GENETIC DAMAGE IN NEW ZEALAND VIETNAM WAR VETERANS

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ABSTRACT

From July 1965 until May 1971, New Zealand Defence Force Personnel fought in the Vietnam War. During this time the United States military forces sprayed more than 76,500,000 litres of phenoxylic herbicides over parts of Southern Vietnam and Laos. The most common herbicide sprayed was known as 'Agent Orange'. All of the Agent Orange sprayed during the Vietnam War was contaminated with 2,3,7,8-tetrachlorobenzo-*para*-dioxin (known simply as TCDD), a known human carcinogen. Since returning to New Zealand more than 30 years ago, New Zealand Vietnam War veterans have expressed concern about the numerous health problems experienced by both themselves and their children. New Zealand Vietnam War veterans attribute these health problems to exposure to Agent Orange while serving in Vietnam.

This study aimed to ascertain whether or not New Zealand Vietnam War veterans have incurred genetic damage as a result of service in Vietnam. The Sister Chromatid Exchange assay (SCE) is a very sensitive and widely applied assay used to detect genetic damage induced by an environmental agent or clastogen. In the current study a group of New Zealand Vietnam War veterans and a control group were compared using an SCE analysis in order to determine if genetic damage had been sustained by the Vietnam War veterans. All participants were screened to reduce the possible influence of factors that could severely impact on findings and to eliminate any bias in the SCE results.

The results from the SCE study show a highly significant difference between the mean of the experimental group and the mean of the control group ($p < 0.001$). This result indicates that New Zealand Vietnam War veterans have sustained genetic damage; this damage can be attributed to service in Vietnam (possibly as a result of exposure to Agent Orange). This result is strong and indicates that further scientific research on New Zealand Vietnam War veterans is required.

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ABBREVIATIONS

In addition to the chemical symbols from the Periodic Table of Elements and the *Système International d'Unitès* (SI), the following abbreviations are used:

2,4-D	**2,4-dichloropheoxyacetic acid**
2,4,5-T	**2,4,5-trichlorophenoxacetic acid**
AhR	**Aryl Hydrocarbon Receptor**
ANCOVA	**Analysis of Covariance Assay**
Arnt	**Aryl Hydrocarbon Receptor Nuclear Translocator**
BrdU	**5-bromo-2-deoxyuridine**
c-metaphse	**Colchicine-treated cell in metaphase**
CINCPAC	**Scientific Advisory Group of the Commander in Chief Pacific**
CF	**Clastogenic Factor**
CYP	**Cytochrome P-450**
dH_2O	**Distilled Water**
DNA	**Deoxyribose Nucleic Acid**
DRE	**Dioxin Response Element**
et al.	***Latin*, and others**
ES	**Effect Size**
FISH	**Fluorescence *in situ* Hybridisation**
G	**Gauge**
h	**Hour**
HIV	**Human Immunodeficiency Virus**
IARC	**International Agency for Research on Cancer**
ICMESA	**Industrie Chimiche Meda Società**
IPCS	**International Programme on Chemical Safety**
MB	**Megabytes**
MqH_20	**Milli-Q Water**
n	**Sample Size**
NHL	**non-Hodgkin's Lymphoma**
p	**Probability**

pers. comm.	**Personal Communication**
PHA	**Phytohaemagglutinin**
PCC	**Premature Chromosome Condensation**
ppm	**Parts Per Million**
ppt	**Parts Per Trillion**
RAM	**Random Access Memory**
rpm	**Revolutions Per Minute**
SCE	**Sister Chromatid Exchange**
Std.	**Standard**
Std. Dev.	**Standard Deviation**
Std. Error	**Standard Error**
SOD	**Superoxide Dismutase**
TCDD	**2,3,7,8-tetrachlorobenzo-*para*-dioxin**
USA	**United States of America**
UV	**Ultra Violet Light**
WBC	**White Blood Count**
w/v	**Weight per volume**

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1 CHAPTER ONE: INTRODUCTION

In the 1950s South East Asia was an area of the globe in severe political turmoil. Emerging from the post-colonial era, nations were attempting to establish their own identity. For reasons beyond the scope of this thesis, New Zealand became embroiled in one of the most bitter wars of the last century outside of the two World Wars: the Vietnam War.

In 1958, several religious and political groups, most notably the North Vietnamese Communists (Viet Cong), revolted against the South Vietnamese government. New Zealand Defence Force Personnel were based in Vietnam from June 1964. In July 1965, New Zealand troops moved into a combatant role supporting the USA in an attempt to stop invasion of South Vietnam by its North Vietnamese neighbours. New Zealand's troops continued to fight in Vietnam for almost 6 years; the last leaving in May 1971. The Australian Task Force base at Nui Dat, in Phuoc Tuy Province, was established in June 1966, and although most New Zealand troops spent some time here, New Zealand soldiers generally served in Long Khanh, Bien Hoa, Binh Duong, Gia Dinh and Hua Nghia provinces as well as Phuoc Tuy province (Irvine, 2003) (Figure 1.1)

During the Vietnam War the United States military forces sprayed an estimated 76,540,964 litres of phenoxylic herbicides (Duchnowicz *et al.*, 2005) over approximately 3.6 million hectares of Vietnamese and Laotian land in order to remove forest cover, destroy crops and clear vegetation from the perimeters of the US bases as part of their military strategy. A consequence of this decision was a legacy of ill health, not only amongst the Vietnamese population themselves, but also in thousands of American, Australian and New Zealand Vietnam War veterans, and their children.

In 1961, the USA government commenced an aerial spraying programme (codenamed Operation "Ranch Hand") of a group of defoliants, the most common of which was known as 'Agent Orange'. The concentration at which herbicides were sprayed by USA forces was more than an order of magnitude greater than that for similar domestic weed control.

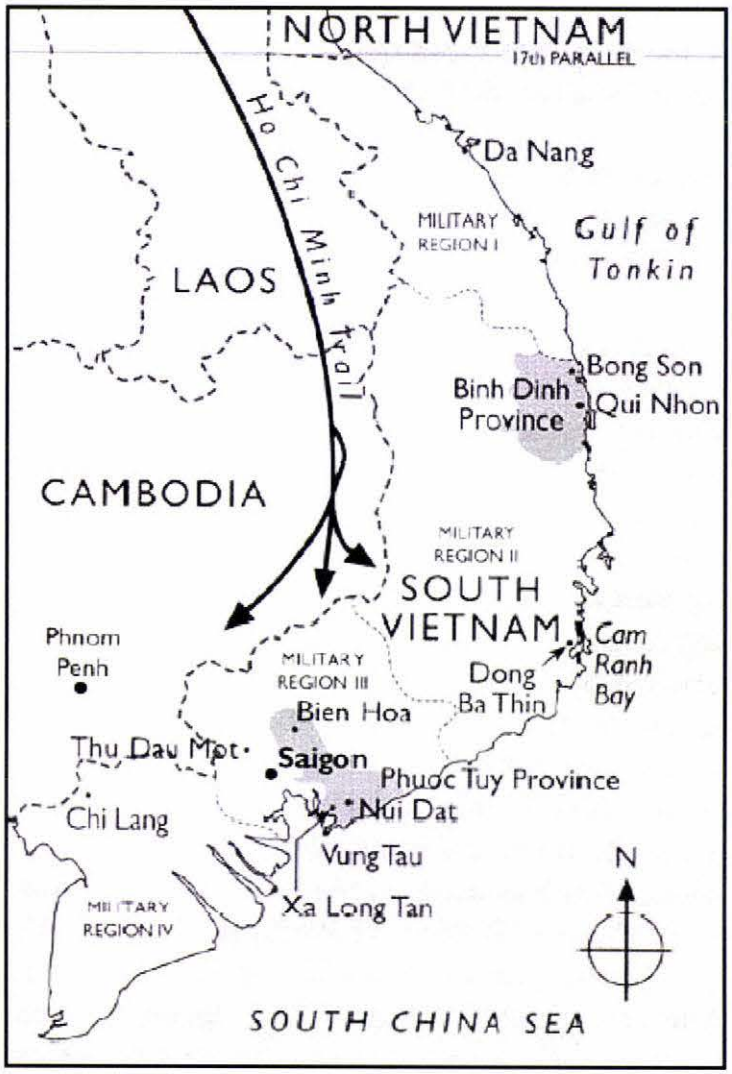

Figure 1.1 South Vietnam 1965-1972
Most New Zealand troops spent some of their time in Vietnam at the Australian Task Force base in Nui Dat, (in Phuoc Tuy Province). The dark colour around Nui Dat indicates the area where New Zealand troops served (Chadwick, 2004)

Between 1961 and 1972 various herbicide mixtures, nicknamed by their coloured identification barrels, were used by the USA and the Republic of Vietnam forces to defoliate forests and mangroves in order to clear perimeters of military installations and to destroy "unfriendly" crops as a tactic for decreasing enemy shelter and food supplies (Stellman *et al.*, 2003).

Operation Ranch Hand dispersed around 95 % of all the herbicides used in Operation Trail Dust, the overall herbicide programme. Other branches of the USA armed services and the Republic of Vietnam forces used hand sprayers, spray trucks, helicopters and boats to disperse the remainder.

Current literature substantiates the view that exposure to Agent Orange and other herbicides can lead to adverse health effects and cause genetic damage in humans (Akhtar *et al.*, 2004; Bukowska, 2004; Duchnowicz *et al.*, 2005; Eriksson *et al.*, 1981; Hardell, 1979; Palmer, 2005; Schecter *et al.*, 1995). With the amount of information that is now available, it is accepted that New Zealand Vietnam War veterans were exposed to Agent Orange and other herbicides during their service in Vietnam. The current study has therefore been established to investigate genetic damage (if any) that has been sustained by New Zealand Vietnam veterans. The Sister Chromatid Exchange Assay (SCE) has been chosen to analyse Vietnam veterans in the current study. The SCE Assay is a reliable and widely applied assay used for detecting genetic damage. This assay has been used successfully in previous studies involving chemical exposure and possible genetic damage (Akin *et al.*, 2005; Arias, 2002; Bhattacharya *et al.*, 2005; Garaj-Vrhorac & Zeljezic, 2001; Iannuzzi *et al.*, 2004; Zober *et al.*, 1993).

The detection of SCE in dividing blood lymphocytes is used to evaluate genetic damage from exposure to environmental genotoxic agents (Sarto *et al.*, 1985; Tucker *et al.*, 1993). Exchanges occur when DNA is replicating after an initial change in the form of DNA base damage (Uggla & Natarajan, 1983). In 2000, the IPCS (International Programme on Chemical Safety) published guidelines for the monitoring of genotoxic effects in humans (Albertini *et al.*, 2000). In defining the significance of the endpoint and application of the sister chromatid exchange assay, the report states "The readily quantifiable nature of SCEs with high sensitivity for revealing toxicant-DNA interaction and the demonstrated ability of genotoxic chemicals to induce a significant increase in SCEs in cultured cells...has resulted in this endpoint being used as an indicator of DNA damage in blood lymphocytes of individuals exposed to genotoxic (agents)." The SCE assay is thus acceptable as an indicator of *in vivo* damage. Futhermore, it is an accepted tenet in the current study that any damage to DNA may lead to ill health and possibly result in intergenerational effects. Follow-up studies on individuals exposed to genotoxic agents have clearly demonstrated the predictive value

of high chromosomal damage for subsequent health risk (Hagmar *et al.*, 1994, 1998, 2001).

1.1 Aim

- **To determine whether or not New Zealand Vietnam veterans have incurred any genetic damage as a result of their service in Vietnam.**

In order to achieve this aim, the following objective is stated: An SCE analysis will be conducted to establish whether or not a sample group of Vietnam veterans have a statistically higher frequency of sister chromatid exchange than a control group of men who did not serve in Vietnam.

1.2 Hypothesis

- **That New Zealand Vietnam veterans have incurred genetic damage as a result of their service in Vietnam.**

The null hypothesis is that New Zealand Vietnam War veterans did not sustain genetic damage. If the null hypothesis is true then we would predict, according to the current objective, that no statistically significant difference in mean SCE frequency between the Vietnam veterans group and the control group would be detected.

2 CHAPTERTWO: LITERATURE REVIEW

2.1 Agent Orange and Health Effects

Over the duration of Operation Ranch Hand, 6 major herbicides were aerially spayed: Agent Pink (approximately 51,000 L); Agent Green (approximately 31,000 L); Agent Purple (approximately 1.8 million L); Agent Orange (unknown volume, but in excess of 50 million L); Agent White (approximately 20.5 million L) and Agent Blue (approximately 4.7 million L).

Approximately 65 % of the herbicides used contained 2,4,5-trichlorophenoxacetic acid (2,4,5-T), and all of the 2,4,5-T used contained 0.5 to 100 ppm of 2,3,7,8-tetrachlorobenzo-*para*-dioxin (known simply as TCDD or Dioxin) as a manufacturing contaminant (Gough, 1991). It is the TCDD that is considered to be the prime cause of detrimental health and genetic effects from these herbicides (Neuberger *et al.*, 1999; Palmer, 2005; Pavuk *et al.*, 2005; Pearce & Mclean, 2005).

Military herbicide operations in Vietnam became a matter of scientific controversy right from their inception. In April 1970 2,4,5-T was banned from most USA domestic uses and from many other countries on the basis of evidence of its teratogenicity[1] (Stellman *et al.*, 2003). Even given this knowledge, the military strategy of using herbicide spray in Vietnam was considered a greater priority at the time.

Estimates of exactly how much TCDD was deposited in Vietnam are based on the volume of 2,4,5-T-containing herbicide sprayed, and on TCDD contamination levels, but are hard to predict. In 1970 when Operation Ranch Hand finally ended, over 3.6 million hectares of forest and villages in Central and Southern Vietnam had been covered with millions of litres of toxic herbicide (Tuyet & Johansson, 2001). Any humans or other living organisms situated in these 3.6 million hectares of forests would have almost certainly come into direct contact with these toxic substances.

[1] A '**teratogen**' is a term used to describe any agent with the potential to cause genetic deformities.

Agent Orange was the most common of the herbicides used by the USA, and comprised a 1:1 mixture of 2,4-dichloropheoxyacetic acid (2,4-D) and 2,4,5-T (Figure 2.1). Agent Orange was used for the longest duration, and was one of the most toxic herbicides used. It was commonly made available in hand-sprayers to be used by soldiers around the perimeters of their camps. No precautions while using Agent Orange were generally enforced, thus giving soldiers the impression that this substance was harmless. Aerial spraying and hand spraying missions not only brought soldiers and the Vietnamese living in the area into direct contact with the toxic herbicide, but also caused contamination of drinking water and many food sources such as fish and crops. Exposure to Agent Orange and the toxic contaminant TCDD therefore occurred very easily during the Vietnam War, and was usually unavoidable. Dai (2000) estimates that during the War about 17 million people living in South Vietnam, and about one million from the North, were directly exposed to TCDD-contaminated herbicides.

In studies conducted by Schecter *et al.* (1995) comparing Vietnam veterans with contemporary veterans who had served elsewhere, TCDD levels were found to be significantly elevated among those who had served in Vietnam.

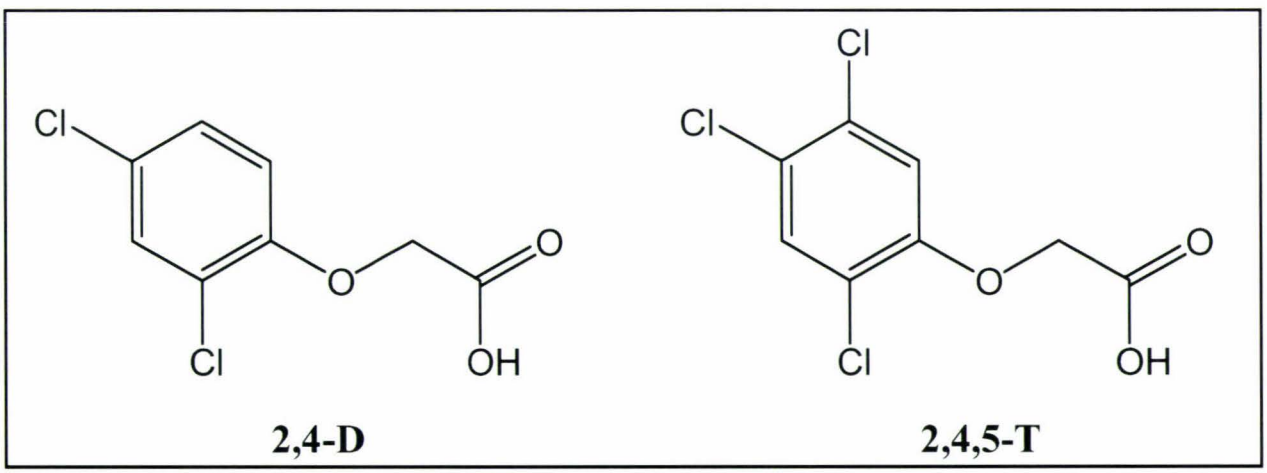

Figure 2.1 Chemical Structure of 2,4-D and 2,4,5-T

Chemical structure of 2,4-dichlorophenoxyacetic acid (left) and 2,4,5-trichlorophenoxacetic acid (right). The herbicide Agent Orange comprised a 1:1 solution of these two chemicals.

2.1.1 2,3,7,8-tetrachlorobenzo-*para*-dioxin (TCDD)

Known simply as TCDD or Dioxin, this chemical is produced as a by-product of many industrial processes. It is a contaminant of particular phenoxylic herbicides that contain 2,4,5-T and have been manufactured and used in many countries through out the world. TCDD is formed during the incomplete combustion of organic material where chlorine is available in the feedstock or in the air supply. It is also produced at trace levels in various industries. Focus on dioxins as contaminants began in the 1940s and 1950s in industrial settings, where manufacture of chlorinated phenoxy herbicides occurred, as a result of exposed workers exhibiting particular health problems (Aylward & Hays, 2003). The ability of TCDD to affect the endocrine system, and toxic effects on experimental animals, prompted several studies into the possible effects of dioxin on humans, especially regarding their reproductive ability. The chemical structure of TCDD is shown in Figure 2.2. TCDD is part of the group of 75 polychlorinated dibenzo-*para*-dioxins. The members of this group are essentially planar, aromatic chemicals characterized by high octonol/water partition coefficients (thermodynamic hydrophilic/lipophilic balance), extremely low water solubility and low vapour pressures (Geyer *et al.*, 2000; Mackay *et al.*, 1992).

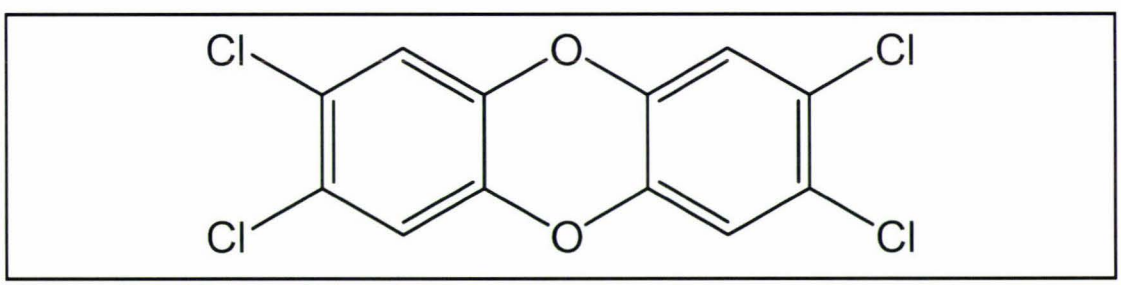

Figure 2.2 Chemical Structure of TCDD

Chemical structure of 2,3,7,8-tetrachlorobenzo-*para*-dioxin (TCDD), a contaminant of 2,4,5-T and thought to be the cause of detrimental health effects from exposure to Agent Orange.

