible to radiation than adults, and that adults show no difference in susceptibility as they age. To better understand how and when this elevated susceptibility of newborns declines with age it would be helpful to assess children and young adults ages 0 – 21 for their responses to radiation. Fetal susceptibility is another interesting question that could be explored in an animal model such as rats. We did not measure cell growth kinetics and



cell concentrations during the 48 hr culture period, and gathering such data in a future experiment may help elucidate an insight into this difference in susceptibility.

## CHAPTER 2

This chapter has been previously published in the journal "Mutation Research: Genetic Toxicology and Molecular Mutagenesis":

Bakmutsky M.V., Oliver M.S., McDiarmid M.A., Squibb K.S., Tucker J.D., 2010. Long term depleted uranium exposure in Gulf War I Veterans does not cause elevated numbers of micronuclei in peripheral blood lymphocytes, Mutation Research 720 (2011) 53–57

### **Long Term Depleted Uranium Exposure In Gulf War I Veterans Does Not Cause Elevated Numbers Of Micronuclei In Peripheral Blood Lymphocytes**

#### INTRODUCTION

Depleted uranium (DU) is a heavy metal that is both radioactive and hazardous due to its chemical properties. Natural U is composed of three U isotopes ( $U^{234}$ ,  $U^{235}$  and  $U^{238}$ ). DU is a by-product of the U enrichment process that natural uranium undergoes in order to extract the  $U^{235}$  isotope for use in nuclear weapons and nuclear fuel production. Thus, DU has a lower  $U^{235}/U^{238}$  ratio and is approximately 40% less radioactive than natural U. DU metal is used for military applications due to its high density, high pyrophoricity, tensile strength that is similar to steel, high availability, and low cost. It is ideal for use in armor piercing munitions because it has self-sharpening properties upon impact allowing it to penetrate armor more effectively than other metals. DU dust is formed upon impact of the projectiles, which is one source of internal inhalation exposure to DU in the battlefield. The other military application of DU is for protective tank armor, which can increase inhalation and ingestion exposures to DU

dust if a munition pierces DU armor during battle (Parkhurst, 2005). Under normal conditions, occupants of tanks with DU armor are also exposed to increased amounts of DU-derived radiation. However, this additional radiation dose is very small and does not constitute a significant health risk (Bleise et al., 2003). The heavy metal toxicity of DU is generally considered to present a greater health hazard than its radioactivity.

External exposure to DU does not present a significant health hazard, but internal exposure via dust particle inhalation and embedded DU fragments may lead to adverse health effects due to both chemical and radiological toxicity. *In vitro* studies in human cell lines treated with soluble DU compounds show increased transformation to tumorigenic phenotypes; several bacterial strains show increased mutagenic activity after DU exposure and animal models implanted with DU pellets developed local tumors (National\_Research\_Council, 2008). These results suggest that DU may increase the risk of cancer (Sztajnkrzyer and Otten, 2004). However, in contrast to the animal and *in vitro* studies, humans exposed to high internal and external doses of DU do not appear to suffer measurable health effects (Dorsey et al., 2009; Marshall, 2008). For 18 years, the Department of Veterans Affairs (VA) has been monitoring the health of Gulf War I Veterans that were exposed to DU during friendly fire incidents. In addition to inhalation and ingestion exposures, about forty percent of this cohort sustained traumatic injury resulting in embedded fragments of DU being retained in soft tissue. During biennial visits to the Baltimore VA Medical Center, the Veterans are monitored for the concentration of DU in their urine, for clinical chemistry measures that assess organ system function with a focus on biomarkers of adverse effects on the renal and reproductive systems, and for hematological, neuroendocrine and bone metabolism

parameters. Additional tests have included measures of chromosomal aberrations and HPRT mutation frequency in blood lymphocytes. To date, no clinically significant DU-related health effects have been observed, even in subjects with the highest urine uranium concentrations (McDiarmid et al., 2004; McDiarmid et al., 2009; McDiarmid et al., 2001a; McDiarmid et al., 2007; McDiarmid et al., 2006; McDiarmid et al., 2000; McDiarmid et al., 2001b; Squibb and McDiarmid, 2006).

Enumeration of micronuclei (MN) in peripheral blood lymphocytes (PBLs) is a well-established cytogenetic method for detecting chromosome damage caused by radiation and chemical exposures in humans (Bonassi et al., 2001). Here we used the cytokinesis-blocked MN assay to measure the number of MN found in the PBLs of DU-exposed Gulf War I Veterans enrolled in the VA monitoring program. The goal of this study was to determine whether DU exposure as measured by urine U concentration results leads to detectable levels of cytogenetic damage. The results indicate that chronic systemic exposure to DU in Gulf War I Veterans with embedded DU fragments does not result in elevated frequencies of MN in peripheral blood lymphocytes compared to the frequencies of MN in Veterans with normal U body burdens.

## MATERIALS AND METHODS

### Recruitment of Subjects

The number of micronuclei present in peripheral blood lymphocytes was measured in blood samples collected from 35 members of the Veteran Administration (VA)'s DU-exposed Gulf War I veteran cohort who participated in the 4-day medical surveillance visit at the Baltimore VA Medical Center (Baltimore, MD) between April and June, 2009. Although all 79 members of this cohort were invited to participate in this surveillance visit, only about half of the total cohort accepted the invitation due to personal, employment or military service schedule constraints. One participant was excluded from this examination of micronuclei because he had previously received Cobalt radiation therapy. Demographics for the group of 35 Veterans that were examined for micronuclei in blood lymphocytes are shown in Table 1. Approximately 43% of the Veterans in this group had evidence of embedded fragments when examined by x-ray.

**Table 1.** Demographic characteristics of the DU follow-up program 2009 medical surveillance visit participants

	2009 Cohort (n=35)	
	N	%
Gender (% males)	35	100
Race		
African American	12	34%
Asian American	1	3%
Caucasian	20	57%
Hispanic	2	6%
Participants with embedded DU fragments	15	43%
Age *	43.62 ± 5.35	

\* Mean age at time of 2009 evaluation (± standard deviation)

## **Blood Collection, Cell Culturing, Slide Preparation and Staining, and Micronuclei Analysis**

Blood was drawn using 6 mL green topped vacutainer tubes containing heparin. The tubes were kept on a tilt shaker at low speed until they were prepared for shipping (within 2 hr). The blood samples were shipped overnight from the Baltimore VA Medical Center to Wayne State University with ice packs to remain cold (approximately 4°C). Samples were stored at 4°C upon arrival for 1-2 hours before culturing.

Lymphocytes were isolated by carefully layering 2 mL of blood diluted with 2 mL Hank's Balanced Salt Solution (HBSS) over 3 mL Lymphocyte Separation Medium (Mediatech, Inc.), then centrifuged at 400 x g at room temperature for 30 minutes. The mononuclear cell layer was transferred to a new tube and mixed with 4 mL HBSS, then centrifuged for 10 minutes at 260 x g at room temperature. The cell pellet was washed twice with 4 mL HBSS and centrifuged for 10 minutes at 260 x g at room temperature. The pellet was then re-suspended in 1 mL RPMI 1640 medium (Hyclone), supplemented with 15% Fetal Bovine Serum (Atlanta Biologicals), penicillin–streptomycin (100 units/mL penicillin G sodium, 100 µg/mL streptomycin in 0.85% saline, Gibco), 0.02 mg/mL PHA (Gibco) and 2 mM L-glutamine (Gibco). A cell count of the 1 mL cell suspension was obtained and cultures were seeded at a concentration of approximately 500,000 cells/mL. Cells were incubated in a fully humidified incubator with 5% CO<sub>2</sub> at 37°C in T25 suspension culture flasks (Corning) for 44 hours, then treated with Cytochalasin B (Sigma–Aldrich) (6 µg/mL final concentration) and cultured for an additional 28 hours for a total culture time of 72 hours. Cells were re-suspended in their culture medium with a transfer pipette to break up cell clumps. The cells were

then spun onto pre-cleaned microscope slides using a cytocentrifuge (Stat-Spin) for 4 min at 1300 RPM. The slides were air dried and fixed in 100% methanol for 15 min, then dried and stored at room temperature until staining. Slides were stained with 10% Giemsa Solution in Sorenson's buffer (67 mM  $\text{Na}_2\text{HPO}_4$ , 67 mM  $\text{KH}_2\text{PO}_4$  pH 6.8) for 15 minutes, rinsed briefly in distilled  $\text{H}_2\text{O}$ , air-dried and then mounted with Permount (Fisher Scientific) and a glass coverslip.

All blood samples were coded prior to shipping to the cytogenetics laboratory. The code was not broken until all the slide scoring had been completed. A total of 2000 binucleated cells were scored from each donor under a light microscope (Nikon Eclipse E200) by 2 trained individuals each of whom scored 1000 cells. Only intact, binucleated cells with clearly distinct nuclei were scored (Fenech et al., 2003); the number of micronuclei (MN) per sample as well as the number of cells with 0, 1, 2, 3, 4, and 5 or more MN were recorded. Binucleated cells containing nucleoplasmic bridges were excluded from scoring. The Nuclear Division Index (average number of nuclei per cell) was also determined for each sample.

### **Urine Uranium Analysis**

At each biennial health surveillance visit, twenty-four hour urine samples are collected from each subject and shipped to the Armed Forces Institute of Pathology, Department of Environmental Toxicologic Pathology (Washington, DC) for analysis of total uranium using a previously described inductively coupled plasma-dynamic reaction cell-mass spectrometer (ICP-DR-MS) method (Ejnik, 2005; McDiarmid et al., 2007). Urine U concentrations are standardized on the basis of urine creatinine concentrations



to obtain micrograms of U per gram of creatinine to account for urine dilution due to water intake and/or dehydration (Karpas, 1998; McDiarmid et al., 2000).

### **Uranium Exposure Metric**

A mean urine U (uU) exposure metric for each participant in this study was calculated using all the uU concentrations obtained for a participant each time they had participated in a surveillance visit at the Baltimore VA between 1994 and 2007. This U exposure metric, labeled mean uU 2007, was used to determine whether a relationship exists between mean uU exposure over the past 18 years and the presence of micronuclei in blood lymphocytes.

### **Statistical Analysis**

The Mann-Whitney U test was used to test for the significance of differences observed between High versus Low U exposed groups established based on each participant's mean uU 2007 value. Historically, the Baltimore VA DU health surveillance program has used a cut-off value of 0.1  $\mu\text{g U/g creatinine}$  for dividing High from Low exposed individuals (McDiarmid et al., 2009). This cut-off point was chosen because it was between the 95% percentile reported by (National Health and Nutrition Examination Survey (NHANES), 2003) for creatinine-adjusted urine U concentrations in U.S. populations with normal exposure to natural U through their diet and drinking water (0.034  $\mu\text{g/g creatinine}$ ) and 0.35  $\mu\text{g/L}$ , a value reported as a uU upper limit in populations living in areas where natural U is elevated in water and food (International

Commission on Radiologic Protection (ICRP), 1974). Differences were considered statistically significant when calculated p values were  $< 0.05$ .

Data were also analyzed using regression analysis. Since micronuclei frequency is a discrete variable created by a count, Poisson or Negative Binomial probability distribution is indicated for multivariate analysis (Ceppi et al., 2010). Poisson distribution can be used when the mean equals the variance but the negative binomial is indicated when this assumption is violated (e.g., by over-dispersion). The use of the Poisson regression to estimate the association of micronuclei abnormality frequency with urine uranium adjusting for age, current smoking and x-ray exposure during the past year was examined using the statistical package STATA version 11 (StataCorp, College Station, Texas). Because of over-dispersion of the data, the results of the equivalent negative binomial distribution are reported.

## RESULTS

A total of 35 Veterans were evaluated for the formation of MN as a function of their urine U concentrations. All subjects were adult males, ranging from 36 to 59 years of age. Table 2 shows the ages of each subject, the mean urine Uranium concentrations calculated for each individual (mean uU 2007) and the MN data obtained from each subject. Cells with 0, 1, 2, 3, 4, 5 and >5 MN were observed.

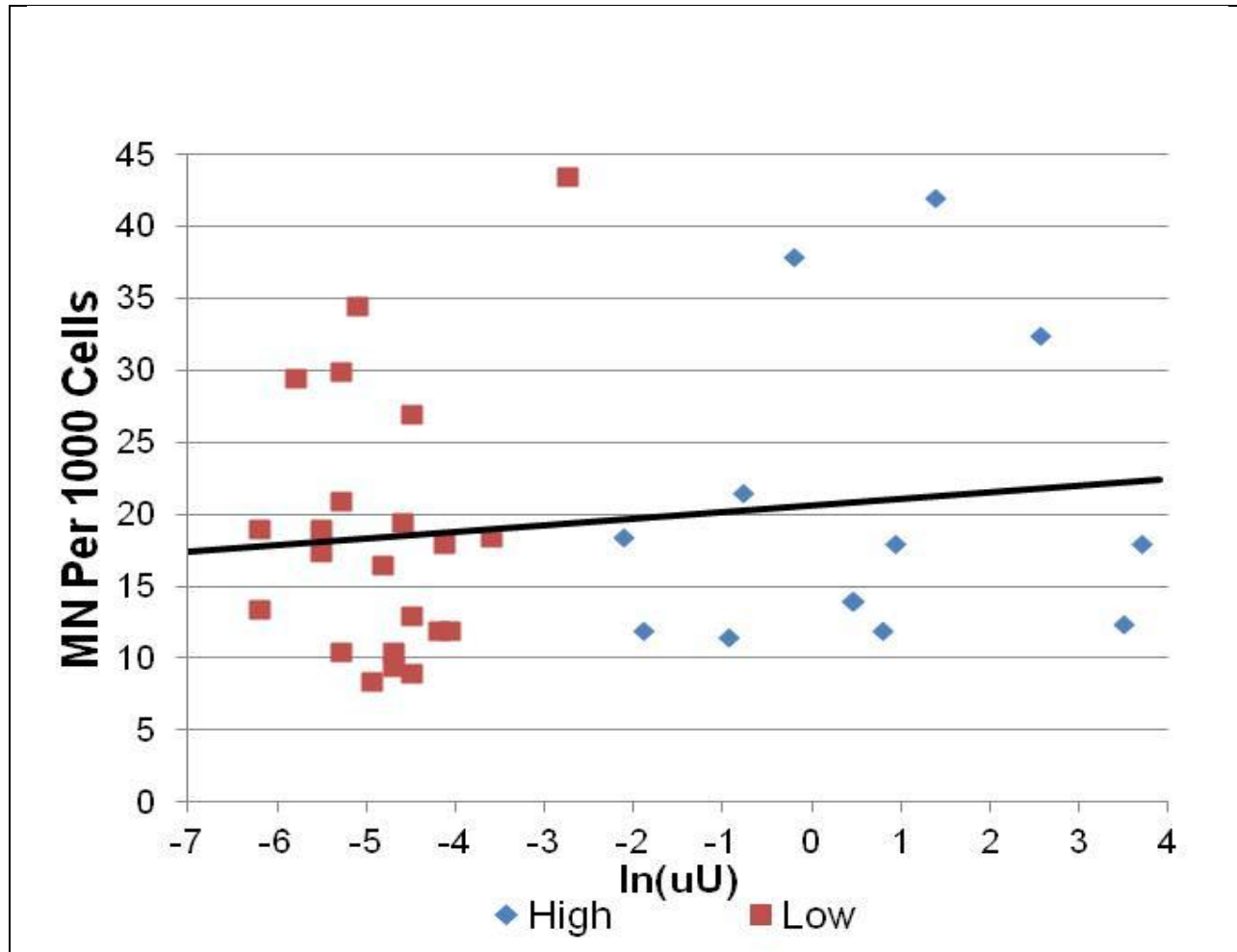
**Table 2.** Urine uranium concentrations and MN data by donor and group.

Donor Code	Urine [U] ( $\mu\text{g U/g cre}$ )	Donor Age	Number of Normal Cells	Number of Cells with MN	Mean MN per 1000 cells	Nuclear Division Index
<b>High exposure group (<math>\geq 0.1 \mu\text{g U/g cre}</math>)</b>						
102	0.81	38.59	1948	52	38.0	1.31
104	32.61	51.47	1976	24	12.5	1.28
105	40.40	41.74	1967	33	18.0	1.48
115	0.12	41.83	1967	33	18.5	1.72
116	0.46	50.49	1963	37	21.5	1.34
122	1.58	45.9	1978	22	14.0	1.39
123	1.56	39.18	1975	25	14.0	1.74
129	2.19	44.03	1980	20	12.0	1.46
145	12.7	56.06	1947	53	32.5	1.15
193	3.98	45.86	1937	63	42.0	1.47
199	2.5	44.81	1971	29	18.0	1.66
201	0.15	40.3	1983	17	12.0	1.62
202	0.39	40.96	1980	20	11.5	1.85
<b>Mean</b>	<b>7.65</b>	<b>44.71</b>	<b>1967</b>	<b>32.92</b>	<b>20.35</b>	<b>1.50</b>
<b>S.D.</b>	<b>13.32</b>	<b>5.24</b>	<b>14.59</b>	<b>14.59</b>	<b>10.42</b>	<b>0.21</b>
<b>Low exposure group (<math>&lt; 0.1 \mu\text{g U/g cre}</math>)</b>						
101	0.064	41.78	1927	73	43.5	1.45
110	0.007	42.49	1989	11	8.5	1.48
119	0.005	48.84	1966	34	21.0	1.54
126	0.016	38.54	1972	28	18.0	1.57
130	0.010	40.37	1963	37	19.5	1.55
134	0.005	42.66	1984	16	10.5	1.63
143	0.027	42.6	1968	32	18.5	1.34
174	0.011	36.73	1976	24	13.0	1.48
176	0.004	52.49	1972	28	17.5	1.51
179	0.011	41.69	1982	18	9.0	1.51
183	0.005	59.83	1952	48	30.0	1.33
186	0.003	41.74	1949	51	29.5	1.48
187	0.002	39.49	1974	26	13.5	1.64
188	0.008	41.71	1977	23	16.5	1.31
190	0.015	44.33	1980	20	12.0	1.45
194	0.011	49.58	1957	43	27.0	1.51
200	0.009	43.85	1982	18	9.5	1.18
204	0.004	38.87	1970	30	19.0	1.44
205	0.006	38.55	1936	64	34.5	1.31
207	0.017	38.19	1978	22	12.0	1.37
210	0.002	42.13	1963	37	19.0	1.34
212	0.009	38.62	1980	20	10.5	1.33
<b>Mean</b>	<b>0.011</b>	<b>42.96</b>	<b>1968</b>	<b>31.95</b>	<b>18.73</b>	<b>1.44</b>
<b>S.D.</b>	<b>0.013</b>	<b>5.43</b>	<b>15.7</b>	<b>15.72</b>	<b>9.15</b>	<b>0.12</b>

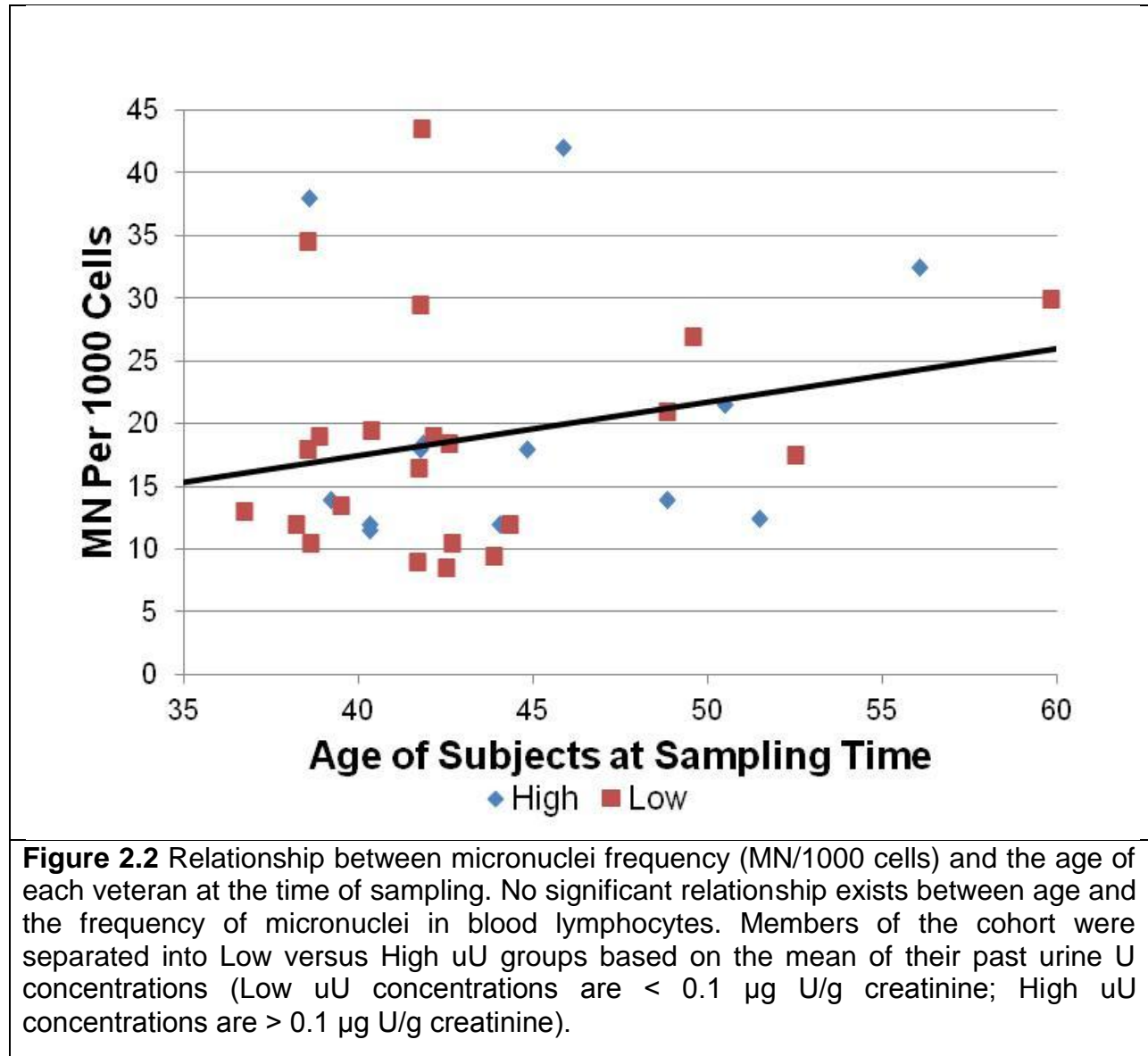
To examine the relationship between MN frequency and U body burden, the participants in this cohort were divided into two groups based on their mean uU2007 exposure metric, with the Low group consisting of all individuals with mean urine U concentrations below 0.1  $\mu\text{g U/g creatinine}$  and the individuals in the High group having concentrations equal to or above 0.1  $\mu\text{g/g creatinine}$ . The value of 0.1  $\text{ug U/g creatinine}$  was chosen as the cut-point between the high and low groups based on its close proximity to the 95th percentile upper limit value reported by NHANES (2003) for the concentration of natural U in urine the U.S. population (0.043  $\text{ug/g creatinine}$ ). Twenty-two (22) samples were placed in the Low group with mean urine [U] ranging from 0.002  $\mu\text{g U/g creatinine}$  to 0.064  $\mu\text{g U/g creatinine}$  while 13 samples in the High group ranged from 0.12  $\mu\text{g U/g creatinine}$  to 40.41  $\mu\text{g U/g creatinine}$ . The effect of a high U burden on two MN outcome measures, 1) the number of cells with MN, or 2) the total number of MN per 2000 cells, was determined by comparing the mean values for these two parameters in the Low versus High groups. The mean number of cells with MN was 32.9 in the High group vs. 32.0 in the Low group; while the average number of MN/1000 binucleated cells in the Low group was 18.7 versus 20.4 for the High group. Statistical analysis of the data using the Mann Whitney test of significant difference indicated that the means of the Low versus High groups were not significantly different at the 0.05 level for either parameter.

Because micronuclei results are discrete variables created by a count, a more acceptable analysis for examining continuous relationships between data is the Poisson or Negative Binomial analysis (Ceppi et al., 2010). No significant relationships were observed between the mean uU2007 exposure metric and MN frequency when mean

uU2007 was examined by itself (Figure 2.1) or when covariates (current smoking, x-rays in the past year and age) were included in the analysis (data not shown). The relationship between the number of cells with MN and age was also examined and is shown in Figure 2.2. There was no significant relationship between MN frequency and age when examined separately or when controlled for uU concentrations, smoking and x-rays in the past year (data not shown).



