

Environmental Factors Affecting the Risk of Breast Cancer.

**A thesis submitted in total fulfilment of the requirements of the
degree of Doctor of Philosophy**

By

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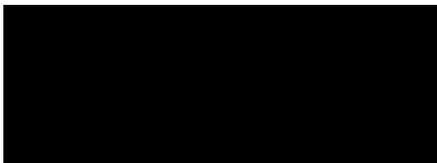
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DECLARATION OF AUTHENTICITY

“I, Cheryl Taylor, declare that the PhD thesis entitled “environmental Factors Affecting the Risk of Breast Cancer” is no more than 100,000 words in length, exclusive of tables, figures, appendices, references and footnotes. This thesis contains no material that has been submitted or accepted previously, in whole or part, for the award of any other academic degree or diploma in any other university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made. Except where otherwise indicated, this thesis is my own work. All work contained (collection of specimens from the hospitals and follow-up, interviews, questionnaire processing, pesticide extraction and analysis, some statistical analyses, cell proliferation assays, soft agar assays and microarrays) in this thesis has been performed by myself, except where due credit has been given”.



✓ C. M. Taylor

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LIST OF ABBREVIATIONS

ACAA2	acetyl-coenzyme A acyltransferase 2
ADI	Acceptable daily intake
AIHW	Australian Institute of Health and Welfare
ALDH6A1	methylmalonate semialdehyde dehydrogenase
ATP5A1	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, alpha subunit, isoform 1 cardiac muscle.
Atyp. Hyp.	Atypical hyperplasia
BBD	benign breast disease
BGN	biglycan
BMI	Body mass index
BRF1	butyrate response factor 1
BSE	breast self examination
CACNA2D2	calcium channel, voltage dependent, alpha2/delta subunit 2
CALN1	calneuron 1
c-chlor	cis isomer of chlordane
Cdk	cyclin dependent kinase
cDNA	complementary DNA
CEA	carcinoembryonic antigen
CEACAM5	carcinoembryonic antigen related cell adhesion molecule 5
CI	confidence interval
cm	centimetre
CPM	carboxypeptidase M
csFCS	charcoal stripped foetal calf serum
DCIS	ductal carcinoma in situ
DDD	1,1-dichloro-2,2-bis(chlorophenyl)ethane
DDE	dichlorodiphenyldichloroethylene
DDT	1,1,1-trichloro-2,2-bis(4chlorophenyl)ethane
DEPC	diethylpyrocarbonate
DES	diethylstilbestrol
DMBA	mammary carcinogen
DME	Dulbecco's enriched modification media
DMSO	dimethylsulfoxide

DNA	deoxyribonucleic acid
E ₁	oestrone
E ₂	17 β -oestradiol
E ₃	oestriol
EDTA	ethylenediamine tetra-acetic acid
EEG	electroencephalogram
EFNB1	ephrin B1
EGF	epidermal growth factor
EPA	Environmental Protection Agency
EPAS1	endothelial PAS domain protein 1
ER	oestrogen receptor
ERT	oestrogen replacement therapy
EST	expressed sequence tag
FCS	foetal calf serum
FLT1	fms-related tyrosine kinase 1
FSH	follicle stimulating hormone
G1	grade 1 cancer
G2	grade 2 cancer
G3	grade 3 cancer
GABA	gamma aminobutyric acid
GnRH	gonadotropin releasing hormone
GRWD	glutamate rich WD repeat protein GRWD
H2BFA	H2B histone family member A
HCB	hexachlorobenzene
HCH	hexachlorocyclohexane
HE	heptachlor epoxide
HPF	High power field
HRT	hormone replacement therapy
IARC	International Agency for Research on Cancer
IGFBP3	insulin like growth factor binding protein 3
IUCC	International Union Against Cancer
kg	kilogram
KIAA0130	KIAA0130 gene product

KIAA0468	KIAA0468 gene product
LCIS	lobular carcinoma in situ
LCN2	lipocalin 2
LH	lutinising hormone
m	metres
ml	millilitres
ml/min	millilitres per minute
mm	millimetres
MCF-7	oestrogen receptor positive breast cancer cell line
MDA-MB-231	oestrogen receptor negative breast cancer cell line
MDA-MB-435	oestrogen receptor negative breast cancer cell line
MIC2	antigen identified by monoclonal antibodies 12E7, F21 and O13.
MRL	maximum residue limit
MYH9	myosin heavy polypeptide 9, non-muscle
ng/g	nanograms per gram
NHL	Non-Hodgkin's lymphoma
NHMRC	National Health and Medical Research Council
NMT2	N-myristoyltransferase 2
Non-prolif	non proliferative benign tissue
Norm	normal tissue
OD	optical density
OR	Odds ratio
o,p'-	ortho para isomer
OXC	oxychlordan
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PR	progesterone receptor
Pre-mp	pre-menopausal
PRKCB1	protein kinase c, beta 1
Prolif.	Proliferative benign tissue
Post-mp	post-menopausal
p,p'-	para para isomer
PSMA3	proteasome subunit alpha type 3

PSPH	phosphoserine phosphatase
PTGES	prostaglandin E synthase
PURA	purine rich element binding protein A
RLT	RNeasy lysis buffer
RNA	ribonucleic acid
RNF11	ring finger protein 11
RPE	RNeasy wash buffer
RPL21	ribosomal protein L21
RPL29	ribosomal protein L29
RR	relative risk
RT-PCR	real time polymerase chain reaction
RW1	RNeasy lysis buffer
SAS	N-acetylneuraminic phosphate synthase; sialic acid synthase
SAT	spermidine/ spermine N1 acetyltransferase
SBR	Scarff Bloom Richardson classification
SC65	nucleolar autoantigen similar to rat synaptonemal complex protein
SD	standard deviation
SDS	sodium dodecyl sulfate
SEER	Surveillance Epidemiology and End Results program
SES	socio-economic status
SGE	Scientific Glass Engineering
SHBG	sex hormone binding globulin
SLC25A6	solute carrier family 25, member 6
SLC31A1	solute carrier family 31, member 1
SMR	standardised mortality rate
SRB	sulforhodamine B
SR-BP1	sigma receptor 1
T47D	oestrogen receptor positive breast cancer cell line
TCA	trichloroacetic acid
TCB	3,3,'4,4'-tetrachlorobiphenyl
t-chlor	trans isomer of chlordane
TCDD	dioxin; 2,3,7,8-tetrachlorodibenzo-p-dioxin
TDLU	terminal duct lobular unit

TGF	transforming growth factor
TGFBI	transforming growth factor beta induced
TNF	tumour necrosis factor
TNFRSF21	tumour necrosis factor receptor superfamily member 21
TNM	tumour nodes metastases system
TNON	trans nonachlor
TRAP	thyroid hormone receptor associated protein
TSPYL	TSPY-like
TTC4	tetratricopeptide repeat domain 4
µg/ml	micrograms per millilitre
µl	microlitres
µM	microMolar
µm	micron
UK	United Kingdom
USA	United States of America
VDR	vitamin D (1,25-dihydroxyvitamin D3) receptor
VEGF	vascular endothelial growth factor
VUT	Victoria University of Technology
WHO	World Health Organisation
ZNF264	zinc finger protein 264

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CHAPTER 1
LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Breast cancer is one of the leading causes of mortality and morbidity amongst females of the Australian population (AIHW, 2000). In 1990- 1992 an average of 7516 women were diagnosed annually with breast cancer in Australia (Kricker and Jelfs, 1996). In the same time frame 2458 women, on average, died from the disease (Kricker and Jelfs, 1996). Breast cancer is relatively rare in women younger than 40 years of age (15 per 100 000 woman- years in 1982- 1992), however the incidence and mortality from breast cancer increase with increasing age (Table 1) (Kricker and Jelfs, 1996).