TCDD behaves as a multi-site human carcinogen, and is thought to induce tumours in humans indirectly (Albertini *et al.*, 2000). The biological mechanism of TCDD-induced carcinogenesis is not completely clear, but appears to be related to the aryl hydrocarbon receptor (AhR) or the aryl hydrocarbon receptor nuclear translocator, known as the Arnt. AhR and Arnt are both transcription factors with DNA-binding

domains (Giri *et al.*, 2004). Schwarz & Appel (2005) reported that high-affinity binding of TCDD and other dioxin-like chemicals to the AhR was the mediating factor for most, if not all, of the toxic responses to these agents. Biochemical and genetic evidence has indicated that the biochemical mechanism of the action of TCDD is mediated by the aryl hyrdrocarbon receptor, a ligand-activated transcription factor. TCDD diffuses across the plasma membrane and binds to the aryl hydrocarbon receptor complex, present in the cytoplasmic compartment (Juan *et al.*, 2006; Ma & Whitlock, 1997) Ligand aryl hydrocarbon receptor complexes are then translocated into the nucleus, and following their association with the nuclear Aryl hydrocarbon receptor nuclear translocator (Arnt) protein, the ligand-aryl hydrocarbon receptor-Arnt complex is able to bind to the dioxin responsive element (DRE) on DNA and initiate transcription of genes including cytochrome P-450 (CYP) (Denison & Whitlock, 1995; Hankinson, 1995; Sogawa *et al.*, 1986). TCDD exposure therefore ultimately causes a change in the expression of a large number of genes. Thus exposure to TCDD results in a broad spectrum of biological responses, including altered metabolism, disruption of the normal hormone signalling pathways, and reproductive and developmental effects.

2.1.2 TCDD Half Life in Humans

The half life of a particular chemical is of great importance for hazard assessment because it allows an estimation to be made of the persistence of a chemical in living aquatic and terrestrial organisms. The half life is the time required to reduce the concentration or body burden of a chemical by one-half in tissue, organ or in the whole organism (Geyer *et al.*, 2000).

It is known that TCDD is very persistent and has a long half-life in organisms (Geyer *et al.*, 2000; Li *et al.*, 1999; Van den Berg *et al.*, 1994). Geyer *et al.* (2002) states that the average half life of TCDD in humans is approximately 2,840 days (7.78 years). A half life as large as this means that New Zealand Vietnam veterans who were exposed to TCDD more than 30 years ago are still very likely to have elevated TCDD levels when compared to non-veterans. As recently as 1995, TCDD blood levels were found to be between 25 and 170 times higher in people living in sprayed areas of Vietnam, compared to people living in unsprayed villages in Northern Vietnam (Palmer, 2005).

2.1.3 Health Effects Caused by Exposure to TCDD

Spraying of Agent Orange during the Vietnam War represents the world's largest ever TCDD contamination to date. Health effects associated with exposure to TCDD have not been fully characterised. In 1997 the International Agency for Research on Cancer (IARC) classified TCDD as a Group 1 human carcinogen, based largely on four highly-exposed industrial cohorts that showed an excess of all cancers (Steenland *et al.*, 1999). The largest of the four groups considered by the IARC is the USA cohort of 5,172 workers at 12 plants that produced chemicals contaminated with TCDD. These workers were exposed to high levels of TCDD. The workers were found to have on average 286 times more TCDD in their blood than the general population. This population was also found to have a 46 % greater mortality rate caused by cancers.

A recent study contrasting cancer incidence rates in white male USA Air Force veterans involved in Operation Ranch Hand with the USA white male population reported increases in prostate cancer and melanoma in Ranch Hand Veterans (Akhtar *et al.*, 2004). Pavuk (2005) reported statistically significant associations between TCDD and all types of cancer in USA Air Force veterans selected as comparisons in the Air Force health study.

Farm and agricultural workers who are exposed to a range of chemicals as part of their job have been the subject of many scientific studies. Illing (1997) found an increased rate of cancer in farmers and agricultural workers that was directly related to their occupational background exposure to organochlorines (including TCDD) and other pesticides. Dich & Wiklund (1998) reported a statistically significant increased risk of prostate cancer among pesticide applicators. In Britain a study was conducted around a pesticide factory, revealing an excess of skin melanoma, lung, stomach, pancreas and prostate cancers (Wilkinson *et al.*, 1997). A series of case-control studies in Sweden have found increased risks of soft-tissue sarcoma and malignant lymphoma among agricultural workers who had been exposed to phenoxy herbicides (Eriksson *et al.*, 1981; Hardell & Sandstrom, 1979).

A cancer study conducted by Saracci *et al.* (1991) found an Odds Ratio of 1.5 for non-Hodgkin's lymphoma (NHL) (99 cases, 95% Confidence Interval 1.1-2.0) for male

veterans who served in Vietnam compared to male veterans who did not (Saracci *et al.*, 1991). An Odds ratio greater than one indicates that the experimental group is more likely to develop cancer than unexposed controls. A similar Australian study (Fett *et al.*, 1987) found the increased relative risk of NHL to be 1.8 (4 cases, 95 % Confidence Interval 0.4-8.0) for service in Vietnam. This result suggests that people who served in Vietnam have a 1.8-fold increased risk of developing NHL compared to those who did not serve in Vietnam.

Research on ex-servicemen from the Vietnam War has shown significant associations between TCDD exposure and certain kinds of cancer, including soft tissue sarcoma (Eriksson & Hardell, 1990; Lynge, 1993), non-Hodgkin's lymphoma (Pearce & Mclean, 2005) and multiple myeloma (Bertazzi *et al.*, 2001). One major investigation that warrants special attention is the on-going study of the residents of Seveso, a small town in Italy. In 1976 an explosion at the ICMESA (Industrie Chimiche Meda Società) chemical plant near Seveso resulted in the highest exposure to TCDD in a residential population to date. The earliest related health effect was chloracne in children who were outdoors and in the path of the toxic cloud (Caramaschi *et al.*, 1981). In the following years other adverse health effects were observed and found to be linked to TCDD exposure. These include spontaneous abortions (Revich *et al.*, 2001), cytogenetic abnormalities (Bertazzi *et al.*, 2001), congenital malformations (Mastroiacovo *et al.*, 1988; Revich *et al.*, 2001), impaired liver function and lipid metabolism (Ideo *et al.*, 1985; Mocarelli *et al.*, 1986).

In one particular study of the Seveso population, Bertazzi *et al.* (2001) discovered a two-fold increase in rectal cancer-induced deaths and an excess of "other" digestive cancer-induced deaths. Lung cancer was also in moderate excess; nearly twice as many lymphohemopoietic neoplasms were observed than expected. Bertazzi *et al.* (2001) also reported particular increases in Hodgkin's disease, multiple myeloma, and acute myeloid leukaemia. All of these cancers are well established as cancers arising from specific genetic malfunction. Moderate to significant increases were also observed in chronic obstructive pulmonary disease and diabetes.

In 1953 a trichlorophenol production facility called BASF in Germany was contaminated with TCDD as a result of an uncontrolled decomposition reaction. Chloracne and other signs of acute toxicity were seen immediately in many employees.

A follow-up study by Zober *et al.* (1993) has shown that employees exposed to TCDD in this industrial accident have experienced a higher incidence of chromatid breaks in peripheral lymphocytes compared to non-exposed workers. These breaks are indicative of genetic damage (Section 2.6) and are here observed forty years after the exposure event.

In addition to known carcinogenic properties, Steenland *et al.* (1999) reported that TCDD exposure is a possible cause of heart disease. Elevated ratios for mortality from heart disease were found in a large multi-country study, including a heavily-exposed Dutch cohort and a German industrial cohort. TCDD offers plausible mechanisms for causing cardiovascular disease, primarily by the alteration of lipid metabolism, although some other mechanisms are currently being studied. Calvert *et al.* (1996) conducted cross-sectional studies on TCDD exposure and have shown an inverse relationship between serum TCDD level and high-density lipoprotein. Steenland *et al.* (1999) also reported a positive relationship with total cholesterol and TCDD exposure.

Exposure to Agent Orange also has major effects on the reproductive system of humans; TCDD is an endocrine-disrupting chemical with a highly toxic effect on the human reproductive system (Rogan & Ragan, 2003). Even at low doses TCDD can seriously disrupt normal reproduction in humans; it can lower fertility, increasing antenatal mortality and the risk of endometriosis, and can also cause many birth defects (Lawson *et al.*, 2004). Egeland *et al.* (1994) conducted a study on male reproductive endocrine function of subjects occupationally exposed to TCDD and found that exposed individuals had lower testosterone and higher gonadotrophin levels in a dose-dependent relationship with increasing serum dioxin concentrations. This may explain the significant alteration in the sex ratio of newborns (in favour of females) that was observed after Seveso (Mocarelli *et al.*, 1996); female conceptions being favoured by low testosterone levels and elevated gonadotrophin levels. In addition to these illnesses, Agent Orange exposure was also found to be associated with the onset of porphyria cutanea tarda[2] (Frumkin, 2003).

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[2] **Porphyria cutanea tarda** is a disorder of heme biosynthesis due to a defective liver enzyme. Symptoms of this disorder include photosensitivity; hepatic dysfunction; discolored teeth, gums and skin; excessive hair; and psychiatric symptoms.

Henriksen *et al.* (1997) performed a cross-sectional medical study of the Operation Ranch Hand participants and found a 50 % higher prevalence of diabetes among those individuals with the highest levels of TCDD in serum, compared with non-exposed control individuals. Steenland *et al.* (2001) found an increased risk of diabetes with TCDD exposure. Kern *et al.*, (2002a) biochemically linked TCDD exposure and diabetes in humans, complementing the many epidemiological studies that had been conducted previously.

According to Spencer *et al.* (1999), the specific deleterious effects associated with exposure to dioxin are believed to be due to species- and tissue-specific expression of dioxin-regulated genes. TCDD is part of a family of halogenated polyaromatic hydrocarbons that are known to be concentrated in adipose tissue, because of their high-lipid solubility. Kern *et al.* (2002a) studied differentiation of human adipocytes after dioxin exposure and concluded that insulin resistance and diabetes were caused by TCDD exposure.

Although the majority of current literature supports the claim that exposure to TCDD causes detrimental health effects, there are some studies that disagree. Most of these studies are related to the reproductive outcome of those exposed to TCDD. Wolfe *et al.* (1995) conducted a study on reproductive outcomes in Vietnam War veterans and found no elevation in the risk of spontaneous abortion or still birth. Some elevations were found in birth defects but these were reported to be non-significant and there was no increase in birth defect severity. Schnorr *et al.* (2001) found no association between paternal TCDD levels at the time of conception and spontaneous abortion or sex ratio among pregnancies fathered by men exposed to TCDD.

Goetz *et al.* (1994) investigated neurological disorders and brain tumours in Vietnam veterans exposed to TCDD; it was concluded that there was insufficient evidence to determine an association between neurological disorders and exposure to TCDD in Vietnam. However, it was found that there was limited evidence to suggest no association between exposure and brain tumours.

2.2 Exposure of New Zealand soldiers to herbicide sprays

Past claims of herbicide exposure have largely relied on anecdotal evidence. More recently however, a major Select Committee report conducted by the New Zealand Government and published in October 2004 has resulted in more convincing information being presented on exactly where New Zealand soldiers were at certain times, which corresponds exactly to times and places of Agent Orange release. The case is no longer circumstantial and the evidence strongly substantiates the claim that New Zealand troops were exposed to Agent Orange. The claim that Vietnam veterans were exposed to herbicide spraying as part of Operation Trail Dust was recognised by the Government in December 2003, 32 years after the New Zealand Vietnam War veterans left Vietnam.

On 3 December 2003 a new report was submitted to Parliament's Health Select Committee (Chadwick, 2004). The Chief of the Defence Force had requested an investigation be conducted into the spraying of herbicides in Phuoc Tuy province in South Vietnam between 1965 and 1971 (Figure 1.1). The report concluded that while serving in Vietnam between 1965 and 1971, New Zealand Vietnam War veterans were exposed to large quantities of the defoliants Agent Orange, Agent Blue[3] and Agent White[4]. It was estimated that at least 1.8 million litres of the defoliants Agent Orange, White and Blue was sprayed in the Phuoc Tuy Province between November 1965 and June 1968 (Taylor, 2003).

Phuoc Tuy Province was the location of the first Ranch Hand missions conducted in January 1962. The head of the Scientific Advisory Group of the Commander in Chief, Pacific (CINCPAC) reported that 227,125 litres of defoliant was sprayed on the Phuoc Tuy Province between December 1965 and January 1966. New Zealand Vietnam veterans were serving in this province during this time (Irvine, 2003).

Yet more evidence that New Zealand War Veterans became exposed to harmful defoliants during their service in Vietnam was presented upon examination of the military command zones which the American commanders divided South Vietnam into:

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[3] **Agent Blue** was a mixture of herbicides containing Cacodylic acid, commonly known as Arsenic. It was used specifically to kill rice in Vietnam.

[4] **Agent White** was a 4:1 mixture of 2,4-D and Picloram. Picloram is a herbicide used on woody plants.

I Corps, II Corps, III Corps and IV Corps (Figure 2.3). New Zealand and Australian units operated exclusively in III Corps. Operation Ranch Hand records show that III Corps received 21,521,614 litres of Agent Orange, 563,852 litres more than the other three zones combined.

The evidence is substantial to support the claim that New Zealand troops were both directly and indirectly exposed to the TCDD-containing herbicide Agent Orange while serving in Vietnam.

2.3 Consequences of Herbicide Exposure in Vietnam

During the 1970s, returned Vietnam Veterans began to report skin rashes, cancers, psychological symptoms, extreme fatigue, congenital abnormalities as well as handicaps in their children, and many other health problems. In the USA some 300,000 veterans have undergone medical tests and an estimated 2,000 children of veterans are suffering from the birth defect spina bifida (Palmer, 2005). A Columbia University study also estimates that up to 4 million people may be directly affected by Agent Orange (Stellman *et al.*, 2003). Most veterans are concerned that Agent Orange exposure might have contributed to these health problems. These concerns helped to initiate a series of scientific studies on Agent Orange and its carcinogenic contaminant TCDD. In addition to polluting the environment, exposure to the toxic TCDD-containing herbicides has been found to cause many diseases, including several types of cancers (Hardell & Eriksson, 1999; Hardell & Sandstrom, 1979; Khuder *et al.*, 1998; Lynge, 1998; Pavuk *et al.*, 2005; Safi, 2002; Saracci *et al.*, 1991), as well as causing increased rates of endometriosis (Igarashi *et al.*, 2005; Rier & Foster, 2003), congenital birth defects (Barrow *et al.*, 2002; Lawson *et al.*, 2004) and other health problems (Aoki, 2001; Lawson *et al.*, 2005; ten Tusscher *et al.*, 2003) in the children of those exposed.

Tuyet and Johansson (2001) conducted a study on Vietnamese women and their husbands who were exposed to Agent Orange during the Vietnam War. The authors found that 66 % of all children had some type of major health problem. Thirty-seven

percent of these children were born with some visible malformation or disability while 27 % had developed a disability during the first year of life. Of the 60 children suffering from health problems, 40 were unable to attend school but were able to help with agricultural work and domestic chores. Twenty children were very severely physically and mentally disabled, and required 24-hour care; needing to be attended to by their parents for every daily need. There were no cases of congenital malformation nor other disabilities among unexposed siblings of the husbands and wives, nor among the children of their siblings.

Giri *et al.* (2004) concluded that exposure to Agent Orange is associated with an increased risk of prostate cancer. Men who had been previously exposed to Agent Orange were at least two times more likely to be diagnosed with prostate cancer as unexposed men. Pavuk *et al.* (2005) also reported that prostate cancer was significantly associated with service in South East Asia (including, or exclusively in, Vietnam).

2.4 USA & Australian Reactions to Agent Orange Exposure

The United States Department of Veteran Affairs now accepts a link between Agent Orange exposure and Hodgkin's Disease, multiple myeloma, non-Hodgkins lymphoma, respiratory cancers (lung, bronchus, larynx and trachea), soft-tissue sarcoma, prostate cancer, chronic lymphocytic leukemia, porphyria cutanea tarda, acute and subacute peripheral neuropathy, and adult-onset diabetes.

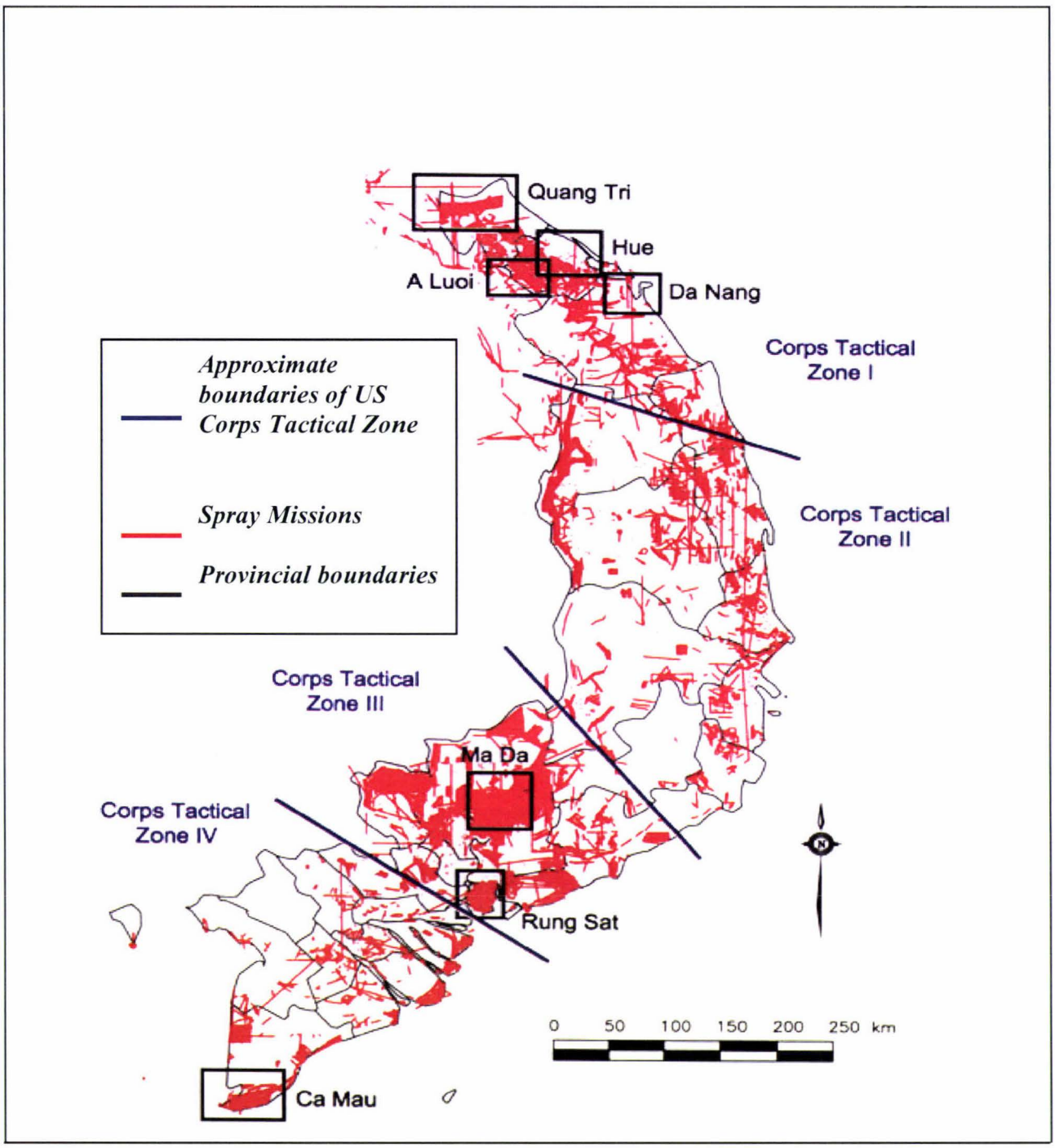

Figure 2.3 Map of Agent Orange Spraying

Map of South Vietnam, showing the aerial herbicide spray missions of Agent Orange from 1965 to 1971 as part of Operation Ranch Hand. New Zealand troops served exclusively in III Corps. III Corps received the greatest volume of Agent Orange.