**Figure 2.1** Relationship between micronuclei frequency (MN/1000 cells) and the natural log of urine uranium concentrations of Gulf War I Veterans exposed to DU. No significant relationship exists between urine U excretion and the frequency of micronuclei in blood lymphocytes. Members of the cohort were separated into Low versus High uU groups based on the mean of their past urine U concentrations (Low uU concentrations are  $< 0.1 \mu\text{g U/g creatinine}$ ; High uU concentrations are  $> 0.1 \mu\text{g U/g creatinine}$ ).



**Figure 2.2** Relationship between micronuclei frequency (MN/1000 cells) and the age of each veteran at the time of sampling. No significant relationship exists between age and the frequency of micronuclei in blood lymphocytes. Members of the cohort were separated into Low versus High uU groups based on the mean of their past urine U concentrations (Low uU concentrations are  $< 0.1 \mu\text{g U/g creatinine}$ ; High uU concentrations are  $> 0.1 \mu\text{g U/g creatinine}$ ).



## DISCUSSION

The results of our study show no difference in MN frequency in the high versus low urine U groups. This conclusion is consistent with other results from the DU surveillance program which has been monitoring the health of these Veterans since the 1991 Gulf War. The surveillance program has not detected any significant health effects that can be attributed to DU exposure, even in those Veterans with DU shrapnel in their bodies (McDiarmid et al., 2009). Urine Uranium levels in most of the Veterans in the DU exposed cohort are similar to the general U.S. population and only remain elevated in Veterans with embedded DU fragments (Dorsey et al., 2009). In a previous assessment of this DU-exposed 'friendly-fire' cohort, a group of Gulf War I deployed, but non- DU-exposed controls was evaluated (McDiarmid et al., 2000). The urine U distribution of the non-DU exposed Veterans was found to be similar to the cohort of Veterans that are DU-exposed but without embedded fragments; these values were also within the normal range for the U.S. population. Thus, using uU as a measure of U body burden, the low uU group within the friendly-fire cohort of DU exposed Veterans provides an appropriate comparison group for the Veterans with embedded DU fragments who have high uU concentrations.

We hypothesized that Veterans with substantial numbers of DU fragments embedded in their bodies may have elevated MN frequencies due to the prolonged and constant systemic DU exposure they experience. DU causes radiation exposure because it is a radioactive metal which naturally emits alpha particles along with beta and gamma radiation during the decay process (Bleise et al., 2003). The alpha particles are high energy but have low penetrating power and they can be a potential internal

hazard when DU is inhaled, ingested or found in wounds. The beta and gamma radiation are generally external exposure hazards, however, the overall radioactivity of DU is very low (Sztajnkrzyer and Otten, 2004). Existing experimental evidence also suggests that DU may be genotoxic based on its chemical characteristics (Coryell and Stearns, 2006; Hartsock et al., 2007; Stearns et al., 2005).

These data do not support our original hypothesis that MN may be elevated in Veterans with a chronically elevated U body burden due to embedded DU fragment retention. A possible physical explanation for this finding is that the embedded fragments are not close enough to the blood supply to cause any visible damage to the passing lymphocytes, either by emission of poorly penetrating alpha particles or by chemical toxicity. If many DU fragments are located close to large blood vessels, it would be more likely that radiation or chemical toxicity effects could be seen in the lymphocytes. The cells in the tissue surrounding the shrapnel would suffer the most damage from DU, and these effects would diminish with distance from the metal. Detailed information about the size, locations, and number of fragments embedded in each individual is not available.

Although no significant relationship between elevated MN and uU concentration was observed in this study, DU exposure may lead to small increases in micronucleus frequencies in some individuals but these increases are too low to be detectable. The effect of DU exposure could be masked by factors that are known to influence MN frequencies, such as age and cigarette smoking. MN frequencies are known to increase with age (Bonassi et al., 2001; Hando et al., 1994), yet we do not see an age effect present in our results, probably due to the relatively small sample size. Cigarette

smoking can also be a factor in heavy smokers. The Human MicroNucleus Project examined the effects of smoking on MN frequencies in multiple studies involving nearly 6000 subjects. These analyses indicate that smokers do not have more MN than non-smokers, and even show a small decrease in MN frequencies. However, heavy smokers (at least 30 cigarettes per day) do show increases which can only be observed in people who are not occupationally exposed to genotoxic agents (Bonassi et al., 2003). We controlled for current smoking (yes/no) in our regression analysis, but did not have information on the number of cigarettes smoked per day for each individual.

Exposure to ionizing radiation can also lead to increased MN frequencies, thus we controlled for past exposure to x-rays as reported by the Veterans. Many received multiple X-rays or other diagnostic procedures following their injuries, however controlling for number of x-rays in the past year did not alter the relationship between MN and mean uU concentrations.

Only one other study has used micronuclei to evaluate individuals with environmental exposure to DU. Krunic et al. (Krunic et al., 2005) recruited individuals from areas of Bosnia and Herzegovina where DU munitions were used during the Balkan conflict. A control population was recruited from West Herzegovina which was not impacted by war activities. Results of this study showed a small increase in the MN frequency in the exposed group compared to controls. However, there was no control for exposure to other genotoxic chemicals, and DU exposure was not directly measured in individual subjects but was assumed based on the presence of DU in environmental matrices (Krunic et al., 2005). Other important differences also exist between their study design and the one we report here. Krunic et al. evaluated individuals thought to be

exposed to DU dust through direct contact and aerosol inhalation, but did not include subjects with embedded shrapnel. Their control group consisted of unexposed individuals while our Low group consisted of individuals with previous exposure to DU but low urine U concentrations.

Depleted Uranium exposure can be external (skin contact) and internal (ingestion, inhalation and embedded fragments) and present both radiological and chemical hazards. However, the levels of exposure that occurred during the 1991 Gulf War did not lead to significant increases in body burdens above non-exposed populations (Bleise et al., 2003) except in the cases involving embedded DU fragments. This study concurs with others showing that the DU exposure that occurred in Gulf War I Veterans with embedded DU fragments does not appear to increase biomarkers of genotoxic damage despite an ongoing elevation of their U body burdens for over 18 years.

## CHAPTER 3

### **Long Term Depleted Uranium Exposure In Gulf War Veterans Does Not Cause Increased Chromosome Aberrations In Peripheral Blood Lymphocytes Measured By Fish Whole Chromosome Painting**

#### **INTRODUCTION**

Depleted Uranium (DU) is a chemically toxic heavy metal with weak radioactive properties that is used extensively by the military in the manufacture of munitions and armor. Its appeal for use in weapons is due to its high density, pyrophoricity, self-sharpening properties, and low cost. Uranium is the heaviest naturally occurring element normally found in soil, rocks and water; it is composed primarily of three isotopes,  $^{234}\text{U}$  (0.005%),  $^{235}\text{U}$  (0.72%) and  $^{238}\text{U}$  (99.27%), that emit alpha particles and beta radiation upon decay. Natural uranium is processed to increase the concentration of the fissile isotope  $^{235}\text{U}$  for use in nuclear fuel and nuclear weapons. The enrichment process results in the production of uranium metal that is depleted in  $^{235}\text{U}$  and  $^{234}\text{U}$ , and consequently has 40% less of the alpha particle emission than natural uranium. During the 1991 Gulf War, DU munitions were used extensively in combat (Sztajnkrzyer and Otten, 2004) and consequently some military personal and civilians were exposed to DU, with the highest exposures accrued to service members involved in DU friendly fire. Exposures involved inhalation of DU dust, skin contact, wound contamination from particulate aerosol and from shrapnel embedded in tissue of those who were wounded, and entry of DU into food and water sources. At that time there were widespread concerns that DU exposure may have health consequences such as organ damage, increased cancer incidence, radiation exposure, and birth defects.

The most common form of DU exposure is through inhalation of particles, which are absorbed in the lungs if they are soluble and pass into blood plasma before being distributed throughout the body (Bleise et al., 2003; Briner, 2010). DU is then deposited in the bone, kidneys and other soft tissues. Only about 10% of inhaled soluble DU is retained in the body. Two-thirds of that will be rapidly excreted in the urine and much of the rest will be excreted over time (Marshall, 2008). This type of exposure is more transient compared to individuals with embedded shrapnel fragments who experience higher body burdens and chronic exposures that have been observed to persist for at least two decades as measured by urine Uranium concentrations (Squibb et al., 2012).

The toxicity and radioactivity of DU have raised questions about its possible genotoxic effects, specifically in individuals suffering chronic exposure from embedded fragments compared to subjects with inhalation-only exposures and normal urine Uranium levels. Accordingly, the Department of Veterans Affairs has been monitoring a cohort of Gulf War I Veterans exposed to DU in friendly fire incidents for almost 20 years for possible health effects from long term DU exposure (McDiarmid et al., In press; Squibb et al., 2012). This cohort suffered inhalation exposures and some experienced wound contamination with dust and embedded DU fragments that cannot be easily removed. Urine Uranium levels have been measured for all attending participants at every biennial visit. Individuals with inhalation-only exposures have urine Uranium levels similar to unexposed individuals from the general population, while subjects with retained embedded shrapnel continue to excrete elevated levels of DU in their urine. The clinical assessment provided by the DU Follow-Up Program includes a detailed medical history, physical examination, clinical chemistry measures, renal and

lung function testing, skin patch tests, and genotoxicity assays. To date, several tests of mutagenicity and clastogenicity have been conducted in this cohort including sister chromatid exchange (SCE), micronuclei, chromosome aberrations, and mutant frequencies of HPRT and PIGA (Bakmutsky et al., 2011; McDiarmid et al., 2011a). Early SCE results were inconsistent as they related to DU exposure and many other markers provided non-statistically significant results with the exception of a possible threshold effect observed for HPRT mutations in the highest urine Uranium exposure group (summarized in McDiarmid et al., 2009). Similarly, the DU Follow-Up Program has found no significant U-related health effects associated with DU exposure in this cohort (Bakmutsky et al., 2011; Dorsey et al., 2009; McDiarmid et al., 2011b; Squibb et al., 2012).

However, analyses of chromosome translocations have not been performed in DU-exposed individuals. Translocations are a common and widely used biomarker for evaluating chronic and acute radiation exposures. Elevated translocation frequencies are a biomarker of effect that may indicate an increased risk of developing cancer. Translocations are an integrating biodosimeter, i.e. they provide a lifetime cumulative measure of the different types of clastogenic exposures a person has had. Translocations are the most persistent of all structural chromosome aberrations. Their relative stability over time allows them to persist for decades. A baseline translocation frequency for nearly 2000 unexposed controls is available for comparison with our DU exposed Veterans (Sigurdson et al., 2008b; Tucker and Luckinbill, 2011), which enables radiation dose estimates to be made on individuals for whom no pre-exposure translocation data are available (Tucker and Luckinbill, 2011).

The purpose of the work described here is to extend the ongoing surveillance effort by the DU Follow-Up Program by evaluating chromosome translocation frequencies using FISH whole chromosome painting.



## MATERIALS AND METHODS

### Recruitment of Subjects, and Blood and Data Collection

For this study, blood samples were collected from 35 members of the Veteran Administration (VA)'s DU-exposed Gulf War I veteran cohort who participated in the 4-day medical surveillance visit at the Baltimore VA Medical Center (Baltimore, MD) between April and June 2011. Although all 80 members of this cohort were invited to participate in this surveillance visit, only about half of the total cohort accepted the invitation due to personal, employment or military service schedule constraints. Approximately 31% of the Veterans in this group had evidence of embedded fragments when examined by plain film X-ray. Each subject provided data via a health questionnaire, which requested their lifetime exposure to X-rays, pack years smoked, and alcohol use. Each participant's questionnaire was reviewed for completeness. The surveillance protocol used in this work was approved by the Baltimore VAMC's and the University of Maryland School of Medicine's IRB programs. Each participant completed an informed consent document.

Blood was drawn from each participant using 6 mL Becton Dickinson Vacutainer Sodium Heparin tubes. The blood samples were shipped overnight from the Baltimore VA Medical Center to Wayne State University with ice packs to maintain the temperature at approximately 4°C. Samples were stored at 4°C upon arrival for 2 hours before culturing.

## Cell Culturing, Slide Preparation and FISH Painting

Eight hundred  $\mu\text{L}$  of blood was dispensed into 10 mL of RPMI 1640 medium (Hyclone), supplemented with 15% Fetal Bovine Serum (Atlanta Biologicals), penicillin–streptomycin (100 units/mL penicillin G sodium, 100  $\mu\text{g}/\text{mL}$  streptomycin in 0.85% saline, Gibco), 2% phytohemagglutinin (Gibco) and 2 mM L-glutamine (Gibco) pre-warmed to 37°C. Cultures were incubated vertically in T25 suspension flasks with vented caps (CELLSTAR) in a humidified chamber with 5%  $\text{CO}_2$  at 37°C for 48 hrs. Cultures were treated with KaryoMAX Colcemid solution (10  $\mu\text{g}/\text{mL}$ , Gibco) at a final concentration of 0.1  $\mu\text{g}/\text{mL}$  after 44 hours in culture to arrest cells in metaphase. After 48 hours cells were gently re-suspended and decanted into 15 mL conical tubes and centrifuged at 1400 RPM for 5 min at room temperature; supernatant was aspirated with a Pasteur pipette. The cell pellets were gently resuspended in 8 mL 75 mM KCL solution pre-warmed to 37°C and incubated for 30 minutes at 37°C. Two mL of freshly prepared fixative (methanol:acetic acid 3:1 v/v) solution was added to each tube, mixed thoroughly and centrifuged at 1400 RPM for 5 minutes. The supernatant was aspirated and the cells were washed with 2 mL of fixative 3 more times. The pellet was resuspended in pure glacial acetic acid and cells were dropped onto cooled microscope slides pre-cleaned with 70% ethanol over a 70°C water bath to obtain metaphase spreads.

Slides were aged overnight at room temperature then stored in  $\text{N}_2$  gas with a desiccant pack at -20°C. Samples were brought back to room temperature and were hybridized with FISH Whole Chromosome Painting kits from Cambio or Applied Spectral

Imaging according to the manufacturers' protocols. Chromosomes 1, 2, 4 were painted with a Cy3 labeled probe and chromosomes 3, 5, 6 probes were labeled with FITC. Slides were mounted with 50  $\mu$ L ProLong Gold antifade reagent with DAPI (Invitrogen) and a glass coverslip. The chromosome paints from both manufacturers were comparable in quality and signal strength.

### Cell Scoring

Samples were coded prior to their arrival at the cytogenetics laboratory at Wayne State University and the code was not broken until all the data were collected. A minimum of 1800 metaphase cells or 1000 cell equivalents (CE; 1 CE = 0.56 metaphase cell) (Tucker, 2010b) were scored for each subject by trained observers using epifluorescent microscopes. Coordinates and digital photographs were taken for all abnormal cells, and every abnormal cell was double checked by an expert cytogeneticist to assure accuracy and consistency in the identification of chromosome aberrations.

All abnormalities in each metaphase cell were recorded using the PAINT nomenclature system (Tucker et al., 1995). Aberrant chromosomes with 2 or more junctions are referred to here as multi-junction chromosomes. Examples of these aberrations include (CBAb), (Bca), (abc), (cBc), (Bcbab), r(Ac) and (AAc), where A, B and C are portions of chromosomes painted blue, red and green respectively, and capital letters indicate material with a centromere. While insertions fit this definition, they

were not included in the multi-junction count. Note that some of these junctions involved adjacent pieces painted in the same color.

### **Urine Uranium Analyses**

At each biennial health surveillance visit, twenty-four hour urine samples were collected from each subject and shipped to the Joint Pathology Center (JPC) Biophysical Toxicology Laboratory (Joint Base Andrews Naval Air Facility, MD) (previously the Armed Forces Institute of Pathology's Department of Environmental Toxicologic Pathology, Washington DC) for analysis of total uranium using a previously described inductively coupled plasma-dynamic reaction cell-mass spectrometer (ICP-DR-MS) method (Ejnik et al., 2005; McDiarmid et al., 2007). Isotopic analysis was also conducted on each sample to determine the  $U^{235}/U^{238}$  ratio (Gray et al., 2012). Urine total Uranium concentrations were standardized on the basis of urine creatinine concentrations to obtain micrograms of U per gram of creatinine to account for urine dilution due to water intake and/or dehydration (Karpas et al., 1998; McDiarmid et al., 2000).

### **Statistical Analyses**

Least squares regression analyses were performed to evaluate the effects of urine Uranium, age, lifetime X-rays, cigarette smoking (in pack-years) and alcohol use on the frequencies of cells with translocated chromosomes, dicentrics, acentric fragments, color junctions, and abnormal cells per 100 CEs. Although Poisson regression is often used when cytogenetic data are being evaluated, here this approach

was problematic because the data did not fit a Poisson distribution, even upon transformation. For this reason all regressions assumed a normal distribution, and two variables, urine Uranium and lifetime X-rays, were  $\log_{10}$  transformed because this helped normalize their variances. The remaining variables were not transformed as this did not help normalize their variances.

Each of the cytogenetic variables was evaluated as the frequency of cells per 100 CEs with the given type of aberration, rather than by the frequency of each aberration type per 100 CEs. There is no difference in these two approaches unless some cells have more than one of the given aberration type. Because the doses observed in this study are very low, cells containing more than one aberration of a given type are rare. Translocations are the most commonly observed aberration type because they are stable and increase in frequency with age (Sigurdson et al., 2008b). The problem with translocations is that they arise in multiple ways and can appear as 1-junction (“1-way”) events, 2-junction (“2-way”) exchanges, and exchanges that are 3-way and 4 (or more)-way. The problems enumerating translocation frequencies for dosimetry have been described in detail (Tucker, 2008a, 2010a). The existence and prevalence of multi-way chromosome exchanges has been demonstrated with studies involving mFISH (Loucas et al., 2013). By evaluating the *frequency* of cells with translocations we have avoided problems associated with the manner in which translocations are enumerated (Tucker, 2008a, 2010a). For consistency, all the other aberration types evaluated here were also analyzed as the *frequency* of cells with the given type of event.

Cells with color junctions include all junctions found in chromosomes with apparently simple exchanges as well as in chromosomes with more than one junction, however, the data from the multi-junction chromosomes were not included in any of the other aberration types.

All statistical analyses were performed using JMP software, version 6.0, SAS Institute Inc.

## RESULTS

A total of 35 Gulf War I Veterans were evaluated for chromosome aberrations with FISH whole chromosome painting. Table 3 shows the demographics of the participants in the 2011 health surveillance visit. All were males, ranging in age from 39 to 62 years of age. Eleven participants (31%) have embedded DU fragments and all but one has higher urine Uranium concentrations compared to donors who do not have fragments. Donor age at time of blood draw, lifetime X-ray exposure, cigarette smoking, alcohol binges and urine Uranium concentrations are listed for each subject in Table 4.

**Table 3.** Demographic characteristics of the DU follow-up program 2011 medical surveillance visit participants.

	2011 cohort (n=35)	
	n	%
Gender (% males)	35	100
Race		
African American	11	31
Asian American	1	3
Caucasian	21	60
Hispanic	2	6
Participants with embedded DU fragments	11	31
Age*	45.94 +/- 5.51	

\* Mean age at the time of 2011 evaluation ( $\pm$  standard deviation)



**Table 4.** Urine Uranium concentration, age, lifetime X-rays received, pack years smoked and alcohol use for 2011 DU Follow-up Program medical surveillance visit participants.

Donor code	Urine Uranium concentration ( $\mu\text{g}$ U/g creatinine)	Donor age	X rays lifetime	Pack years	Alcohol use
9-102	0.3682	41	15	13.34	no
9-104	39.9574	54	20	8.5	no
9-105	9.0231	44	21	2.5	no
9-110	0.0054	44	100	0	no
9-115	0.1131	44	25	0	no
9-116	0.0828	52	35	0	no
9-119	0.0009	51	11	0	no
9-120	0.0059	53	20	0	no
9-121	0.2116	49	10	0	no
9-122	1.2307	48	100	26.25	yes
9-123	2.5329	41	20	0	no
9-126	0.0066	40	100	16.5	yes
9-129	1.4165	46	30	0	no
9-130	0.0037	42	50	0	no
9-145	11.0453	58	24	2	no
9-174	0.0020	39	51	2.63	yes
9-176	0.0045	55	25	8	yes
9-179	0.0040	44	100	48.39	no
9-182	0.0125	54	17	39.03	no
9-183	0.0047	62	31	6.49	no
9-186	0.0039	44	6	0	no
9-187	0.0024	41	no data	0	no
9-190	0.0058	46	5	0	no
9-193	2.8308	48	20	0	no
9-194	0.0036	52	75	13.71	no
9-198	0.0031	46	60	0	no
9-199	0.9826	47	3	7.94	no
9-200	0.0034	46	20	0	no
9-204	0.0028	41	10	0	no
9-205	0.0011	41	15	6.66	no
9-210	0.0030	44	5	7	yes
9-212	0.0094	41	no data	17	yes
9-219	0.0057	48	30	0	no
9-220	0.0125	43	18	28.99	no
9-221	0.0331	43	no data	0	no
Mean	2.00	46.6	33.5	7.3	
S.D.	7.03	5.6	30.1	11.9	

A minimum of 1800 metaphase cells per donor were analyzed for chromosome damage. The numbers of normal cells, abnormal cells, color junctions and aberrant chromosomes including translocations, dicentrics, acentric fragments, insertions, centric and acentric rings, and multi-junction chromosomes observed per donor, are shown in Table 5. A total of 479 translocated chromosomes and 55 dicentric chromosomes were observed in the 64,193 metaphase cells evaluated in these 35 subjects. Translocated chromosomes are the predominant aberration type seen in this study because they are more stable than all other types of aberrations. Insertions are thought to have similar stability but are much more rare; only 5 were observed here. All donors were checked for possible clones of abnormal cells (Johnson et al., 1999) but none were found.