Table 1. Breast cancer incidence and mortality in Australia between 1987 and 1992.

Age group (years)	Incidence (woman- years)	Mortality (woman- years)
up to 40	15 per 100 000	3 per 100 000
40- 49	138 per 100 000	30 per 100 000
50- 69	207 per 100 000	69 per 100 000
70 +	267 per 100 000	125 per 100 000

(Data adapted from Kricker and Jelfs, 1996)

Breast cancer is a group of related conditions, affecting the breast, which is characterised by differing microscopic appearance and biological behaviour (Coe and Steadman, 1995; Davis and Bradlow, 1995). There is still uncertainty as to the causes of breast cancer, however, there are some factors that are thought to increase one's likelihood of developing the disease. These factors include age, gender, family history of the disease especially if there are first degree relatives affected, history of benign breast disease, age at menarche, age at menopause, number of full term pregnancies and the use of hormone therapies. Some researchers suggest that as the incidence of breast cancer is rising, and coincides with increased industrialisation, they suggest that environmental pollutants may contribute to breast cancer risk (Epstein, 1994; Davidson, 1998; DeBruin and Josephy, 2002). Some researchers overseas suggest that exposure to

organochlorine pesticides, such as DDT, may increase the risk of breast cancer (Dewailly *et al.*, 1994; Guttus *et al.*, 1998; Bagga *et al.*, 2000) while others suggest that there is no association (Falck *et al.*, 1992; Krieger *et al.*, 1994; Moysich *et al.*, 1998). There are many methods which can be used to assess pesticide exposure; assaying biopsies or blood for pesticide residues, followed by an interview with the recruited subject to assess breast cancer risk factors; animal models or organ and cell culture systems, utilising breast cancer cells and assessing for a cytotoxic or a genetic alteration in response to environmental contaminant exposure.

This chapter provides a detailed review of the literature, which will highlight the reasons why this research is important. To understand how a cancer may develop, it is important to firstly look at the physiology of the breast and how the various structured components within the breast are coordinated, along with the hormones cycling around the body, to achieve functionality. This will be addressed in section 1.2 of this chapter.

An important part of understanding how a disease functions, is to review the causes (or the aetiology) of the disease which will be addressed in section 1.3 of this chapter. Since it has been speculated that only a small percentage of breast cancer cases are caused by the factors that will be addressed in section 1.3, section 1.4 will address the possibility of organochlorine pesticides increasing the breast cancer risk in the remainder of those affected.

The possible mechanisms behind the speculated increase in risk of breast cancer attributable to organochlorine pesticide exposure, via the various testing methods available, will be addressed in section 1.5. The information presented in sections 1.1 through to 1.5 will be integrated, to highlight the relevance of this study, in section 1.6.

1.2 ANATOMY, PHYSIOLOGY AND CANCER OF THE BREAST

1.2.1 Anatomy and Physiology

The female human breast has a distinctive protuberant form, unlike other primates, whose breasts are comparatively flat (Haagensen, 1986). The upper edge of the breast is situated in line with the second or third rib, whilst the lower edge is located approximately at the sixth or seventh costal cartilage (cartilage which connects the front ends of most ribs to the sternum) (Haagensen, 1986). The medial border of the breast is located at the edge of the sternum and the lateral border on the anterior axillary line (Figure 1) (Haagensen, 1986).

The normal female adult breast consists of a mixture of epithelial and stromal elements. The epithelial elements of the breast contain a series of branching ducts, which extends from the nipple, and terminates into the functional units of the breast, the lobules (DiSaia, 1993; Hayes, 1993). Each breast is composed of 15-20 lobules, containing a cluster of alveoli, which are responsible for the secretion of milk during lactation (Vander *et al.*, 1970; Hayes, 1993). The stroma contains variable amounts of interspersed adipose tissue and fibrous connective tissue, which constitutes most of the breast volume in a non-lactational state (Carola *et al.*, 1992; DiSaia, 1993; Hayes, 1993). There are no muscles in the breast, but the breast is supported by a group of Cooper's ligaments that overlay the pectoralis muscles and the anterior serratus muscles of the chest (Osborne, 1991).

Blood and lymph vessels are also interspersed throughout the breast. As the breast is located immediately beneath the dermis, it is drained of waste material and dead cells by the dermal lymphatics (Haagensen, 1986). These lymphatics form part of the main lymphatics of the body, and run laterally until they are joined by tributaries, which subsequently run to the axilla (the armpit) (Haagensen, 1986).