US Department of the Army (Irvine, 2003)

The USA Government also accepts a link between Agent Orange and spina bifida in children of male veterans and a link between all birth defects that are not caused by familial disorder, birth-related injury, or foetal or neonatal infirmity in children of female veterans. Compensation and health care are provided to veterans and children of veterans suffering from these illnesses (Chadwick, 2004; Irvine, 2003).

According to Irvine (2003), the Australian Department of Veteran Affairs does not accept a link between Agent Orange and these health problems; yet they give the benefit of the doubt to veterans on a case-by-case basis. They also provide treatment to any Vietnam veteran with cancer. The Australian Department of Veteran Affairs (while still not accepting a link) in partnership with the Department of Health and Ageing, provides treatment to children of Vietnam veterans born with cleft lip or palate, spina bifida, acute myeloid leukemia, and adrenal gland cancer.

2.5 Detection of Genetic Damage

Although concerned about the direct health effects of Agent Orange, Vietnam War Veterans are most fearful of long term genetic damage, with the possibility of this damage being passed on to their children and further generations. Several molecular assays are now available to researchers to determine if genetic damage has occurred in humans. These assays include the COMET assay (Singh *et al.*, 1988) to measure DNA degradation directly (Olive & Banath, 1992), Fluorescent *in situ* Hybridisation (Albalwi *et al.*, 1997) to detect chromosomal translocations, the Micronucleus Assay (Scott *et al.*, 1999) to measure the efficiency of DNA repair, G2 assay (Slijepcevic, 1992) to measure DNA repair specifically in the G2 phase of the cell cycle, and the Premature Chromosome Condensation Assay (PCC assay) to measure chromosome breaks at all stages of the cell cycle (Cheng *et al.*, 1993).

However, one of the most sensitive and widely applied tests for clastogenicity[5] is the Sister Chromatid Exchange (SCE) assay. Due to the time constraints on the current study it was only possible to conduct one of the cytogenetic tests mentioned here. SCE was chosen due to its high sensitivity and success rate in studies on clastogens.

2.5.1 Determination of Genetic Damage using SCE Assay

As noted earlier (Section 1), a statistically significant increase in the average SCE frequency, using an experimental and a matched control group, is indicative of genetic damage (Albertini *et al.*, 2000). The SCE cytogenetic test is used to visualise the number of SCEs in cells, and is considered a bioindicator of any genetic damage that has been sustained by the subject. It is therefore a test that indicates the harmfulness of particular chemicals.

Changes in genetic material induced by environmental mutagens may produce harmful genetic effects on human health, causing mutations in sex cells and somatic cells. Sex cell mutations create a genetic risk for hereditary diseases and congenital defects while somatic cell mutations result in various diseases including cancer (Kaioumova & Khabutdinova, 1998). The SCE cytogenetic test has been previously applied successfully to animals and humans to determine whether or not genetic damage has been incurred as a result of exposure to dioxins and other chemicals (Akin *et al.*, 2005; Arias, 2002; Bhattacharya *et al.*, 2005; Garaj-Vrhovac & Zeljezic, 2001; Iannuzzi *et al.*, 2004; Zober *et al.*, 1993). Zober *et al.* (1993) show that the SCE technique is suitable to evaluate possible genotoxic effects of particular chemicals even if exposure occurred many years ago. It is therefore appropriate that the SCE test be performed as a test for ascertaining the extent of genetic damage, if any, sustained by New Zealand Vietnam Veterans as a result of exposure to herbicides while serving in Vietnam. The results of the current study will add to the body of knowledge so far gathered on the genetic health status of Vietnam War veterans.

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[5] A **'clastogen'** is the term used to describe any environmental agent which results in damage to DNA. A clastongen may or may not be a 'mutagen' (resulting in mutations directly), a 'teratogen' (resulting in genetic deformities) or a 'carcinogen' (resulting in cancer).

2.5.2 TCDD Exposure using the SCE Assay

It was mentioned previously that in 1953 a trichlorophenol production facility called BASF in Germany was contaminated with TCDD as a result of an uncontrolled decomposition reaction. Zober *et al.* (1993) conducted a cytogenetic study scoring chromosome aberrations and SCEs in 27 of the exposed BASF workers with current TCDD blood lipid concentrations exceeding 40 parts per trillion (ppt). The control group comprised 28 unexposed control individuals of similar age. It was found that there was an increased rate of SCE per cell ($p = 0.051$) and a higher percentage of cells with more than 10 SCEs ($p = 0.064$) in the exposed group. These results indicate that exposure to TCDD induces genetic damage in humans. This is not surprising in the light of an *in vitro* study using human lymphocytes, which showed that TCDD is highly genotoxic in humans (Nagayama *et al.*, 2002). Genetic damage was found to occur at very low TCDD levels, only about ten times higher than normal background levels. In a related study, Arias (2002) conducted an SCE study on chicken embryos after exposure to 2,4-D. The author endeavoured to assess the extent of genetic damage that may be incurred from exposure to agricultural chemicals, especially the genotoxic potential of 2,4-D. The author found that prolonged exposure to 2,4-D does not increase SCE frequency, although interestingly once the 2,4-D had been made into its commercial herbicide form the data obtained indicated a dose-dependent increase in SCE frequency. This result therefore indicates a contamination with TCDD that occurs during the formation of the commercial herbicides as the real cause of the detrimental results seen. This study provides substantiating evidence that the herbicides sprayed over Vietnam during Operation Ranch Hand were genetically damaging.

In another study, Kaioumova & Khabutdinova (1998) investigated the effect of dioxin-containing products on the cytogenetic characteristics of peripheral blood lymphocytes of herbicide plant workers, using control groups consisting of people with no professional contact to herbicides. The herbicide plant produced both 2,4,5-T and 2,4-D from 1961 until 1969. Besides chloracne in the workers involved in production, associated disorders of liver, nervous system and lipid metabolism impairment were also observed. Chloracne was observed to affect the exposed workers for as long as 26 years. The authors found that the mean incidence of cells with chromosome aberrations was two-fold higher in the herbicide plant workers than the mean incidence level of the

control groups ($p < 0.05$). The estimation of chromosome aberration incidence in the peripheral blood lymphocyte cultures makes it possible to reveal the mutagenesis level in somatic cells. The authors concluded that the herbicides 2,4,5-T and 2,4-D have mutagenic effects in humans.

2.6 The Sister Chromatid Exchange Assay

J.H. Taylor (1958) was the first to observe an apparent recombination event between two sister chromatids, during his autoradiographic work. By growing cells in the presence of radioactive thymidine, J.H Taylor's work resulted in genetically identical sister chromatids that were physically different. Changes in the sister chromatids were observed by the exchange between the radioactive chromatid and the non-radioactive chromatid. Therefore, this exchange was determined to have occurred after the replication that created them. Autoradiography did not provide the resolution to allow the nature of this exchange to be studied with precision.

In 1972, Zakhavov and Egolina worked to increase the observable resolution of sister chromatid exchanges. It was found that chemically distinct sister chromatids could be produced by growing lymphocytes in bromodeoxyuridine (BrdU). BrdU is a thymidine analogue that is incorporated into the DNA at replication in a similar manner to Taylor's radioactive thymidine. Using BrdU, a powerful technique now called Sister Chromatid Exchange (SCE) has been developed in order to study these exchanges with greater precision. With the greater resolution afforded by this technique it has now been determined that SCEs occur by a reciprocal exchange of DNA between assumed identical loci of the two sister chromatids of a duplicated chromosome, presumably at a replication fork (Rodriguez-Reyes & Morales-Ramirez, 2003). The Sister Chromatid Exchange technique relies on BrdU incorporation into the replicating DNA. The reaction of chromatids with the fluorescent dye Hoechst is quenched by the presence of BrdU. Visually this is observed as a decrease in fluorescence, or a decrease in staining with Giemsa (Figure 2.4).

The incidence of sister chromatid exchanges in a cell increases due to many variables including age, smoking, some medications, and exposure to any substances that cause damage to DNA. A wide variety of agents that are known to cause chromosome breaks have also been found to induce SCE. Numerous studies have utilised SCE analysis to explore the extent of genetic damage caused by environmental agents. Rowland & Harding (1999) found that SCE frequency increased in female smokers between the ages of 16 and 25 years, compared to a non-smoking control group. A study conducted on methamphetamine abusers also found significant increases in SCE frequencies (Li *et al.*, 2003). Iannuzzi *et al.* (2004) reported increases in the frequencies of SCEs in sheep flocks that had been exposed to specific dioxins in herbicides. Exposure to pesticides, iodine-131, wood dust, and many other environmental factors have also shown increases in SCE frequency when compared with matched controls (Elavarasi *et al.*, 2002; Sonmez *et al.*, 1997; Zeljezic & Gavaj-Vrhovac, 2002). These studies highlight the usefulness of SCE analysis in the biomonitoring of human populations exposed to a variety of agents.

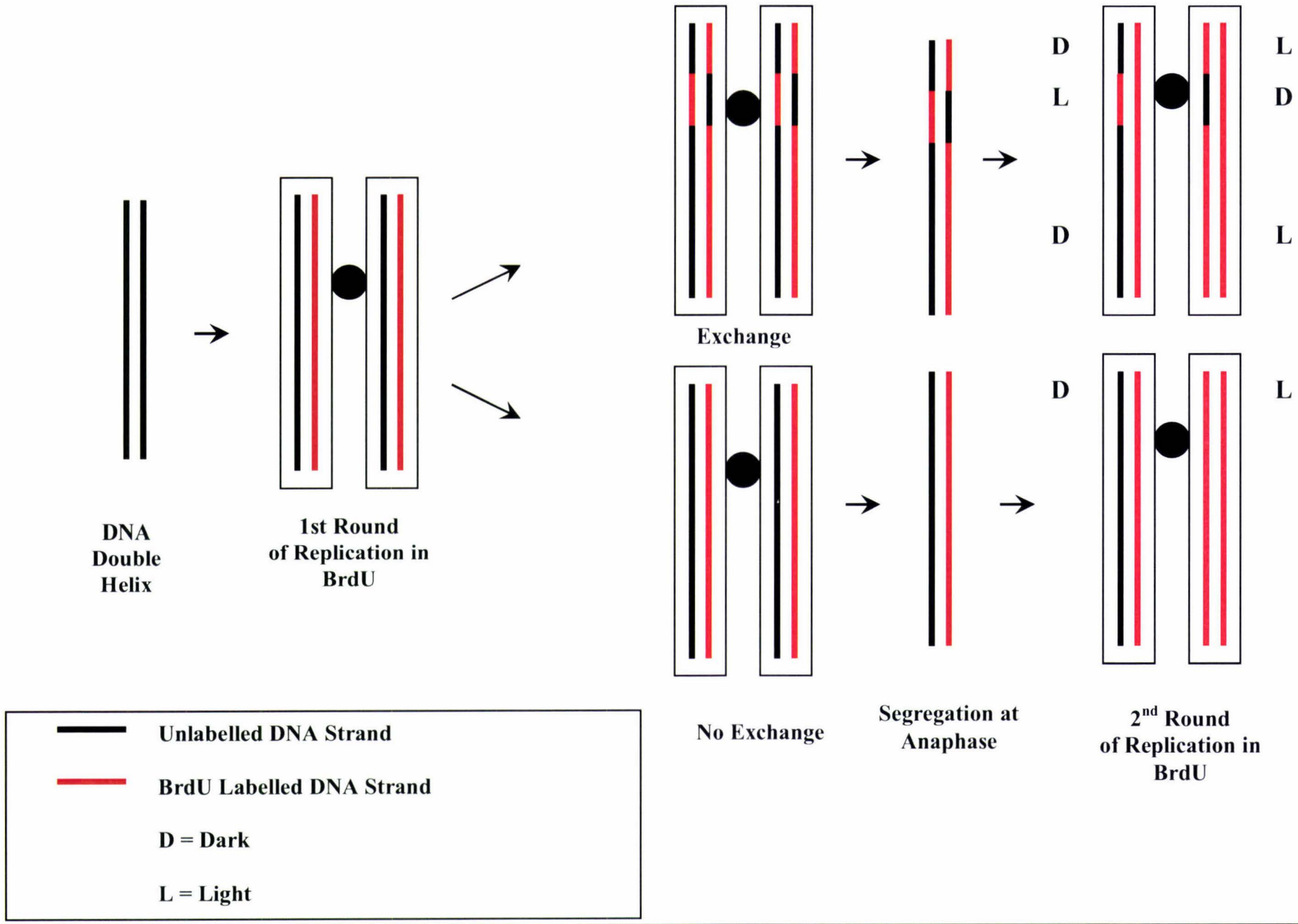
The background SCE levels in human chromosomes quoted in SCE studies vary internationally because the living environment of human populations varies enormously; ethnic differences are also possible. The way in which the SCE assay is conducted also differs between laboratories, which can lead to further variations in SCE frequency. There is no internationally accepted level for normal or background SCE frequency, therefore valid comparisons between studies cannot be made. This can lead to debate regarding the harmfulness of a substance. However, a major report on population monitoring using cytogenetic techniques (Carrano & Natarajan, 1988) states that the baseline SCE frequency in human peripheral lymphocytes averages about 7 - 10 per cell in non-exposed individuals. Evidence of genetic damage is accepted if the number of SCEs in an experimental group is more statistically significant than a selected control group (Albertini *et al.*, 2000). A significant increase in SCE frequency is accepted as an indication that the DNA of a target group has been damaged in some way. Any damage to DNA is universally accepted as being detrimental to a person's well-being.

The stimulation for SCE to occur is still largely debated. Although the exchange process is not fully understood, SCE appears to be a consequence of errors in DNA

replication on a damaged template at the replication fork. The primary determination of genetic damage is therefore established by whether the SCE frequency observed in the study group and control group show a statistically significant difference.

Figure 2.4 A schematic illustration of The Sister Chromatid Exchange Assay.

The first round of replication in the presence of BrdU results in BrdU incorporation into one strand of each DNA duplex. When BrdU is incorporated into only one DNA strand of a Sister Chromatid it is termed unifilar. When both strands are the same for their BrdU incorporation it is termed bifilar. A Sister Chromatid Exchange may occur after the first or second round of replication before separation at anaphase. The exchange can be detected after a second round of replication in BrdU when treated with Hoechst solution and then Giemsa, as the bifilar chromatid appears much lighter than the unifilar chromatid. A sister chromatid exchange is observed as an exchange of light and dark between two chromatids.


DNA
Double
Helix
1st Round
of Replication in
BrdU
Exchange
No Exchange
Segregation at
Anaphase
2nd Round
of Replication in
BrdU
D
L
D
L
D
L
D
L
Unlabelled DNA Strand
BrdU Labelled DNA Strand
D = Dark
L = Light

3 CHAPTER THREE : MATERIALS AND METHODS

3.1 Buffers and Solutions

All materials used for this study were stored at room temperature unless otherwise stated.

Bromodeoxyuridine Solution

Bromodeoxyuridine (BrdU) (Sigma)	0.00614 g
Milli-Q Water (MqH_2O)	100 mL

The BrdU solution was freshly prepared on the day of use. BrdU was weighed and placed in an autoclaved 5 mL bottle (Duran). A sterile syringe (Terumo) was then used to deliver autoclaved water to the bottle, avoiding contamination. The resulting solution was mixed on a magnetic stirrer for one hour or until completely dissolved.

Colchicine 0.05 % (w/v)

The Colchicine solution was stored refrigerated at 4 °C and could be reliably stored in this way for one year.

Fixative

Glacial Acetic Acid	1 Part
Methanol	3 Parts

The Fixative was freshly prepared on the day of harvesting. Fixative was kept on ice while in use.

Hoechst Stock Solution

Hoechst 33258 (bisbenzimide) (Sigma)	10 mg
dH_2O	20 mL

The Stock Solution was made to a concentration of 0.5 mg/mL. This Stock Solution can be stored for up to 6 months at 4 °C in the dark. The Hoechst Stock Solution is very sensitive to light so to avoid light exposure the bottle was wrapped in aluminium foil.

Hoechst Working Solution

The Hoechst working solution was prepared by diluting the Hoechst Stock Solution 100 fold in Sorensen's Buffer. A one-step dilution was performed to obtain a final Hoechst concentration of 5 µg/mL using 0.1 mL of Stock Solution diluted in 10 mL of Sorensen's Buffer. The resulting Working Solution could be stored for up to three months in the dark at 4 °C. The Hoechst Working Solution is also very light sensitive, so to avoid light exposure the bottle was wrapped in aluminium foil.

KCl

KCl (BDH)	0.075 M

The KCl solution was mixed on a magnetic stirrer until completely dissolved. Sterility was not required.

MacIlvaine's Buffer (pH 7.0)

$C_4H_6O_7.1H_2O$ (BDH)	0.1 M
$Na_2HPO_4.2H_2O$ (BDH)	0.2 M

Each ingredient was dissolved in half the total volume and then the solutions were combined to form MacIlvaine's Buffer. The pH was adjusted to 7.0 (HCl , NaOH).

Phytohaemagglutinin (PHA) (Gibco)

The PHA was prepared as per manufacturers instructions. The solution could be stored at - 20°C for one year.

Sorensen's Buffer (Merck)

The Sorensen's Buffer was prepared as per manufacturer's instructions. These buffer tablets ensured correct buffer formation; sterility was not required.

2 x SSC (pH 8.0)

NaCl (BDH)	0.3 M
$Na_3C_6H_5O_7.2\ H_2O$ (Merck)	0.3 M

Each ingredient was dissolved in half the total volume and then the solutions were combined to form 2 x SSC. The pH was adjusted to 8.0 (HCl, NaOH).

3.2 Stain

Giemsa Stain

Giemsa Stain Solution (BDH)	8 mL
Sorensen's Buffer (Section 3.1)	72 mL

The Giemsa Stain Solution was stored at 4 °C. Before making the stain the Giemsa Stain Solution was removed from the refrigerator and allowed to warm to room temperature. The bottle was swirled before use to ensure homogeneity of the solution. The Sorensen's Buffer was added to the 8 mL aliquot and mixed well. The Giemsa stain was used immediately after preparation.

3.3 Cell Culture Media

The cell culture media was prepared in bulk and stored frozen at -20 °C. Kept in these conditions the culture media could be stored for up to 6 months.

Sister Chromatid Exchange (SCE) Cell Culture Media

Wellcome Media (Media 199) (Gibco)	5 mL
AB Serum (Gibco)	1 mL
Phytohaemagglutinin (Section 3.1)	0.1 mL
Bromodeoxyuridine Solution (Section 3.1)	0.05 mL

In order to ensure sterility all steps were performed in the biohazard hood.
The manufacturer's instructions of Media 199 were followed in order to make Wellcome media. Media was mixed on a magnetic stirrer at room temperature until powder was completely dissolved.

The resulting media was vacuum filtered through a positive flow filter to sterilise (Stericup/Steritop Filter Unit, Millipore, 0.22 µm pore size). To heat activate the AB Serum, the thawed solution was placed in a 57 °C water bath for 45 minutes. A positive-flow filter was also used to sterilise the AB Serum following heat activation.