**Table 5.** Number of metaphase cells scored and types of aberrant chromosomes observed in individual participants.

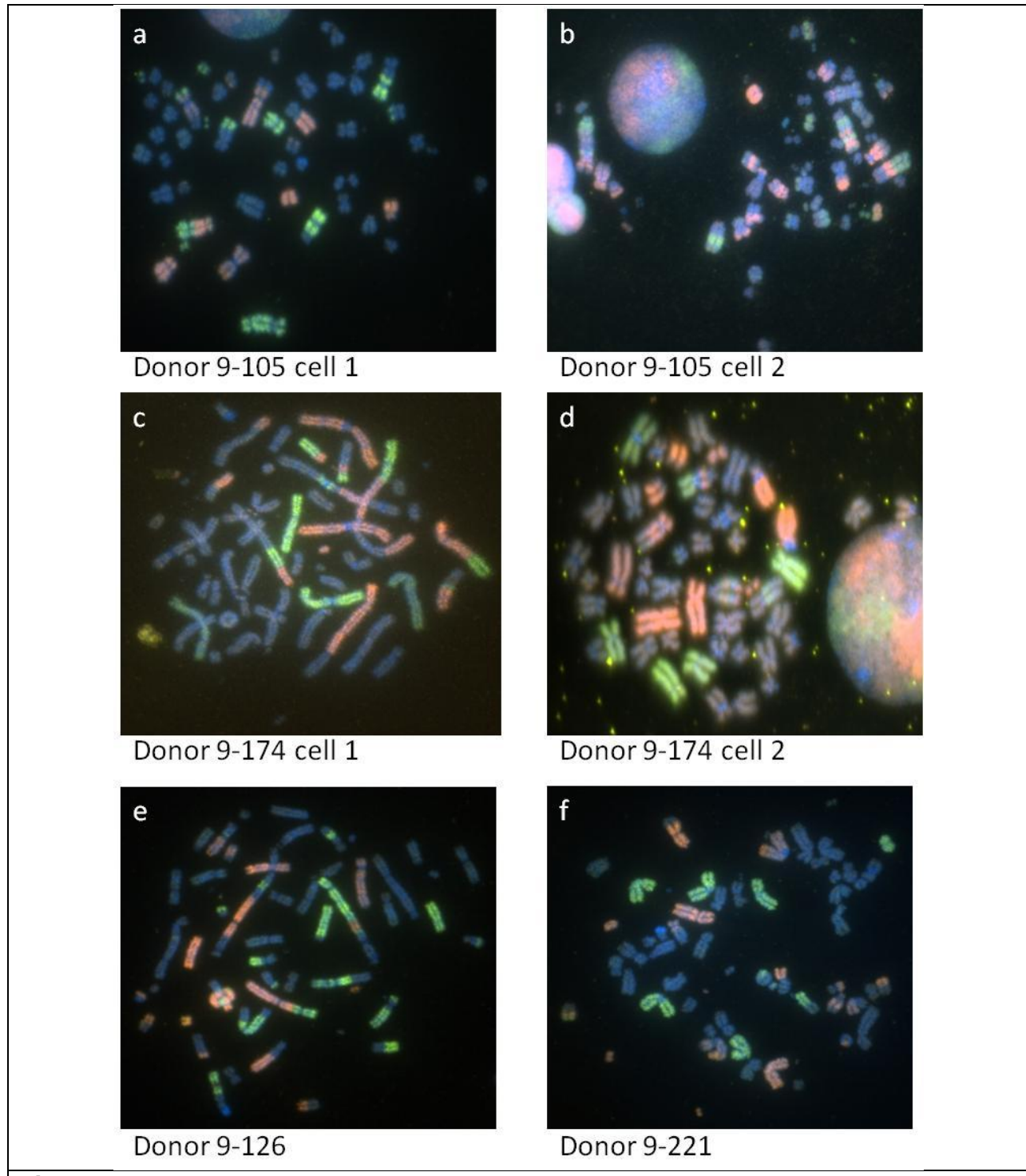
Donor code	Total metaphase cells scored	Normal cells	Aberrant cells	Color junctions	Translocated chromosomes	Dicentrics	Acentric fragments	Insertions	Rings	Multi-junction chromosomes
9-102	1815	1810	5	9	8	1	0	0	0	0
9-104	1820	1805	15	24	20	2	4	0	0	1
9-105	1805	1798	7	23	13	2	7	0	0	3
9-110	2000	1994	6	8	7	1	2	0	0	0
9-115	1804	1799	5	8	8	0	1	0	0	0
9-116	1813	1801	12	26	22	2	1	0	0	1
9-119	1800	1791	9	24	16	3	2	0	0	2
9-120	1812	1800	12	21	16	2	2	1	0	0
9-121	1804	1797	7	16	15	0	1	0	0	0
9-122	1813	1796	17	30	27	1	4	0	0	0
9-123	1806	1800	6	11	10	0	1	0	0	0
9-126	1802	1789	13	20	14	2	5	1	0	0
9-129	1817	1812	5	10	8	1	1	0	0	0
9-130	1867	1852	15	30	23	2	3	0	0	1
9-145	1814	1792	22	50	28	8	10	1	0	1
9-174	1814	1809	5	35	8	1	8	1	0	7
9-176	1817	1814	3	5	4	1	0	0	0	0
9-179	1812	1800	12	22	18	2	2	0	0	0
9-182	1808	1803	5	10	10	0	0	0	0	0
9-183	1804	1790	14	22	18	2	3	0	0	0
9-186	1809	1802	7	11	10	1	1	0	0	0
9-187	2386	2380	6	12	10	2	0	0	0	0
9-190	1807	1796	11	25	15	2	2	0	0	3
9-193	1809	1803	6	9	9	0	1	0	0	0
9-194	1809	1791	18	39	24	6	7	1	0	1
9-198	1809	1802	7	14	14	0	1	0	0	0
9-199	1807	1799	8	8	9	1	6	0	0	2
9-200	1813	1805	8	11	10	0	1	0	0	0
9-204	1805	1799	6	12	12	0	0	0	0	0
9-205	1822	1814	8	13	12	1	0	0	0	0
9-210	1814	1809	5	9	7	1	1	0	0	0
9-212	1814	1803	11	15	15	0	3	0	0	0
9-219	1807	1803	4	6	3	2	2	0	0	0
9-220	1817	1800	17	30	26	2	4	0	0	0
9-221	1818	1811	7	27	10	4	10	0	3	4
Total	64,193	63,869	324	645	479	55	96	5	3	26
Mean	1834	1825	9	18	14	2	3	0	0	1
S.D.	101.72	102.42	4.69	10.53	6.52	1.69	2.81	0.36	0.51	1.50

Some cells contained chromosomes with multiple junctions that did not readily fit into any aberrant chromosome category. A total of 14 cells containing 26 multi-junction chromosomes were found in 11 different donors (Table 6). Uncommon aberrations such as centric and acentric two-color rings and a chromosome with 5 color junctions were observed. Six cells with 10 or more color junctions were found in four donors (Figure 3.1). Two of these 6 cells had too many color junctions to count reliably and were excluded from further analyses, the other four cells were included in these analyses. These highly damaged cells did not show any association with urine Uranium concentration. Table 7 presents the number of cells with each type of chromosome aberration, and includes the aberrations from the 4 highly damaged cells in which color junctions could be reliably counted.

**Table 6.** Multi-junction chromosomes observed.

Donor code	# of cells with multi-junction chromosomes	# of multi-junction chromosomes	Multi-junction chromosomes*
9-104	1	1	(Bca)
9-105	1	3	(cBc), (ABc), (CaC)
9-116	1	1	(AAc)
9-119	2	2	(aCabC), (AAc)
9-130	1	1	(CAb)
9-145	1	1	(Bcbab)
9-174	2	7	(Acb), (abc), (BcA), (AbaCa), (CbA), (Acbcab), (CBAb)
9-190	2	3	(aBa), (bAb), (Cba)
9-194	1	1	(bCa)
9-199	1	2	(AcB), (cAca)
9-221	1	4	(Abc), (bAba), r(ab), r(Ac)
Total	14	26	

\* Where A, B and C chromosomes are labeled blue, red and green respectively.



**Figure 3.1** Highly damaged cells, which could be termed *rogue*, each with more than 10 color junctions found in this study. The donor number is indicated beneath each image. Multi-junction chromosomes found in cells a, c, d, and f were scored for aberrations, which are listed in Table 4. The aberrations from these four cells are included in the analyses reported here. Cells b and e were not included in these analyses because the types and numbers of their aberrations could not be reliably determined.

**Table 7.** Number of cells with each type of chromosome aberration.

Donor code	Color junctions	Translocated chromosomes	Dicentrics	Acentric fragments	Insertions	Rings	≥ 10 color junctions	Multi-junction chromosomes
9-102	5	4	1	0	0	0	0	0
9-104	11	11	2	4	0	0	0	1
9-105	6	6	1	1	0	0	2 <sup>a</sup>	1
9-110	5	4	1	2	0	0	0	0
9-115	4	4	0	1	0	0	0	0
9-116	12	11	2	1	0	0	0	1
9-119	9	9	3	2	0	0	0	2
9-120	11	9	2	2	1	0	0	0
9-121	7	7	0	1	0	0	0	0
9-122	16	14	1	3	0	0	0	0
9-123	6	6	0	1	0	0	0	0
9-126	10	8	2	5	1	0	1 <sup>b</sup>	0
9-129	5	4	1	1	0	0	0	0
9-130	15	13	2	3	0	0	0	1
9-145	21	16	6	8	1	0	0	1
9-174	4	4	1	3	1	0	2	2
9-176	3	3	1	0	0	0	0	0
9-179	12	10	2	2	0	0	0	0
9-182	5	5	0	0	0	0	0	0
9-183	13	11	2	3	0	0	0	0
9-186	6	5	1	1	0	0	0	0
9-187	6	6	2	0	0	0	0	0
9-190	11	10	2	2	0	0	0	2
9-193	5	5	0	1	0	0	0	0
9-194	17	13	6	5	1	0	0	1
9-198	6	6	0	1	0	0	0	0
9-199	4	5	1	4	0	0	0	1
9-200	7	7	0	1	0	0	0	0
9-204	6	6	0	0	0	0	0	0
9-205	8	7	1	0	0	0	0	0
9-210	5	4	1	1	0	0	0	0
9-212	8	8	0	3	0	0	0	0
9-219	4	2	2	2	0	0	0	0
9-220	16	14	2	4	0	0	0	0
9-221	6	3	4	4	0	1	1	1
Total	295	260	52	72	5	1	3	14
Mean	8	7	1	2	0	0	0	0
S.D.	4.48	3.65	1.48	1.80	0.36	0.17	0.38	0.65

<sup>a</sup> One of these 2 cells had too many color junctions to count.

<sup>b</sup> This cell had too many color junctions to count

To compare aberration frequencies among the subjects, metaphase cell counts were converted to cell equivalents (CEs). Table 8 shows the total number of CEs scored per donor, and the frequency of each type of aberration per 100 CEs. The frequencies of aberrant cells and translocated chromosomes per 100 CEs ranged from 0.29 to 2.17, and 0.30 to 2.76, respectively. The frequencies of insertions and rings in this data set are very low, therefore these aberration types were not evaluated further.



**Table 8.** Aberrations per 100 Cell Equivalents (CEs)\*.

Donor code	CEs scored	Aberrant cells	Multi-junction chromosomes	Color junctions	Translocated chromosomes	Dicentrics	Acentric fragments	Insertions	Rings
9-102	1016	0.49	0.00	0.89	0.79	0.10	0.00	0.00	0.00
9-104	1019	1.47	0.10	2.35	1.96	0.20	0.39	0.00	0.00
9-105	1011	0.69	0.10	2.28	1.29	0.20	0.69	0.00	0.00
9-110	1120	0.54	0.00	0.71	0.63	0.09	0.18	0.00	0.00
9-115	1010	0.49	0.00	0.79	0.79	0.00	0.10	0.00	0.00
9-116	1015	1.18	0.10	2.56	2.17	0.20	0.10	0.00	0.00
9-119	1008	0.89	0.20	2.38	1.59	0.30	0.20	0.00	0.00
9-120	1015	1.18	0.00	2.07	1.58	0.20	0.20	0.10	0.00
9-121	1010	0.69	0.00	1.58	1.48	0.00	0.10	0.00	0.00
9-122	1015	1.67	0.00	2.95	2.66	0.10	0.39	0.00	0.00
9-123	1011	0.59	0.00	1.09	0.99	0.00	0.10	0.00	0.00
9-126	1009	1.29	0.00	1.98	1.39	0.20	0.50	0.10	0.00
9-129	1018	0.49	0.00	0.98	0.79	0.10	0.10	0.00	0.00
9-130	1046	1.43	0.10	2.87	2.20	0.19	0.29	0.00	0.00
9-145	1016	2.17	0.10	4.92	2.76	0.79	0.98	0.10	0.00
9-174	1016	0.49	0.20	3.45	0.79	0.10	0.79	0.10	0.00
9-176	1018	0.29	0.00	0.49	0.39	0.10	0.00	0.00	0.00
9-179	1015	1.18	0.00	2.17	1.77	0.20	0.20	0.00	0.00
9-182	1012	0.49	0.00	0.99	0.99	0.00	0.00	0.00	0.00
9-183	1010	1.39	0.00	2.18	1.78	0.20	0.30	0.00	0.00
9-186	1013	0.69	0.00	1.09	0.99	0.10	0.10	0.00	0.00
9-187	1336	0.45	0.00	0.90	0.75	0.15	0.00	0.00	0.00
9-190	1012	1.09	0.20	2.47	1.48	0.20	0.20	0.00	0.00
9-193	1013	0.59	0.00	0.89	0.89	0.00	0.10	0.00	0.00
9-194	1013	1.78	0.10	3.85	2.37	0.59	0.69	0.10	0.00
9-198	1013	0.69	0.00	1.38	1.38	0.00	0.10	0.00	0.00
9-199	1012	0.79	0.10	0.79	0.89	0.10	0.59	0.00	0.00
9-200	1015	0.79	0.00	1.08	0.98	0.00	0.10	0.00	0.00
9-204	1011	0.59	0.00	1.19	1.19	0.00	0.00	0.00	0.00
9-205	1020	0.78	0.00	1.27	1.18	0.10	0.00	0.00	0.00
9-210	1016	0.49	0.00	0.89	0.69	0.10	0.10	0.00	0.00
9-212	1016	1.08	0.00	1.48	1.48	0.00	0.30	0.00	0.00
9-219	1012	0.40	0.00	0.59	0.30	0.20	0.20	0.00	0.00
9-220	1018	1.67	0.00	2.95	2.56	0.20	0.39	0.00	0.00
9-221	1018	0.69	0.10	2.65	0.98	0.39	0.98	0.00	0.29
Total	35,948								
Mean	1027								
S.D.	56.96								

\* 1 CE = 0.56 metaphase.

Table 9 shows the frequencies of cells with translocated chromosomes, color junctions, dicentrics and acentric fragments per 100 CEs; these are the dependent variables that were used in the regression analyses to determine whether DU exposure and other risk factors had an effect on chromosome aberration frequencies. The independent variables assessed were urine Uranium concentration, age, lifetime x-ray exposure, pack years smoked, and alcohol use. Each dependent variable was evaluated separately with each independent variable and the results are shown in Table 10. Urine Uranium concentration, X-rays, smoking, and alcohol use showed no statistically significant relationship with any type of chromosome aberration. Age was significant with 4 of the 5 aberration types; the only variable that was not significant was cells with acentric fragments per 100 CEs. A significant increase of cells with translocated chromosomes with age is evident in Figure 3.2. Similar significant increases are seen with dicentrics (Figure 3.3), color junctions (Figure 3.4) and abnormal cell frequencies (Figure 3.5).

**Table 9.** Frequency of cells with various aberrations per 100 CEs\*.

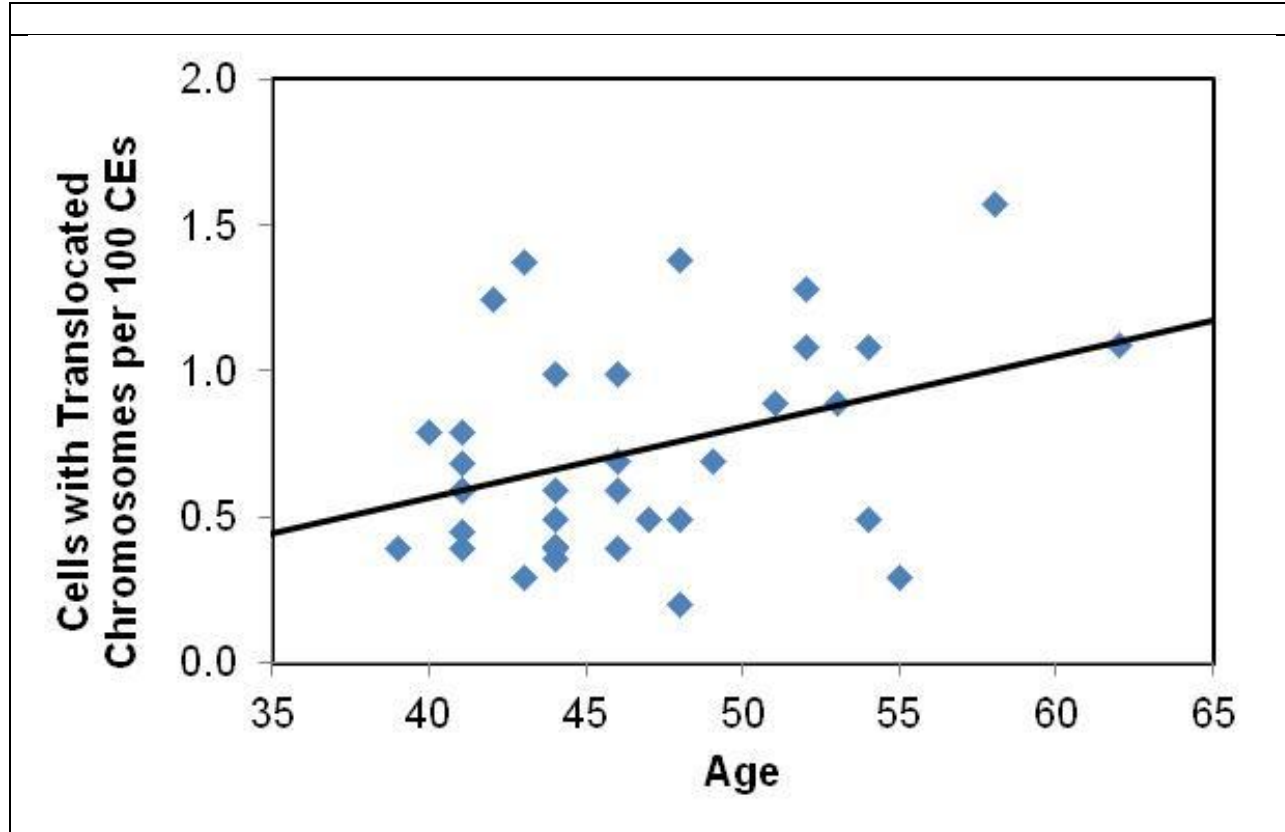
Donor code	Color junctions	Translocated chromosomes	Dicentric	Acentric fragments	Rings	Multi-junction chromosomes
9-102	0.49	0.39	0.10	0.00	0.00	0.00
9-104	1.08	1.08	0.20	0.39	0.00	0.10
9-105	0.59	0.59	0.10	0.10	0.00	0.10
9-110	0.45	0.36	0.09	0.18	0.00	0.00
9-115	0.40	0.40	0.00	0.10	0.00	0.00
9-116	1.18	1.08	0.20	0.10	0.00	0.10
9-119	0.89	0.89	0.30	0.20	0.00	0.20
9-120	1.08	0.89	0.20	0.20	0.00	0.00
9-121	0.69	0.69	0.00	0.10	0.00	0.00
9-122	1.58	1.38	0.10	0.30	0.00	0.00
9-123	0.59	0.59	0.00	0.10	0.00	0.00
9-126	0.99	0.79	0.20	0.50	0.00	0.00
9-129	0.49	0.39	0.10	0.10	0.00	0.00
9-130	1.43	1.24	0.19	0.29	0.00	0.10
9-145	2.07	1.58	0.59	0.79	0.00	0.10
9-174	0.39	0.39	0.10	0.30	0.00	0.20
9-176	0.29	0.29	0.10	0.00	0.00	0.00
9-179	1.18	0.99	0.20	0.20	0.00	0.00
9-182	0.49	0.49	0.00	0.00	0.00	0.00
9-183	1.29	1.09	0.20	0.30	0.00	0.00
9-186	0.59	0.49	0.10	0.10	0.00	0.00
9-187	0.45	0.45	0.15	0.00	0.00	0.00
9-190	1.09	0.99	0.20	0.20	0.00	0.20
9-193	0.49	0.49	0.00	0.10	0.00	0.00
9-194	1.68	1.28	0.59	0.49	0.00	0.10
9-198	0.59	0.59	0.00	0.10	0.00	0.00
9-199	0.40	0.49	0.10	0.40	0.00	0.10
9-200	0.69	0.69	0.00	0.10	0.00	0.00
9-204	0.59	0.59	0.00	0.00	0.00	0.00
9-205	0.78	0.69	0.10	0.00	0.00	0.00
9-210	0.49	0.39	0.10	0.10	0.00	0.00
9-212	0.79	0.79	0.00	0.30	0.00	0.00
9-219	0.40	0.20	0.20	0.20	0.00	0.00
9-220	1.57	1.38	0.20	0.39	0.00	0.00
9-221	0.59	0.29	0.39	0.39	0.10	0.10

\* 1 CE = 0.56 metaphase.

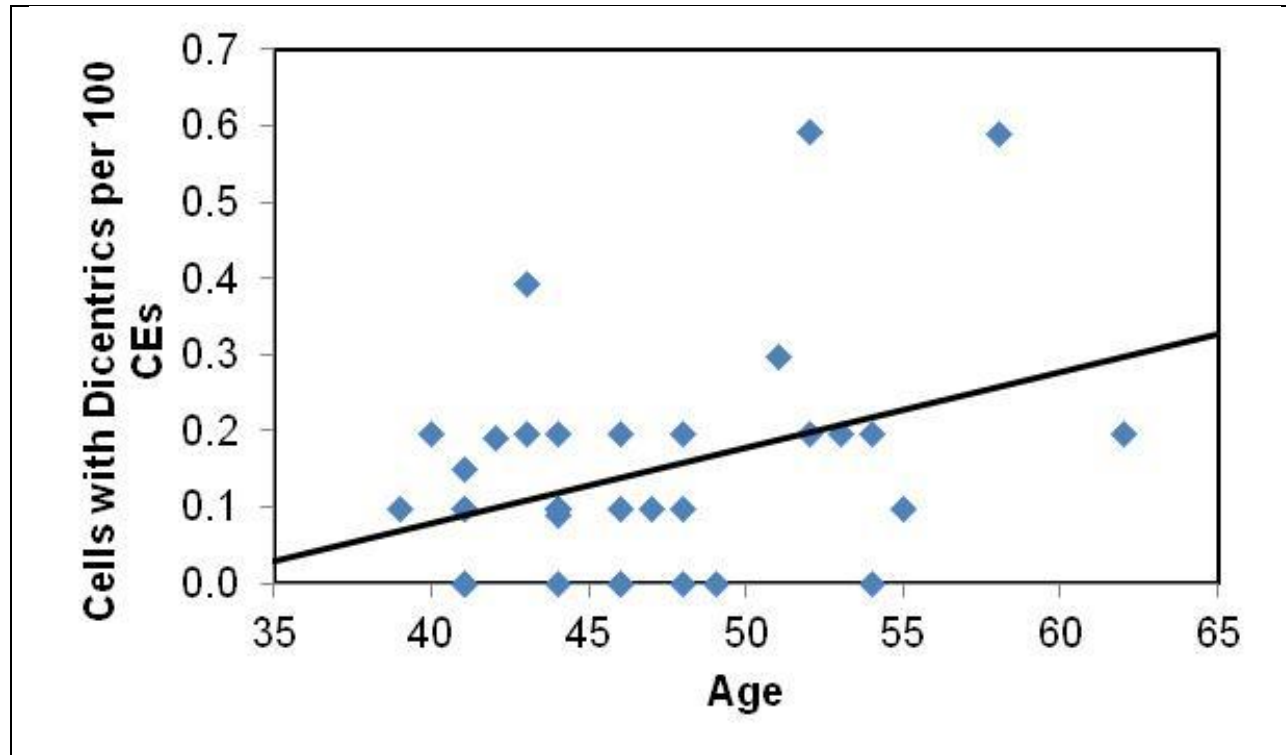
**Table 10.** Summary of P-Values from Least-Square Regression Analyses

Cytogenetic endpoints per 100 CEs	Independent variables (evaluated separately)				
	Age	Urine Uranium concentration ^	Lifetim e X- rays^	Pack- years	Alcohol Use
Cells with translocated chromosomes	0.0261	0.425	0.179	0.089	0.630
Cells with dicentrics	0.0243	0.948	0.262	0.908	0.581
Cells with acentric fragments	0.135	0.176	0.172	0.481	0.496
Cells with color junctions	0.0227	0.460	0.090	0.099	0.484
Abnormal cells	0.0260	0.303	0.107	0.091	0.662