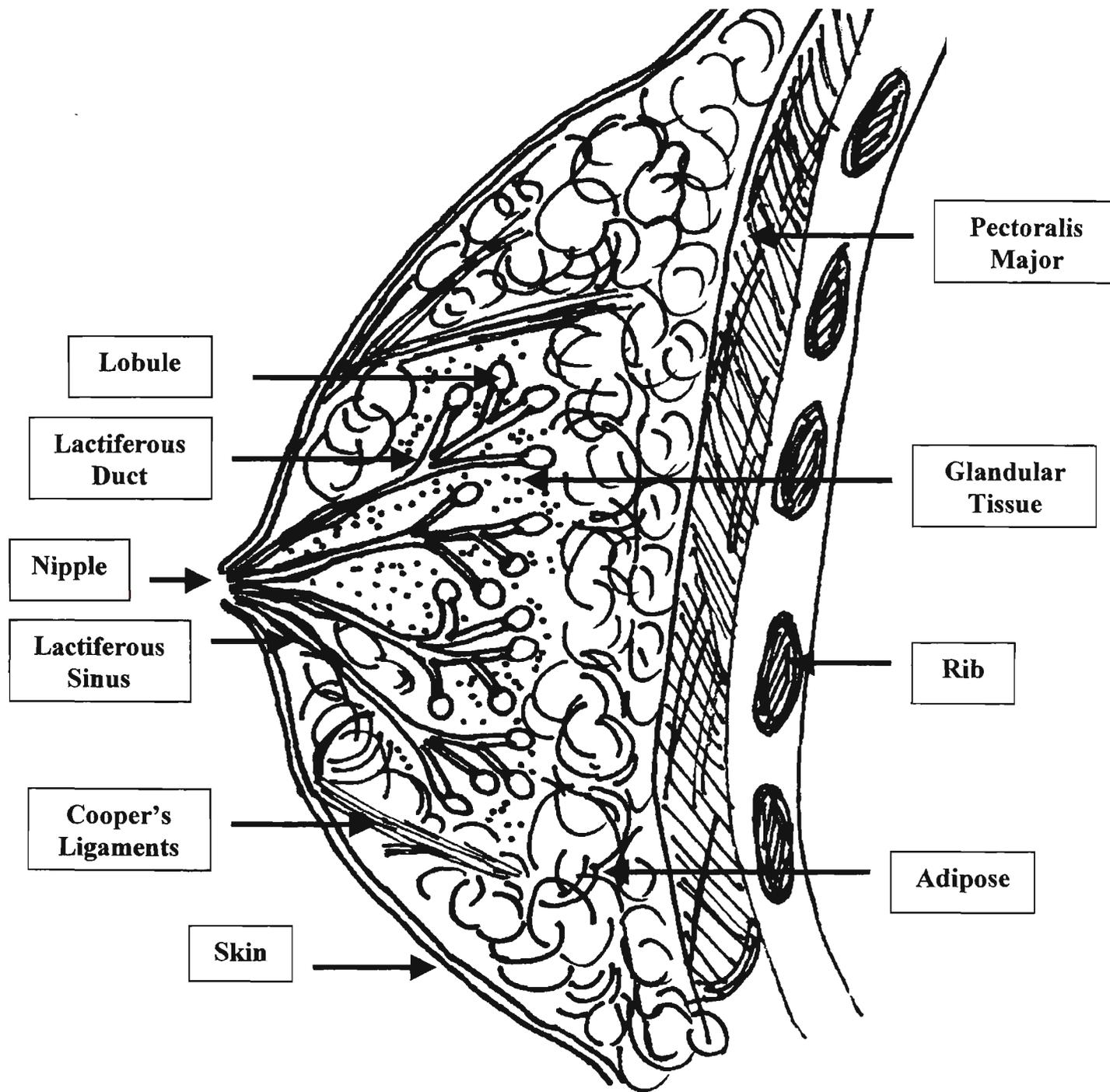


Figure1, Drawing of a fully developed, female breast.

Approximately 3% of the lymph from the breast drains into the internal mammary chain of lymph glands and 97% flows to the axillary nodes (Hultborn *et al.*, 1955). This would help to explain why the axillary lymph nodes are considered to be an important route in the spread of primary mammary carcinoma.

The breast also consists of an areola and a nipple, comprised mainly of smooth muscle. The muscle within these structures functions to contract the areola and to compress the base of the nipple, thus making the nipple erect, smaller and firmer, and ready to empty milk from the ducts (Haagensen, 1986). The areola and nipple also have a marked pigmentation, which is more obvious in younger women than older women. It is suspected that this variation is related to varying oestrogen levels, as the intensity of pigmentation fades with an increase in age, until menopause is reached, and can be intensified at any time with the administration of exogenous oestrogens (Haagensen, 1986).

1.2.2 Development of the breast through the lifespan

At birth, the epithelial portion of the female breast consists of a small number of branching ducts stemming from the nipple (Hayes, 1993; Speroff *et al.*, 1994). During pre-pubertal development, these ducts exhibit slow but progressive growth and branching (Hayes, 1993). In males, breast development discontinues at this stage.

The ovarian cycling of sex steroid hormones stimulates breast development during puberty. The onset of puberty (around the age of 10 to 12 years) sees a change in the pattern of release of gonadotropin releasing hormone (GnRH), which stimulates the anterior pituitary to release follicle stimulating hormone (FSH) and luteinising hormone (LH) (Osborne, 1991; Seeley *et al.*, 1995). At puberty these hormones not only increase in concentration but also establish a cyclic pattern of release. FSH and LH stimulate the immature ovarian follicles to mature and release oestrogens, primarily in the form of 17 β -oestradiol (Vorherr, 1974; Osborne, 1991). In turn these oestrogens act on the uterine cells to bring about progesterone secretion, both of which also establish a cyclic pattern of secretion, known as the ovarian menstrual cycle. The surge of oestrogen into

the body at this age stimulates the maturation of the genital organs and the maturation and differentiation of the tissues of the breast. Many physiological changes occur in the female body during puberty, which continue throughout a woman's reproductive life. The first 5 days of the menstrual cycle is associated with mild haemorrhage of the uterine epithelium (Seeley *et al.*, 1995). The days preceding menses up to the 14th day of the cycle are known as the follicular phase of the cycle, as this is the period of growth and maturation of the ovarian follicles. This phase is also known as the proliferation phase as the uterine mucosa and the glandular epithelium of the breast undergo rapid proliferation, from the influence of high oestrogen concentrations. The 14th day of the menstrual cycle is marked by a slight decline in oestrogen and the gradual elevation of progesterone, known as ovulation. The days following ovulation, up to the 28th day of the menstrual cycle are characterised by the transformation of the developing follicles into corpus luteum cells (the luteal phase of the cycle). This is also the secretory phase of the menstrual cycle, whereby the uterine glands secrete higher levels of oestrogen (producing a bi-phasic pattern of excretion throughout the cycle) and progesterone. The peak mitotic activity of the breast takes place in this phase (Ferguson and Anderson, 1981). In the breast, oestrogens stimulate the growth of ductal epithelium, as well as the proliferation of terminal ducts, which precede breast lobules later in breast development (Vorherr, 1974). As a result of the growth of ductal tissue, the volume and elasticity of connective tissue increases and fat deposition is enhanced (Vorherr, 1974). Growth hormone, prolactin, insulin and adrenal glucocorticoids are also important for growth of the ductal system, as each hormone is known to play a role in protein metabolism (Guyton, 1986).

During pregnancy, ductal, lobular and alveolar growth occurs, from the stimulation of luteal and placental sex hormones, placental lactogen, prolactin and chorionic gonadotropin (Osborne, 1991). Prolactin, along with insulin and hydrocortisone, stimulates epithelial growth of the secretory glands, which in turn initiates the formation of milk proteins required for the nutrition of the offspring (Turkington, 1972; Guyton, 1986). In the second half of pregnancy, the breast increases in volume as a result of the dilation of alveoli with colostrum, and hypertrophy of myoepithelial cells, connective and adipose tissue (Osborne, 1991). After parturition the placental hormones are withdrawn, and prolactin, growth hormone, insulin and cortisol initiate the conversion

of mammary epithelial cells from a presecretory to a secretory state (Guyton, 1986; Osborne, 1991). After birth, the breasts enlarge due to the accumulation of milky secretions in the alveoli. Initially, colostrum is secreted, which contains a mixture of lactoglobulin, fatty acids, phospholipids, and fat-soluble vitamins (Osborne, 1991). All these components transfer passive immunity and are of nutritional value to the newborn. The release of prolactin is maintained by suckling while the synthesis of oxytocin from the pituitary acts on the myoepithelial cells to aid in ejecting milk from the alveoli into the ducts.

Under the influence of placental oestrogen, the ductal system increases to the point where the breast is mainly comprised of epithelial elements and very little stroma (Guyton, 1986; Hayes, 1993). This condition continues during lactation. Progesterone, growth hormone, prolactin, insulin and adrenal glucocorticoids, cause the growth of the lobules and the development of the secretory characteristics in the cells of the alveoli (Guyton, 1986). In a postlactational state, the epithelial elements of the breast decrease and the stroma becomes the main constituent of the breast (Hayes, 1993).