The Media, AB Serum and PHA were all placed in sterile 8 mL Falcon™ Culture tubes (Becton Dickinson) and stored frozen at -20 °C until required.

The BrdU was not added prior to freezing but was added fresh upon use of each culture tube. A 0.22 µm Acrodisc syringe tip filter (Pall) was used for delivery of BrdU into culture tubes to ensure sterility.

To prepare frozen culture tubes for use, tubes were removed from -20 °C freezer and placed in a 37 °C hot water bath until completely thawed.

3.4 Experimental Design

3.4.1 Ethical Considerations

Ethics approval to conduct the study was sought and given by the Massey University Human Ethics Committee and the Wellington Human Ethics Committee. Letters of approval from these committees can be found in Appendix I.

3.4.2 Selection of Participants

In designing the current study, matched controls were required to be selected and this involved careful planning. Controls were matched for both age and occupation. The variable of interest was exposure to Agent Orange. Therefore, matched controls were sought who differed from the veterans group only with regard to this variable. The 'healthy solider' effect (Bross & Bross, 1987) was of primary consideration when matched controls were chosen. For this reason all matched controls were current or ex-army personnel. This ensured that any 'healthy soldier' effect bias was eliminated. The mean age for the veterans group was 62.5 years, while the mean age for the controls group was 57 years. This is approximately a five year difference in age. This difference is not expected to make a difference in SCE frequency, however it was possible to statistically control for any influence.

Initial contact with the veterans was made through the Association of Vietnam Veterans via the Returned Servicemen's Association. Names and contact details of volunteers were obtained by Vietnam Veteran representatives; participants were then approached by researchers to seek their consent to take part in the study. Names of participants to be part of the control group were given to the researcher by a New Zealand Army representative after participant permission was gained.

The following research protocol, which evolved after considerable consultation with human researchers and advice from Ethics Committees, was adopted. Adherence to this protocol ensured that the current study was methodologically robust.

Once participants were confirmed and had issued verbal consent, they were sent out an Information Sheet, Consent Form and Personal Questionnaire (Appendix II, III & IV). The Personal Questionnaire gathered information about participants' life events and general health. Completion of a questionnaire by all participants was necessary in order for the researcher to take into account any other factors that may cause chromosome damage, other than service in Vietnam. It was arranged for a blood sample to be taken at the volunteers' convenience. The Personal Questionnaire and Consent Form were collected from participants at the time of blood collection.

3.4.3 Data collection methods

For each participant a 10 mL blood sample was drawn from a vein in the arm by venipuncture (21 G ¾ Vacutainer™, BD) into heparinized Vacutainer™ tubes (BD). This blood withdrawal was undertaken by a fully-qualified phlebotomist under sterile conditions. The blood samples were cultured immediately, then used to make a chromosome preparation of dividing lymphocytes.

Each blood sample was coded with a unique numerical code to identify the individual without revealing the sample's original source (veteran or control). This code was not decoded until all analysis for that year was completed.

3.5 Blood Manipulation

For the SCE assay, sterility is essential to prevent contamination of samples. For this reason all blood manipulations were performed in the biohazard hood shown in Figure 3.1. The biohazard hood was sterilised prior to use by swabbing with 70 % ethanol and exposure to UV light for 1 hour. The biohazard hood was reserved solely for work with human blood, removing the risk of bacterial contamination from microbial work. Gloves were swabbed with 70 % ethanol and the surface of the blood vial was swabbed with 70 % ethanol skin preparation pads (BDH) prior to the removal of blood using a sterilised 1 mL syringe (Terumo).

Once samples were prepared for a period of culturing, the blood samples were all placed in the same 37 °C incubator illustrated in Figure 3.2. The incubators' heat is generated by six 150 W light bulbs; the temperature is controlled by an external thermostat.

The chamber where samples are put for incubation is blackened out to prevent light from affecting the samples' growth. Samples are placed on a rack in the incubator at an angle of 20 °, as this angle provides the optimal surface area for growth.

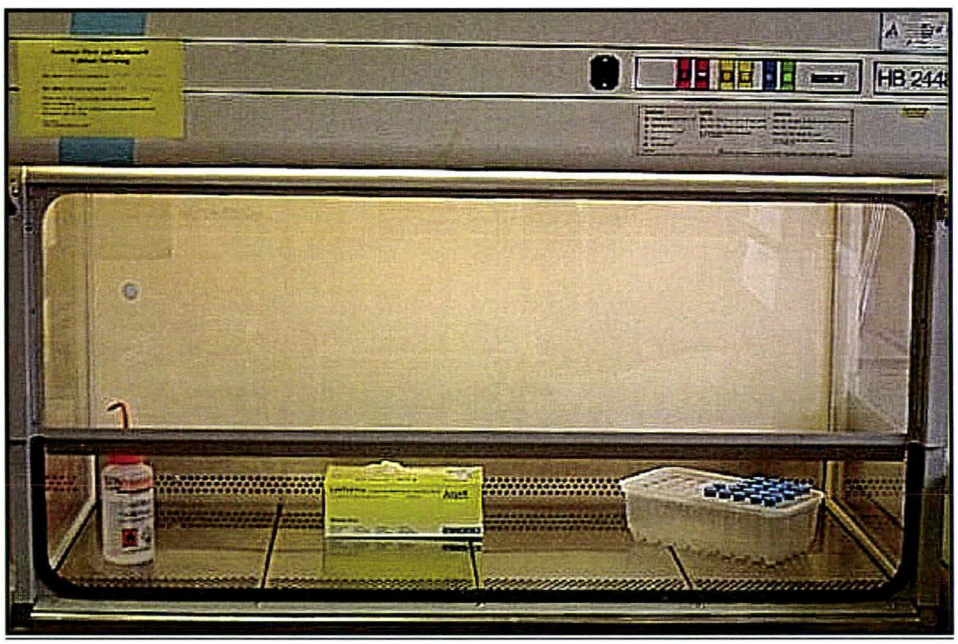


Figure 3.1 A Photograph of the Biohazard Hood
The Biohazard hood was used for all blood manipulations to avoid contamination. The system can be sealed off and exposed to the UV light unit, which sterilises the surface.

3.6 Sister Chromatid Exchange

3.6.1 Sister Chromatid Exchange Culturing

To ensure the number of lymphocytes in each culture tube was constant, a lymphocyte count was taken using the Coulter JT Whole Blood Counter (Appendix V).

Blood was added to the previously prepared culture tubes (Section 3.3) with a 1 mL sterile syringe (Terumo) to a final concentration of 2.96 x 10^6 lymphocytes per 6 mL culture tube. At high concentrations, Bromodeoxyuridine (BrdU) has the capability to induce SCEs, therefore, the lymphocyte count must be kept constant to ensure the results are standardised across all samples, reducing internal variation. Exactly 0.05 mL of 0.01 M BrdU (Section 3.1) was then added to each culture tube through a 0.22 µm Acrodisc pore syringe tip filter (Pall). Three culture tubes were established for each individual to cover the risk of accidental damage to one tube.

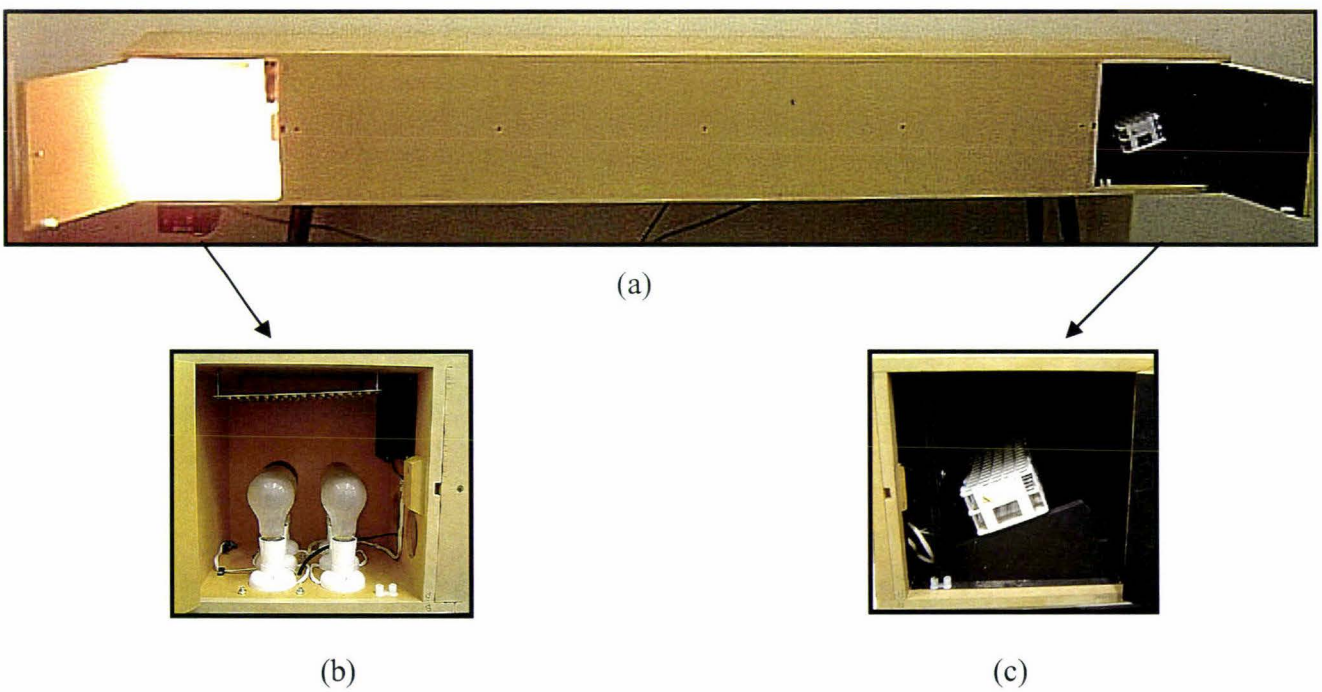


Figure 3.2 Incubator Used for Blood Culturing
The 37 °C incubator apparatus (a). The light bulbs are situated on the left hand side (b), and the culture tubes were placed at the opposite end of the apparatus (c) for the period of incubation. A door seals all light from the culture tubes preventing SCEs being induced by light exposure.

3.6.2 Sister Chromatid Exchange Harvesting

After approximately 71 hours, culture tubes were removed from the incubator. One hundred microlitres of 0.05 % colchicine was added to each tube. The tubes were inverted several times to ensure the colchicine was evenly mixed, and returned to the incubator for 1 hour to allow the cells to arrest mitosis at metaphase. It was important that this incubation took place in the dark to prevent any inducement of SCEs due to light exposure. Although sterility was no longer important at this step, the colchicine addition was performed in the biohazard hood to protect both the sample and the researcher.

Once the hour incubation was completed the culture tubes were removed from the incubator, mixed gently, then centrifuged for 10 minutes at 100 rpm. The resulting supernatant was removed to 1 cm above the pellet using a positive flow vacuum flask. The pellet and remaining supernatant were mixed thoroughly with a vortex stirrer for 5 seconds to avoid clotting. Five millilitres of 0.075 M KCl (warmed to 37 °C) was added to the remaining pellet to lyse the erthrocytes. Tubes were mixed gently by inversion 6 - 8 times and incubated for precisely 10 minutes in a 37 °C water bath to activate osmosis, which causes the lymphocytes to swell. Tubes were centrifuged for a second time at 1000 rpm for 10 minutes.

The supernatant was discarded (to 1 cm above the pellet) using a vacuum flask, and the remaining pellet re-suspended with a vortex stirrer for 3 seconds. Five millilitres of 6 % acetic acid was added slowly to each of the tubes while under constant agitation. Six percent acetic acid is added as a pre-fixative step. This pre-fixative step is essential; allowing the cells to adapt to a low dose of acetic acid before a high dose is delivered. The culture tubes were left to stand at room temperature for 5 minutes. Following pre-fixation, a centrifugation of 20 minutes at 1000 rpm was required to pellet the lymphocytes. Supernatant was then removed as described previously.

Approximately 5 mL of ice-cold fixative (1:3 glacial acetic acid / methanol) was then added to the pellet of the last centrifugation, mixed, and centrifuged for 10 minutes at 1000 rpm, followed by removal of the supernatant and the addition of more fixative.

Addition of fixative, centrifugation, and supernatant removal was repeated twice. After the final centrifugation with fixative, the supernatant was removed to 5 mm above the pellet (without disturbing the pellet).

The resulting lymphocyte pellet was drawn up into a Pasteur pipette and dropped from a height of approximately 40 cm onto 5 pre-cleaned microscope slides (frosted 25 mm x 75 mm, BIOLAB). This height ensured the metaphases were spread for visualisation. Slides were left to air-dry at room temperature.

3.6.3 Sister Chromatid Exchange Staining

The Sister Chromatid Exchange Staining is known as Fluorescence-plus-Giemsa-staining. The protocol for this staining was a modification of methods devised by Wolf & Perry (1975). The slides prepared from harvesting were completely set before staining occurred, usually by storage at room temperature for 3 days or more. The prepared slides were soaked in Sorensen's buffer solution (Section 3.1) for 5 minutes, then rinsed with distilled water and left to air-dry.

To prevent light exposure, the staining was performed in the dark. Each slide received seven drops of the light sensitive reagent Hoechst working solution (Section 3.1), and a coverslip (BIOLAB) placed over each slide. Hoechst staining photosensitizes degradation of BrdU-stimulated DNA, creating single-strand nicks. The slides were left for 30 minutes to allow the Hoechst stain to intercalate. The slides were washed with Sorensen's buffer and distilled water to remove the coverslips. Slides were mounted with eight drops of McIlvaine's buffer (Section 3.1), and a coverslip placed on top. McIlvaine's buffer prevents chromosomal damage. Slides were exposed to UV light (356 nm) for 2.5 hours, at a distance of 10 cm. After the 2.5 h exposure, the coverslips were removed by rinsing with Sorensen's buffer and distilled water. The samples were no longer light sensitive and the remaining steps were not performed in the dark.

Slides were immediately incubated in 2 x SSC (Section 3.1) for 20 minutes at a temperature of 65 °C. This incubation is performed to elute small DNA fragments.

Once the slides had dried they were stained with fresh Giemsa for approximately 8 minutes before rinsing in Sorensen's buffer. The required duration of staining often differed, and it was common practice for one slide to be stained first in order to correct the staining time. Air-dried slides were mounted in DPX (BDH) and allowed to set for at least two days.

3.6.4 Sister Chromatid Exchange Analysis

Following the completion of the Fluorescence-plus-Giemsa-staining, analysis of slides could occur. The examination of 50 – 100 metaphase spreads is the conventional number of cells scored for SCE (Elavarasi *et al.*, 2002; Kasuba *et al.*, 1999; Villarini *et al.*, 1998). In the current study, 50 consecutive second mitotic metaphase cells that showed good chromosome morphology, the complete complement of 46 chromosomes, differentially-stained sister chromatids, no overlapping chromosomes and no indistinguishable sister chromatids, were selected from each participant.

To analyse the metaphases, images were viewed using an Olympus BX51 microscope, under 1000 X magnification. The images were captured using an Optronics MagnaFIRE SS99802 digital camera with MagnaFIRE frame-grabbing software on a 2 GHz Pentium 4 computer with 256 MB of RAM and a 128 MB video card, illustrated in Figure 3.3.

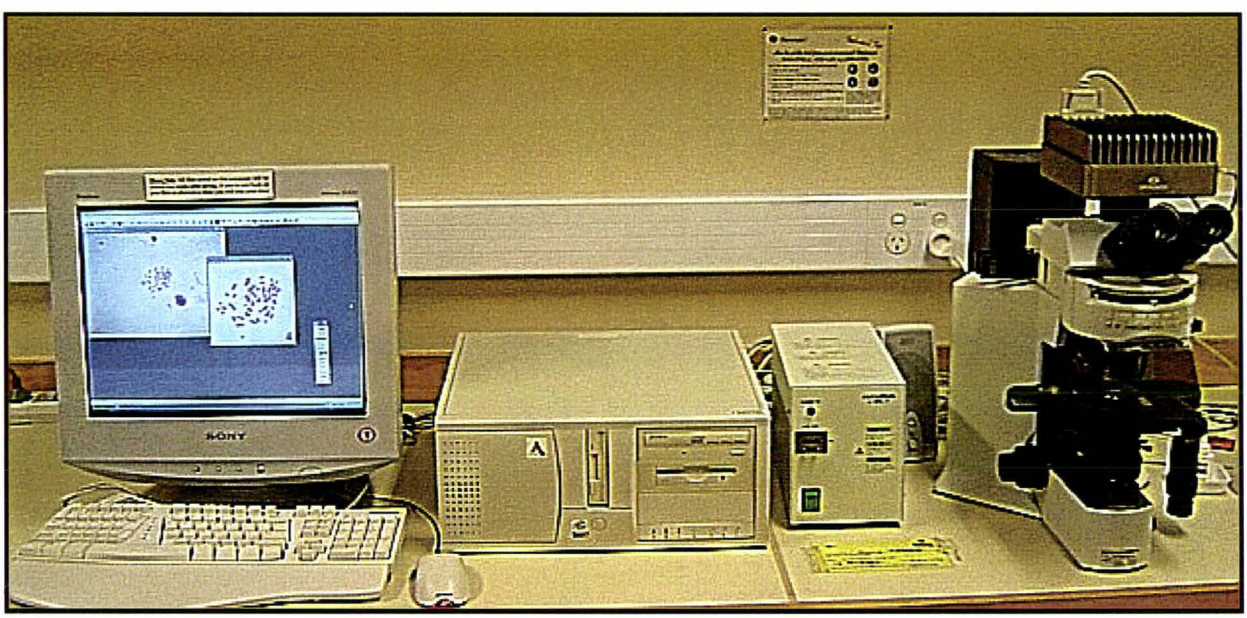


Figure 3.3 The Olympus BX51 fluorescent microscope.
The microscope is situated at the right hand side of the figure. On top of the microscope is the Optronics MagnaFIRE SS99802 digital camera. The computer has the software required for the image analysis.

When scanning for metaphase spreads, steps were taken to ensure no bias when deciding which metaphase spreads were chosen for analysis. The scanning for metaphase spreads commenced at one end of the slide and progressed sequentially to the other side, with images of all identified metaphase spreads taken, until the required number was reached. This sequential method both ensured the lack of bias and prevented the occurrence of duplicated images.

Analysis was performed in Microsoft Paint, with a mark made on each chromosome when counted to ensure accuracy of scoring (Figure 3.4). If a metaphase spread did not meet the specified criteria no further analysis was performed.