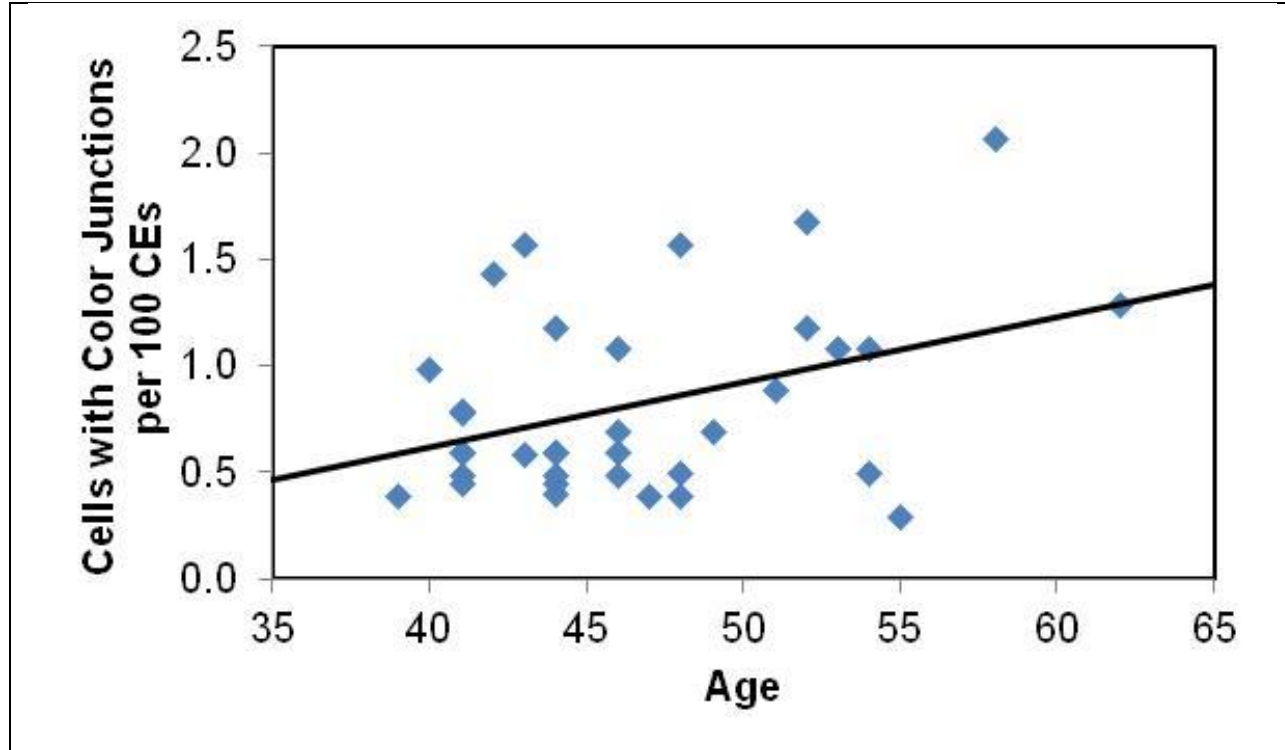
^ log10 transformed



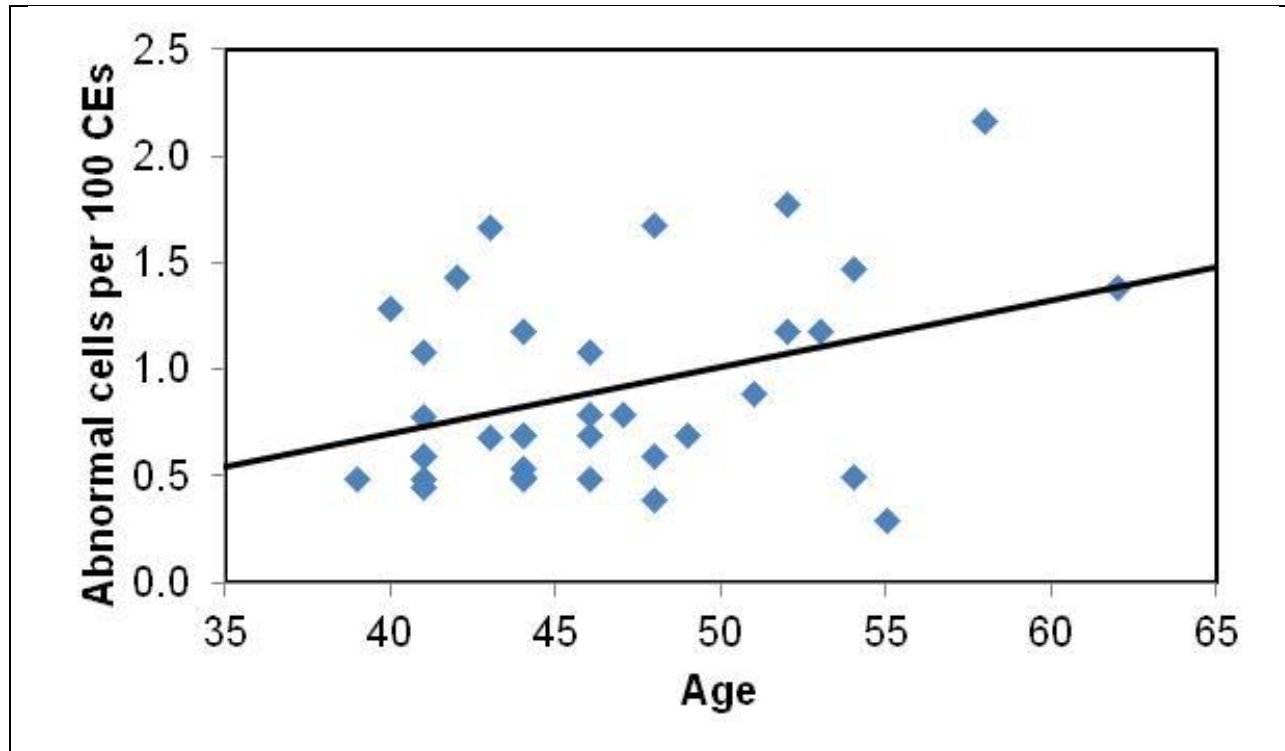
**Figure 3.2** Relationship between the number of cells containing translocated chromosomes per 100 CEs and Veterans' age at the time of sample collection. The line is the best fit least squares regression ( $p = 0.026$ ).



**Figure 3.3** Relationship between the number of cells containing dicentric per 100 CEs and Veterans' age at the time of sample collection. The line is the best fit least squares regression ( $p = 0.024$ ).



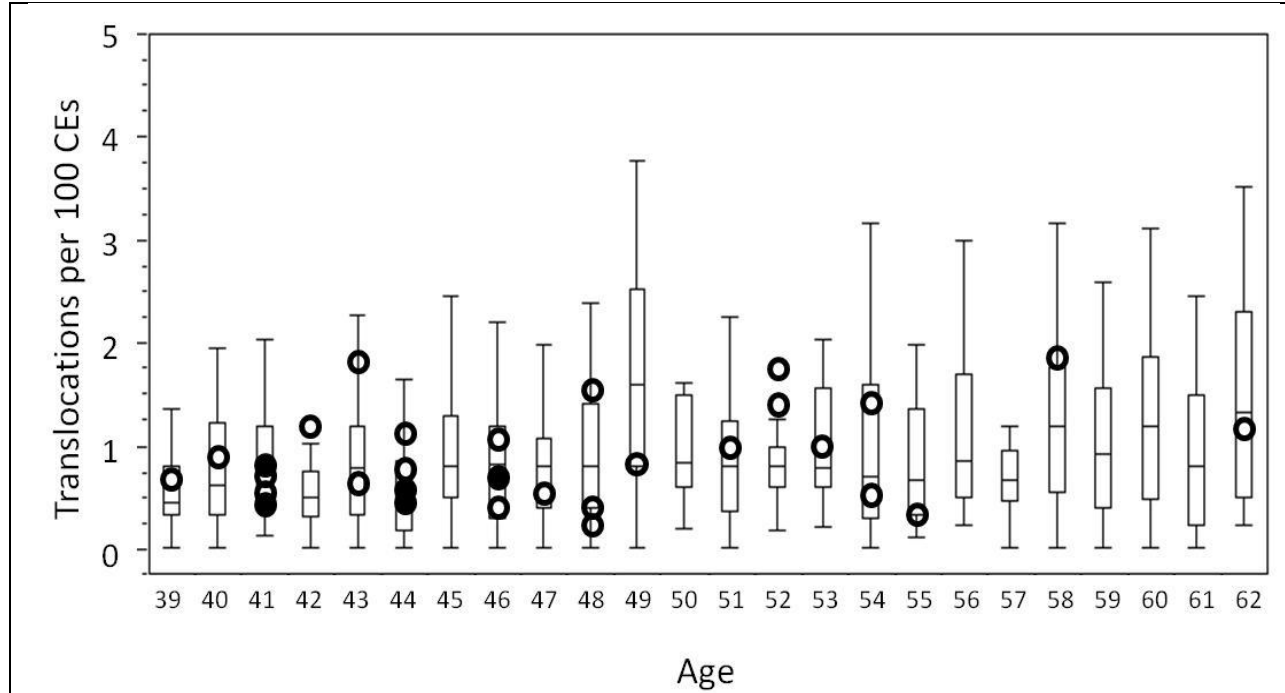
**Figure 3.4** Relationship between the number of cells containing color junctions per 100 CEs and Veterans' age at the time of sample collection. The line is the best fit least squares regression ( $p = 0.023$ ).



**Figure 3.5** Relationship between the number of abnormal cells per 100 CEs and Veterans' age at the time of sample collection. The line is the best fit least squares regression ( $p = 0.026$ ).



The translocation frequencies observed in this study were compared to the translocation frequencies in 1933 normal healthy individuals from around the world (Sigurdson et al., 2008b). Figure 3.6 shows that the translocation frequencies in the DU exposed individuals reported here are very similar to this international population. This evidence further supports the results of our analyses which show that urine Uranium concentration is not significantly associated with chromosome damage in these subjects.



**Figure 3.6** Baseline translocation frequencies per 100 CEs for a population of 1933 normal healthy individuals from around the world (Sigurdson et al., 2008) compared to the frequencies found in the 35 DU-exposed veterans. The figure was adapted from (Tucker and Luckinbill, 2011). Black circles represent DU-exposed individuals in this study; open circles represent one individual while filled circles represent 2 individuals with the same value. “Translocations” for this figure were counted as 1 reciprocal translocation = 1 translocation, and 1 translocated chromosome = 1 translocation; all other translocations data in this paper are presented as translocated chromosomes. The Veterans in this study are well within the normal background frequencies of translocations and are not elevated due to long term DU exposure.

## DISCUSSION

No correlations between chronic DU exposure and chromosome aberrations were observed in this study. The results of this work are consistent with previous studies showing no U-related health effects resulting from exposure to depleted uranium in this population (Squibb et al., 2012). Clear trends were seen with respect to age, which is a well known factor associated with increased chromosome aberrations. Similarly, X-rays, smoking and alcohol use did not show any correlation with chromosome damage. The numbers of X-rays are reported by the subjects from memory and may not be as accurate as desired, and the cumulative X-ray doses are not known. Even if the total X-ray doses were known, they would probably be too low to have a measurable effect on chromosome aberration frequencies. In addition, the population sample size of 35 individuals makes it hard to discern small effects such as those arising from smoking and alcohol use, so it is not surprising that these variables (Ramsey et al., 1995; Sigurdson et al., 2008b) showed no statistical significance.

Urine Uranium levels remain elevated in Veterans with embedded fragments, indicating the existence of persistent localized chemical and radiological exposure, however the genotoxic effects of DU, if any, are too small to be observed by our methods with a population this size. In our previous work, the cytokinesis block micronucleus assay was used to enumerate micronuclei (MN) in this same population (Bakmutsky et al., 2011). Thirty participants were analyzed both in that study and in the present effort. No statistically significant correlation was observed between the frequencies of micronucleated cells and any type of chromosome aberration (data not

shown). As in the present work, no association was observed between DU exposure and MN levels in our previous study.

Health risk estimates resulting from DU exposure of U.S. soldiers during the 1991 Gulf War have been evaluated mathematically (Marshall, 2008), who developed models for cancers, birth defects, radiation doses, kidney toxicity, neurotoxic effects and bone damage. The results for radiation doses received from DU are well under the allowed lifetime limits for radiation workers. The theoretical increased risk from DU exposure on cancer development is 1.4% for highly exposed Veterans with retained fragments; the US average risk of developing fatal cancer is 24% (Marshall, 2008), so this theoretical increase is likely too low to be observed. Most individuals in this DU cohort did not experience such high exposures, nor do they have large retained fragments, so the long-term health risks based on the currently available data, including that presented here, are probably negligible. The risk of radiation or chemically induced birth defects is also not significant, according to this modeling, and neither is long term kidney damage. The results of this study are consistent with the findings of the DU Follow-Up Program.

A small study by Schroder et al. (Schroder et al., 2003) examined chromosome aberrations in 16 Veterans deployed in the Gulf and/or Balkans who described possible but undocumented DU inhalation exposures. Study volunteers were not tested for levels of uranium in urine, thus it was difficult to establish whether DU exposure had actually occurred. The authors describe an increase in the frequencies of dicentric and acentric ring chromosomes compared to laboratory controls, however other causative factors were not examined.

Many *in vitro* studies have evaluated the clastogenic, carcinogenic and reproductive effects of DU in cell lines and animal models. DU was shown to be a potential human bronchial carcinogen based on *in vitro* exposures causing chromosome instability and aberrations in cultured bronchial epithelial cells (LaCerte et al., 2010; Xie et al., 2010). The kidney is thought to be a target organ for DU toxicity. Exposure of rat kidney mitochondria to uranyl acetate caused damage to the mitochondrial outer membrane, and impairment of the electron chain as well as oxidative stress (Shaki et al., 2012). Renal anemia was induced in rats exposed by ingestion to DU for 9 months which resulted in kidney deterioration and a 20% reduction in erythrocyte counts (Berradi et al., 2008). Rats exposed to 4 months of DU-contaminated food showed genotoxic and reproductive effects both in the F<sub>0</sub> and F<sub>1</sub> generations (Hao et al., 2009, 2012). All the rats in the above studies were exposed to DU in their food, which is a different route of exposure than the inhalation and wound contamination experienced by Veterans. Food contamination is an unlikely source of exposure for most human populations because environmental contamination by DU is localized and confined to where DU munitions were used. Levels and isotopic composition of uranium in food samples from the Balkan region where DU-containing weapons were widely employed were found to be comparable to those in other European regions where DU was not used (Carvalho and Oliveira, 2010).

Micronucleus formation was investigated in mouse embryonic fibroblasts (MEFs) exposed either to enriched uranium (EU) or DU to compare their genotoxic profiles (Darolles et al., 2010). Both DU and EU were shown to induce MN formation, however DU-induced MN contained centromeres, indicating that DU can be classified as

aneugenic as well as clastogenic. While the clastogenic effects of EU were mainly due to its radiotoxicity because of the correlation between ionizing radiation doses received by cells and the number of micronucleated cells, the effect of DU was due to its chemotoxicity where no such correlation between radiation dose and MN formation could be established.

Another study presented evidence that normal background gamma radiation could interact with embedded DU particles in the human body to produce an enhanced dose of radiation to the surrounding tissue due to the photo-electric absorption of the radiation by DU metal. However, the radiation dose from these DU particles has been determined to be negligible (Pattison, 2013) and not likely to raise the lifetime cancer risk significantly.

We observed 6 cells with unusually high amounts of chromosome damage, each of which had more than 10 color junctions. These types of cells have been termed multidamaged or rogue cells in the literature (Neel et al., 1992; Sevan'kaev et al., 1993). No clear-cut definition of rogue cells exists, but they are generally defined as cells with excess chromosome-type damage with a prevalence of acentric fragments and multicentric chromosomes. The damage in these cells varies from a few breaks to cases where the number of aberrations cannot readily be determined due to their complexity, such as in 2 of the 6 cells found here. Rogue cells appear to be non-randomly distributed in individuals. Two participants in our study had 2 such cells and the other 2 subjects had one each. Rogue cells appear to be transient in individuals, more rogue cells may or may not be found in subsequent sampling (Neel et al., 1992). Rogue cells show no correlation with radiation exposure and are just as readily found in

controls as in radiation exposed individuals (Lazutka, 1996; Mustonen et al., 1998; Sevan'kaev et al., 1993). The direct causes of rogue cells are still unknown but this phenomenon has been observed in many cytogenetic studies around the world (Mustonen et al., 1998). We observed no association between urine Uranium levels and the incidence of heavily damaged or rogue cells in our population.

Here we have shown there is no correlation between any type of structural chromosome aberration and urine Uranium levels, smoking, or alcohol use in US Veterans of Gulf War I. However, age was found to be significantly associated with most types of chromosome damage. These results indicate that long term depleted uranium exposure does not cause increased rates of chromosome damage in Gulf War I Veterans.

## CHAPTER 4

### CYTOGENETIC SUSCEPTIBILITY TO IONIZING RADIATION IN NEWBORNS AND ADULTS

#### INTRODUCTION

Structural chromosome aberrations are a well known biomarker of exposure to clastogenic agents such as ionizing radiation and can be helpful in determining cancer susceptibility rates. Chromosome damage is also a biomarker of the effect of clastogens on human cells. Aberrations such as translocations are useful and important for evaluation of chronic exposures. Translocations are the most stable chromosome rearrangement because they are compatible with cell division. For this reason translocations can persist for years and have been used to assess chronic and temporally-displaced exposure (Jones et al., 2001; Tucker et al., 1997). Cells containing unstable aberrations such as dicentrics, fragments, and rings are eliminated quickly because they are strongly selected against in mitosis, but they are a useful marker of recent acute exposures.

Baseline chromosome aberration frequencies, especially translocations, are well known to increase with age (Ramsey et al., 1995; Sigurdson et al., 2008b). Aberrations accumulate in older individuals due to many possible factors such as lifetime cumulative low level exposures to external and internal environmental agents, telomere shortening (Soler et al., 2009), free radicals, accumulation of misrepaired genome lesions, and cellular senescence (Sabin and Anderson, 2011). Cells with chromosome aberrations may not necessarily develop into cancer cells (Mladinic et al., 2010) but increased aberration levels are well known indicators of increased cancer risk (Bonassi et al.,



2008; Hagmar et al., 1998). Radiation exposures, a common cause of aberration formation, increase the overall lifetime risk of developing cancer as seen in atomic bomb survivors (Pierce and Mendelsohn, 1999). Cancer risks in background and exposed individuals both increase with age. Children exposed to radiation have a higher lifetime risk for developing cancers due to having more rapidly dividing cells that can propagate DNA damage such as chromosome aberrations throughout a longer remaining lifetime than an adult (Shah et al., 2012). When an adult is exposed to ionizing radiation, their induced cancer risk remains relatively stable and does not decline as age at exposure increases (Shuryak et al., 2010).

Radiation-induced cancer risk is a consequence of DNA damage but cannot be directly extrapolated from the effects of radiation-induced chromosome aberrations. However, not much is known about whether cellular responses to ionizing radiation change as a factor of the ageing process. Rodent models exposed to radiation at different stages of life show a higher sensitivity for ionizing radiation at the birth to weaning period and decreasing in adulthood (Hattis et al., 2004).

To our knowledge, this is the first cytogenetic study that has systematically evaluated changes in susceptibility to ionizing radiation for people of different ages. Here we show that cord blood obtained from newborns is significantly more susceptible to radiation-induced structural chromosome aberrations than is peripheral blood obtained from adults. Among adults however, no significant change in response to radiation was observed with age. The findings of this work may influence future risk assessment for ionizing radiation exposure in people of all ages.

## MATERIALS AND METHODS

### Recruitment of Subjects and Blood Collection

Prior approval for this use of human subjects was obtained from the Wayne State University (WSU) Human Investigations Committee for both adult and newborn donors. Adult subjects were recruited from the WSU campus and surrounding areas. Subjects filled out a health questionnaire (provided in Appendix A) which inquired about age, gender, smoking and alcohol use. Peripheral blood samples were drawn into 6 mL Vacutainer Sodium Heparin tubes (Becton Dickinson) at the Campus Health Center by a registered nurse. Samples and questionnaires were coded immediately and transported to the cytogenetics laboratory within 30 minutes following phlebotomy.

Blood samples from full-term newborns were collected by a nurse or physician from the fetal side of the placenta and / or from umbilical cords (hereafter referred to simply as “cord” blood) into 6 mL BD Vacutainer Sodium Heparin tubes with prior consent of the newborns’ mothers. All deliveries occurred at the Hutzel-Webber Hospital, Detroit Medical Center, which is affiliated with WSU. After collection the samples were kept at 4°C for less than 24 hours before transporting the tubes in an insulated container via a 5 minute walk to the Gershenson Radiation Oncology Center at the Karmanos Cancer Institute.

## Cell Culturing, Irradiation, Slide Preparation And FISH Painting

Adult blood samples were split into five T25 suspension flasks with plug caps (CELLSTAR) and transported in an insulated container by car to the Gershenson Radiation Oncology Center, a distance of approximately 1 km from the cytogenetics laboratory. Cord blood samples were also split into the same number and type of flasks at the Radiation Oncology Center. All flasks were acutely irradiated with Cobalt-60 gamma rays at 1, 2, 3 and 4 Gy. The 0 Gy (control) flasks traveled with the other samples but were not irradiated.

All samples were then transported back to the cytogenetics laboratory, where 800  $\mu$ L of blood was dispensed into T25 flasks containing 10 mL of RPMI 1640 medium (Hyclone), supplemented with 15% Fetal Bovine Serum (Atlanta Biologicals), penicillin–streptomycin (100 units/mL penicillin G sodium, 100  $\mu$ g/mL streptomycin in 0.85% saline, Gibco), 2% PHA (Gibco) and 2 mM L-glutamine (Gibco) that had been pre-warmed to 37°C. Two T25 suspension flasks with vented caps (CELLSTAR) were set up for each dose and donor. Culture flasks were placed upright in a fully humidified incubator with 5% CO<sub>2</sub> at 37°C for 48 hrs. KaryoMAX Colcemid solution (10  $\mu$ g/mL, Gibco) was added at a final concentration of 0.1  $\mu$ g/mL 4 hours prior to the end of culture to arrest cells in metaphase. The cells were then gently re-suspended and decanted into 15 mL conical tubes, and centrifuged at 1400 RPM for 5 min at room temperature. The supernatant was aspirated and the cell pellets gently resuspended in 8 mL 75 mM KCL solution pre-warmed to 37°C and incubated for 30 minutes at 37°C. Two mL of freshly prepared fixative (methanol:acetic acid 3:1 v/v) solution was added to

each tube, mixed well and centrifuged at 1400 RPM for 5 minutes. The supernatant was again aspirated and the cells were washed three times with 2 mL of fixative. To make metaphase spreads the pellet was resuspended in a few drops of pure glacial acetic acid and cells were dropped onto ethanol-cleaned cold glass microscope slides over a 70°C water bath.

Slides were aged overnight at room temperature and subsequently stored in N<sub>2</sub> gas with a desiccant pack at -20°C. As needed, slides were returned to room temperature and hybridized with FISH Whole Chromosome Painting kits from Cambio or Applied Spectral Imaging following the manufacturers' protocols. Chromosomes 1, 2, and 4 were hybridized with a Cy3 labeled probe and chromosomes 3, 5, and 6 were simultaneously labeled with FITC. Slides were mounted with 50 µL ProLong Gold antifade reagent with DAPI (Invitrogen) and a glass coverslip and left to cure for 24 hours at 4°C. The chromosome paints from both manufacturers were tested and found to be comparable in quality and signal strength.

### **Slide Coding and Cell Scoring**

Individual slides for each donor and dose were coded after hybridization and before being scored. Approximately 1800, 900, 540, 180 and 180 metaphase cells, which correspond to 1000, 500, 300, 100 and 100 cell equivalents (1 CE = 0.56 metaphase cells) (Tucker, 2010b) were scored for each subject for the 0, 1, 2, 3 and 4 Gy doses, respectively. All slides were scored by trained observers using epifluorescent microscopes. Coordinates and digital photographs were recorded for all abnormal cells.

Every chromosome aberration in each abnormal cell was double checked by an expert cytogeneticist to assure accuracy and consistency across slide readers in the identification of chromosome damage.

All structural chromosome abnormalities were initially recorded using the PAINT nomenclature system (Tucker et al., 1995). Adjacent pieces of a chromosome labeled in different colors were recorded as color junctions. Each chromosome containing one centromere and one color junction was counted as a translocated chromosome. Dicentrics, acentric fragments, insertions, centric and acentric ring chromosomes and abnormal cells were also recorded. Aberrant chromosomes with 2 or more junctions are referred to here as multi-junction chromosomes; examples include (ACb), (BCaC), (CbC), (CABA), where A, B and C are portions of chromosomes painted blue, red and green respectively, and capital letters indicate chromosome material with a centromere (Tucker et al., 1995). Many multi-junction aberrant chromosomes were found at the higher doses, and these were divided into their corresponding simple aberrations for use in subsequent analyses. For example, a chromosome identified as (AbC) was counted as 2 translocated chromosome events (Ab) and (bC), and (CBa) was counted as a dicentric (CB) and a translocated chromosome (Ba). While insertions fit the definition of a multi-junction chromosome, they were categorized separately.

A mitotic index for each donor was obtained using control (0 Gy) slides stained with Giemsa. Scoring for all 30 samples was performed by one well-trained observer under a light microscope.