After menopause, the glandular elements decrease further, with a marked reduction in the number of lobules (Hayes, 1993). In some cases the lobules disappear completely, with only the ducts remaining (Hayes, 1993). Fibrous connective tissue, extending from the fascia, also diminishes, whilst adipose tissue accumulation in the breast increases (Hayes, 1993).

1.2.3 Hormones and the regulation of breast development

Many steroid hormones regulate the development of the breast. Oestrogen and progesterone, play a major role in breast cell proliferation, while prolactin and glucocorticoids are thought to be involved in breast cell differentiation. Only through binding to intracellular receptor proteins can a hormone-mediated response proceed. Oestrogen, for example, binds to the oestrogen receptor (ER), to form a homodimer complex, which in turn binds, with high affinity, to oestrogen response elements (or transcription factors) (Fuqua, 1994; Habel and Stanford, 1993). The binding of the

oestrogen complex to the response elements then influences gene expression of oestrogen-responsive genes, such as the progesterone receptor and some growth factors. Alterations to binding or oestrogen levels may enhance transcriptional activation and the overexpression of genes such as c-fos, c-jun and c-myc (Davis *et al.*, 1997; Telang *et al.*, 1997). These hormone responsive genes, under normal circumstances are positive regulators of growth, but in situations of altered oestrogen stimulus, may promote increased proliferation and lead to a cancerous phenotype (Telang *et al.*, 1997). Growth factors, such as epidermal growth factor (EGF) and transforming growth factor (TGF) also play an important role in regulating mammary cell growth. Reports by Snedeker and DiAugustine (1996) suggest that oestradiol enhances the synthesis of TGF α in normal mammary cells. However the overexpression of TGF α , initiated by altered oestrogens in the mammary gland has been shown to promote neoplastic and hyperplastic growths of the alveoli and terminal ducts in virgin female and pregnant transgenic mice (Matsui *et al.*, 1990).

Oestrogen and progesterone receptor levels have been found to be lower in normal breast tissue compared with that in breast tissue showing evidence of benign and malignant breast lesions (Ricketts *et al.*, 1991). Research by Ricketts and colleagues (1991) suggests that the cells of normal breast tissue only expresses 7% and 19% of oestrogen and progesterone receptors respectively, compared to the levels expressed for benign dysplasia (16% for oestrogen receptor and 31% for progesterone receptors). These data suggest that the overexpression of ER and PR may be linked to cell proliferation and thus an increased risk of developing breast cancer (Ricketts *et al.*, 1991; Davis *et al.*, 1997). Early studies reported by Bassler (1970) suggest that oestrogens and progesterones can affect the DNA synthesis phase of the cell cycle, in the nuclei of mouse mammary gland epithelium. In the normal resting mammary gland of mice, the DNA synthesis phase lasts approximately 20.7 hours; if mice were treated with oestrogen and progesterone for 3 days, DNA synthesis was shortened to 10.7 hours (Bassler, 1970). DNA synthesis was further shortened to 8.8 hours if the mice were treated with oestrogen and progesterone for 2 to 3 weeks, thus suggesting that oestrogen and progesterone can accelerate DNA synthesis in the breast (Bassler, 1970). This illustration indicates that oestrogen promotes the growth of cells in the breast and therefore oestrogen is likely to be an important factor involved in the development of

breast cancer. A study by Hertz (1985) suggests that malignant tissue has a phenotype consisting of excessive mitotic activity, very similar to that of tissue, which has been induced to proliferate by oestrogen treatment. Research reported by Snedeker and DiAugustine (1996) suggested that oestrogen has a direct effect on the cells of the mammary gland and thus can regulate the development of breast cancer. When pellets containing 17β -oestradiol (the most potent and biologically active oestrogen) were implanted near the mammary gland of castrated rodents the results indicated that oestrogen stimulated the regrowth of the ducts without having any effects on other glands. On the other hand, Snedeker and DiAugustine (1996) showed that the development of spontaneous breast tumours in mice could be abolished by ovariectomy, which was due to a reduction in production of ovarian oestrogen.

Early research by Siiteri and colleagues (1974) indicated that the ratio of the various oestrogen forms was an important factor in understanding the role of oestrogen in breast and endometrial cancer development. In the absence of 17β -oestradiol (E_2) secretion from the ovaries, oestrone (E_1) and oestriol (E_3) are the major oestrogens in circulation of the body. Oestrone, if not a product of oestradiol hydroxylation, is a precursor oestrogen converted from androstenedione and is the principal oestrogen available to target tissues in patients likely to develop breast cancer (Siiteri *et al.*, 1974; Davis *et al.*, 1997). Oestriol is a breakdown product of oestrone in the liver, but has low oestrogenic and carcinogenic activity. In the absence of E_2 , E_1 and E_3 compete for binding to the oestrogen receptor, but both bind with poorer affinity compared to E_2 (Siiteri *et al.*, 1974). Long-term exposure of the breast to oestrone has indicated that this oestrogen does provide the necessary stimulus to breast cells to develop cancer (Siiteri *et al.*, 1974). Research combined from nine prospective studies carried out world-wide (The Endogenous Hormones and Breast Cancer Collaborative Group, 2002) suggest too that the risk of breast cancer is increased with increasing concentrations of endogenous sex hormones (total estradiol, free estradiol, non-sex hormone-binding globulin-bound estradiol, estrone, estrone sulfate, androstenedione, dehydroepiandrosterone, dehydroepiandrosterone sulfate and testosterone). Conversely, high levels of oestriol have been detected in populations with a low incidence of breast cancer, suggesting that oestriol may have a protective effect against cancer development (Siiteri *et al.*, 1974; Korenman, 1980). This hypothesis was thought to explain the protection afforded by full

term pregnancy. Pregnant women have high concentrations of urinary oestriol in relation to the other oestrogens, therefore the carcinogenic effect of oestrone is reduced (Siiteri *et al.*, 1974; Ziegler *et al.*, 1997). Obesity has been shown to increase the conversion of androstenedione to oestrone, thereby increasing the carcinogenic potential. Liver disease also increases the level of circulating oestrone, which is likely to be due to the inadequate conversion to oestriol (Siiteri *et al.*, 1974). Alternatively it has been suggested that the enzymatic pathway which results in the formation of different oestrone metabolites from unbound oestradiol, may be associated with increased breast cancer risk (MacMahon *et al.*, 1973; Davis *et al.*, 1997; Rudel, 1997; Ziegler *et al.*, 1997). Several researchers have proposed that oestradiol can either be converted into 2-hydroxyoestrone (a weak oestrone) by the insertion of a hydroxyl group at the second carbon or into 16 α -hydroxyoestrone (a potent oestrone) with the aid of cytochrome P450 hydroxylases (Davis *et al.*, 1997; Liehr, 1997; Telang *et al.*, 1997). Studies reported by Davis and colleagues (1997) suggest that mammary epithelial cells exposed to 16 α -hydroxyoestrone resulted in genotoxic DNA damage and increased cellular proliferation. In contrast the addition of 2-hydroxyoestrone inhibited the expression of a transformed phenotype (Davis *et al.*, 1997). In vitro laboratory experiments have shown that a naturally occurring phytoestrogen, indole-3-carbinol, was able to inhibit carcinogenic transformation due to the enhancement of 2-hydroxyoestrone production (Davis *et al.*, 1993; Davis *et al.*, 1997). Moreover, chemicals used in the environment to control pests have also been found to interact with the oestrogen receptor by mimicking the action of endogenous oestrogens in animal models and cell cultures (Colborn *et al.*, 1993; Davis *et al.*, 1993). Results presented by McDougal and Safe (1998) suggest that environmental chemicals such as DDT, are able to induce carcinogenic transformation by enhancing 16 α -hydroxyoestrone production.