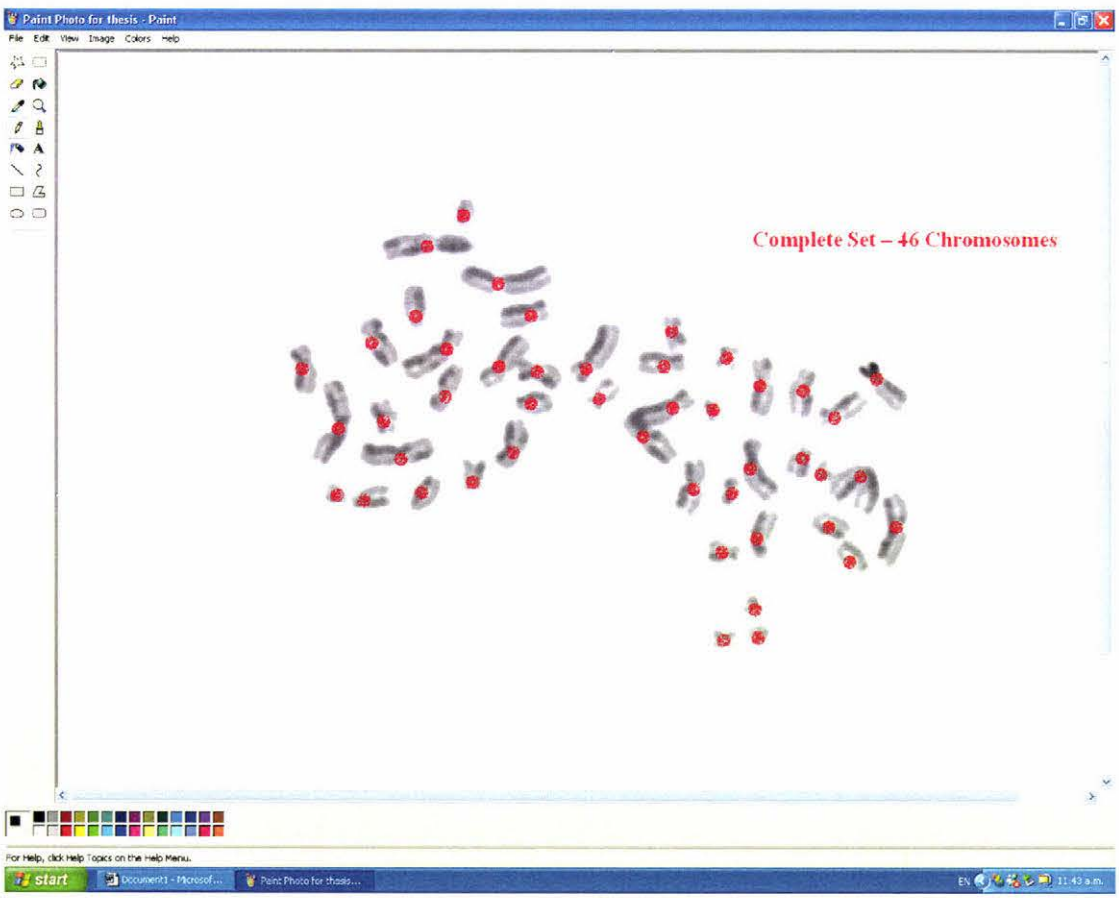


Figure 3.4 A representation of method used to count Chromosomes.

The spray-paint function was used to mark each chromosome so that each was only counted once. If a full complementation of 46 non-overlapping chromosomes was present, the metaphase spread was analysed for sister chromatid exchanges.

The number of sister chromatid exchanges were scored with the assistance of ImagePro Software (MediaCybernetics). ImagePro Software is able to enhance the contrast and brightness of an image to make it easier to score. The ImagePro Software is demonstrated in Appendix VI. When scoring, each exchange between the dark chromosomes and the light chromosomes was counted (Figure 3.5), and the results were recorded for future statistical analysis.

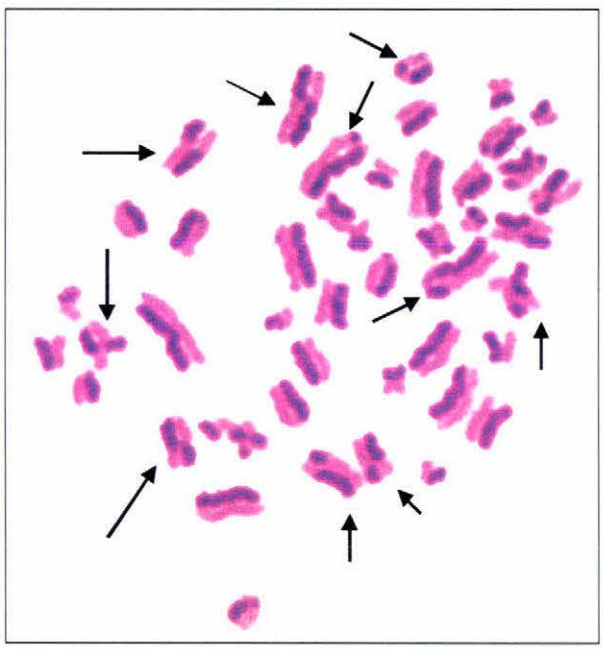


Figure 3.5 A demonstration of how sister chromatid exchanges were scored.
Each arrow on the spread indicates a sister chromatid exchange, it is easily visualised here as an exchange between light and dark stained chromatids. This image show a total of 18 SCEs.

4 CHAPTER FOUR: RESULTS

4.1 SCE Analysis

The Sister Chromatid Exchange Assay (SCE) is a very sensitive and widely applied assay to study genetic damage induced by an environmental agent or clastogen. In the current study a group of New Zealand Vietnam veterans and a control group were compared using an SCE analysis, with the aim of exploring the possibility of genetic damage in the experimental group.

The SCE analysis initially included 25 men in each of the veterans and control group, a total of 50 participants. The personal questionnaire (Appendix IV) was completed and returned to the researcher by 47 of these participants. Of the remaining three men, two failed to return a questionnaire by the conclusion of the study, and one returned an incomplete questionnaire that was unsuitable for use in statistical analysis for the current project. Hence 24 veterans and 23 control individuals made up the study group. All results and statistical analyses for the current study were therefore calculated using this group of 47 individuals only.

A total of 50 c-metaphase spreads were analysed (Section 3.6.4) for each participant. The types of metaphases rejected were those comprising less than 46 chromosomes, BrdU incorporated into all strands, mixed BrdU incorporation, and normal block staining (chromatids with no BrdU incorporated). Appendix VII details these metaphase staining patterns. A total of 2,500 metaphase spreads were located, photographed and scored taking a duration of 18 months.

The raw data and statistical description of means for all 47 participants is given in Appendix VIII. The descriptive statistics for the mean SCE rates for both the veteran and the control data sets are displayed in Table 4.1; the overall mean SCE for the veterans group was 10.99 SCEs/cell compared with 8.24 SCEs/cell for the control group. The difference between the two groups was 2.75 SCEs/cell. These data are also illustrated in Figure 4.1, where the graphical representation allows the difference between the two groups to be more easily seen.

	Mean SCE/Cell	Std. Deviation	N
Veteran Group	10.99	3.05	24
Control Group	8.24	1.10	23

Table 4.1 SCE Analysis Results
Descriptive statistics for the mean SCE rates of the veteran and control group. Each set of statistics was calculated from the 50 c-metaphase spreads analysed for each participant.

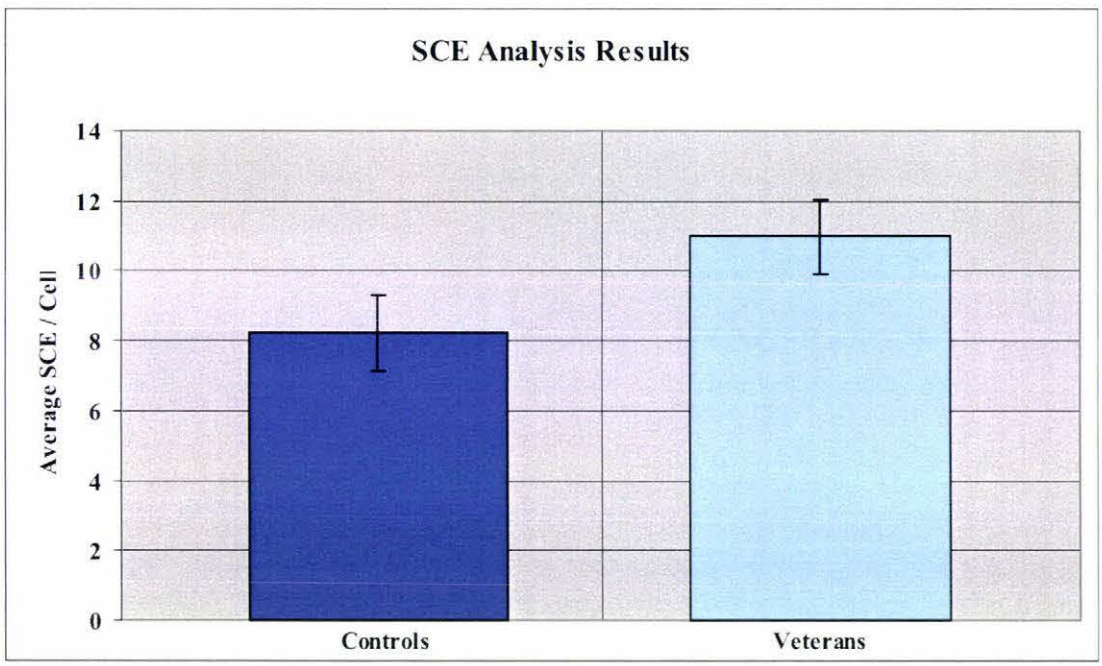


Figure 4.1 SCE Analysis Results
Graphical representation illustrating the descriptive statistics for the SCE rates (Table 4.1). The increase in average SCE per cell between the two groups can be clearly seen. The 95% confidence intervals are indicated on the error bars; the confidence intervals do not overlap.

4.1.1 Intra-group Variability

During analysis of the data it became evident that the two test groups did not have equal variances. This is graphically displayed in Figure 4.2, as the spread of data for the veterans group is much larger than that seen for the controls.

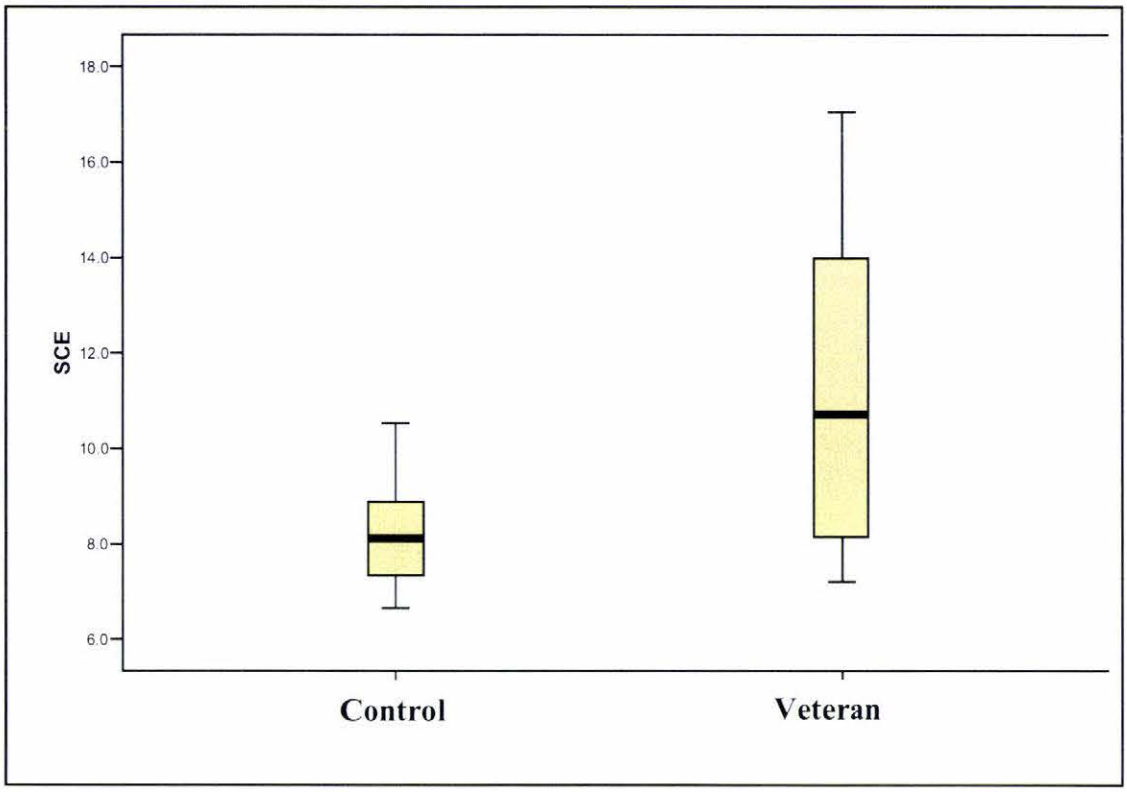


Figure 4.2 Stem & Leaf Plot illustrating spread
Graphical representation illustrating the spread of data for each of the veterans group and controls groups. The large difference in spread of data between the two groups can be clearly seen. There is only 34.7 % overlap of SCE scores between the two groups.

An examination of the raw data from each of the veterans and controls was conducted in an attempt to find whether the veterans data consisted of a small number of outliers that were affecting the overall mean for the veterans group, or rather, if the overall spread of the veterans SCE data was simply larger than that of the control group. Figure 4.3 shows the overall spread of all 47 participants.

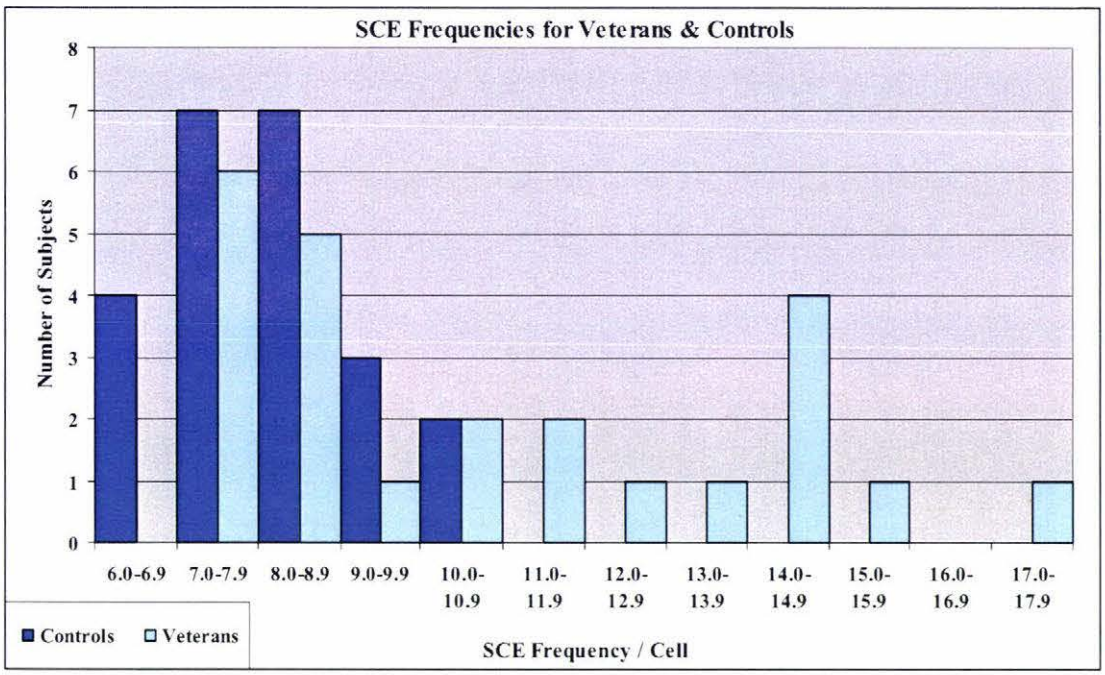


Figure 4.3 Plot of SCE Raw Data
The raw data (SCE averages) for each of the 24 veterans and 23 controls being statistically analysed. This plot clearly displays the difference in spread between the two groups. It is evident from this plot that the difference in means between the two groups is not due to a few high value outliers.

After examination of Figure 4.3 it is evident that although the veterans group does possess one very high value (17.04 SCEs / cell), the overall spread of this group is much higher than the control group. A Levene's test of variances was conducted and showed $p < 0.0001$. Therefore, the difference in variance between the two groups is not significant and is thus causing no effect.

4.1.2 Independent t-test & Statistical Effect Size (ES)

Further statistical analysis was required, because it was necessary to ascertain whether the difference seen between the means of each test group was statistically significant. An independent *t*-test (Keppel & Zedeck, 1989) was conducted on the data (equal variances **not** assumed) and the difference was found to be highly statistically significant, $t(45) = 4.071$, $p < 0.001$

Upon obtaining highly significant results from the statistical analysis, it was necessary and informative to calculate the Effect Size (ES) of the results obtained. The Cohen's *d* statistic gives a measure of the ES of a difference in means (Cohen, 1988). The value obtained for the current study was 1.266, which represents a large ES. This result also indicated that the two data sets only overlapped by 34.7 %.

4.2 Confounding Factors

The current literature available on the SCE assay suggests that there are many factors that can influence the rate of SCE in humans. To ensure the validity of the current study it was absolutely paramount to statistically control for any factors that may be influencing SCE results.

4.2.1 Analysis of Covariance Assay (ANCOVA)

An analysis of covariance assay (Pallant, 2001) was conducted on the data, examining the effect, if any, that the three main confounding factors have on the means, and thus the difference between the veteran and the control groups.

Pearson correlations were conducted to ascertain whether any correlations between the covariates existed, that is, to find if any of the covariates were highly correlated (Pallant, 2001). The results of this analysis (Table 4.2) indicate that there are no significant correlations between any of the three covariates. Therefore, all three were included in the ANCOVA.

The covariates tested were:

- Participant age (as of 31 December 2005)
- Cigarette/cigar Smoking (number of units per day)
  One unit was defined as 1 cigarette or 1 cigar
- Average Alcohol Consumption (standard drinks per week)

All assumptions for the ANCOVA were tested, and none were violated.

CORRELATIONS					
		Age	**Smoking**	**Alcohol**	**F-Value**
Age	Pearson Correlation		0.10	- 0.12	
	P Value		0.49	0.43	**0.44**
	N		47	47	
Smoking	Pearson Correlation			0.39	
	P Value			0.79	**0.09**
	N			47	
Alcohol	Pearson Correlation				
	P Value				**0.47**
	N				

Table 4.2 Pearson Correlations
The descriptive statistics for Pearson Bivariant Correlation analysis conducted to ascertain any significant correlations between covariants used in ANCOVA

The results from the ANCOVA allowed new estimates of the means for SCE rates of both the veteran and control groups to be made, removing any effects induced by the covariates. Complete results from the ANCOVA are detailed in Table 4.3.

Table 4.4 shows the estimated means for each group after correction for Participant Age, Smoking Rate and Alcohol Consumption. When comparing these values with the initial means calculated from raw data (Table 4.1) it is observed that only a minimal change in means has occurred after removing the effects of the confounding factors. The F values for Participant Age, Smoking Rate and Alcohol Consumption are 0.437, 0.093, and 0.470 respectively. When F is less than 1, the covariant has no effect on the means of the groups. Therefore, the covariates being analysed have no effect on the means of the two test groups (veteran or control), and the t-test conducted on the original SCE data (Section 4.1) is accurate.

Source	df	Mean Square	F	*p* - value
Age	1	2.44	0.44	0.51
Smoking	1	0.51	0.09	0.76
Alcohol	1	2.62	0.47	0.50
Total Group	1	69.27	12.41	0.001
Error	42	5.58		
Total	47			
Corrected Total	46			

Table 4.3. Complete ANCOVA Results
The effect that each covariate is having on SCE frequency can be clearly seen by looking at the F- and *p*-values. None of the values are significant and therefore we can say that these factors have no effect on SCE frequency in the current study.

The 95 % confidence intervals for the estimated means show no overlap between the veterans and the control group, indicating that the difference in the means is highly significant.

	Mean	Std. Error	95 % Confidence Interval	
			Lower Bound	Upper Bound
Veterans	11.05	0.53	9.99	12.12
Controls	8.18	0.54	7.08	9.27

Table 4.4. Statistically Corrected Results
Estimated statistics calculated using ANCOVA analysis. The estimated means are only minimally different from the original means, this indicates that the three confounding factors being analysed are having no effect on the SCE rates obtained.