## Statistical Analysis

Univariate and multivariate regression analyses were performed to determine whether age is a significant factor in individual susceptibility to radiation-induced chromosome damage. Initial exploratory analyses were performed for each of the dependent variables, i.e., the frequencies per 100 CEs of translocated chromosomes, dicentrics, acentric fragments, rings, insertions, color junctions, and abnormal cells, which were regressed in multivariate analyses against the independent variables of dose, dose-squared, and age. As expected, dose and dose-squared were major factors in determining the frequency of each cytogenetic endpoint. In most of these analyses the effect of age was also found to be statistically significant, except for rings and insertions, for which comparatively fewer events were seen. Subsequent regression analyses incorporated an interaction term for age and dose (or dose-squared) and this interaction term was in many cases also found to be significant.

We then sought to determine which dose(s) (i.e., 0, 1, 2, 3, and / or 4 Gy) were responsible for the age effect. Here, regression analyses for each aberration type were performed individually with each dose using age as the sole independent variable. The frequencies of all aberration types except insertions and rings were found to *increase* with age in the control (0 Gy) group, consistent with published reports (Ramsey et al., 1995; Sigurdson et al., 2008b). In contrast, aberration frequencies in the irradiated samples generally *declined* with age. The slopes of the regression lines in the control (0 Gy) group were in the opposite direction from those of the irradiated samples and we wanted to evaluate the frequencies of events induced by radiation to better understand the susceptibility to ionizing radiation in our samples. To obtain the frequency of induced

events we subtracted the frequency of events in the control (0 Gy) group for each cytogenetic endpoint from the total frequencies of cytogenetic events. We then regressed these induced values for each endpoint with age as the sole independent variable for each dose.

The effects of other independent variables (gender, ethnicity, cigarette smoking, and alcohol use) were evaluated using univariate regression analyses.

All analyses were performed using JMP software, version 6.0, SAS Institute Inc.

## RESULTS

A total of 20 healthy adult and 10 newborn cord blood samples were evaluated with FISH whole chromosome painting for chromosome damage after receiving acute doses of 0 (control), 1, 2, 3 or 4 Gy of Cobalt-60 gamma rays. Table 11 shows the demographics of the study population including age at time of blood draw and gender. For the adult donors, self-reported race, pack years smoked, and alcoholic drinks per week are also provided. Cord blood donors' chromosomal sex was determined by the presence or absence of a Y chromosome in FISH painted metaphase cells counterstained with DAPI. No other data were obtained from the mothers of the newborns in regards to ethnicity, drinking, or smoking during pregnancy.



**Table 11.** Demographic characteristics of the 30 study participants.

Donor #	Age	Gender	Ethnicity	Pack years	Alcohol drinks per week
<b>Adults</b>					
1	26	M	Asian	0 <sup>a</sup>	25
2	35	M	African-American	0	6
3	44	M	White	0 <sup>b</sup>	1
4	56	F	White	0	1.5
5	67	F	White	24	0.1
6	78	F	White	20	1
7	22	F	African-American	6 <sup>c</sup>	8
8	48	M	Asian	0	0
9	75	M	White	2	14
10	39	M	Mexican-American	0	3
11	67	M	White	0	5
12	68	F	White	0	0
13	62	M	African-American	40	6
14	57	F	African-American	0	2
15	24	M	African-American	3 <sup>c</sup>	10
16	29	M	White	2.5	2
17	32	M	White	0	0.5
18	47	M	African-American	15	6.5
19	53	M	African-American	0	4
20	49	F	White	3.5	12
<b>Cord Bloods <sup>d</sup></b>					
21	0	F	-	-	-
22	0	M	-	-	-
23	0	F	-	-	-
24	0	M	-	-	-
25	0	M	-	-	-
26	0	F	-	-	-
27	0	F	-	-	-
28	0	M	-	-	-
29	0	M	-	-	-
30	0	F	-	-	-

<sup>a</sup> Occasional pipe/hookah use.

<sup>b</sup> Occasional chewing tobacco use.

<sup>c</sup> Years of smoking, amount of cigarettes per day not specified.

<sup>d</sup> Data for ethnicity, and pack-years and alcohol use during pregnancy were not obtained.

Table 12 shows the number of metaphase cells scored by dose group for adults and for newborns. The numbers of translocated chromosomes, dicentrics, acentric fragments, color junctions, insertions, centric and acentric rings that were observed are also shown. Metaphase cells were converted to cell equivalents (1 CE = 0.56 metaphase cell) to enable direct comparisons to be made of frequencies of different types of aberrations among donors.

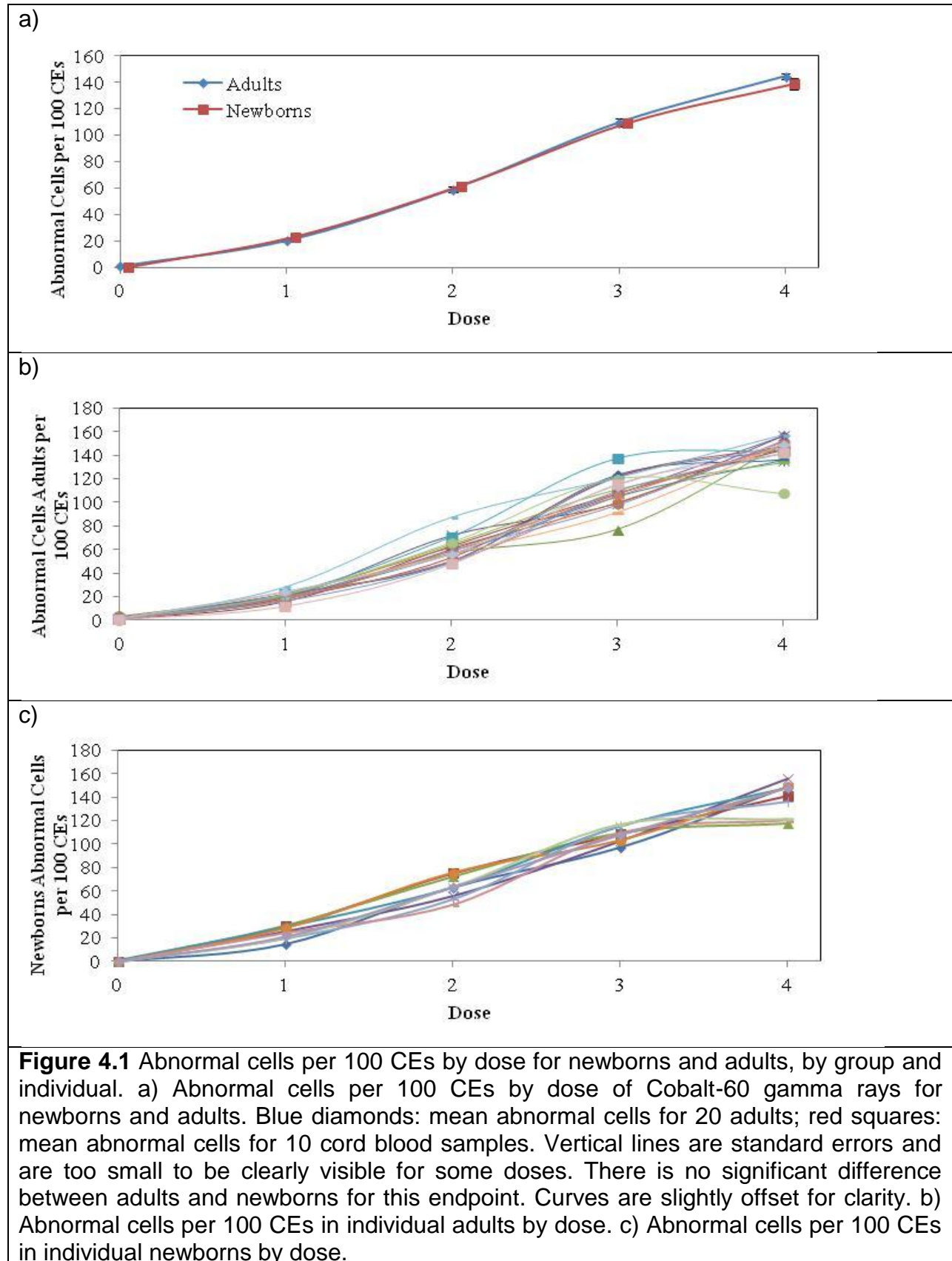
**Table 12.** Number of normal and abnormal cells, and number of structural chromosome aberrations for adults and newborns.

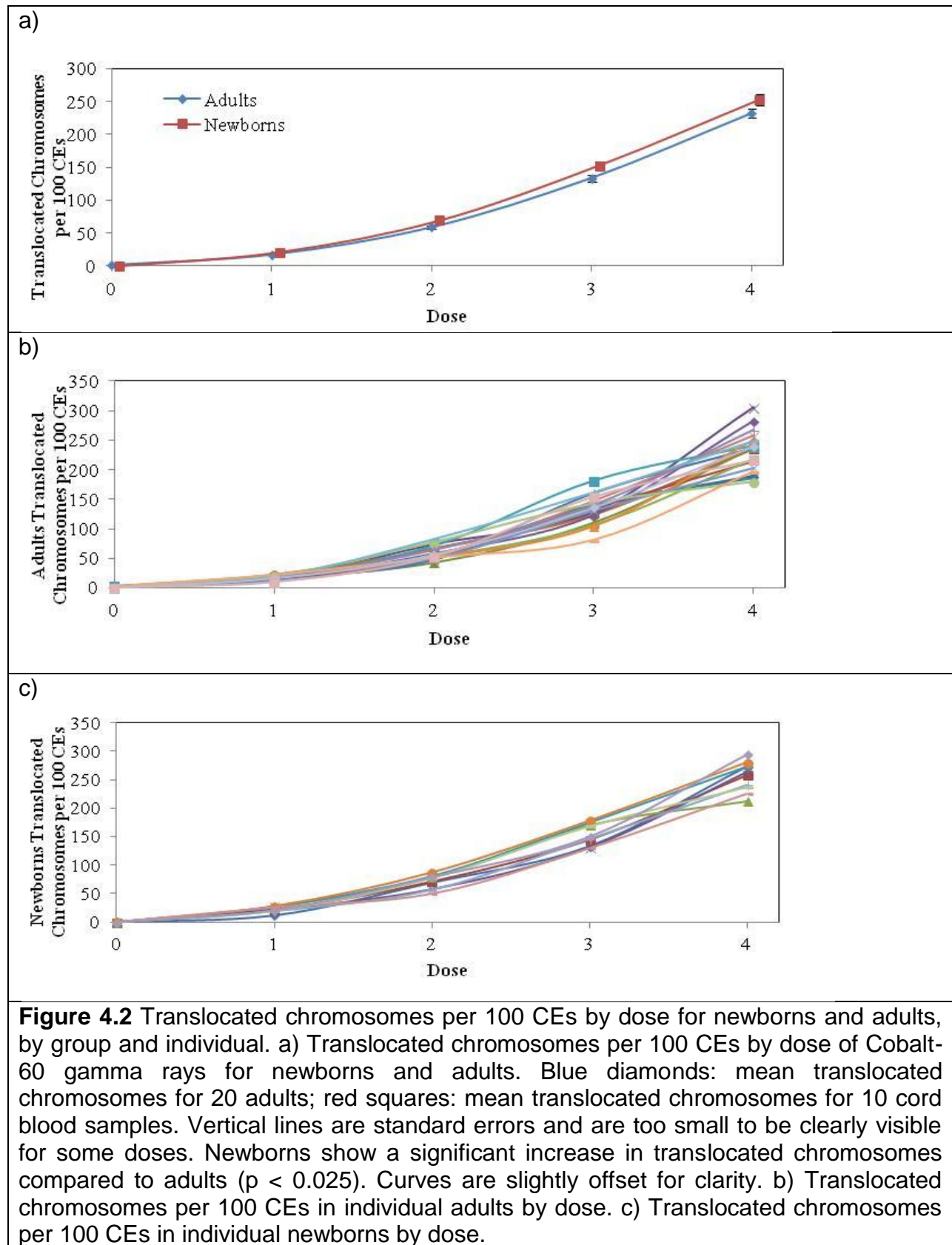
Dose (Gy)	# of metaphase cells scored	Cell equivalents scored <sup>^</sup>	Normal Cells	Abnormal Cells	Translocated chromosomes*	Dicentrics*	Acentric Fragments*	Color Junctions	Insertions	Centric rings	Acentric rings
<b>Adults</b>											
0	42,183	23,622	41,969	214	278	40	60	348	4	5	1
1	19,500	10,920	17,304	2,196	1,885	688	1,302	3,330	19	60	13
2	11,526	6,455	7,725	3,801	3,769	1,589	2,885	7,080	44	105	35
3	4,108	2,213	1,630	2,478	3,027	1,393	2,423	5,636	49	87	19
4	3,812	2,134	731	3,081	4,946	2,133	4,187	9,696	136	169	25
Total	81,129	45,344	69,359	11,770							
<b>Cord Bloods</b>											
0	18,560	10,394	18,543	17	8	3	11	14	0	1	0
1	9,401	5,265	8,131	1,270	1,158	282	738	2,146	10	15	2
2	5,508	3,084	3,555	1,953	2,180	571	1,527	4,073	19	34	5
3	1,950	1,092	761	1,189	1,668	472	1,246	3,195	20	30	6
4	1,967	1,102	439	1,528	2,822	820	2,026	5,405	75	47	10
Total	37,386	20,936	31,429	5,957							
Grand Total	118,515	66,280	100,788	17,727							

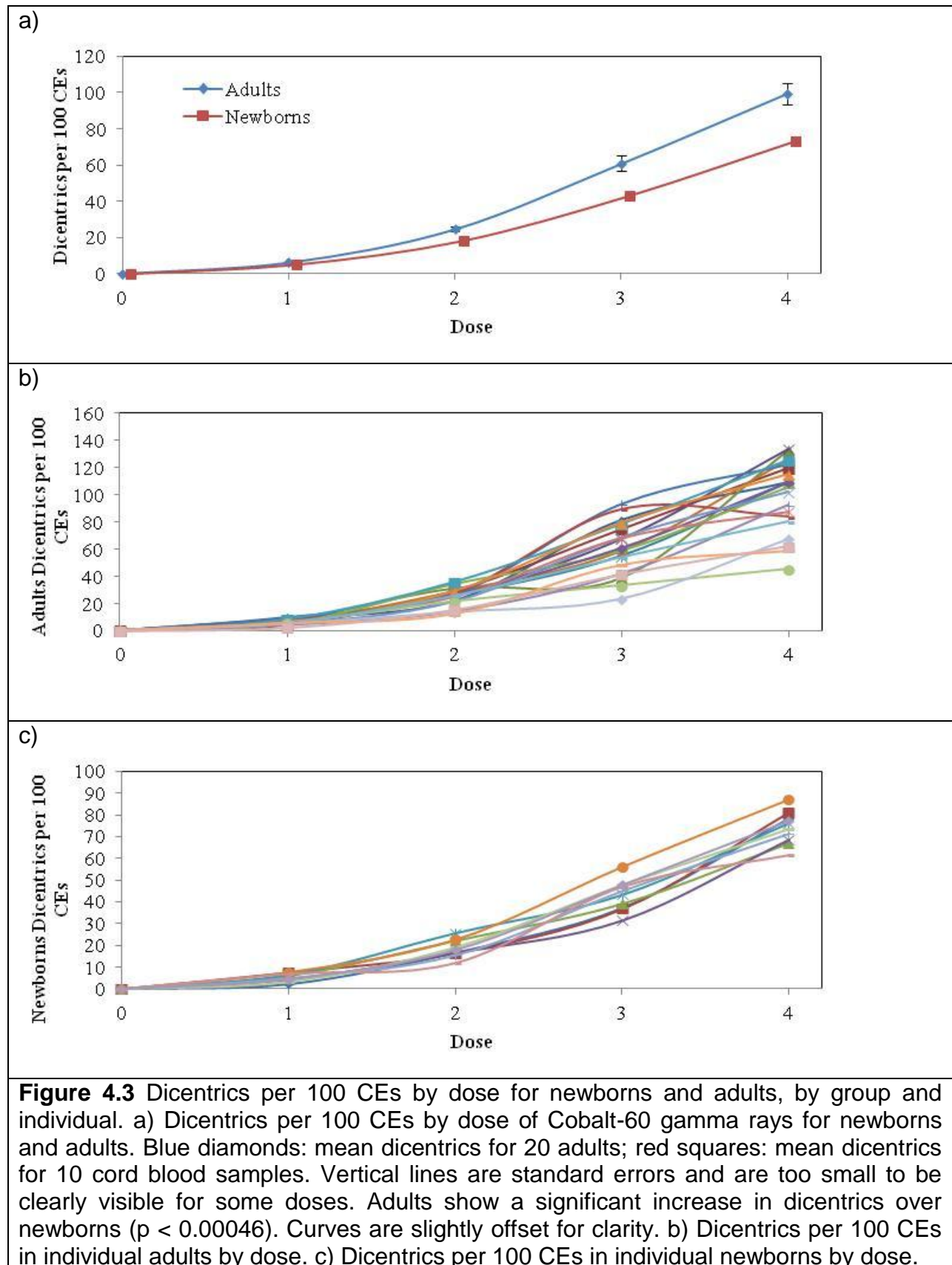
<sup>^</sup> 1 Cell equivalent = 0.56 metaphase cell

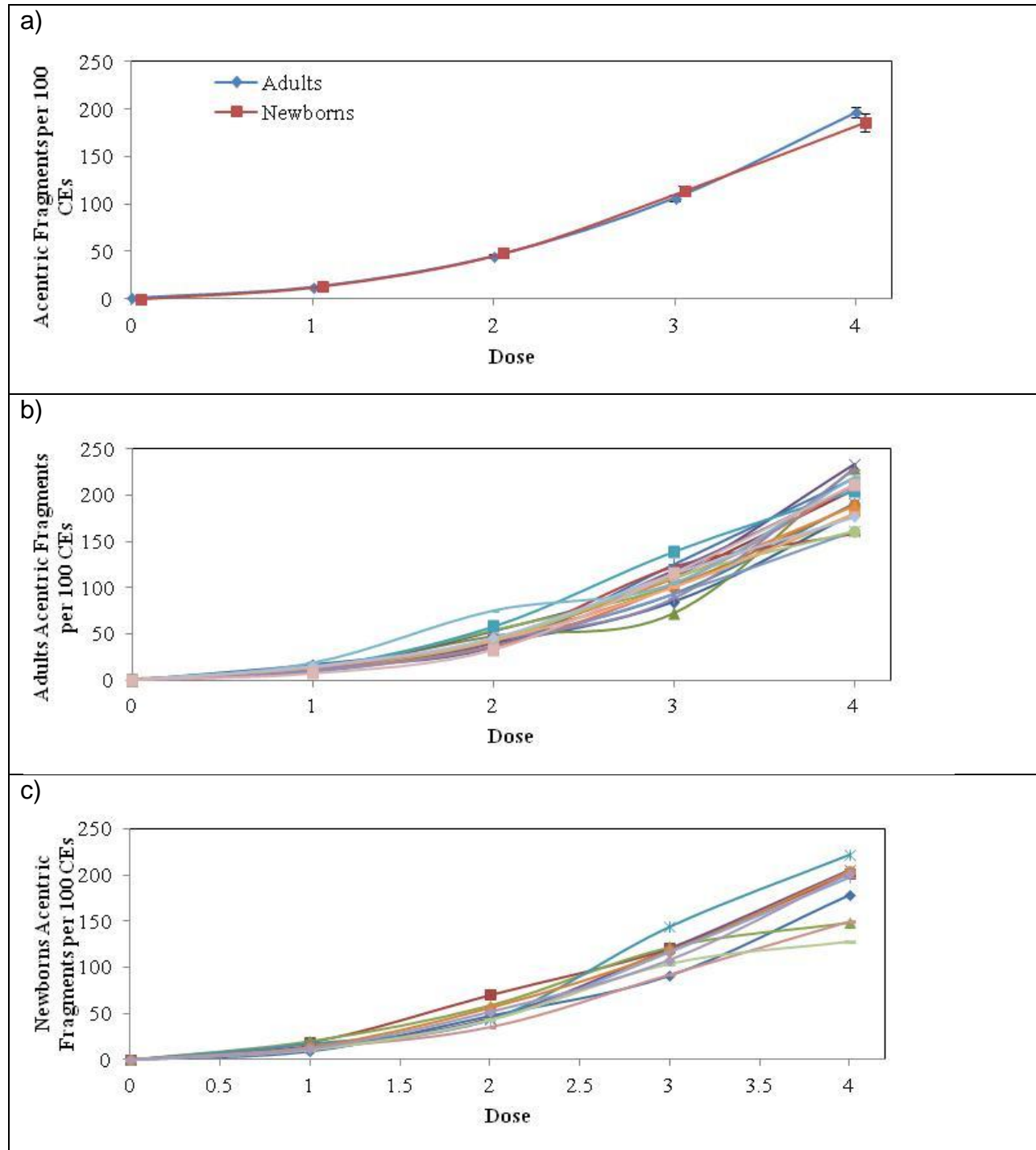
\* Includes data from multi-junction chromosomes

Figures 4.1-4.7 show the dose response curves for different cytogenetic endpoints for individual donors and as a summary comparison between adult and cord blood donor groups. Figure 4.1 shows that adults and newborns have similar numbers of abnormal cells after radiation exposure. Newborns as a group have significantly more translocated chromosomes (Figure 4.2) and color junctions (Figure 4.5) than adults but fewer dicentrics (Figure 4.3) and rings (Figure 4.7). Acentric fragments and insertions show no difference between the groups (Figures 4.4 and 4.6 respectively). Mitotic indices were determined for all control (0 Gy) samples (Figure 4.8). Newborns have significantly more mitotic cells compared to adults which likely explains some of the differences between the dose response curves.