1.2.4 Disease progression

Several studies have suggested that there is a continuum of events linking benign breast disease with breast cancer development (Black *et al.*, 1972; Rywlin, 1984; Dupont and Page, 1985; Bodian, 1993). This continuum of events is based on the theory that carcinogenesis is a multistep process involving initiating or promoting factors, such as

mutagens, which may invoke a transformation of normal cells into malignant cells (Rywlin, 1984).

The study of benign breast disease is central to understanding what happens when women are diagnosed with breast cancer. Discovering a lump or abnormality within the breast, regardless of whether the condition is pathologically confirmed as benign or malignant, carries the same emotional and sexual significance. Approximately eighty percent of breast lesions are benign (Hockenberger, 1984). But the term “benign” covers a wide spectrum of pathologically defined lesions, which could predispose a woman to breast cancer development at varying rates. A more descriptive nomenclature has been proposed, which has created more homogenous and prognostically relevant categories, and can be used to give better estimates of breast cancer risk for women with benign breast disease (Dupont and Page, 1985; Bodian, 1993). These categories are based on the degree of epithelial proliferation and are as follows: non-proliferative lesions, proliferative lesions without atypia and atypical hyperplasias of both lobular and ductal types. Non-proliferative lesions consist of fibroadenomas, cysts, apocrine metaplasia and lesions with mild hyperplasia. Generally these lesions are well-defined clinical lumps, usually between two and four epithelial cell layers within ducts, without any invading capacity (Goehring and Morabia, 1997). Research by Dupont and Page (1985) suggests that there is no evidence of any increased risk of breast cancer with lesions lacking a proliferative component. However other researchers would argue that some types of non-proliferative lesions, in particular fibroadenomas, have an associated increased risk of breast cancer (Fleming *et al.*, 1982; Bodian, 1993). Proliferative lesions without atypia consist of moderate or florid hyperplasia, intraductal papilloma and sclerosing adenosis. These lesions tend to exist in greater than four epithelial cell layers and have a tendency to bridge the ducts (Goehring and Morabia, 1997). Atypical hyperplasias of ductal and lobular types have some features of in situ carcinomas (Goehring and Morabia, 1997). Studies by Dupont and Page (1985) have found that the relative risk of breast cancer development tends to increase through the continuum of events for carcinogenesis (relative risk of 1.0 for non-proliferative lesions, relative risk of 1.9 for proliferative lesions without atypia and relative risk of 5.3 for atypical hyperplasias).

Breast cancer is a group of related conditions, characterised by differing microscopic appearance and biologic behaviour, in which the cells of the breast escape the usual constraints of replication, and grow and divide rapidly and uncontrollably (Coe and Steadman, 1995; Davis and Bradlow, 1995). It is believed that this escape from the replication cycle involves the accumulation of mutations, usually in genes that regulate cell division and the accurate replication of DNA (Davis and Bradlow, 1995). Hormones and other substances located in close proximity of the cell can stimulate abnormal cell multiplication. Like benign breast conditions there are differing degrees of severity, each of which have their own associated prognosis and appropriate treatment.

As the cancer cell continues to grow and divide, it forms a mass of cells known as a malignant tumour or neoplasm, which may remain confined within the basement membrane of the duct or lobule (Vander *et al.*, 1970; Hayes, 1993; Coe and Steadman, 1995). If the breast cancer remains within the basement membrane and does not invade surrounding tissue or metastasise to distant organs it is said to be in situ (non-invasive) (Sainsbury *et al.*, 1994; Coe and Steadman, 1995). In situ lesions are of two types; ductal carcinoma in situ (DCIS), also known as intraductal carcinoma, and lobular carcinoma in situ (LCIS) (Hayes, 1993). DCIS is characterised by malignant epithelial cells that are contained within the mammary ducts, without the involvement of the basement membrane (Coe and Steadman, 1995). LCIS is predominantly located in the lobules. These two lesions differ from each other by clinical features, morphology and biologic behaviour (Table 2). However, as the tumour increases in size, it adopts the ability to draw on the body's nutrient supplies and invade (or infiltrate) the normal adjacent tissue (Vander *et al.*, 1970; Coe and Steadman, 1995).

Invasive carcinomas also consist of a heterogeneous group of lesions. The most common type is infiltrating ductal carcinoma (also known as infiltrating carcinoma of no special type or infiltrating carcinoma not otherwise specified), which are usually hard, grey masses that invade the adjacent tissue (Hayes, 1993; Coe and Steadman, 1995). In the time frame between 1986 and 1990, ductal carcinoma was the most commonly reported histopathologically grouped cancer in women in Australia (80.9 % of the total breast cancer cases) (Kricker and Jelfs, 1996). From microscopic observation

and cytological features, infiltrating carcinomas can be divided into three grades: well differentiated (grade I), moderately differentiated (grade II), and poorly differentiated (grade III) (see discussion in clinical pathology of breast cancer) (Hayes, 1993).

Table 2, Comparison of Ductal Carcinoma In Situ (DCIS) and Lobular Carcinoma In Situ (LCIS).

Classification criteria	DCIS	LCIS
Location	ducts	lobules
How is the condition usually detected	incidental finding, abnormality in mammogram, nipple discharge, palpable mass, Paget's disease	incidental finding
Cell size	medium or large	small
Pattern of detection	comedo, cribriform, micropapillary, papillary, solid	solid
Calcification	present or absent	absent
Risk of subsequent invasive cancer	high	low
Location of subsequent invasive cancer	ipsilateral	ipsilateral or contralateral

(Adapted from Hayes, 1993)

Infiltrating lobular carcinomas are the second most common type of invasive carcinomas (7.7% of the total breast cancer cases) (Kricker and Jelfs, 1996). These lesions are characterised by small tumour cells that infiltrate the stroma (Hayes, 1993). Other variations of invasive carcinoma include medullary, mucinous, tubular and papillary cancers, all of which have a better prognosis than infiltrating ductal carcinoma

(Hayes, 1993). These histopathologically defined breast cancers only account for a small proportion of the total breast cancer cases documented in Australia (1.7%, 1.8%, 1.2% and 0.5% respectively) (Krickler and Jelfs, 1996). More uncommon forms of invasive carcinomas of the breast include metaplastic and adenoid cystic carcinomas.