5 CHAPTER FIVE: DISCUSSION

New Zealand Defence Force Personnel began fighting in the Vietnam War in 1965 and continued to serve there for almost 6 years; the last leaving in 1971. During the Vietnam War the United States military forces sprayed thousands of litres of phenoxylic herbicides over approximately 3.6 million hectares of Vietnamese and Laotian land. The most common defoliant sprayed was known as 'Agent Orange'. Agent Orange was contaminated with TCDD, a known human carcinogen. Since returning from Vietnam more than 30 years ago, New Zealand Vietnam veterans have expressed concerns about the numerous health problems experienced by both themselves and their children. They attribute these health problems to exposure to Agent Orange while serving in Vietnam.

The analysis of Sister Chromatid Exchanges (SCEs) in peripheral blood lymphocytes has been widely used for investigating humans exposed to harmful environmental agents in order to establish whether or not they have sustained genetic damage (Akin *et al.*, 2005; Arias, 2002; Bhattacharya *et al.*, 2005; Elavarasi *et al.*, 2002; Garaj-Vrhorac & Zeljezic, 2001; Iannuzzi *et al.*, 2004; Pitarque *et al.*, 2002 Silva *et al.*, 1996; Sonmez *et al.*, 1997; Stolley *et al.*, 1984; Tucker *et al.*, 1993; Uggla & Natarajan, 1983; Zeljezic & Gavaj-Vrhovac, 2002; Zober *et al.*, 1993). Hundreds of studies similar to these have been conducted. SCEs are considered a sensitive cytogenetic endpoint of testing the genotoxic risk associated with exposure to mutagenic and carcinogenic agents.

In the current study, a Sister Chromatid Exchange analysis was conducted on a group of New Zealand Vietnam War veterans. The aim of conducting this study was to ascertain whether or not New Zealand men who served in the Vietnam War have sustained any genetic damage that can be attributed to service in Vietnam and exposure to the herbicide Agent Orange. A second group of men was also selected for SCE analysis; this group had not served in the Vietnam War and therefore not been exposed to Agent Orange; these men were known as the matched control group.

All participants were screened to reduce the possible influence of factors that could severely impact on findings and to eliminate any bias in the SCE results. A further precaution to eliminate bias from the study was to conduct the SCE analysis blindly.

The results from the sister chromatid exchange assay show a highly significant difference between the mean of the experimental group and the control group. A difference between these two groups was expected if genetic damage had been sustained by the veterans group while serving in Vietnam. However, a statistical analysis was required to ascertain whether the difference seen between the two groups was statistically significant or possibly just due to coincidence. It was essential to determine what confounding factors may be acting on individuals in the current study and perhaps altering the results, leading to inaccurate conclusions. Therefore, a close examination of the confounding factors known to have an effect on SCE rates was conducted.

5.1 Confounding Factors

It is possible that there are factors other than service in Vietnam that may affect the SCE results obtained in the current study. These confounding factors could therefore be causing the difference seen between the groups. Careful consultation of current literature revealed that the biggest confounding factor with regard to SCE studies is cigarette smoking, with smokers consistently having significantly higher SCE rates compared to non-smokers (Barale *et al.*, 1998; Biro *et al.*, 2002; Burkvic *et al.*, 1998; Burgaz *et al.*, 1998; Karaoguz *et al.*, 2005; Lambert *et al.*, 1982; Lazutka *et al.*, 1992; Sardas *et al.*, 1991; Testa *et al.*, 2005). Karaoguz *et al.* (2005) conducted an SCE study investigating the association of SCE rates with cigarette smoking and alcohol consumption. Both cigarette smoking and alcohol consumption were found to be associated with increased SCE rates. This finding was supported by that of Popp *et al.* (1994) who also found a positive correlation between alcohol consumption and SCE rates. Lazutka *et al.* (1992) conducted a study on the effects of cigarette smoking, alcohol consumption, and age on SCE in human lymphocytes. Although age was only found to affect SCE rate to a small extent ($p = 0.057$), both cigarette

smoking and alcohol consumption were found to have significant associations with SCE rates ($p = 0.0231$ and $p = 0.0001$ respectively).

Despite the small effect of age reported by Lazutka *et al.* (1992), age has been consistently reported as having a significant association with SCE rates in humans (Burgaz *et al.*, 1998; Gulten *et al.*, 2002; Kaul *et al.*, 2001; Kelsey *et al.*, 1992). Kelsey *et al.* (1992) state that important confounding factors that could affect results are: age, alcohol intake, current smoking, and leukocyte count. The leukocyte count in the current study was kept constant for each individual (Section 3.6) and therefore did not act as a confounding factor. Another confounding factor that caused significant differences in past SCE studies was the sex of individuals (male or female). Women were found to have consistently higher SCE rates than men (Brown *et al.*, 1983; Das *et al.*, 1985; Fenech & Morley, 1985; Richard *et al.*, 1993; Wulf *et al.*, 1986). However, the fact that the current study only involved male individuals meant this factor was able to be dismissed. The review of current literature clearly indicated three confounding factors that needed to be statistically corrected for: age, cigarette smoking and alcohol consumption; all have the potential to affect SCE results. Information on age, smoking rates and average alcohol consumption of participants was available in the personal questionnaires (Appendix IV). Appendix IX gives complete results for these factors.

When investigating confounding factors it is important to note that the SCE assay only detects damage that is being caused by what is in the bloodstream at the time the blood is drawn. Therefore, it is only necessary to correct for those confounding factors which are having an effect currently. Cigarette smoking is a good example of this; as (mentioned above) cigarette smoking is known to increase SCE frequency in humans (Barale *et al.*, 1998; Biro *et al.*, 2002; Burkvic *et al.*, 1998; Burgaz *et al.*, 1998; Karaoguz *et al.*, 2005; Lambert *et al.*, 1982; Lazutka *et al.*, 1992; Sardas *et al.*, 1991; Testa *et al.*, 2005). However, it is important to note that SCE frequency is only elevated by **current** cigarette smoking. Approximately six months after cessation of cigarette smoking, SCE frequencies return to normal (Lazutka, *pers. comm.*).

Bender *et al.* (1988) conducted a major study of a large human population sample, 353 healthy employees of the Brookhaven National Laboratory with data obtained from scoring 16,898 cells for sister chromatid exchanges. This study showed no

significant difference in SCE frequency between former smokers and non-smokers. This finding was supported by that of Shaham *et al.* (2001) who reported a mean number of SCEs / chromosome that was negligibly higher in current smokers (0.25) than non-smokers or past smokers who scored identical frequencies of 0.24. Therefore although some participants in the current study may have smoked earlier in life, the SCE assay will not show any damage from this past smoking, providing it was at least 6 months prior to the blood used in the current study being drawn. Examination of all personal questionnaires revealed that all non-smokers in both the veteran and control group had not smoked for a minimum of 12 months. Similarly, some participants in the current study may have consumed large amounts of alcohol as teenagers, but it is only necessary to examine their current alcohol intake.

Although every effort has been made to correct for all confounding factors, which could be affecting SCE rates, it is possible that one or more confounding factors have been overlooked due to the fact that they are unknown to the researcher. It appears likely, for the data obtained, that there is some factor(s) involving the experimental group that has resulted in them showing higher average SCE rates compared to New Zealand army personnel who did not serve in Vietnam. By controlling for all known confounding factors, we can assume this difference is caused by service in Vietnam.

5.2 Statistical Analysis

The independent *t*-test that was conducted gave a *p*-value of less than 0.001, indicating that the difference in means between the experimental and the control groups was highly statistically significant. This result indicates that New Zealand Vietnam veterans have sustained genetic damage as a result of service in Vietnam. Further investigation into the results was conducted providing more statistical data to support this finding.

5.2.1 Intra-group Variability

When analyzing the descriptive statistics for the raw SCE data, it became apparent that although there was a significant difference between the veteran and the control groups, the spread of the data within these groups was also very different. The standard deviation for the control group was 1.10, the veterans group had a standard deviation of 3.05, almost three times higher than that of the control group. It was therefore necessary to investigate the spread of data in each of these groups to try and ascertain the reason for this large difference. It was possible that the higher standard deviation and perhaps the higher mean seen in the veterans group was due to a small number of very high SCE frequencies skewing the distribution. The average SCE frequency per cell for each of the veterans and controls was plotted (Figure 4.3) to investigate the spread of these groups and compare them with one another. Figure 4.3 illustrates the difference in spread between the two groups. It can be seen that the difference in standard deviation between the groups is due to the overall spread of data being greater in the veterans group compared to the control group; the large difference in spread between the two groups is not due to a small number of outliers that are skewing the veterans group distribution, although the distribution is slightly skewed.

Note that out of the 24 veterans included in this analysis, 12 have higher SCE frequencies than the highest SCE frequency recorded for the entire control group (10.54 SCEs/cell). Therefore, half of all veterans studied have a higher SCE frequency than the highest obtained SCE frequency among the controls. This illustrates the fact that the experimental group have much higher SCE rates as a group when compared to the control group and the statistical analysis reflects this. The difference in variance does not affect the *t*-test results because it was conducted without assuming equal variances. The 95 % confidence intervals for the estimated means show no overlap between the veterans and the control group (Figure 4.1), giving further evidence that the difference in the means is significant, despite the difference in spread. It is also important to note that the estimated statistics show very similar values for the standard error of each distribution respectively. This again reiterates the fact that the difference between the two groups is very significant.

5.2.2 Statistical Effect Size

The Effect Size (ES) is a measure of the magnitude of a treatment effect; in the case of the current study a measure of the magnitude of exposure effect. The ES is independent of sample size. Cohen (1988) defined any ES of 0.8 or larger as being a large effect. The ES obtained for the current study, 1.226, is therefore a large ES, and indicates that the effect observed (perhaps caused by exposure to Agent Orange), is large.

5.2.3 Analysis of Covariance

The ANCOVA results showed that there was no correlation between any of the 3 covariates chosen for analysis. Therefore, no covariate was having an effect on any other. This was important to ascertain, because it indicated that all three of these covariates may be acting as confounders on the SCE rates obtained for the experimental and control group. It was therefore essential to statistically control for the three covariates. Estimates of the means for the experimental and control group were able to be made after removing any effects from the three covariates. The F-values calculated were each less than 1 and therefore the assumption was made that none of the covariates were having a significant effect on the means of the two groups.

5.2.4 Conclusions

- A *t*-test on SCE results shows a highly statistically significant difference between a group of New Zealand Vietnam veterans and a group of matched controls $p < 0.001$)

- Following statistical correction for influence of confounding factors, the difference in mean between the two groups remains highly significant ($p < 0.001$)

5.3 Clastogenic Factors

Sister Chromatid Exchanges are induced by exposure to genotoxic agents. However, SCE only gives an indication of what is present in the blood stream at the time of conducting the assay. Therefore, the genotoxic agent must still be present in the blood to measure genetic damage that has been caused by this agent. The measurements of TCDD half-life in humans indicate that humans exposed to TCDD more than 30 years ago would probably still have TCDD present in their body today; however, the presence of TCDD may also cause the production of clastogenic factors. TCDD does not damage DNA directly (Chan *et al.*, 2004), and it is possible that exposure to TCDD can induce clastogenic factors that may contribute to the DNA damage seen in the New Zealand Vietnam veterans.

Clastogenic Factors were first described by radiobiologists in the plasma from people irradiated accidentally or for therapeutic reasons (Goh & Sumner, 1968; Hollowell & Littlefield, 1968). The idea was introduced that breaks in normal human chromosomes were induced by a transferable substance in the plasma of irradiated persons exposed to total-body irradiation (Goh & Sumner, 1968). These so-called clastogenic factors (CFs) were found also in plasma from atomic bomb survivors, with clastogenic activity persisting for up to 31 years after radiation exposure occured (Pant & Kamada, 1977). However, it is not exclusively radiation exposure that can cause the formation of CFs; Emerit *et al.* (1994) reported that clastogenic factors are also observed in patients with chronic inflammatory diseases, HIV-infected patients, asbestos workers, and in the congenital breakage syndromes Ataxia telangiectasia, Blooms syndrome and Fanconi's anemia. It is accepted that CFs are a cause of carcinogenesis in humans (Emerit, 1994), and this is an area where more research is required.

Clastogenic factor formation and clastogenic factor action are mediated by super oxide radicals and stimulate further super oxide production by competent cells (Emerit *et al.*, 1997). The involvement of super oxide in CF-induced chromosome damage was suggested by the protective effect of superoxide dismutase (SOD). Added to the culture medium, SOD reduces chromosomal instability in cultures from

irradiated patients cells and protects the cells of healthy subjects (Obe & Beek, 1982). This effect results in the self-sustaining and long-lasting process of clastogenesis. The life-cycle of CF formation and CF action results in a shift of the pro-antioxidant balance of the organism toward the pro-oxidant side. It is therefore possible that antioxidants may be protective as anti-clastogens and consequently as anti-carcinogens. CFs are considered to be biomarkers of oxidative stress. Their clastogenic action is due to the formation of lipid peroxidation products (Emerit *et al.*, 1991), unusual nucleotides of inosine (Auclair *et al.*, 1990) and cytokines such as tumour necrosis factor alpha (Emerit *et al.*, 1995) released into the plasma as a consequence of increased super oxide production.

5.3.1 Clastogenic Factors & Oxidative Stress

As mentioned above, CFs are considered to be biomarkers of oxidative stress (Emerit *et al.*, 1991). A number of congenital conditions include oxidative stress as a phenotype, these include the congenital breakage syndromes mentioned above as well as xeroderma pigmentosum and two frequent congenital conditions: Down syndrome and cystic fibrosis. Another clinical feature of these disorders is cancer susceptibility. Pagano *et al.* (1998) reported that the onset of oxidative stress is related to excess formation or defective detoxification of reactive oxygen species (ROS), and therefore the formation of CFs. It is well accepted that HIV-1 infected patients have an oxidative imbalance, and therefore also possess CFs within their plasma (Buhl *et al.*, 1989; Dworkin *et al.*, 1986; Eck *et al.*, 1989; Favier *et al.*, 1994; Folkers *et al.*, 1988; Fuchs *et al.*, 1994; Sonnerborg *et al.*, 1988; Staal *et al.*, 1992). There are also studies that have investigated the presence of CFs in individuals who have been exposed to particular chemicals that are thought to cause deleterious outcomes in humans.

Maffei *et al.* (2005) conducted a study on traffic police in Bologna Italy, who were exposed to airborne benzene in the atmosphere. Benzene is of concern because of its haematoxicity and leukaemogenic hazards, probably due to clastogenic factors. During the redox cycling of benzene metabolites reactive oxygen species are generated (Snyder, 2002). This is almost certainly the reason that benzene exposure produces CFs in humans. This study concluded that traffic police in Bologna had

significantly higher DNA damage than indoor workers. It is possible that there are many more environmental factors that individuals are currently exposed to that cause the formation of CFs.

In summary, oxidative stress causes CFs, and CFs in turn cause DNA damage; any DNA damage is universally considered to be detrimental to human health.

5.3.2 TCDD Causes Oxidative Stress

Effects of TCDD exposure have been the subject of intensive investigations during the past 30 years (Mandal, 2005). This is not surprising given that TCDD has the potential to disrupt multiple endocrine pathways (Grassman *et al.*, 1998). However, despite the vast amount of investigation, the mechanism of TCDD toxicity is not well defined. Binding of TCDD causes activation of the AhR, and this activation mediates a transcriptional response for genes regulated by this transcription factor. Recently TCDD has been reported to produce oxidative stress through cytochrome P-450 1A induction in vascular endothelium, resulting in local circulatory failure and apoptosis in the dorsal midbrain of zebrafish embryos (Chen *et al.*, 2004; Dong *et al.*, 2002). El-Tawil & Elsaieed (2005) found that TCDD exposure resulted in oxidative stress in multiple tissues and different animal species. Hassoun *et al.* (2001) found that TCDD induced super oxide formation, lipid peroxidation and DNA damage in the hepatic and brain tissues of rat. In addition, complex changes in the oxidative stress enzymes activity of both adipocytes and liver were found by Kern *et al.* (2002b). It is therefore widely accepted that TCDD induces oxidative stress in humans and animals.

5.3.3 New Zealand Vietnam Veterans & Clastogenic Factors

It is accepted that New Zealand Vietnam veterans were exposed to TCDD-contaminated Agent Orange (Section 2.2). It is also well supported that TCDD exposure causes oxidative stress (Chen *et al.*, 2004; Dong *et al.*, 2002; El-Tawil & Elsaieed, 2005; Hassoun *et al.*, 2001; Kern *et al.*, 2002b), and that oxidative stress induces CF formation (Buhl *et al.*, 1989; Dworkin *et al.*, 1986; Eck *et al.*, 1989; Favier *et al.*, 1994; Folkers *et al.*, 1988; Fuchs *et al.*, 1994; Pagano *et al.*, 1998;

Sonnerborg *et al.*, 1988; Staal *et al.*, 1992;). The possibility therefore exists that the DNA damage indicated by the results of the current study can be attributed to formation of CFs due to oxidative stress induced in Vietnam War veterans as a result of exposure to TCDD. Genetic damage as a result of CFs is self-sustaining and long lasting. Therefore, if New Zealand Vietnam veteran's sustained genetic damage as a result of exposure to Agent Orange, that damage would almost certainly be continuing today. The half-life of TCDD in the human body is estimated to be approximately 7.78 years (Geyer *et al.*, 2002) and therefore it would still be carried in the bodies of those exposed more than 30 years ago, feeding the cycle of oxidative stress in these veterans. This could then be an explanation for the many health problems that are commonly reported by Vietnam veterans, especially the high incidence of cancer. The same patterns in health problems are seen in Vietnamese people who were exposed to Agent Orange during the Vietnam War, as well as other groups of people who have been exposed to large concentrations of TCDD, such as those exposed in Seveso (Palmer, 2005).

Although CFs cannot be passed onto future generations, genetic damage sustained as a result can be. Therefore, this may provide an explanation for the many health problems reported in children of Vietnam veterans. This damage can be passed onto the next generation and future generations, causing similar detrimental health effects for generations to come.

5.4 Overall Summary

It is important to consider that statistical significance of genetic damage should be interpreted cautiously with regard to the biological significance. The SCE assay cannot be applied as a diagnostic tool. Although it is a sensitive and powerful technique for detecting genetic damage, it cannot predict specific health outcomes. However, genetic damage to any degree has the potential to result in adverse health effects.

In the current study, a significantly higher frequency of SCE was observed in a sample group of New Zealand Vietnam War veterans compared to a matched control group of New Zealand army and ex-army personnel. Elevated frequency of SCE in a target group is an accepted indicator of clastogenicity/genotoxicity.

5.4.1 Conclusion

According to the guidelines published by the ICPS (Section 1 of this thesis) defining the significance of the endpoint of the SCE assay as a bioindicator of genetic damage, and detailed further in Sections 2.5 and 2.6, it is possible to draw the following conclusion from the results obtained from the current study:

- **New Zealand Vietnam veterans have experienced a significant measure of genetic damage as a result of service in Vietnam.**

6 CHAPTER SIX: RECOMMENDATIONS

It is acknowledged by the author that time constraints of a two year project have resulted in the small sample size of the current study. However, the strong results that have been obtained are interpreted as being indicative of genetic damage in New Zealand Vietnam War veterans and should be seen as an alert signal.

6.1 New Zealand Vietnam War Veterans

The current study has obtained highly significant results and strong evidence that New Zealand Vietnam War veterans have sustained genetic damage as a result of service in Vietnam. The results therefore warrant a larger study of New Zealand Vietnam War veterans. A larger study would consist of a significantly larger sample size (minimum of 50 veterans and 50 controls) than the current study. In addition to a larger sample size, a number of cytogenetic assays, such as those mentioned in Section 2.5 should be used for analysis.