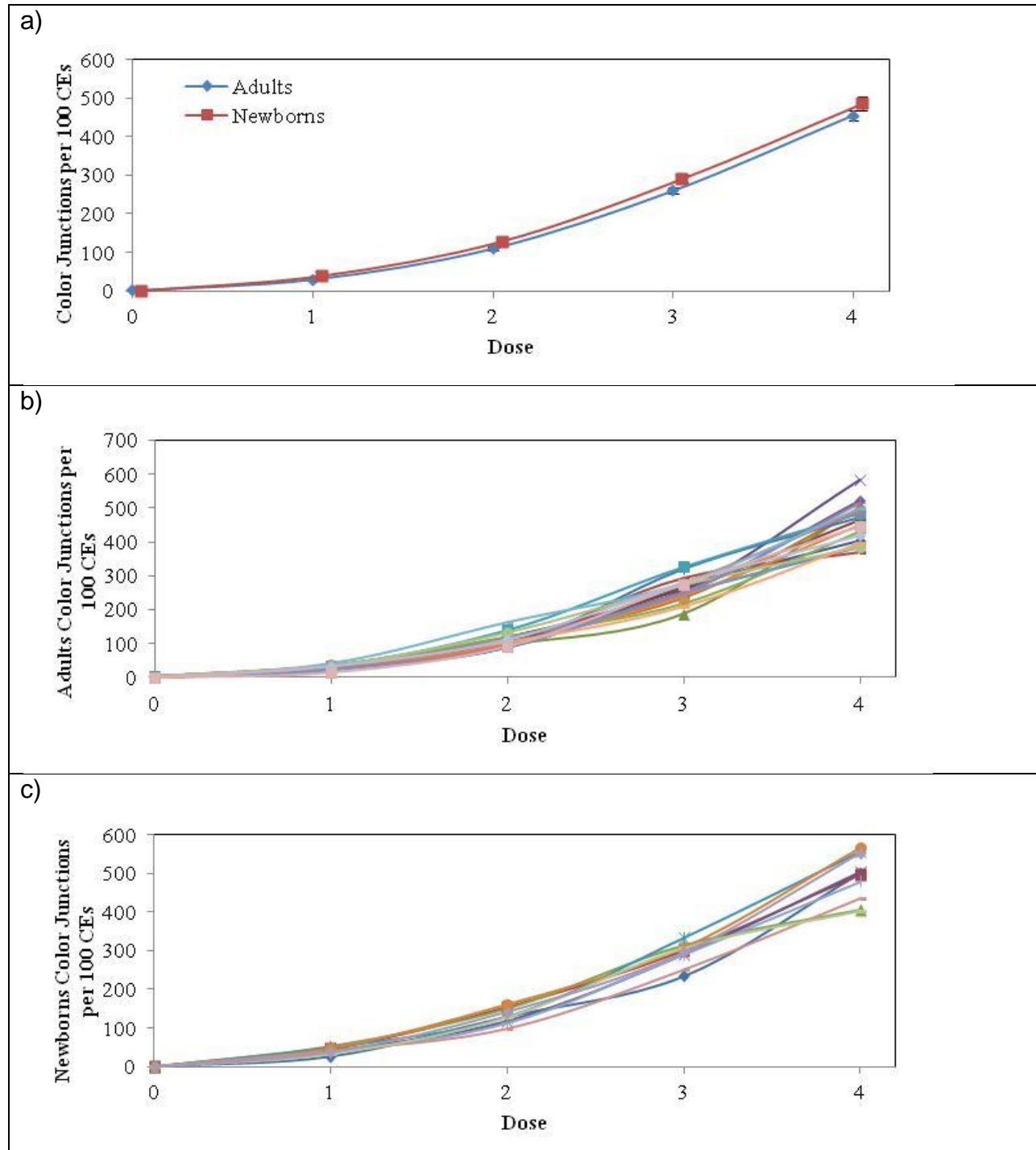




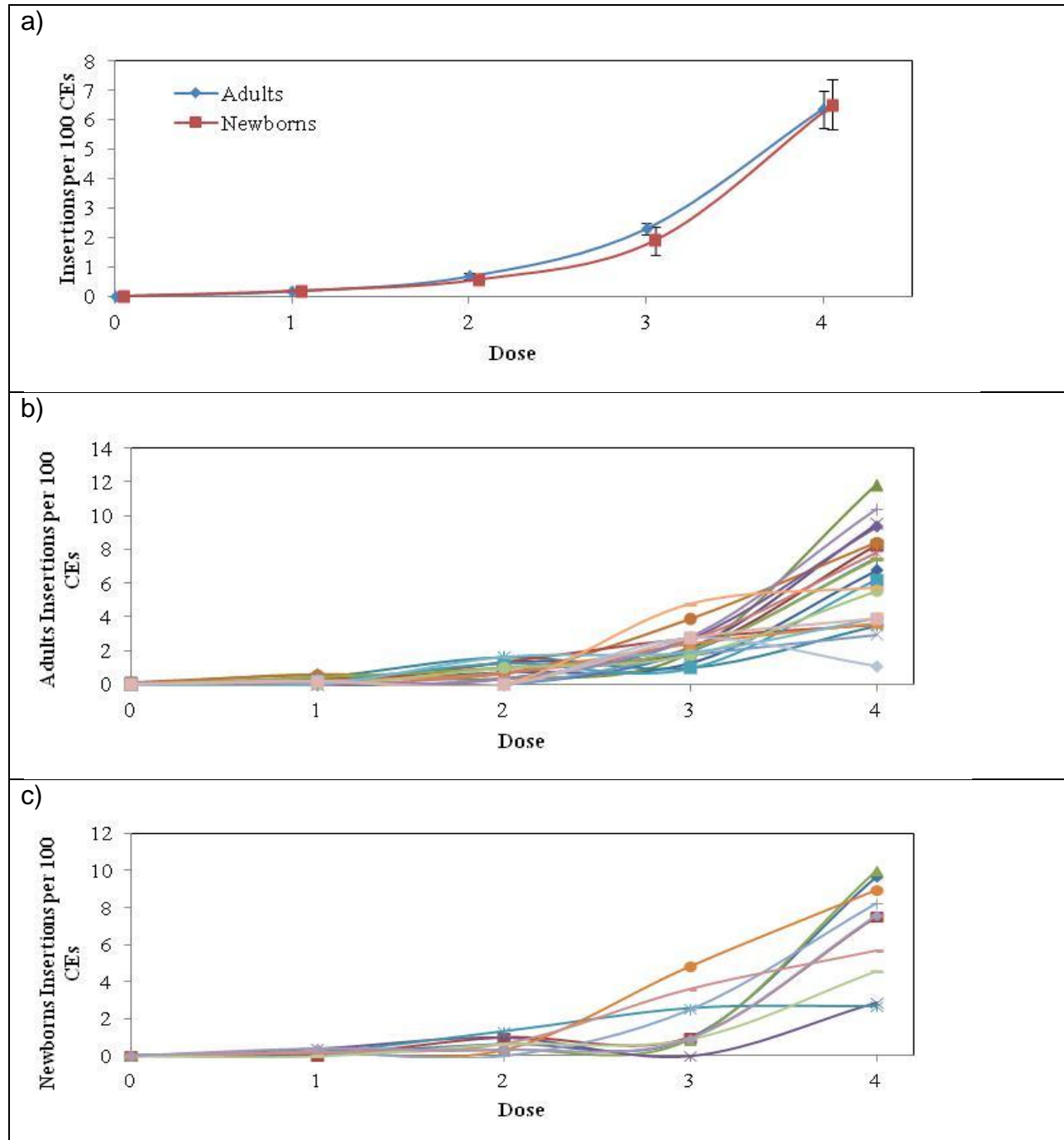


**Figure 4.4** Acentric fragments per 100 CE by dose for newborns and adults, by group and individual. a) Acentric fragments per 100 CE by dose of Cobalt-60 gamma rays for newborns and adults. Blue diamonds: mean acentric fragments for 20 adults; red squares: mean acentric fragments for 10 cord blood samples. Vertical lines are standard errors and are too small to be clearly visible for some doses. There is no statistically significant difference between adults and newborns for this endpoint. Curves are slightly offset for clarity. b) Acentric fragments per 100 CE in individual adults by dose. c) Acentric fragments per 100 CE in individual newborns by dose.

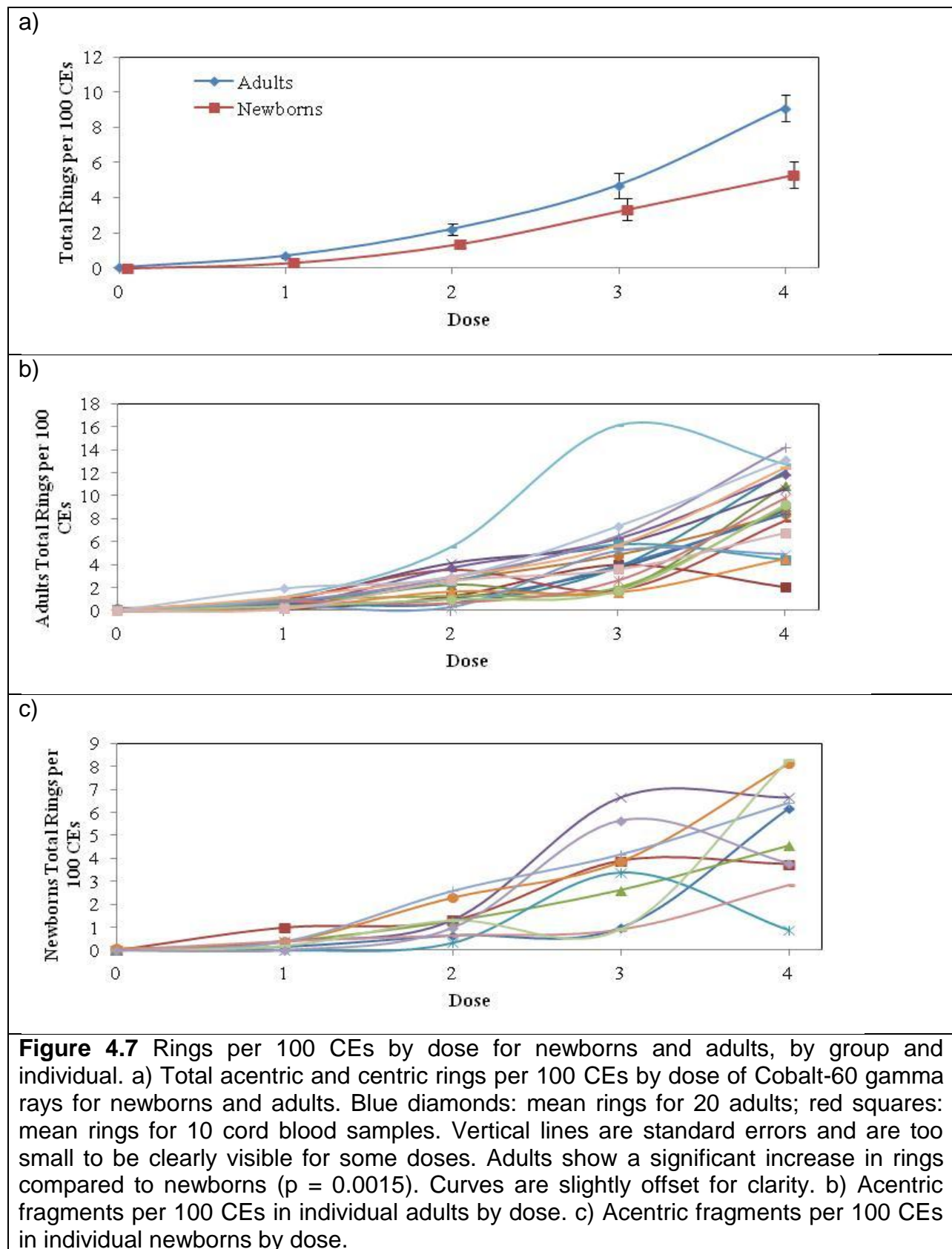


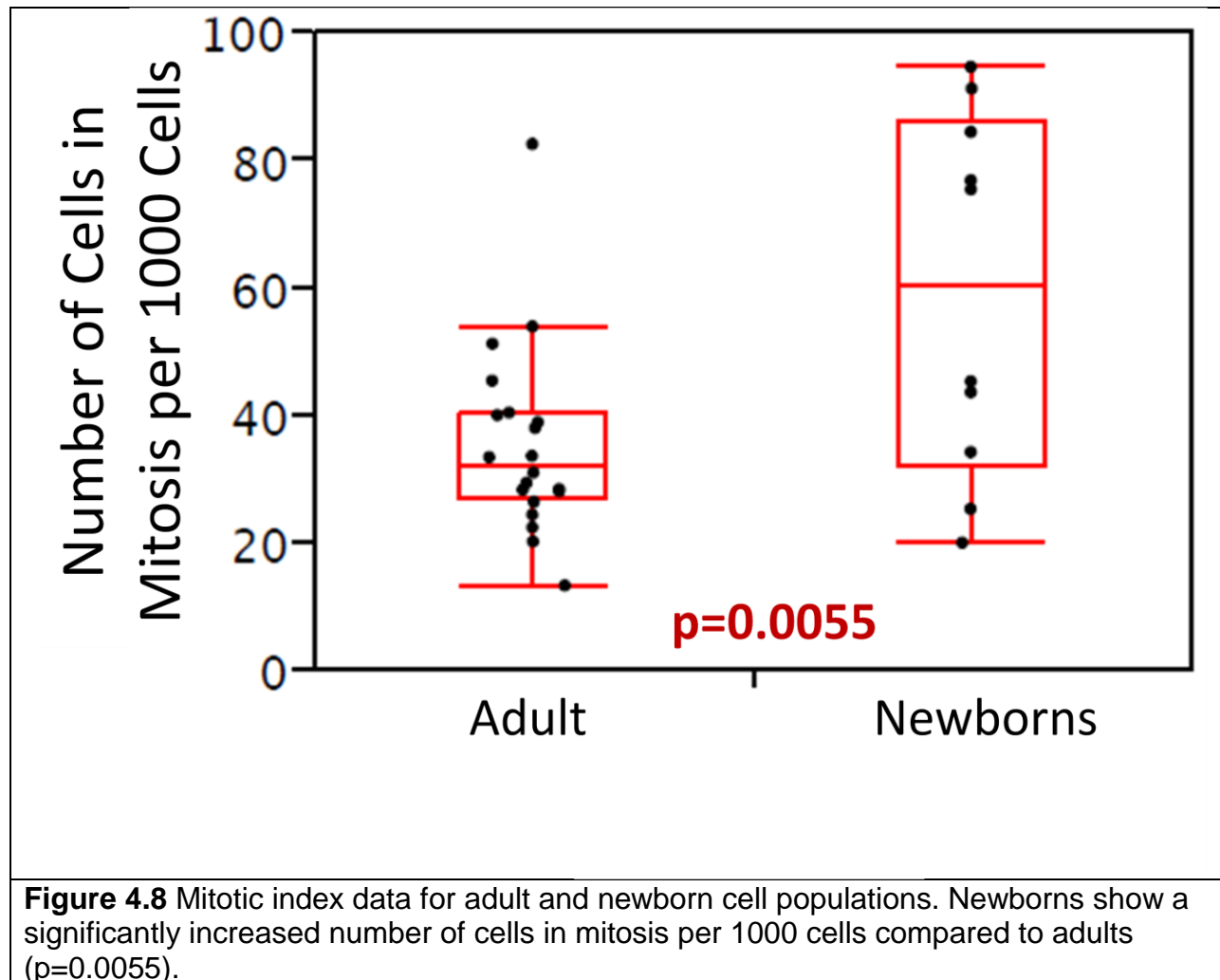


**Figure 4.5** Color junctions per 100 CE's by dose for newborns and adults, by group and individual. a) Color junctions per 100 CE's by dose of Cobalt-60 gamma rays for newborns and adults. Blue diamonds: mean color junctions for 20 adults; red squares: mean color junctions for 10 cord blood samples. Vertical lines are standard errors and are too small to be clearly visible for some doses. There is a significant increase in color junctions for newborns compared to adults ( $p < 0.0046$ ). Curves are slightly offset for clarity. b) Color junctions per 100 CE's in individual adults by dose. c) Color junctions per 100 CE's in individual newborns by dose.

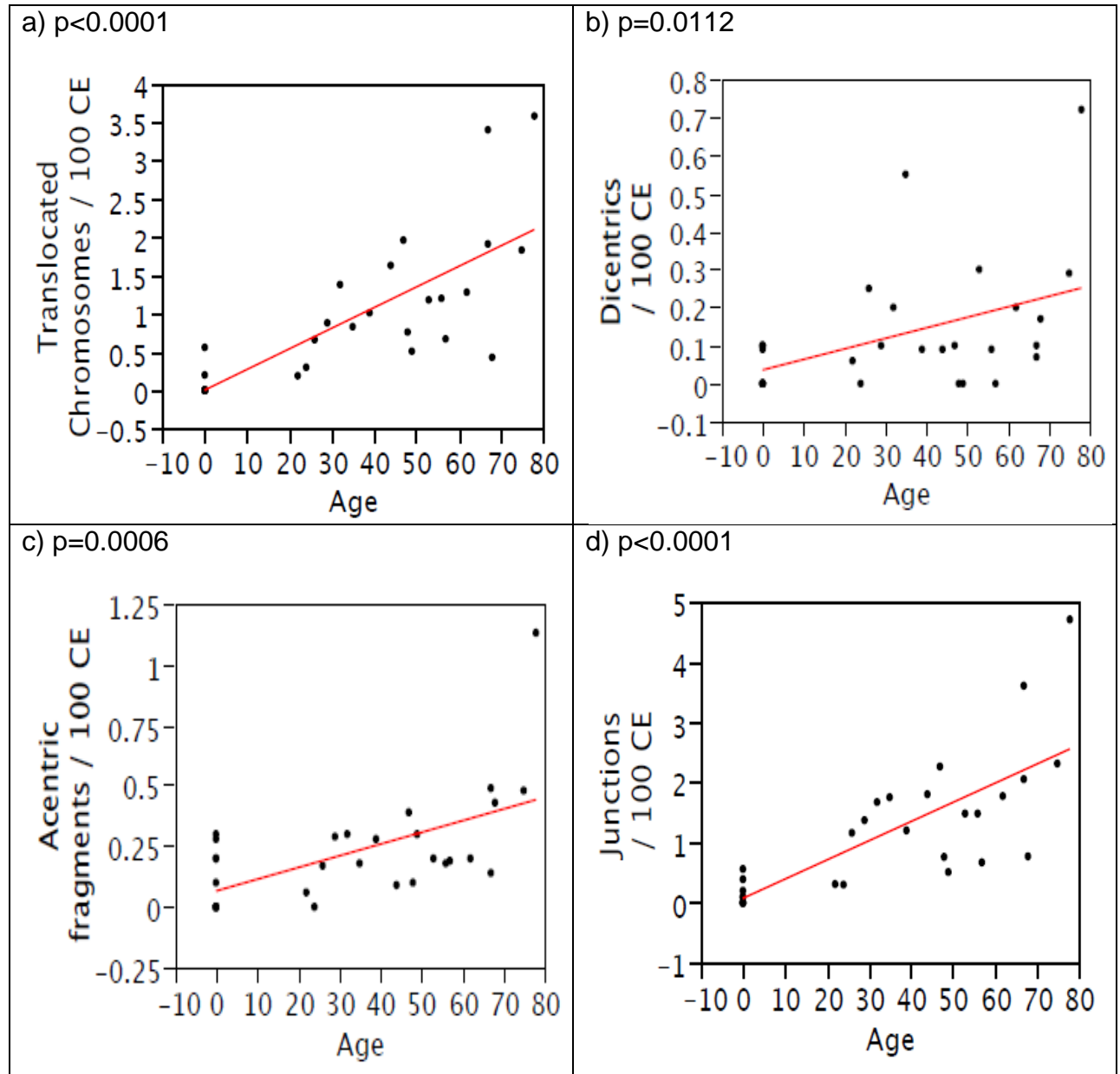


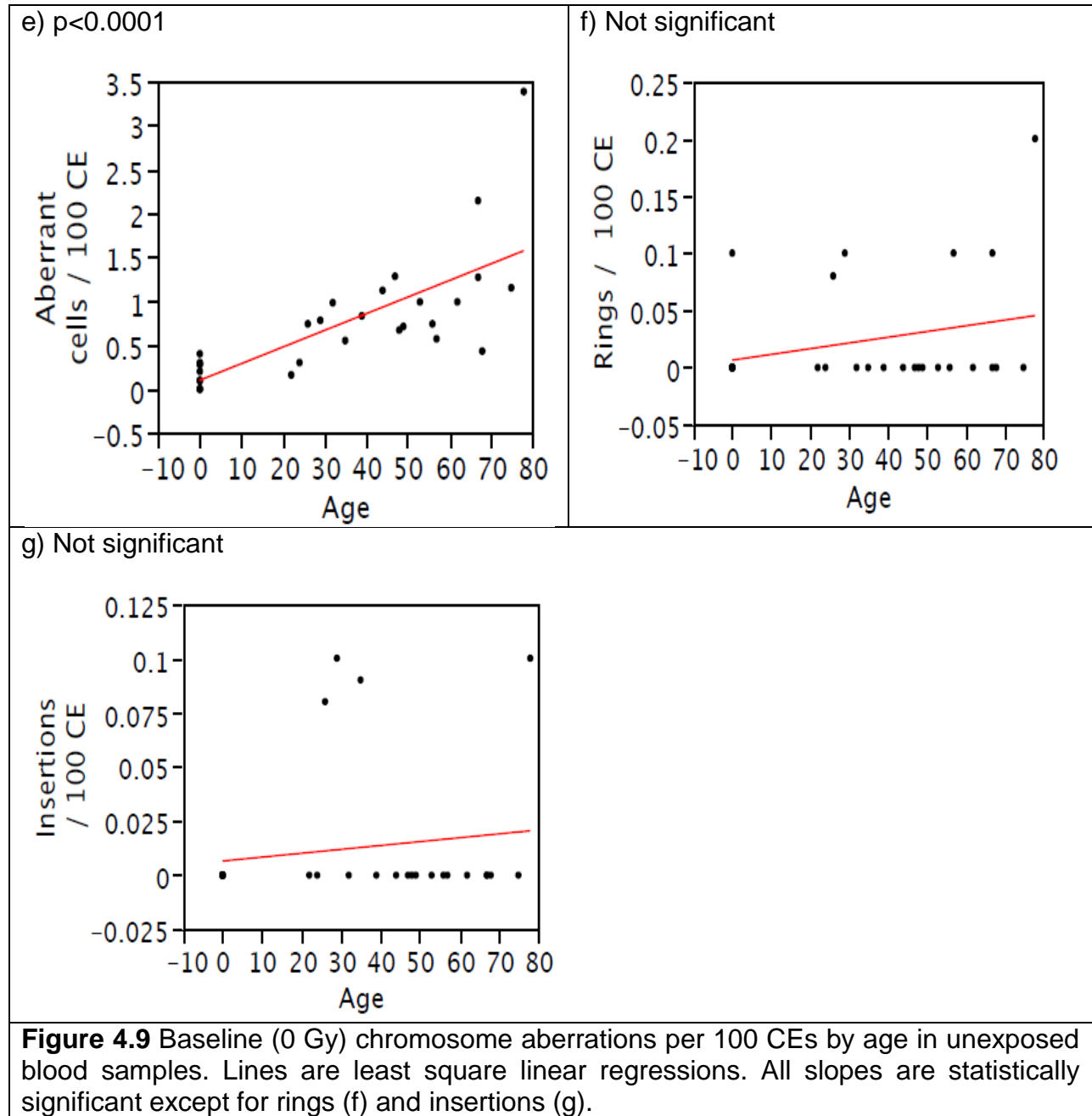
**Figure 4.6** Insertions per 100 CE by dose for newborns and adults, by group and individual. a) Insertions per 100 CE by dose of Cobalt-60 gamma rays for newborns and adults. Blue diamonds: mean insertions for 20 adults; red squares: mean insertions for 10 cord blood samples. Vertical lines are standard errors and are too small to be clearly visible for some doses. There is no statistically significant difference in the levels of insertions between adults and newborns. Curves are slightly offset for clarity. b) Insertions per 100 CE in individual adults by dose. c) Insertions per 100 CE in individual newborns by dose.