When the cancer cells break away from the site of origin and penetrate the basement membrane of the epithelium, they enter the bloodstream or lymphatics located in connective tissue and may metastasise to distant organs and form secondary tumours. The major route of metastases via the lymphatic system is through the axillary nodes. Once these nodes have been involved in metastasis, there is the capacity of the primary breast cancer to extend into the central lymphatic terminus at either side of the base of the neck and “empty” the cancer cells into the venous stream (Haagensen, 1986). These cancer cells can then be carried through the heart to the lungs, where they fill the alveoli and may block and permeate the lymphatics, and grow into the pulmonary vein or the pleura (Haagensen, 1986). Tumour fragments that may break loose from the infected pulmonary vein are then carried off, back to the heart and may enter the bloodstream, to be carried throughout the body. Organs with a rich blood supply, such as the liver, spleen, adrenals and bone, are the targets for blood-borne metastases (Haagensen, 1986). Cancer cells can migrate to the liver through the hepatic artery and do not produce any obvious signs of metastases until an advanced stage. Cancer cells destined for bone, can break off from the pulmonary vein or can reach bone through the vertebral system of veins, where the cancer cells enter directly into the spine, pelvic bones and the skull (Haagensen, 1986).

1.2.5 Clinical pathology of breast cancer

When cancer is present, a number of tests are performed to assess the behaviour of the cancer, and to determine the most effective treatment. Some routine tests include, determining the size of the tumour and whether it has spread to other organs, the levels of oestrogen and progesterone receptors, and lymph node involvement.

1.2.5.1 Tumour Stage.

Once an invasive breast cancer is diagnosed, the extent of the disease is assessed, using the tumour node metastases (TNM) system of the International Union Against Cancer (IUCC) (Stockdale, 1988; Sainsbury *et al.*, 1994). The TNM system of classification is a way of clinically staging the cancer and does not require large amounts of pathological material (Venter, pers. comm.). The TNM system reflects the anatomical extent of the disease and is based on; T, the size of the primary tumour, extension of the tumour to the chest wall, extent of accumulation of serum in the tissues, skin and breast ulceration, or satellite nodules; N, the condition of the regional lymph nodes, on the basis of palpability, suspicion of malignancy and location; and M, the involvement of distant metastases based on physical and clinical studies (Stockdale, 1988; Sainsbury *et al.*, 1994). Once a TNM classification is available for a tumour, the tumour is then classified into a clinical stage; stage I, II, III, or IV (Table 3) (Sainsbury *et al.*, 1994). Survival from breast cancer is largely dependent on the stage at presentation, and the prescription of appropriate treatment is based on stage.

Table 3, The correlation of the tumour, nodes, metastases (TNM) system and the International Union Against Cancer (IUCC) system of classification for tumours.

UICC stage	TNM classification
I	T ₁₋₂ , N ₀ , M ₀
II	T ₁₋₂ , N ₁ , M ₀
III	T ₃₋₄ , N ₂ , M ₀
IV	T ₃₋₄ , N ₂ , M ₁

Key of TNM classification terms for breast cancer (for Table 3)

Abbreviated term	Definition
T ₀	No evidence of primary tumour
T _{is}	Cancer in situ or Paget's disease with no tumour mass
T ₁	Tumour 2cm or less
T ₂	Tumour between 2 and 5cm
T ₃	Tumour more than 5cm
T _{4A}	Tumour of any size with extension to chest wall

T _{4B}	Tumour of any size with involvement of skin
T _{4C}	Both 4A and 4B
T _{4D}	Inflammatory cancer
N ₀	No regional axillary lymph node metastasis
N ₁	Palpable ipsilateral axillary lymph nodes
N ₂	Fixed ipsilateral axillary lymph nodes
M ₀	No evidence of distant metastases
M ₁	Distant metastases

(Sainsbury *et al.*, 1994)

1.2.5.2 Tumour Grade.

Histological grading for breast cancer was first reported in 1925 (Greenough, 1925). On microscopic examination, a tumour can be graded according to the degree of differentiation of the tumour from adjacent “normal” cells. The most common grading system used by pathologists is the Scarff, Bloom, and Richardson (SBR) classification and is usually used as a preference to tumour staging. This system evaluates a tumour according to the degree of glandular formation, nuclear heterogeneity, and frequency of mitoses (Haagensen, 1986; Le Doussal *et al.*, 1989; Sainsbury *et al.*, 1994). The SBR grade is derived from the following;

- (i) the degree of differentiation is evaluated according to the ability of the tumour to form glandular, tubular or papillary structures. If these characteristics are observed throughout the tumour, the tumour is coded 1, if they are seen in some of the tumour, the tumour is coded 2, and if these characteristics are not seen, the tumour is coded 3;
- (ii) the nuclear heterogeneity is evaluated and coded 1 if the nuclei are regular or similar to normal breast epithelial cells, coded 2 if the nuclei are intermediate, and coded 3 if the nuclei are distinctly irregular or distorted;
- (iii) the mitotic index is calculated by looking at ten fields in the tumour periphery under medium power of a microscope to identify areas most abundant in mitosis. If the mitotic index is less than or equal to 1 mitosis/ High Power Field (HPF)

the tumour is coded 1, 2 mitoses/ HPF is coded 2, and areas with more than 2 mitoses per HPF are coded 3 (Le Doussal *et al.*, 1989).

To obtain a SBR grade, the codes from the above three categories are added (Table 4).

Table 4, Scores and definitions pertaining to each tumour grade.

Score	Tumour Grade	Definition
3-5	1	well-differentiated
6&7	2	moderately-differentiated
8&9	3	poorly-differentiated

Grade 1 tumours are small, round, have regular nuclei and very few mitoses. Conversely, grade 3 tumours are large, have irregular nuclei and have many mitoses (Elledge and McGuire, 1993). Survival studies show that grade 1 tumours have a good prognosis, and thus a good response to treatment, whilst grade 3 tumours would have a poor prognosis and the response to treatment would be less successful (Le Doussal *et al.*, 1989; Elledge and McGuire, 1993).

Histological grade is also related to the recurrence rate of tumours. Women with poorly differentiated tumours are more likely to have tumours recurring (22% recurrence at 3 years) compared to women with well-differentiated tumours (6% recurrence at 3 years) (Le Doussal *et al.*, 1989).

1.2.5.3 Oestrogen and progesterone receptors.

The oestrogen and progesterone receptor assays are considered to be essential tools for the assessment, prognosis and treatment of breast cancer (Stockdale, 1988). A large proportion of breast cancers (approximately 50 to 85%) and all oestrogen-responsive tissues contain cytoplasmic proteins (receptors) that specifically bind oestrogen and progesterone (Stockdale, 1988; Donegan, 1992). These oestrogen and progesterone

receptors are shuttled between the cytoplasm and the nucleus, where they form a homodimer complex with oestrogen or progesterone, bind to transcription factors and elicit a hormone dependent response (Stockdale, 1998). Oestrogen and progesterone have been shown to be involved in the promotion of proliferation and cell differentiation in normal breast epithelium, and the study of the mode of action of these hormones and their respective receptors is a key to understanding the effects of these on the breast (Pike *et al.*, 1993).