6.2 Children of New Zealand Vietnam War Veterans

The current study has detected genetic damage in New Zealand Vietnam War veterans and this finding merits a similar scientific investigation of the children of New Zealand Vietnam War veterans to ascertain if these children have inherited genetic damage. Inherited genetic damage can then be passed on to the next generation, possibly causing detrimental health effects through many generations to come.

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APPENDIX I: HUMAN ETHICS APPROVAL

The following is the letter received from the Massey University Ethics Committee, granting ethical approval.

Massey University

OFFICE OF THE ASSISTANT
TO THE VICE-CHANCELLOR
(ETHICS & EQUITY)
Private Bag 11 222
Palmerston North
New Zealand
T 64 6 350 5573
F 64 6 350 5622
humanethics@massey.ac.nz
www.massey.ac.nz

7 April 2005

Louise Edwards
[redacted]
PALMERSTON NORTH

Dear Louise

Re: HEC: PN Application – 04/29
Vietnam Veterans Study – a preliminary investigation into possible genetic damage

Thank you for your letter received on 7 April 2005.

On behalf of the Massey University Human Ethics Committee: Palmerston North I am pleased to advise you that the changes you have made to your application are approved. Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

A reminder to include the following statement on all public documents: *"This project has been reviewed and approved by the Massey University Human Ethics Committee, Palmerston North Application 04/29. If you have any concerns about the ethics of this research, please contact Dr John G O'Neill, Chair, Massey University Campus Human Ethics Committee: PN telephone 06 350 5799 x 8635, email humanethicspn@massey.ac.nz".*

You should now forward the changes to the appropriate Health and Disability Ethics Committee for their approval, along with a copy of this letter. Please forward a copy of the HDEC approval to this office once received, along with a record of any changes requested.

Yours sincerely

JoNeill

pp

Mr Paul Green, Acting Chair
Massey University Campus Human Ethics Committee: Palmerston North

cc Dr Al Rowlands
IMBS
PN462

Prof Jeremy Hyams
IMBS
PN462

The following is the letter received from the Manawatu Whanganui Ethics committee, granting ethics approval.

Manawatu Whanganui Ethics Committee
C/- Palmerston North Hospital
P.O. Box 5203
Palmerston North
Phone/Fax (06) 356 7773
Email: mwethics@xtra.co.nz

7th July 2004

Louise Edwards and Dr Al Rowland
Institute of Molecular BioSciences
Massey University
Private Bag 11 222
Palmerston North

Study Title: Vietnam Veterans Study – a preliminary investigation into possible genetic damage.
Ethics Reference No: 04/05/013

Dear Louise and Dr Rowland

The above study has been given ethical approval by the Manawatu/Whanganui Ethics Committee.

Approved Documents
Protocol No 04/05/013 dated May 2004
Amendment No 1 dated June 2004
Information sheet version 2 dated June 2004
Consent form version 1 dated May 2004

Accreditation
This Committee is accredited by the Health Research Council and is constituted and operates in accordance with the Operational Standard for Ethics Committees, March 2002.

Progress Reports
The study is approved until June 2005. A final report is required at the conclusion of the study. If the study is not completed within 12 months a progress report is required from the researcher.

Amendments
All amendments to the study must be advised to the Committee prior to their implementation.

General
It should be noted that Ethics Committee approval does not imply any resource commitment or administrative facilitation by any healthcare provider within whose facility the research is to be carried out. Where applicable, authority for this must be obtained separately from the appropriate manager within the organisation.

Please quote the above reference number in all correspondence relating to this study.

Please note a new version of the application form (EA0502) is now available either by email from the Administrator or from the Health Research Council website, www.hrc.govt.nz. Form EA0699 will not be accepted after 31 December 2002.

Yours sincerely

Sheryl Kirikiri
Administrator
Manawatu/Whanganui Ethics Committee
centralethics@xtra.co.nz

Manawatu/Whanganui Ethics Committee
P.O. Box 5203
Ph 06/ 350 8199
Email: centralethics@xtra.co.nz

APPENDIX II: INFORMATION SHEET

The following are the information sheets supplied to every possible participant. There was a separate information sheet for the veterans and the controls.

VIETNAM VETERANS STUDY – PRELIMINARY INVESTIGATION INTO POSSIBLE GENETIC DAMAGE

INFORMATION SHEET (for Controls)

My name is Louise Edwards, the person who will be conducting this research. Thank you for expressing an interest in helping me with this Vietnam Veterans' Pilot Study, a study I now invite you to take part in. The purpose of this letter is to tell you more about the study and what you would be asked to do should you wish to be involved. Before you agree, you should read the following information back- grounding the purpose of the study and your involvement in it, should you consent to take part.

The aim of this study is to find out if the genetic material of men who served duty in Vietnam during the Vietnam War in the 1960s and 70s has been adversely affected. I will be conducting this research for my Master of Science under the supervision of Dr Al Rowland in the Institute of Molecular Biosciences at Massey University. Al is an expert in human cell analysis whose research team is currently conducting a major study of possible genetic damage in New Zealand nuclear test veterans. We can be contacted by telephone, Al at (06) 3569099 Ext 7977, and Louise at (06) 3569099 Ext 5413.

The prime purpose of the study is to examine the DNA (your genetic fingerprint) in your blood cells to see if some of the genetic material in those cells is different from a group of Vietnam War Veterans. A relationship is known to exist between DNA damage and ill health. In other words, as DNA damage increases so does the **risk** for some disorders increase. I would like to emphasize, however, that **if I find evidence of suspected abnormal levels of damage to your DNA, it definitely does not mean you are sick, or even likely to get sick.** Rather, it is an alert signal that there is an increased risk for ill health. **Any results of DNA damage that I might find should not be used by anyone, including your doctor, as a diagnostic result.** By comparing veterans who served in Vietnam with other men who did not (control group), I can determine whether the level of DNA damage observed in the group of Vietnam veterans is greater than the control group.

There is an important point I wish to emphasize. Because I have to complete this research by October 2005, time permits me to study only a small sample of men (25 veterans and 25 control volunteers). This means that the sample size is rather small for the results to be of significance, one way or the other, unless some major damage is found. But it's a start and the study could grow into a larger investigation. The results obtained even from this preliminary study will be very useful as a base to start from and it will allow me to gain valuable research experience in an exciting field under the guidance of Dr Rowland. I can reassure you that Dr Rowland has trained me fully and that he will look at all the data I collect very carefully.

Taking a small blood sample from your arm (about 2 teaspoons) will take only a few minutes and will be done either at your home or somewhere convenient for you, by Chris Kendrick from Massey University. There is a minor risk associated with giving blood, such as excessive bleeding or fainting, but Chris is authorised to take human blood samples and is fully trained to deal with emergencies.

I will also be collecting blood samples from a group of men of about your age who served in the Armed Forces and also served in Vietnam. The group you will be part of are known in science jargon as "matched controls". The idea of selecting "matched controls" is to isolate the one factor which may have caused genetic damage, namely service in Vietnam. The control men did not serve in Vietnam, whereas the other group did. In all other respects your life experiences should be similar. If I detect any difference between the Vietnam veterans and the "matched controls" with the genetic test I will be applying, then this would open the door for further study to find out why.

All participants (veterans and matched controls) will also be required to fill out a short questionnaire. This is an essential part of the study so that I can ensure that any differences found are a result of service in Vietnam, not anything else. The questionnaire should take no longer than 10-15 minutes and can be filled out at home in your own time – there is no rush.

To sum up, if you take part in my study, I would like you to provide a single blood sample. Your total involvement should take no more than a 20 minutes to collect the blood sample, and complete the questionnaire, on top of any traveling time and of course, you have the right to decline to take part, and even if you do agree to "sign on"; you have the complete freedom to withdraw at anytime. You are in control! The only risk you are exposed to is having blood taken from a vein in your arm. The risk to you is negligible and no higher than having your own medical laboratory do this for some other purpose. If any DNA damage is detected in you which is at an unusually high level, then you will be advised you to see your doctor.

Surplus blood will be destroyed by high heat treatment (autoclaving) once the blood cultures have been established. If you have any cultural concerns as to how your blood should be disposed, then please bring this to my attention and your wishes will be paramount within legal guidelines for the disposal of blood. I do need you to provide your name but I will give you a code number so that no-one will be able to identify your blood culture. All personal information that could identify you will be kept in a locked cupboard in a locked room in Dr Rowland's laboratory. **Your name will not be disclosed.**

Finally and most importantly, you have clear and distinct rights if you should decide that you want to take part in this study. You have the right:

- to decline to participate;
- to refuse to answer any particular questions;
- to withdraw from the study at any time;
- to ask questions about the study at any time during participation;
- to provide information on the understanding that your name will not be used unless you give permission to the researcher;
- to be given access to a summary of the findings of the study when it is concluded.

Please ring either Louise or Al if you have any concerns whatsoever about this study. We will be freely available to you at any time.

Louise Edwards 06 3569099 X 5413 Al Rowland 06 3569099 X 7977

This project has been reviewed and approved by the Massey University Human Ethics Committee, Palmerston North, Application 04/29. If you have any concerns about the conduct of this research, please contact Professor Sylvia V Rumball, Massey University Campus Human Ethics Committee: PN, telephone 06 350 5249, email humanethicspn@massey.ac.nz

VIETNAM VETERANS STUDY – PRELIMINARY INVESTIGATION INTO POSSIBLE GENETIC DAMAGE

INFORMATION SHEET (for Veterans)

My name is Louise Edwards, the person who will be conducting this research. Thank you for expressing an interest in helping me with this Vietnam Veterans' Pilot Study, a study I now invite you to take part in. The purpose of this letter is to tell you more about the study and what you would be asked to do should you wish to be involved. Before you agree, you should read the following information back- grounding the purpose of the study and your involvement in it, should you consent to take part.

The aim of this study is to find out if the genetic material of men who served duty in Vietnam during the Vietnam War in the 1960s and 70s has been adversely affected. I will be conducting this research for my Master of Science under the supervision of Dr Al Rowland in the Institute of Molecular Biosciences at Massey University. Al is an expert in human cell analysis whose research team is currently conducting a major study of possible genetic damage in New Zealand nuclear test veterans. We can be contacted by telephone, Al at (06) 3569099 Ext 7977, and Louise at (06) 3569099 Ext 5413.

The prime purpose of the study is to examine the DNA (your genetic fingerprint) in your blood cells to see if some of the genetic material in those cells is different from a control group of men who served in the Armed Forces but did not serve in Vietnam. A relationship is known to exist between DNA damage and ill health. In other words, as DNA damage increases so does the **risk** for some disorders increase. I would like to emphasize, however, that **if I find evidence of suspected abnormal levels of damage to your DNA, it definitely does not mean you are sick, or even likely to get sick.** Rather, it is an alert signal that there is an increased risk for ill health. **Any results of DNA damage that I might find should not be used by anyone, including your doctor, as a diagnostic result.** By comparing veterans who served in Vietnam with other men who did not (control group), I can determine whether the level of DNA damage observed in the group of Vietnam veterans is greater than the control group.

There is an important point I wish to emphasize. Because I have to complete this research by October 2005, time permits me to study only a small sample of men (25 veterans and 25 control volunteers). This means that the sample size is rather small for the results to be of significance, one way or the other, unless some major damage is found. But it's a start and the study could grow into a larger investigation. The results obtained even from this preliminary study will be very useful as a base to start from and it will allow me to gain valuable research experience in an exciting field under the guidance of Dr Rowland. I can reassure you that Dr Rowland has trained me fully and that he will look at all the data I collect very carefully.

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I will also be collecting blood samples from a group of men of about your age who also served in the Armed Forces about the same time that you did, but did not serve in Vietnam. In science jargon, these men will be called "matched controls". The idea of selecting 'matched controls' is to isolate the one factor which may have caused genetic damage, namely service in Vietnam. The control men did not serve in Vietnam, whereas you did. In all other respects your life experiences should be similar. If I detect any difference between the Vietnam veterans and the "matched controls" with the genetic test I will be applying, then this would open the door for further study to find out why.

All participants (veterans and matched controls) will also be required to fill out a short questionnaire. This is an essential part of the study so that I can ensure that any differences found are a result of service in Vietnam, not anything else. The questionnaire should take no longer than 10-15 minutes and can be filled out at home in your own time – there is no rush.

To sum up, if you take part in my study, I would like you to provide a single blood sample. Your total involvement should take no more than a 20 minutes to collect the blood sample, and complete the questionnaire, on top of any traveling time and of course, you have the right to decline to take part, and even if you do agree to "sign on"; you have the complete freedom to withdraw at anytime. You are in control! The only risk you are exposed to is having blood taken from a vein in your arm. The risk to you is negligible and no higher than having your own medical laboratory do this for some other purpose. If any DNA damage is detected in you which is at an unusually high level, then you will be advised you to see your doctor.

Surplus blood will be destroyed by high heat treatment (autoclaving) once the blood cultures have been established. If you have any cultural concerns as to how your blood should be disposed, then please bring this to my attention and your wishes will be paramount within legal guidelines for the disposal of blood. I do need you to provide your name but I will give you a code number so that no-one will be able to identify your blood culture. All personal information that could identify you will be kept in a locked cupboard in a locked room in Dr Rowland's laboratory. **Your name will not be disclosed.**

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- to decline to participate;
- to refuse to answer any particular questions;
- to withdraw from the study at any time;
- to ask questions about the study at any time during participation;
- to provide information on the understanding that your name will not be used unless you give permission to the researcher;
- to be given access to a summary of the findings of the study when it is concluded.

Please ring either Louise or Al if you have any concerns whatsoever about this study. We will be freely available to you at any time.

Louise Edwards 06 3569099 X 5413 Al Rowland 06 3569099 X 7977

This project has been reviewed and approved by the Massey University Human Ethics Committee, Palmerston North, Application 04/29. If you have any concerns about the conduct of this research, please contact Professor Sylvia V Rumball, Massey University Campus Human Ethics Committee: PN, telephone 06 350 5249, email humanethicspn@massey.ac.nz

APPENDIX III: CONSENT FORM

The following is the consent form given to every participant in the study.

VIETNAM VETERANS STUDY
CONSENT FORM

I, ... (print name), agree to have approximately 10ml of blood withdrawn from my body by a qualified person for the sole purpose of culturing my blood lymphocytes for DNA examination.

I have read the accompanying Information Sheet concerning this study and fully understand the issues outlined in them. I further acknowledge that the withdrawal of a sample of my blood for Louise Edwards in Dr Rowland's laboratory at Massey University is for the express purpose of examining my DNA for genetic damage.

I agree to participate in this project under the conditions set out in the Information Sheet and on the understanding that I have the right:

- to decline to participate;
- to refuse to answer any particular questions;
- to provide information to the researcher on the understanding that my name will not be used without my permission;
- to insist that all results concerning me will remain confidential except for public disclosure of possible chromosome damage;
- of access to any results concerning me at any time;
- to withdraw form this research programme at any time in which case all results concerning me will be returned immediately;
- to provide information on the understanding that this information will be used only for this research and publications arising from this research.

Signature: ..... Date: .....

This project has been reviewed and approved by the Massey University Human Ethics Committee, Palmerston North, Application 04/29. If you have any concerns about the conduct of this research, please contact Professor Sylvia V Rumball, Massey University Campus Human Ethics Committee: PN, Phone 06 350 5249, Email humanethicspn@massey.ac.nz

APPENDIX IV: PERSONAL QUESTIONNAIRE

The personal questionnaire, required to be completed by every participant in the study.

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 investigator of this study (Louise Edwards). The answers you provide may have a direct bearing on the interpretation of my results. Therefore, I ask that you kindly provide me with information that is accurate as you can give.
Thank you, Louise

Name: .....

Address:.....
.....
.....

Contact Phone: .....(Home) .....(work)

Code Number (given by Louise): .....

Date: ...../...../.....

Personal History

1. Date: ...../...../.....

2. What is your birth date? ...../...../.....
(Month) (Day) (Year)

Present and Past Occupational History

3. Are you currently employed? ❒ YES ❒ NO

4. Do you consider that you were exposed to Agent Orange when you served Vietnam?

.....

5. How long did you serve in Vietnam?

.....

6. What type of work have you been engaged in since serving in Vietnam?

.....

.....

.....

.....

.....

.....

Exposure History (work and non-work related)

7. Have you ever been exposed to any of the following in your job?

		When were you first exposed? (month , year)	When were you last exposed? (Month, Year)	How long in terms of days, months, or years in total were you exposed?
Asbestos	❒ YES→ ..... ❒ NO			
Radiation **(excluding X-rays)**	❒ YES →..... ❒ NO			
Coal Products	❒ YES→ ..... ❒ NO			
Dust (such as wood, leather)	❒ YES → ..... ❒ NO			
Pesticides, Herbicides	❒ YES → ..... ❒ NO			
Petroleum Products	❒ YES → ..... ❒ NO			
Dyes	❒ YES → ..... ❒ NO			
Solvents	❒ YES → ..... ❒ NO			
Other Chemicals (specify in question No. 8)	❒ YES → ..... ❒ NO			

8. List the names of any specific hazardous substances to which you know you were exposed by either breathing or direct skin contact at work either in the last year or within the past ten years.

In the last year (12 months)	How frequently exposed on a monthly average	Within the past 10 years	How frequently exposed on a monthly average
.....			

9. Please list **any chemical exposure you experienced** in the last year while practicing a hobby or other activities either at home or in other non-occupational settings. Refer back to the list in question No. 7 but do not limit your answers to only these substances.

In the last year (12 months)	How frequently exposed on a monthly average	Within the past 10 years	How frequently exposed on a monthly average
.....			
.....			
.....			
.....			

Medical History

9. Have you taken any medication prescribed by a doctor in the past 1 year

10. (for example, blood pressure pills, antibiotics, insulin, tranquilizers, etc.)?

❒ YES ❒ NO

If yes, please indicate below:

Type of medication	Dose	Frequency?	Time period: Began (month)	Ended (month)
.....				
.....				
.....				
.....				

11. Have you taken any non-prescription medication in the past 1 year (for example, aspirin, antacid, anti-histamines, sedatives, or other drugs)?

❒ YES ❒ NO

If yes, please indicate below:

Type of medication	Dose	Frequency?	Time period: Began (month)	Time period: Ended (month)
.....				
.....				

Code Number .....

2. Do you take any vitamins **or other health supplements** currently, or have you in the past 6 months?

❒ YES ❒ NO

If yes, please indicate below:

What kind of vitamins:	Dose	Frequency
.....		
.....		
.....		
.....		

13. Have you ever had any of the following illnesses?

Cancer	❒ YES	❒ NO
Hepatitis	❒ YES	❒ NO
Glandular Fever	❒ YES	❒ NO
Herpes	❒ YES	❒ NO
HIV/AIDS	❒ YES	❒ NO
Meningitis	❒ YES	❒ NO
Bacterial or viral infection	❒ YES	❒ NO
Cardiovascular disease	❒ YES	❒ NO
Diabetes	❒ YES	❒ NO
Other major illness	❒ YES	❒ NO

If yes, please specify what illnesses, when you were ill, and indicate treatment.

Illness	Period of illness (month, year to month, year)	Treatment
.....		
.....		
.....		
.....		

14. List any other illnesses and their treatments you have experienced in the past 12 months (these should include colds, flu, etc.)

Illness	Period of illness (month, year to month, year)	Treatment
.....		
.....		
.....		
.....		

15. List any vaccination which you have received in the past 12 months .

Type of vaccination	Date administered
.....	
.....	
.....	
.....	

16. **List any diagnostic radiology (X-ray or CT scan), radiotherapy or administration of a radioactive substance, other than dental** you have received in the past 10 years.