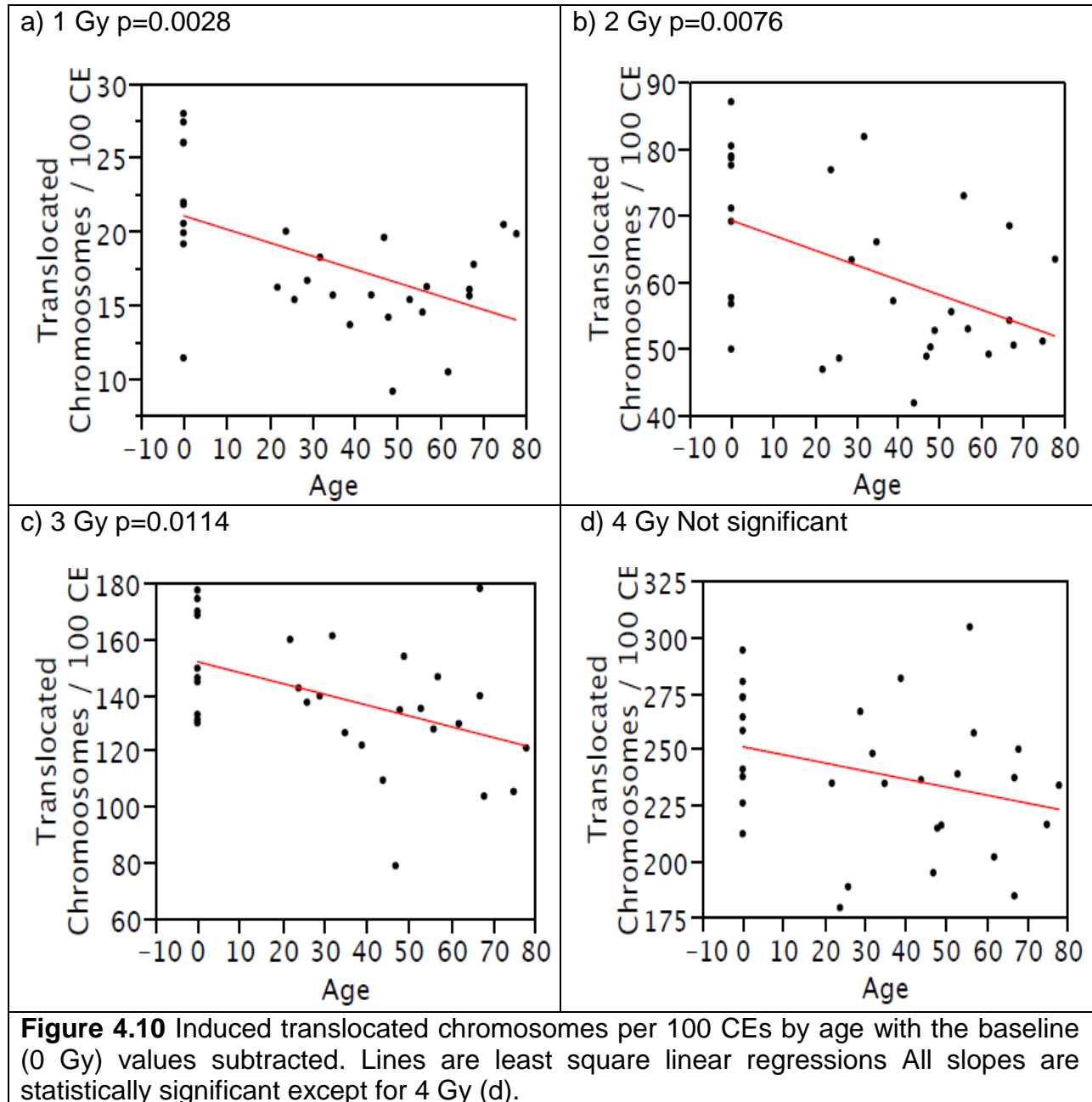




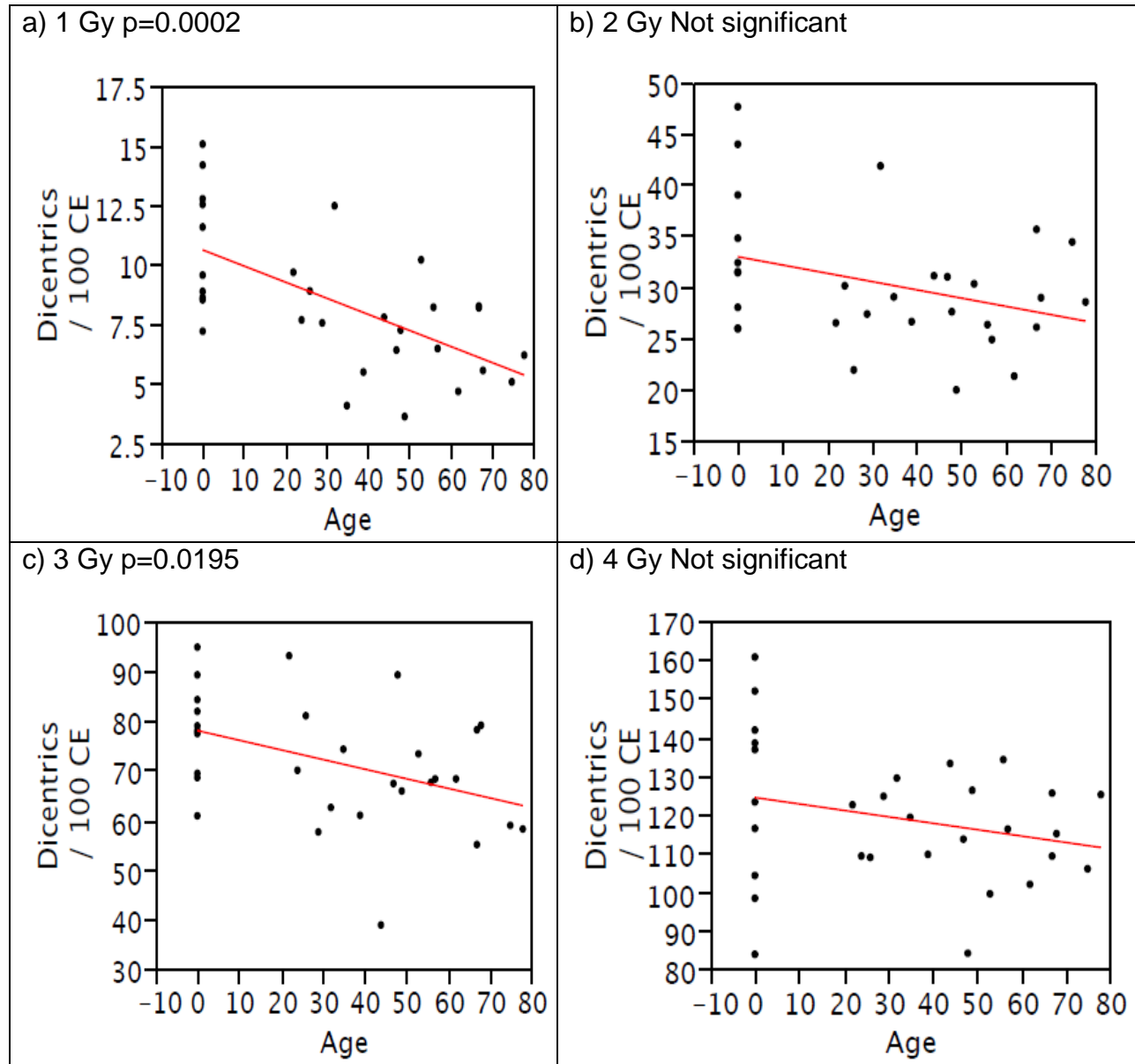
Baseline chromosome aberration frequencies by age for unexposed samples (0 Gy) are shown in Figure 4.9. Statistically significant increases in translocated chromosomes, dicentrics, acentric fragments, color junctions and abnormal cells are seen here with respect to age, which are consistent with previous published studies (Ramsey et al., 1995; Sigurdson et al., 2008b). Figures 4.10–4.16 show the aberration frequencies by age at each radiation dose. Since these frequencies decline with age and baseline (i.e. unirradiated) frequencies increase with age, the frequencies of induced aberrations were obtained by subtracting the frequencies of aberrations in unirradiated cultures from the total aberrations to obtain a clearer view of the susceptibility to radiation by age. Even though age is a statistically significant factor when the baseline data are not subtracted, the effect of age is enhanced when induced values are evaluated. Cord blood samples show increased susceptibility to chromosome damage after irradiation compared to adults receiving the same dose.



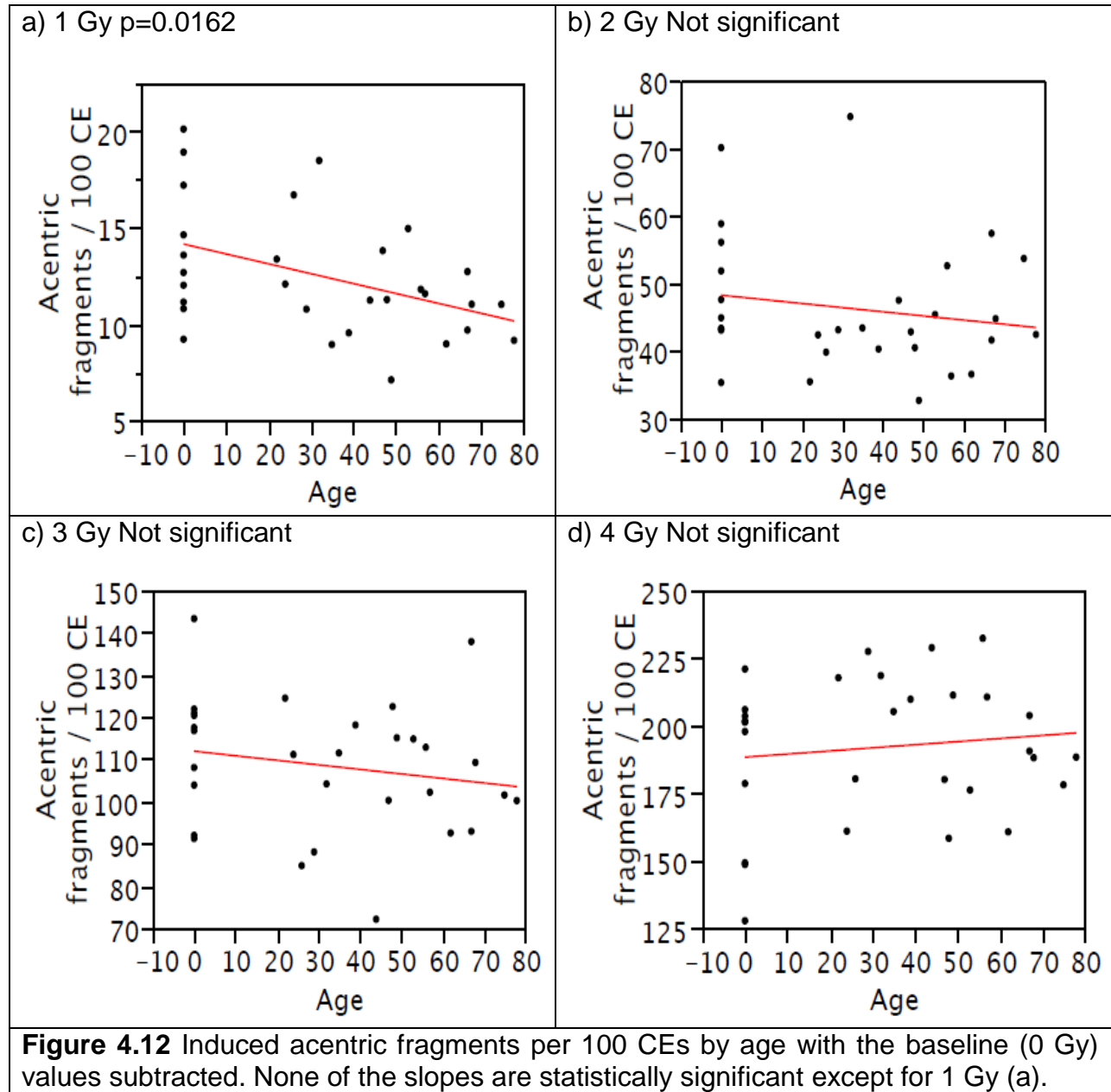


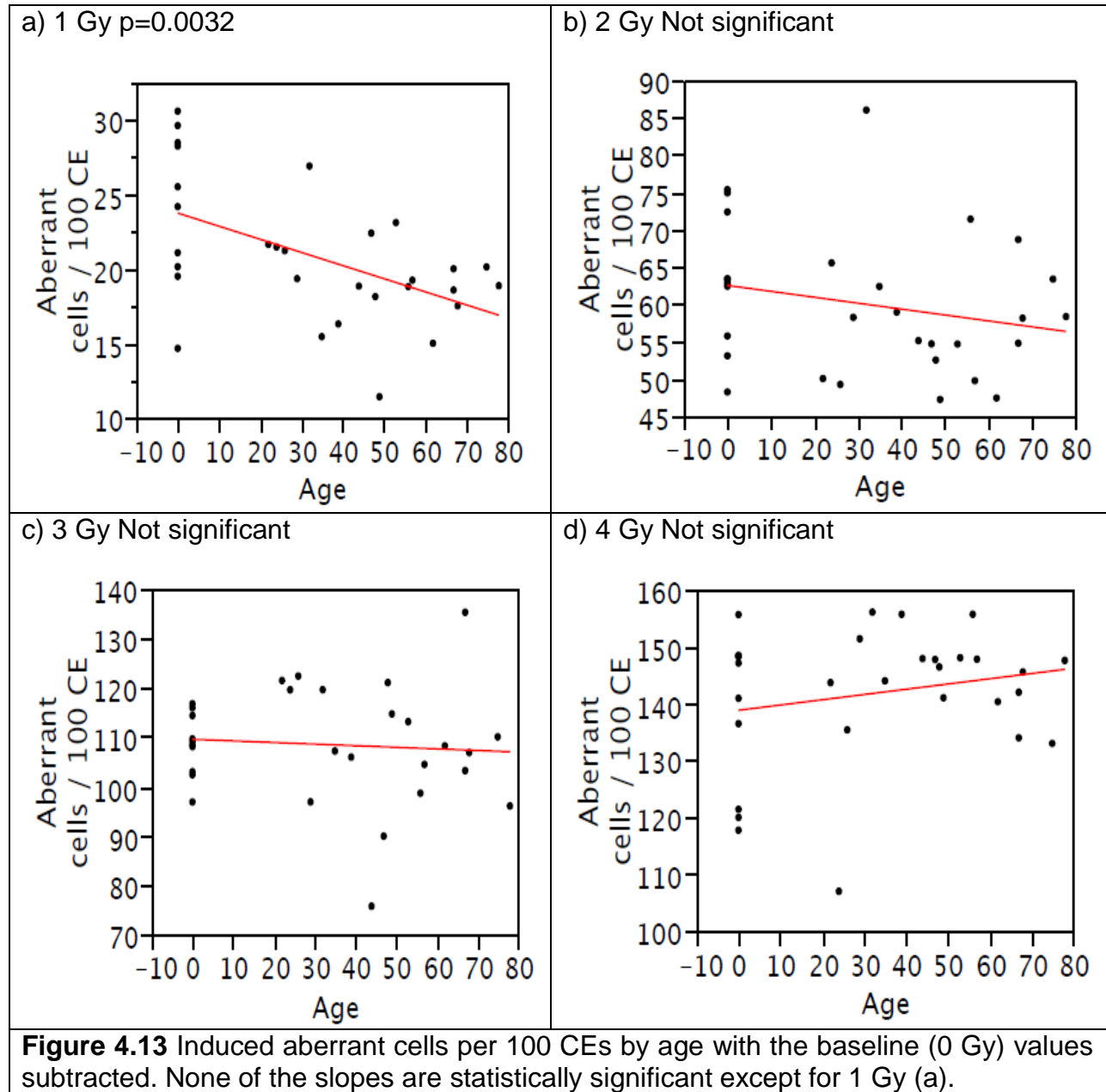


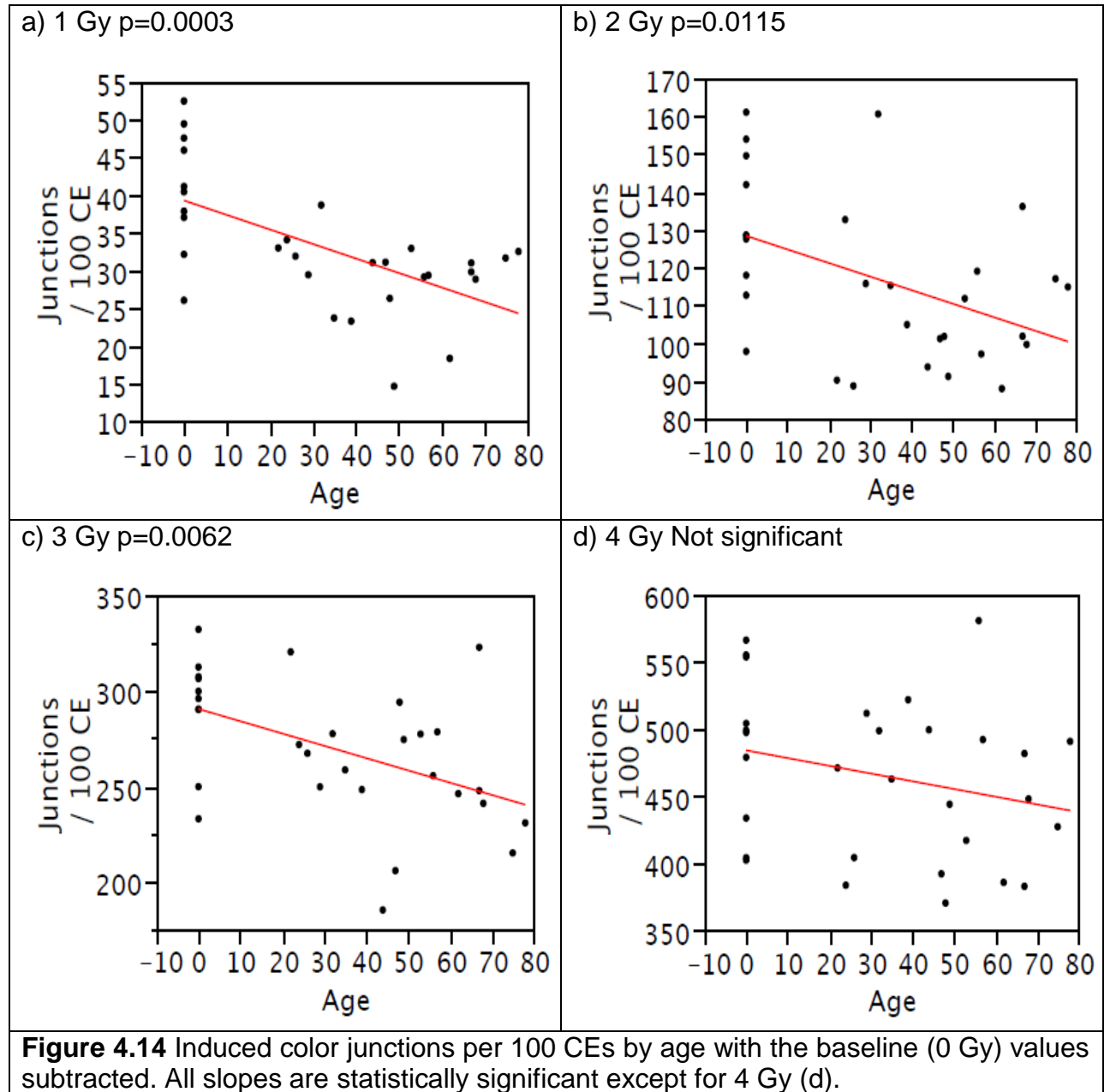


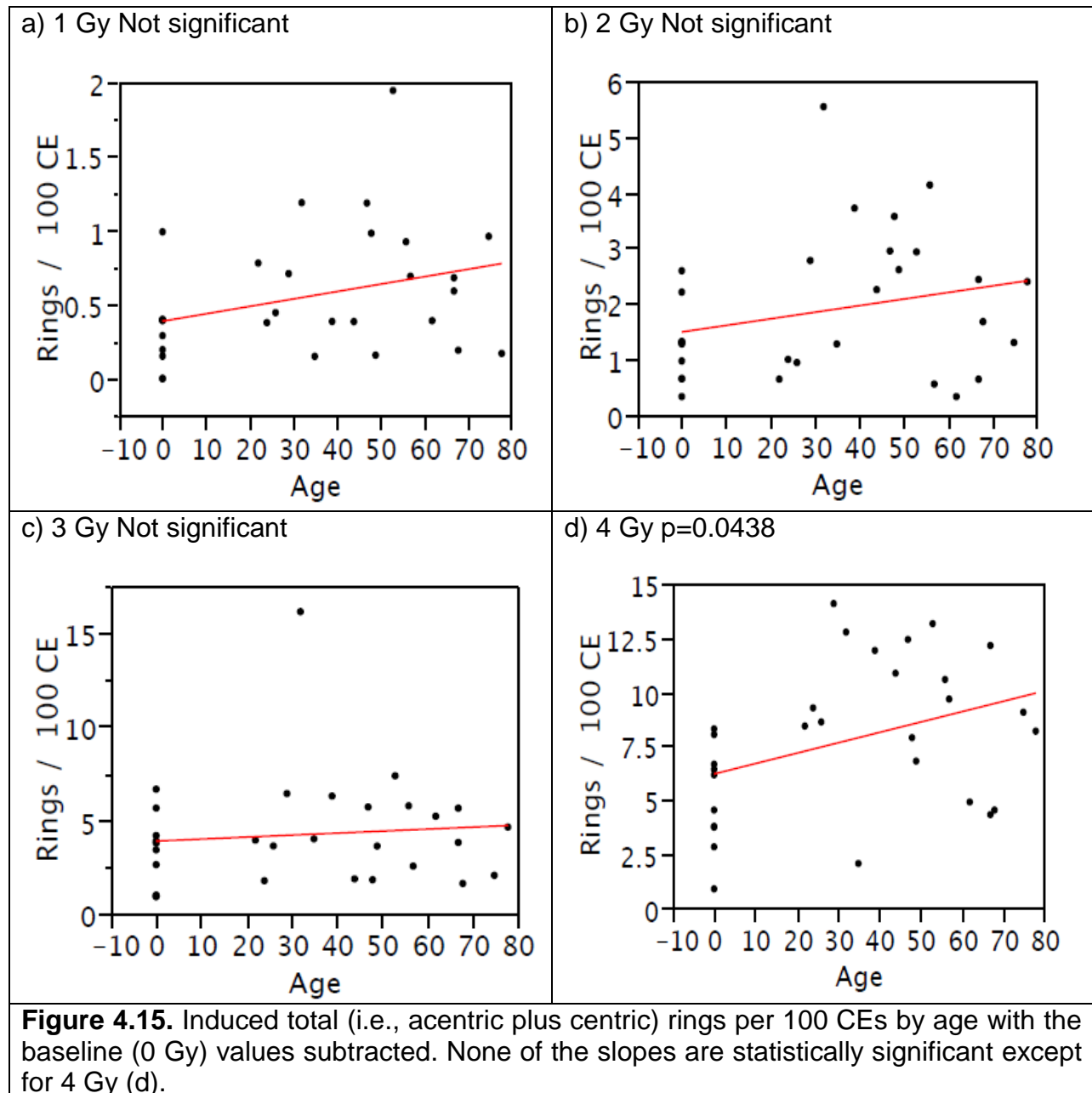


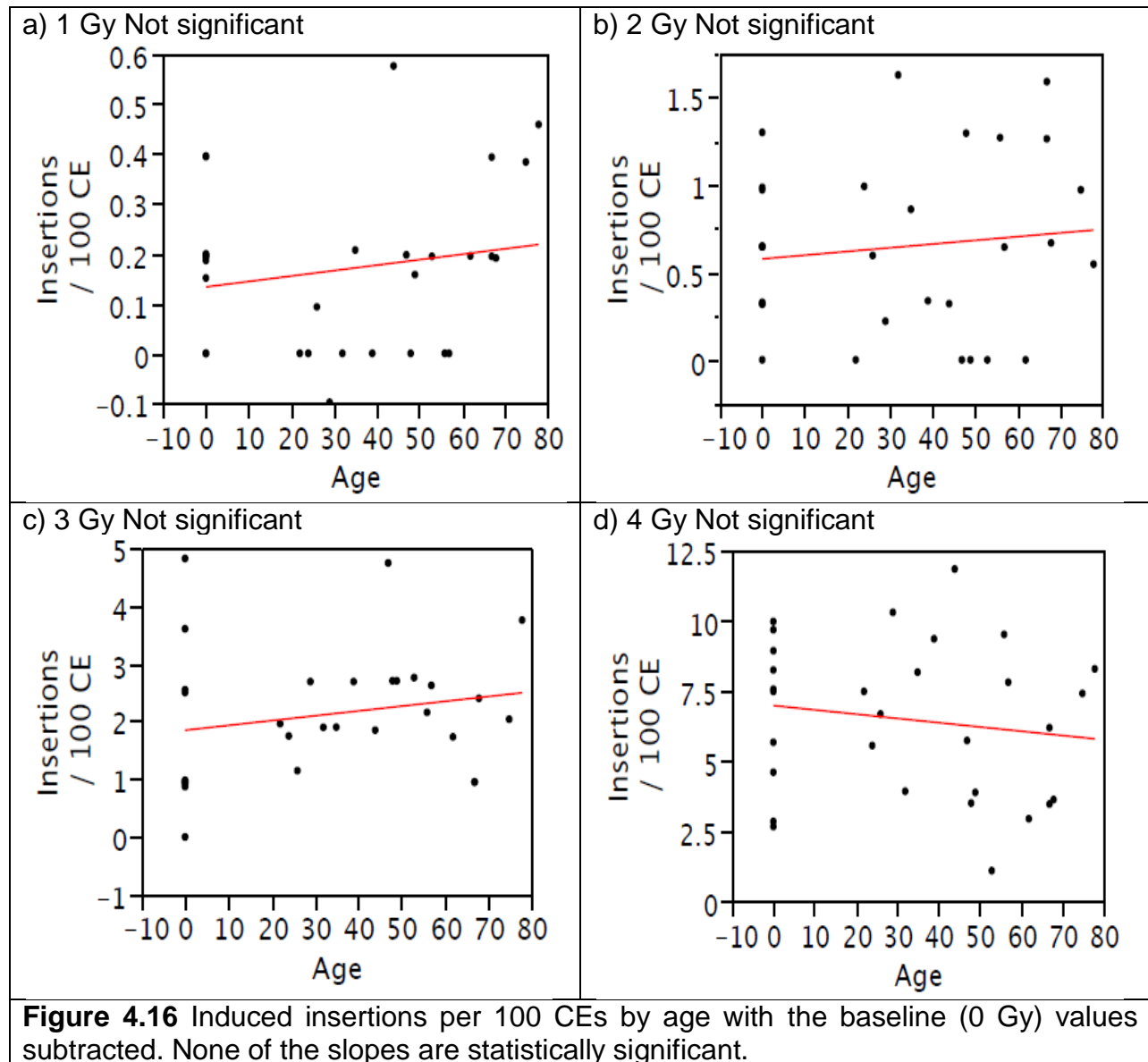
**Figure 4.11** Induced dicentric per 100 CEs by age with the baseline (0 Gy) values subtracted. Two slopes are statistically significant: 1 Gy (a) and 3 Gy (c); two are not significant: 2 Gy (b) and 4 Gy (d).