Oestrogen receptors (ER) and progesterone receptors (PR) are present in higher concentrations in breast cancer tissue than in “normal” breast tissue, and are thus significantly important for planning treatment (Stockdale, 1988). Patients with breast cancers that are shown to be ER positive, respond favourably to hormone treatments such as tamoxifen, in approximately 60-65% of cases (Stockdale, 1988; Donegan, 1992). Patients with negative ER assays have a less than 10% response rate to hormone therapy (Stockdale, 1988; Donegan, 1992; Elledge and McGuire, 1993). The presence of a PR positive result, in addition to a positive ER result, increases the likelihood of a favourable response to hormone treatment. Progesterone receptors are only found to be positive in approximately 5% of cases if the ER are negative (Donegan, 1992).

1.2.5.4 Tumour size.

The size of the primary tumour and the involvement of axillary nodes (which, combined, constitute the stage of the disease) in cancer development, are the most important indicators of prognosis (Stockdale, 1988). A good prognosis is associated with a small tumour (less than 1cm in diameter); whilst a poor prognosis accompanies a large tumour (a diameter greater than 5cm) (Stockdale, 1988; Carter *et al.*, 1989; Donegan, 1992).

Tumour size correlates with the risk of recurrence, up to 30 years after the primary diagnosis (Elledge and McGuire, 1993). Results from the SEER program (Surveillance, Epidemiology and End Results program of the National Cancer Institute) suggest that if tumours are less than 1cm in diameter and have not progressed from the initial site of development, then there is a relatively high chance of survival, after 5 years, from the time of primary diagnosis (Ries *et al.*, 1983; Carter *et al.*, 1989; Donegan, 1992). From

the same program, in comparison, tumours of greater than 5cm in diameter, have an 82% chance of survival after 5 years from the initial time of diagnosis (Ries *et al.*, 1983; Carter *et al.*, 1989).

1.2.5.5 Treatment options.

The appropriate treatment for breast cancer varies according to the severity of the disease, tumour size, lymph node involvement and the status of oestrogen and progesterone receptors. There are four main components of treatment; surgery, radiotherapy, chemotherapy and hormone therapy. Treatment of the tumour commences with surgery, and then other treatments may follow once the extent of disease has been assessed.

Surgery is a form of localised treatment, which aims to remove the breast tumour itself. The extent of surgery depends on the severity and localisation of the disease. Breast conservation therapy involves the removal of the cancer, a small margin of healthy tissue surrounding the tumour and some of the axillary lymph nodes, while leaving the bulk of the breast intact. However for more extensive cancers which are distributed throughout the breast or are ill defined, or involve the nipple or overlying skin, a mastectomy is usually advised. A mastectomy involves the removal of all the breast tissue, with only the pectoralis muscles remaining (Engelking and Kalinowski, 1996).

Radiotherapy utilises ionising radiation, which affects DNA and may cause mutations in relatively normal tissue. Conversely, rapidly dividing cancer cells are more susceptible to ionising radiation, than normal cells, and are therefore killed or inhibited from dividing. The radiation dose is calculated to cause maximal damage to the tumour whilst having minimal effect on normal cells. Radiation therapy is usually a post-operative measure used for the control of residual cancer that may have been undetected at the time of surgery, and to reduce the rate of locoregional recurrences (Engelking and Kalinowski, 1996; Holli *et al.*, 2001). Occasionally, though, radiation therapy may be used before surgery to reduce the size of the tumour (Engelking and Kalinowski, 1996).

Chemotherapy and hormone therapies are forms of adjuvant systemic treatments, usually prescribed in addition to surgery. The aim of these therapies is to increase the

chance of long-term survival without the recurrence of any metastases after local treatment (Stockdale, 1988). Hormone therapy is considered first in the line of therapies used for breast cancer as it is the least toxic of the systemic treatments. Hormone therapy for breast cancer is based on anti-oestrogenic activity, as it antagonises the action of oestrogen, by blocking the oestrogen receptor and inactivating gene expression (Fuqua, 1994; Montano *et al.*, 1996). Tamoxifen is a widely used form of hormone treatment and is known to possess agonist (has the ability to produce the same effect as oestrogen) and antagonist (neutralises the action of oestrogen) properties depending on cell type and the nature of the oestrogen receptor (Montano *et al.*, 1996). Research by Montano and colleagues (1996) investigated the structure-activity relationships of the ER and found that the ER possesses regions (hormone binding domains) which are sensitive to change and can discriminate between oestrogen and anti-oestrogens binding. A single amino acid change conferred by mutation, can result in an altered binding affinity, so that the receptor recognises anti-oestrogens as agonists and oestrogen as an antagonist (Montano *et al.*, 1996). Tamoxifen usually exerts its effects by reducing the uptake of oestrogen by breast cells, by blocking the oestrogen receptor, which in turn reduces tumour growth. Generally, tamoxifen best benefits women whose tumours are pathologically defined as oestrogen receptor positive and well differentiated (grade 1), and if the patient is postmenopausal (Stockdale, 1988; Malycha, 1993). However a changed physiological phenotype of the ER may explain some of the tamoxifen resistance seen in some patients receiving this treatment and may help explain why only a small proportion of women with oestrogen receptor negative tumours have an effective outcome with tamoxifen treatment.

Chemotherapy is best suited for women whose tumours may be oestrogen receptor negative, women with metastatic breast cancer, or in women who may have life-threatening complications as a result of metastatic spread.

The unfortunate reality with breast cancer research (and the funding which dictates the course of research) is that it is primarily focused on treatment and early detection, rather than primary prevention through the assessment of causes and risk factors (Weisburger and Rall, 1972). More research needs to be dedicated to determining the mechanisms that are involved in the control of growth of tumours and what causes the cells to

multiply more rapidly. Therefore it is important to investigate the epidemiology of disease. The following is an account of the epidemiology of breast cancer.

1.3 EPIDEMIOLOGY OF BREAST CANCER

There are a number of studies by different research groups with differing study design that have explored the factors that are thought to increase the risk of breast cancer. Table 5 lists some of the studies, the risk factors that have been explored in relation to breast cancer and the main outcomes of these factors in association with breast cancer risk. From these studies there are a number of established risk factors which have been identified for breast cancer, that help to predict which individuals of the population would be at increased risk of developing the disease. These sorts of risk factors provide information about the pathogenesis of the disease, which in turn affects treatment and can lead to improved prevention.

1.3.1 Gender and Age

From the available evidence, breast cancer is predominantly a disease, which develops in women although in rare circumstances the condition can be diagnosed in males. It seems likely that oestrogen has some role in the development of breast cancer, which would explain why there is almost a 100 -fold difference in breast cancer incidence between males and females. However the difference in incidence may be because in females estradiol is able to exert a direct biological effect on breast cells, whereas in males testosterone needs to be converted to estradiol before exerting any biologic effect (Endogenous Hormones and Breast Cancer Collaborative Group, 2002). In 1996, there were 2557 newly diagnosed incidences of breast cancer among Victorian women, whilst amongst the Victorian male population, there were 17 newly diagnosed cases (Giles *et al.* (eds), 1999). This condition is also relatively rare in young women who are younger than 40 years of age. Some 161 cases were diagnosed in Victorian women under the age of 40 years during 1996, and the incidence increases with increasing age (Table 6) (Giles *et al.* (eds), 1999).