Reason for X-rays	Year received
.....	
.....	
.....	
.....	

17. Have you ever had any dental X-ray? ❒ YES ❒ NO

If yes, within:
- ❒ the last month
- ❒ the last 6 months
- ❒ the last 6-12 months
- ❒ over one year

18. Have you had any surgery during the past year?

Date	Reason
.....	
.....	
.....	
.....	

19. Give dates you have had any high fevers during the past year.

Date	Associated illness	Medication taken
.....		
.....		
.....		
.....		

Smoking History

20. If you previously smoked cigarettes, cigars or a pipe, but have now given up, when did you last smoke? .....

21. Do you currently smoke cigarettes? ❒ YES ❒ NO

If yes, how many **cigarettes** do you smoke each day?

- ❒ **1 – 10 cigarettes**
- ❒ **11 – 20 cigarettes**
- ❒ **21 – 30 cigarettes**
- ❒ **More than 30 cigarettes**

If you smoke more than 30 cigarettes each day, please state how many:

.....

22. Do you currently smoke cigars? ❒ YES ❒ NO

If yes, how many cigars do you smoke each day?

❒ 1 cigar ❒ 2-3 cigars ❒ 4 or more cigars

23. Do you currently smoke a pipe? ❒ YES ❒ NO

If yes, how many pipesful do you smoke each day?

- ❒ 1 pipeful
- ❒ 2-3 pipesful
- ❒ 4 or more pipesful

24. Do you smoke any substance other than tobacco ? ❒ YES ❒ NO

Diet History (should only reflect current habits)

25. Do you eat vegetables? ❒ YES ❒ NO

26. Do you eat meat? ❒ YES ❒ NO

27. Do you use diet sweeteners? ❒ YES ❒ NO

How much per day or week? .....

28. Do you use diet drinks? ❒ YES ❒ NO

How many per day or week? .....

29. Pertinent comments concerning diet not covered by above questions, e.g., special diet such as high protein, low carbohydrate, etc.

.....

.....

.....

30. Do you drink coffee? ❒ YES ❒ NO

If yes, how much / day? .....

Decaffeinated? ❒ YES ❒ NO

31. Do you drink tea? ❒ YES ❒ NO

If yes, how much/day? .....

32. Do you drink beer? ❒ YES ❒ NO

If yes, please indicate your average weekly beer consumption:

❒ 1-6 cans (375 ml) a week or less
❒ 7-12 cans a week
❒ 13-24 cans a week
❒ more than 24 bottles a week.

33. Do you drink wine? ❒ YES ❒ NO

If yes, please indicate your average weekly wine consumption:

❒ 1-4 glasses a week or less
❒ 5-8 glasses a week
❒ 9-16 glasses a week
❒ more than 16 glasses a week.

34. Do you drink other alcoholic beverages (excluding beers and wines)?

❒ YES ❒ NO

If yes, please indicate type and average weekly consumption:

❒ 1-4 glasses a week or less.
❒ 5-8 glasses a week
❒ 9-16 glasses a week
❒ more than 16 glasses a week.

Genetic History

35. **Are you aware of any birth defects, inherited diseases, a family history of cancer, or any other inherited diseases** which affect your parents, brothers, sisters, or their children?

❒ YES ❒ NO

If yes, please specify .....

.....

.....

.....

Thank you for your time.

APPENDIX V: WHITE BLOOD CELL COUNT

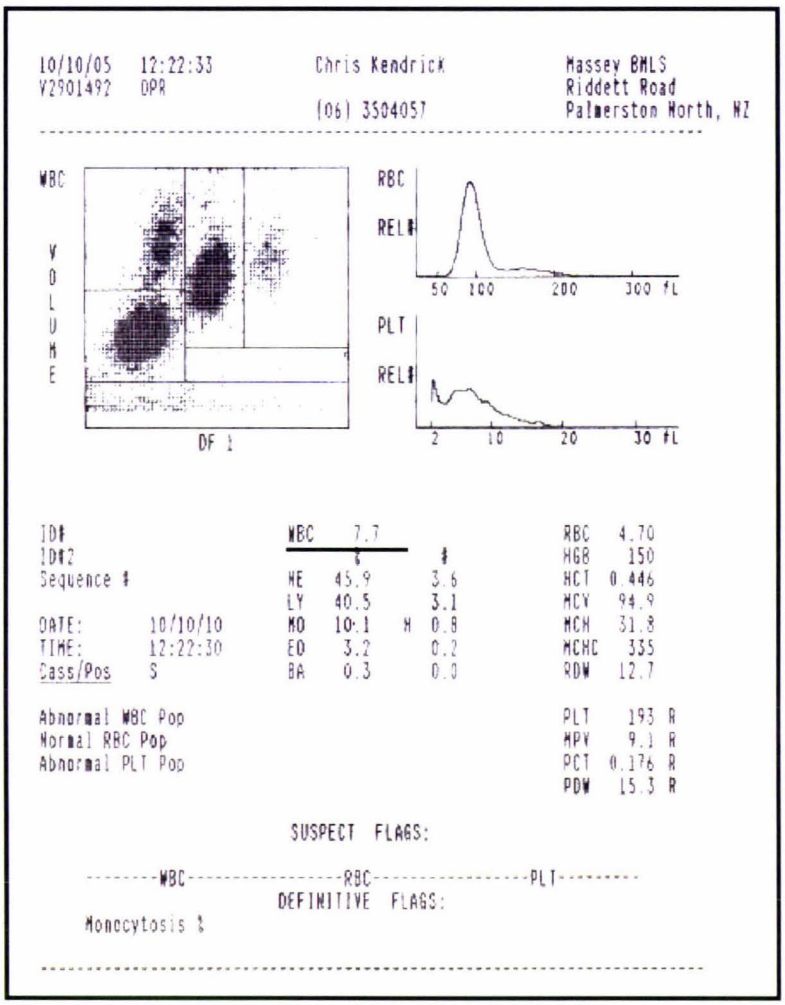

Figure 5.1 An example of a WBC printout
A printout of the characteristic results obtained from the differential blood count performed by the Coulter JT Whole Blood Counter and analysis computer. The White Blood Count (WBC) underlined is the number of interest.

APPENDIX VI: Image Pro Plus SOFTWARE

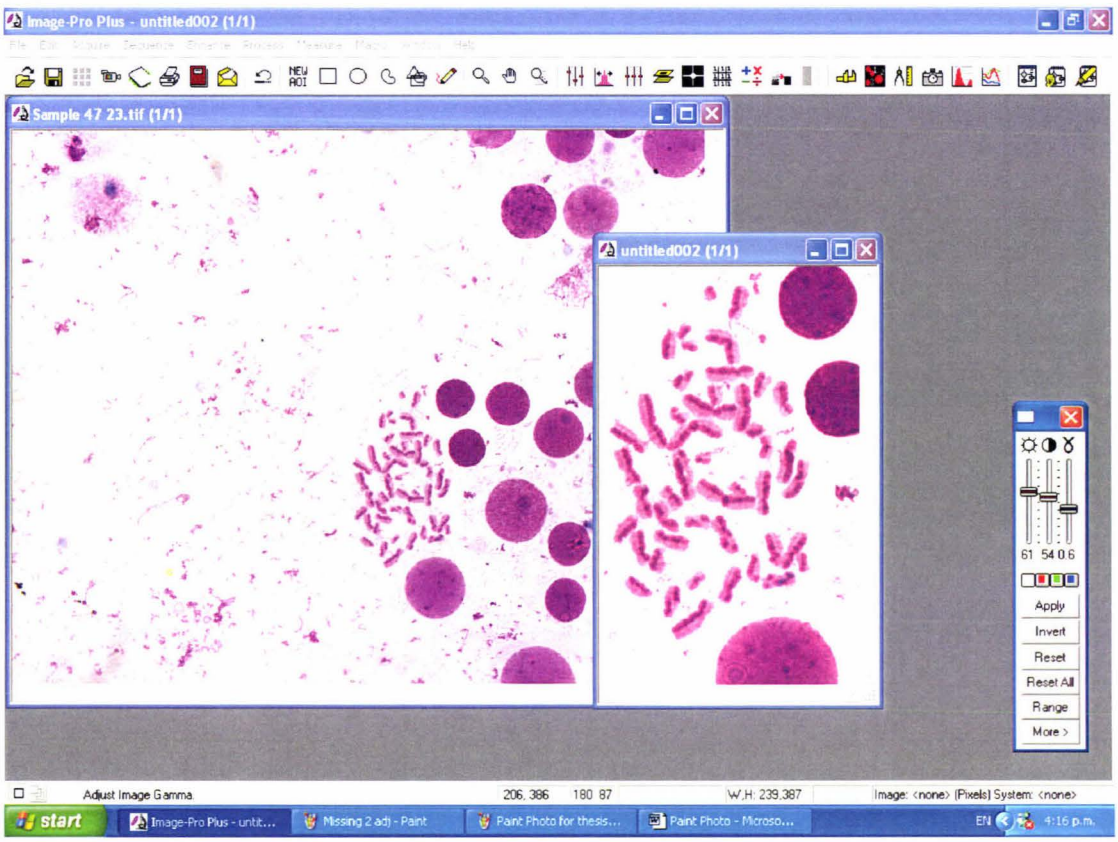

Figure 6.1 A screen view of the ImagePro Plus Software

A view of ImagePro Plus when utilized to enhance the SCE images for scoring. An image taken using the MagnaFIRE software was opened in ImagePro Plus. To clarify the exchanges, the contrast adjustment toolbar was used (shown in the bottom right corner) to reduce background exposure and to increase the contrast between light and dark chromatids.

APPENDIX VII: SCE METAPHASES

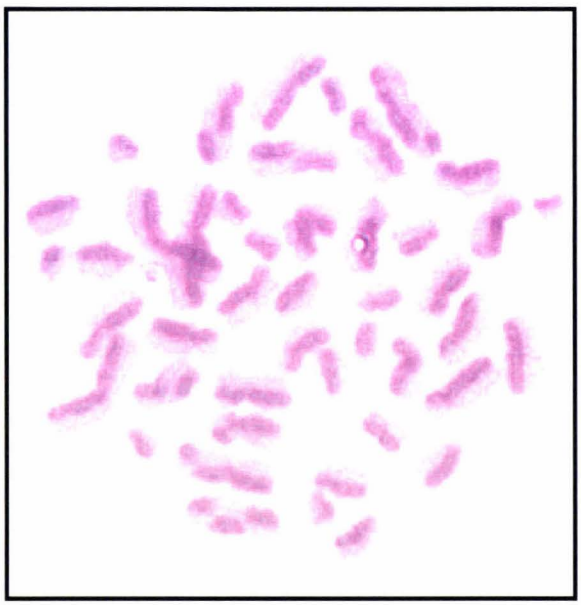

Figure 7.5a

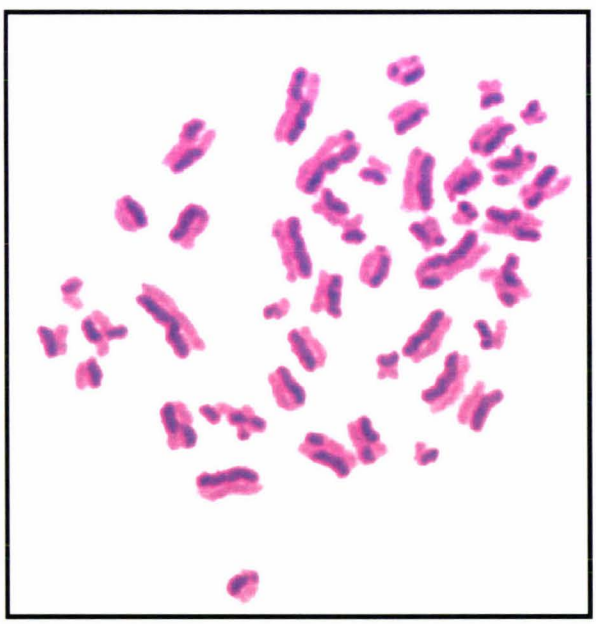

Figure 7.5b

Figure 7.1 Two examples of metaphase spreads suitable for SCE analysis. Each metaphase spread is complete (46 chromosomes) and differential staining has been successful, resulting in one chromatid of each chromosome appearing dark and the other light when viewed down a microscope (1000 X). Note the difference in staining intensity as Figure 7.4a and Figure 7.5b are taken from different slide preparations and therefore have slightly different staining intensities.

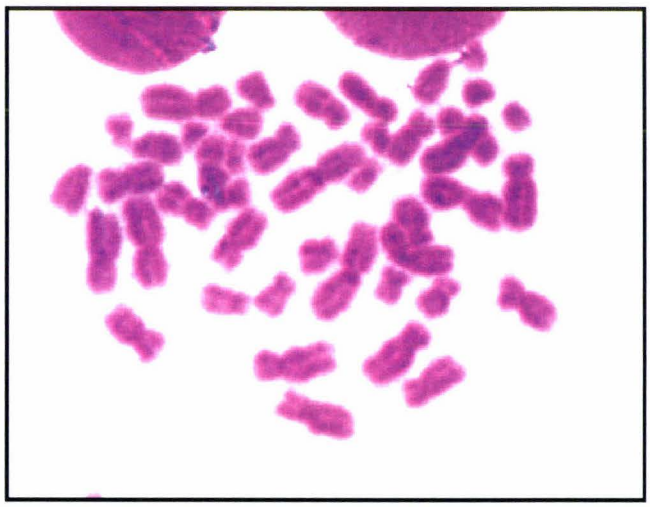

Figure 7.1 Giemsa Block Staining
Metaphases sometimes appeared as normal Giemsa stained metaphases as shown in this figure. This can arise by the lack of incorporation of BrdU during culturing or the lack of differential staining during the staining procedure (1000 X).

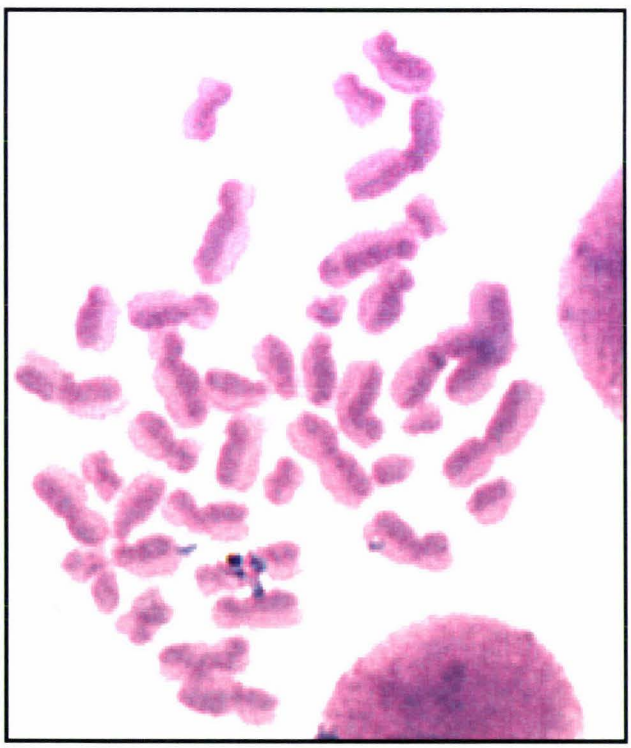

Figure 7.2 An Incomplete Complement of Chromosomes
The chromatids were often differentially stained but lacking the complete chromosome complement, this metaphase contains only 41 chromosomes. When this occurred the metaphase could not be scored for SCEs. It is likely that this may have resulted from the dispersal of chromosomes during slide preparation (1000 X).

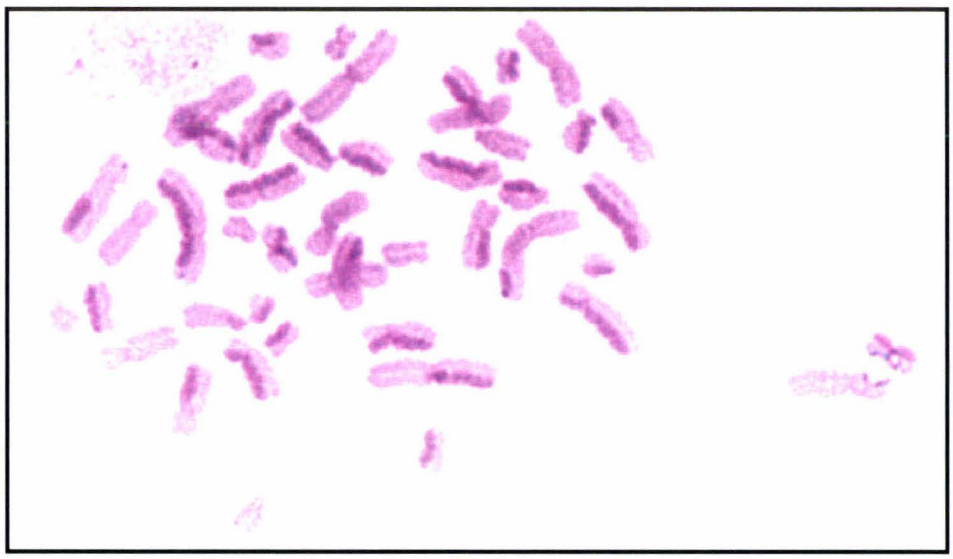

Figure 7.3 A mixed complement of BrdU incorporation
The metaphase spread contains chromosomes that are not uniformly stained. Note: some chromatid pairs are differentially stained, while others are similarly stained (1000 X). It is also possible to obtain metaphase spreads where BrdU is incorporated into both strands of every chromosome. This occurs when cells undergo DNA replication more than twice during the culturing period in the presence of BrdU.

APPENDIX VIII: SCE RESULTS

The complete SCE results for all participants are included on the CD Rom below.
(on back cover)

APPENDIX IX: CONFOUNDING FACTORS

CONTROLS:

ID	SCE	Age	Smoking	Alcohol
002	8.88	57	0	6
007	7.16	53	0	21
008	6.66	55	0	6
009	8.8	64	0	9
013	8.8	75	0	6
015	6.96	66	0	6
017	7.22	59	0	19
019	8.44	61	0	4
020	8.88	64	0	22
036	7.46	53	0	3
037	6.96	61	0	6
038	8.74	53	0	4
040	10.04	49	0	16
041	7.5	52	0	9
042	7.7	58	15	10
043	7.74	50	0	3
044	8.12	55	0	10
045	7.72	56	0	9
046	10.54	55	5	9
047	9.84	54	0	15
048	9.14	53	0	47
049	9.36	56	0	15
050	6.84	51	0	6

VETERANS:

ID	SCE	Age	Smoking	Alcohol
001	8.5	69	0	10
004	7.52	64	0	10
005	8.86	59	25	4
006	7.26	60	0	18
010	7.2	63	5	9
011	7.84	61	35	10
014	8.1	73	0	1
016	8.2	63	0	12
018	7.34	62	0	21
021	9.76	62	0	13
022	10.86	76	0	19
023	14.4	63	5	13
024	13.88	58	0	1
025	11.58	60	0	16
026	8.88	62	0	18.5
027	13.14	71	15	13
028	10.56	57	0	15
029	11.58	59	0	0
030	17.04	62	0	6
031	14.5	57	15	28
032	12.94	57	0	24
033	14.1	57	0	9
034	15.2	68	15	4
035	14.52	58	15	34

Table 9.1 Complete Results for Confounding Factors

Results for age, rates of smoking and alcohol intake in the veterans and controls group. The SCE column indicates SCEs per cell, Age is in years as of December 2005, smoking is in units per day (one unit is equivalent to one cigarette or one cigar)and alcohol is in standard drinks per week. These results were used for the statistical analysis.