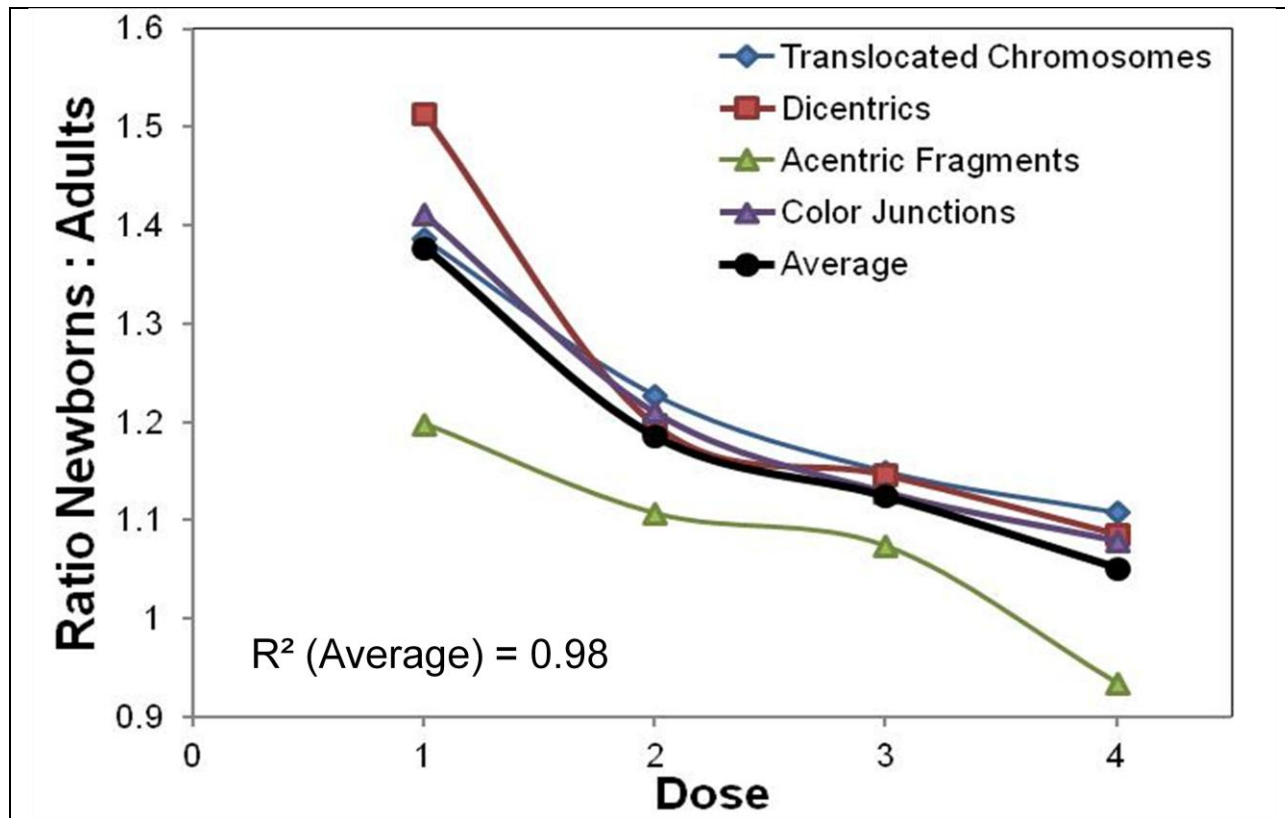






Induction of chromosome damage is only significant by age when the newborn group is included. When adult data are evaluated separately without the newborns, none of the cytogenetic endpoints show any statistical significant effect with age. The 4 Gy dose is not significant for any endpoint, which is most likely due to the variability among individual donors (see panels b and c in Figures 4.1-4.7) but follows the same downward trend as the other doses. The age effect is still generally present when the 4 Gy dose, both 3 and 4 Gy doses, and when the 2, 3 and 4 Gy dose groups are dropped from the analyses (data not shown), indicating that the age effect is not confined to the high doses. There is no statistically significant effect of gender, race, smoking, or alcohol use on any of the cytogenetic endpoints (data not shown).

The magnitude of the increased susceptibility in newborns is shown in Figure 4.17 which shows the ratio of aberrations in newborns compared to adults at each dose. Ratios equal to 1 indicate an equal response to radiation, i.e., no difference in susceptibility, while ratios greater than 1 indicate an increase in susceptibility. The highest susceptibility is seen at 1 Gy where newborns have a 38% increase in induced aberrations compared to adults.



**Figure 4.17** Ratios of induced chromosome aberrations in newborns compared to adults at each dose. The ratios of the frequencies of translocated chromosomes, dicentric, acentric fragments and color junctions are shown as well as the average of these 4 endpoints (black line). The average susceptibility in newborns is greater than 1 at all 4 doses, i.e., 1.38 at 1 Gy, 1.19 at 2 Gy, 1.12 at 3 Gy and 1.05 at 4 Gy.



## DISCUSSION

The major findings of this work improve our understanding of the susceptibility of people of different ages to ionizing radiation. Our results indicate that newborns have elevated susceptibility to radiation-induced chromosome damage in peripheral blood lymphocytes compared to adults, and suggest that the induction of structural chromosome damage is an inherent component of this susceptibility. We also show that susceptibility to radiation-induced chromosome damage does not change with age among adults. These findings support previous work showing that children are at higher risk of developing cancer associated with radiation exposure (Kleinerman, 2006; Sadetzki and Mandelzweig, 2009).

The existence of increased susceptibility in newborns and the absence of an age effect in adults both challenge and simplify risk estimation for people of different ages. Even though an age effect was not evident among adults, inter-individual variation due to intrinsic genetic factors may still influence radiation responses (Schnarr et al., 2007). The findings reported here suggest that the change in susceptibility with age appears to occur between birth and adulthood. The question remains at what age children change in their susceptibility to radiation-induced damage and whether this change is gradual or occurs at specific developmental milestone(s) such as puberty. One study showed that children aged 0.4 to 9 years had increased dicentric yields directly after a low dose exposure to ionizing radiation via a CT scan compared to children aged 10 to 15 years where no significant increase was observed (Stephan et al., 2007). Their work is consistent with our findings of increased susceptibility in newborns and also points to a decrease in susceptibility by the time a child reaches approximately 10 years of age.

The frequencies of all baseline (0 Gy) aberrations except rings and insertions were found to increase significantly with age, which is consistent with published observations (Ramsey et al., 1995; Sigurdson et al., 2008b). These results clearly show that this study has enough statistical power to confirm a known response even with a relatively modest sample size. Cigarette smoking has been previously shown to increase the frequencies of chromosome aberrations (Sigurdson et al., 2008b) but not every study has observed this effect, e.g., (Bennett et al., 2010), a disparity which may be attributable to differences in the amount of smoking among these populations. In the present study we did not observe a significant smoking effect.

There is some debate in the literature about the definition of a “translocation” when reporting chromosome aberrations induced by adverse exposure. Translocations have historically been viewed as a reciprocal exchange of genetic material between exactly 2 chromosomes such that each resulting chromosome contains one centromere and one segment of the other chromosome; this aberration arises due to mis-repair of double strand breaks and is commonly called a “reciprocal translocation”. At increasing doses of radiation, often more than 2 DNA double strand breaks occur simultaneously in a single cell resulting in more complex rearrangements called multi-way exchanges. We did observe and count reciprocal translocations but cells with multiple aberrations that included one or more non-reciprocal translocations were commonly seen, especially at the higher doses. Here we have used the term *translocated chromosome* to denote structurally rearranged chromosomes that have a single centromere. These translocated chromosomes arise from multi-way exchanges (Giannico et al., 2009) resulting from mis-repair and can become very complex (Loucas and Cornforth, 2001;

Tucker, 2010b; Tucker et al., 1995). Our approach for categorizing individual rearranged chromosomes has been described previously (Tucker et al., 1995). Here we cannot elucidate the mechanism(s) of formation of these complex aberrations, but we can clearly see the results of the mis-repair by cells in the form of these exchanges.

There are too few occurrences of ring chromosomes and insertions to see any significant change with age. Rings do exhibit a statistically significant effect at the 4 Gy dose, but here their frequencies increased with age compared to the other aberration types which declined with age. There are very few rings and insertions at the lower doses, so to characterize the relationship between rings and age we would either need data from doses higher than 4 Gy or we would need to score many more cells at the lower doses. Mechanistically, the cytogenetic counterpart of ring chromosomes is inversions, but inversions could not be observed with the painting method used here. If we had been able to see inversions as well as rings, combining these aberration frequencies might have provided enough statistical power at the lower doses to see an age effect.

When the adults were evaluated separately without the newborns, no age-related differences were observed in the frequencies of induced chromosome aberrations. This finding points to a change in susceptibility somewhere between birth and young adulthood rather than a gradual change over the years from birth to senescence. The lack of an age effect among adults is perhaps the result of the completion of growth and development. More data are needed at ages 70 and above to evaluate the effect, if any, that senescence presents on the susceptibility to radiation at the older end of the age spectrum.

When the frequencies of induced aberrations in the irradiated samples (1 – 4 Gy) were evaluated within each dose group, we observed age-related increases at 1, 2, and 3 Gy. At 4 Gy the slopes of the regression lines were generally in the same direction as the slopes from the lower doses, although statistical significance was not achieved. Rings were the only chromosome aberration type to show statistically significant changes with age at 4 Gy. The cells encountered the most damage at this dose and probably experienced negative selection during the 48 hr culture period, either dying or undergoing cell cycle delay which prevented them from being able to enter metaphase where they could be analyzed. In future studies regarding this question, doses of 0, 1, 2 and 3 Gy may be sufficient.

The dose response curves we generated showed statistically significant differences between newborns and adults in the frequencies of translocated chromosomes, dicentrics, rings, and color junctions. Translocated chromosomes are the most stable of all aberrations and for this reason have much greater persistence through cell division than dicentrics and rings. Translocated chromosomes are the best cytogenetic biomarker for detecting cellular responses to radiation when differences in cell growth rates may exist between samples, e.g., between peripheral blood from adults and cord blood from newborns. We observed increased frequencies of translocated chromosomes in newborns compared to adults confirming our conclusions of increased susceptibility of newborns to radiation. More dicentrics were seen in cells from adults than from newborns because the two types of donor cell populations differ in their growth rates. Newborn peripheral blood lymphocytes have a significantly higher mitotic index than adult peripheral blood lymphocytes, which indicates they are dividing

faster. Therefore cells with dicentrics are lost more quickly in newborns. The faster growing cells undergo stronger negative selection due to the unstable nature of dicentric chromosomes, leaving fewer total dicentrics in newborn samples. Rings are also unstable aberrations. We observed fewer rings in newborns than adults, which is consistent with the data for dicentrics. Color junctions include data from several aberration types, and since newborns are more susceptible to radiation they show slightly elevated levels over adults because of the increased total damage in their cells.

Phenotypic and physiological differences are known to exist between adult and umbilical cord blood samples. Cord blood contains hemopoietic stem cells that have a much higher proliferation rate than adult stem cells in peripheral blood (Holmes et al., 2009). Cord blood also has almost three times as many total lymphocytes as adult blood (Beck and Lam-Po-Tang, 1994) but the lymphocytes are immunologically immature and vary in their immunophenotypes analyzed with cell surface markers (Lopez et al., 2009). Our observations are consistent with this evidence in that in our cord blood samples exhibited an increased concentration of cells in culture and greater numbers of cells in metaphase than the adult blood samples; our data for unirradiated controls show that the mitotic indices in the older donors were substantially lower than in the cord bloods.

One of the two major findings of this work, the increased susceptibility of newborns to radiation, is summarized in Figure 4.17. At the lowest dose used in this study (1Gy), newborns showed a 38% increase in the average frequency of induced chromosome aberrations. This number declines with dose and the susceptibility difference is not discernible at the highest dose of 4 Gy. Most radiation exposures are

due to environmental and medical procedures that involve doses well below 1 Gy. We cannot extrapolate the graph or speculate on the susceptibility effect at doses below 1 Gy, but it would be the next logical step in the future of this work.

Mean telomere length in hemopoietic and mature immune cells decreases with age and differs in fetal and newborn samples. Newborns have greater telomere loss compared to fetuses (Holmes et al., 2009). Comparing such changes in fetal and newborn lymphocytes for their susceptibility to ionizing radiation would be interesting, although obtaining viable human fetal tissue is ethically problematic. Future directions for this work could include children and young adults aged 0 to 21 years to determine how and when the change in radiation susceptibility declines after birth. A parallel approach would be to use an animal model such as rats where blood samples from all stages of life, including the fetus, could be obtained.

The work presented here describes the increased cytogenetic susceptibility of newborns to ionizing radiation compared to adults. The increase in structural chromosome aberrations after radiation exposure at a young age could contribute to the known increase in cancer risks associated with childhood exposure. Since susceptibility does not appear to change with age once adulthood is reached, radiation risk assessments on adults do not need to take age into consideration. Further work is necessary to understand better the magnitude and developmental time frame of this susceptibility effect in children.

**APPENDIX A**PERSONAL QUESTIONNAIRE

Please read the following questions carefully and answer them as thoroughly and accurately as possible. The information you give will not be associated with your name in any public document and will be known only to the principal investigators in charge of this study. The answers you provide may have a direct bearing on the interpretation of the results of this study. Therefore, we ask that you kindly cooperate fully by providing correct information.

Thank you very much for your cooperation in furthering the cause of biomedical research.

1. Name:

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Last (family)

First (given)

Middle initial

Home phone number: \_\_\_\_\_ Cell phone number: \_\_\_\_\_

Home Address: \_\_\_\_\_

Street

apt. number

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City

State / Province

ZIP code

2. To be filled in by principal investigator:

Code Number: \_\_\_\_\_

Date: \_\_\_\_\_

If additional space is needed for the completion of an answer, please write on the back of the page and identify the remaining part of the answer with the question's number.

### GENERAL INFORMATION

3. Today's Date: \_\_\_\_\_

Month Day Year

4. a) What is your age? \_\_\_\_\_ (in years)

b) What is your birth date? \_\_\_\_\_

Month Day Year

5. Specify your sex (circle one): Male Female

6. How would you describe your ethnicity?

\_\_\_\_\_

7. What is (was) your most recent occupation?

\_\_\_\_\_

\_\_\_\_\_

8. What other occupations have you had in the last 20 years?



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MEDICAL HISTORY

9. Have you taken any medication prescribed by a doctor **in the past one year** (for example, blood pressure pills, antibiotics, insulin, tranquilizers, muscle relaxants, etc.)?

\_\_\_ YES \_\_\_ NO

If YES, please indicate:

Type of Medication	Dose	How Frequently Per Day	Began (Month)	Ended (Month)

10. Have you ever had a major illness? \_\_\_ YES \_\_\_ NO

If YES, please specify the type of disease(s), when you were ill, and indicate the treatment you received.

Illness	Beginning (month/yr)	Ending (month/yr)	Treatment Received

11. Have you had a blood transfusion **in the last one year?**

\_\_\_ YES      \_\_\_ NO

If YES, when? \_\_\_\_\_

12. Have you had any surgery in **the last one year?**

\_\_\_ YES      \_\_\_ NO

If YES, what kind of surgery? \_\_\_\_\_

When? \_\_\_\_\_

13. Have you **ever** received a tissue transplant?

\_\_\_ YES      \_\_\_ NO

If YES: \_\_\_\_\_ Organ      \_\_\_\_\_ Bone marrow

When? \_\_\_\_\_

14. Have you **ever** been diagnosed with cancer?

\_\_\_ YES      \_\_\_ NO

If YES, what type of cancer? \_\_\_\_\_

Please indicate the year(s) and describe the treatment you received:

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15. Have you had any of the following diagnostic or therapeutic procedures **in the last 10 years?**

a) X-rays for:

i. Broken bones?      \_\_\_ YES      \_\_\_ NO

If YES, please list:

# of X-rays	Year received

ii. Dental? \_\_\_\_\_ YES \_\_\_\_\_ NO

If YES, please list:

# of X-rays	Year received

iii. Chest? \_\_\_\_\_ YES \_\_\_\_\_ NO

If YES, please list:

# of X-rays	Year received

iv. Other? \_\_\_\_\_ YES \_\_\_\_\_ NO

If YES, please list:

# of X-rays	Year received

b) Mammograms? \_\_\_\_\_ YES \_\_\_\_\_ NO

If YES, please list the years received:

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c) CT scans? \_\_\_\_\_ YES \_\_\_\_\_ NO

If YES, please list:

# of scans	Year received

d) Radiation therapy? \_\_\_\_\_ YES \_\_\_\_\_ NO

If YES, please list:

# of treatments	Duration	Year received

e) Chemotherapy? \_\_\_\_\_ YES \_\_\_\_\_ NO

If YES, please list:

# of treatments	Duration	Year received

f) Other diagnostic procedures involving radiation? \_\_\_\_\_ YES \_\_\_\_\_ NO

If YES, please list:

Procedure	# of times received	Year

16. Have you ever been exposed to any other potentially hazardous agents such as pesticides, herbicides, insecticides, dyes, solvents, petroleum products, etc.?

\_\_\_\_\_ YES \_\_\_\_\_ NO

If YES, please describe:

Type of agent	When were you first exposed? (month/yr)	When were you last exposed? (month/yr)	How long were you exposed in total?


### SMOKING HISTORY

17. a) Have you ever smoked or used any tobacco products?    \_\_\_ YES \_\_\_ NO

If NO: Proceed to question #18.

If YES: How long did you smoke? \_\_\_\_\_ in years

b) Do you smoke or use any tobacco products now?    \_\_\_ YES    \_\_\_ NO

If YES proceed to 17c.

If NO: When did you stop smoking, or stop using tobacco products?

\_\_\_\_\_

month            year

Proceed to 17h.

c) Do you currently smoke cigarettes?    \_\_\_ YES    \_\_\_ NO

If YES: How many packs do you smoke each day? \_\_\_\_\_

d) Do you currently smoke cigars?            \_\_\_ YES    \_\_\_ NO

If YES: How many cigars do you smoke each day? \_\_\_\_\_

e) Do you currently smoke a pipe (including hookah)? \_\_\_ YES    \_\_\_ NO

If YES, how many pipes do you smoke each day? \_\_\_\_\_

f) Do you currently chew tobacco? \_\_\_\_\_ YES \_\_\_\_\_ NO

If YES, how many times per day do you chew? \_\_\_\_\_

g) Do you currently use snuff? \_\_\_\_\_ YES \_\_\_\_\_ NO

If YES, how many times per day do you use it? \_\_\_\_\_

h) If you do not smoke now, what did you smoke in the past?

\_\_\_\_\_ cigarettes How many per day? \_\_\_\_\_

\_\_\_\_\_ cigars How many per day? \_\_\_\_\_

\_\_\_\_\_ pipes (including hookah) How many per day? \_\_\_\_\_

### ALCOHOL CONSUMPTION HISTORY

18. a) Do you drink beer? \_\_\_\_\_ YES \_\_\_\_\_ NO

If yes, please indicate your average weekly beer consumption:

\_\_\_\_\_ cans/bottles per week

b) Do you drink wine? \_\_\_\_\_ YES \_\_\_\_\_ NO

If yes, please indicate your average weekly wine consumption:

\_\_\_\_\_ glasses per week.



19. Do you drink liquors other than beer and wine?

\_\_\_\_ YES \_\_\_\_ NO

If yes, please indicate your average weekly consumption for  
other liquors.

\_\_\_\_ shots per week

**THANK YOU VERY MUCH FOR YOUR PARTICIPATION IN THIS STUDY!**

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**ABSTRACT****CYTOGENETIC ANALYSIS OF DEPLETED URANIUM EXPOSURE AND AGE-DEPENDENT SUSCEPTIBILITY TO IONIZING RADIATION**

by

**MARINA V. BAKHMUTSKY****May 2013****Advisor:** Dr. James D. Tucker**Major:** Biological Science**Degree:** Doctor of Philosophy**Chapters 2 and 3:**

Depleted Uranium (DU) is a high-density heavy metal that has been used in munitions since the 1991 Gulf War. DU is weakly radioactive and chemically toxic and long term exposure may cause adverse health effects. This study evaluates genotoxic effects of DU exposure in Gulf War I Veterans as a function of uranium (U) body burden by measuring chromosome damage in peripheral blood lymphocytes with the cytokinesis blocked micronucleus assay (CBMN) and fluorescence *in situ* hybridization (FISH) whole chromosome painting. Study subjects are Gulf War I Veterans exposed to DU during friendly fire incidents in 1991 involving DU munitions which resulted in inhalation and ingestion exposure to small particles of DU and soft tissue DU fragments from traumatic injuries. The Veterans are enrolled in a long term health surveillance program at the Baltimore VA Medical Center.

**Evaluation of subjects using the CBMN assay:**

Blood was drawn from 35 exposed male Veterans aged 36 to 59 years, then cultured and evaluated for micronuclei (MN). The participants were divided into two exposure groups, low and high, based on their mean urine Uranium concentrations. Poisson regression analyses with mean urine U concentrations, current smoking, X-rays in the past year and donor age as dependent variables revealed no significant relationships with MN frequencies. Our results indicate that ongoing systemic exposure to DU in Gulf War I Veterans with embedded DU fragments does not induce significant increases in MN in peripheral blood lymphocytes compared to MN frequencies in Veterans with normal urine Uranium levels.

**Evaluation of subjects using FISH whole chromosome painting assay:**

Blood was drawn from 35 exposed male Veterans aged 39 to 62 years, then cultured and harvested for metaphase chromosome analyses. Chromosomes 1, 2, and 4 were painted red and chromosomes 3, 5, and 6 were simultaneously labeled green. At least 1800 metaphase cells per subject were scored. Univariate regression analyses were performed to evaluate the effects of log(urine Uranium), age at time of blood draw, log(lifetime X-rays), pack-years smoked and alcohol use against frequencies of cells with translocated chromosomes, dicentrics, acentric fragments, color junctions and abnormal cells. No significant relationships were observed between any cytogenetic endpoint and log(urine Uranium) levels, smoking, or log(lifetime X-rays). Age at the time of blood draw showed significant relationships with all endpoints except for cells with acentric fragments. These results indicate that chronic exposure to DU does not induce significant levels of chromosome damage in these Veterans.

**Chapter 4:**

To determine the extent to which age influences individual susceptibility to ionizing radiation, blood samples were collected from 20 adults and from the umbilical cords of 10 newborns. Samples were acutely exposed to Cobalt 60 gamma rays to doses of 0 (control), 1, 2, 3 and 4 Gy. Cells in metaphase were labeled with whole chromosome paints and evaluated for structural chromosome aberrations. Regression analyses were used to evaluate the frequencies of each of the major classes of structural aberrations to determine whether susceptibility to radiation was dependent upon age. Compared to adults, blood from newborns showed statistically significant increases in translocated chromosomes, dicentrics, and color junctions for doses from 0 to 3 Gy, but not at 4 Gy. When adults were considered alone, no significant changes in radiation susceptibility were observed with age. The increased susceptibility of newborns to ionizing radiation, and the absence of any change in susceptibility during adulthood, should be relevant when making radiation-exposure risk assessments.

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- 1) **Bakhmutsky M.V.**, Oliver M.S., McDiarmid M.A., Squibb K.S., Tucker J.D., 2010. Long term depleted uranium exposure in Gulf War I Veterans does not cause elevated numbers of micronuclei in peripheral blood lymphocytes, Mutation Research 720 (2011) 53–57
- 2) McDiarmid M.A., Albertini R.R., Tucker J.D., Vacek P.M., Carter E.W., **Bakhmutsky M.V.**, Oliver M.S., Engelhardt S.M., Squibb K.S., 2011. Measures of Genotoxicity in Gulf War I Veterans Exposed to Depleted Uranium, Environmental and Molecular Mutagenesis 52:569-581 (2011)