Table 5, Summary of studies investigating some of the risk factors for breast cancer.

Authors	Country	Type of study	Number of participants	Main risk factors studied	Conclusive remarks
Helmrich <i>et al.</i> , 1983	USA, Canada & Israel	Hospital based case-control	1185 cases 3227 controls	Menarche parity menopause BMI, BBD family history	<ul style="list-style-type: none"> - Lower risk with late (>15 years) menarche among pre-mp women - Risk increases with increasing age at first birth. High parity (>5 children) reduces risk - Risk increases with increasing age at menopause - High BMI assoc. with high risk among post-mp women - High risk with history of BBD - Family history increases risk
Rosner <i>et al.</i> , 1994	USA	Nurse's Health Study Cohort	91,523 women followed for 14 years	Parity menopause	<ul style="list-style-type: none"> - Age at first birth and subsequent births has long term influence on breast cancer incidence - Among parous women, early age at subsequent births = protective, independent of age at first birth
MacMahon <i>et al.</i> , 1970	USA, Wales, Taiwan, Brazil, Japan, Greece & Yugoslavia	Case-control	4000 cases 13000 hospital controls	Parity SES	<ul style="list-style-type: none"> - Women aged <18 years at first birth have 1/3 the risk of women who delay it until 35 years or older - Subsequent births, even if at an early age = no or very little protective effect - Age at first birth = more important than number of births

Authors	Country	Type of study	Number of participants	Main risk factors studied	Conclusive remarks
Lambe <i>et al.</i> , 1994	Sweden	Case-control	12666 cases 62121 age matched controls	Parity	<ul style="list-style-type: none"> - Uniparous women at higher risk than nulliparous women for up to 15 years after childbirth - Higher risk for older women (35 and over) at first birth
Stavraky & Emmons, 1974	Canada	Case-control	95 pre-mp 278 post-mp cases 106 pre-mp 480 post-mp controls	Parity menarche menopause oral contraceptives	<ul style="list-style-type: none"> - High risk with first birth at or over 20 years of age - Increased risk with increasing age at first birth for post-mp women - Significant decrease in risk with late menarche in pre-mp women - High risk with older age at natural menopause - High risk for long time between menarche and first birth - High risk for pre-mp women who began use of oral cont. over 25 years of age

Authors	Country	Type of study	Number of participants	Main risk factors studied	Conclusive remarks
Colditz <i>et al.</i> , 1996	USA	Nurse's Health Study Cohort	89132 women followed for 13 years	Family History Reproductive factors	<ul style="list-style-type: none"> - Women with family history had different assoc. with reproductive factors than women with no family history: - Little protection from risk with late age at menarche - No protection from breast cancer risk with multiple births - 50% higher risk if early age at first birth in women with a family history
Siskind <i>et al.</i> , 1989	Australia	Case-control	459 cases 1091 electoral roll controls	Lactation	<ul style="list-style-type: none"> - Weak inverse assoc. between history of breastfeeding and risk - Decrease in risk with breastfeeding after first birth - Slight increase in risk for lactations < 1 month, low risk for lactations 1-3 months, weak increase in risk if 9 or > months
London <i>et al.</i> , 1990	USA	Nurse's Health Study Cohort	89413 nurses followed over 10 years	Lactation	<ul style="list-style-type: none"> - No independent assoc. between lactation and risk - Short periods of lactation do not reduce risk among younger women

Authors	Country	Type of study	Number of participants	Main risk factors studied	Conclusive remarks
Plu-Bureau <i>et al.</i> , 1994	France	Cohort	1150 women with BBD	Oral contraceptives against all other established risk factors	<ul style="list-style-type: none"> - Progestagen use and duration of use were not assoc. with increased risk - 19-nortestosterone derivative of progestagen assoc. with lower risk. - Linear decreasing trend of RR with duration of use
White <i>et al.</i> , 1994	USA	Population based case-control	747 cases 961 population controls	Oral contraceptives	<ul style="list-style-type: none"> - No increased risk with use of oral contraceptives - Small increased risk with long duration of use among women 35 years and younger - Small increase in risk with use beginning within 5 years of menarche - Small increase in risk with use of high progestin pills for at least 1 year
Steinberg <i>et al.</i> , 1990	NA	Meta-analysis	16 studies	ERT	<ul style="list-style-type: none"> - Risk increased after 15 years of oestrogen use. But risk did not increase until after 5 years of use - Women with family history and had ever used ERT had a slightly higher risk

Authors	Country	Type of study	Number of participants	Main risk factors studied	Conclusive remarks
Newcomb <i>et al.</i> , 1995	USA	Population based case-control	3130 cases 3698 controls	HRT	<ul style="list-style-type: none"> - No risk in women using HRT - No clear increase in risk with 15 or more years of use - Risk did not vary with menopausal type, family history, history of BBD or alcohol intake
Rohan <i>et al.</i> , 1988	Australia	Population based case-control	451 case control pairs	Diet	<ul style="list-style-type: none"> - Little variation in risk for intake of energy, protein and total fat - A non-uniform decrease in risk for fibre intake - Risk significantly reduced with increasing intake of β-carotene
Bernstein <i>et al.</i> , 1994	USA	Case-control	545 case control pairs	Physical exercise	<ul style="list-style-type: none"> - Women who maintain 1-3 hours/week of exercise reduce risk of pre-mp cancer by 30%, of that of inactive women
Longnecker <i>et al.</i> , 1988	NA	Meta-analysis	21 studies	Alcohol intake	<ul style="list-style-type: none"> - Intakes of 24grams/day or more strongly increased risk - Evidence of dose-dependent assoc. between alcohol and risk - Assoc. stronger in follow-up studies than case-control

Authors	Country	Type of study	Number of participants	Main risk factors studied	Conclusive remarks
Baghurst & Rohan, 1994	Australia	Case-control	451 cases 451 population based controls	Dietary fibre intake	<ul style="list-style-type: none"> - Highly significant reductions in risk assoc. with increasing intake of components of total dietary fibre - Dietary fibre intake may be protective against breast cancer
Collaborative Group on Hormonal Factors in Breast Cancer, 2002a	NA	Prospective case-control & cohort	50302 cases 96973 controls from 47 epidemiol. studies	Breastfeeding	<ul style="list-style-type: none"> - In absence of b.feeding, each birth reduces RR of breast cancer by 7% - RR reduced with increasing duration of b.feeding – RR per 12months of b.feeding being 4.5%
Collaborative Group on Hormonal Factors in Breast Cancer, 1996	NA	Prospective case-control & cohort	53297 cases 100239 controls from 54 epidemiol. studies	Hormonal contraceptives	<ul style="list-style-type: none"> - Slight increase in risk in women currently using or who used in past 10years - Cancer diagnosed in women ever used oral cont. less likely to have spread beyond breast
Collaborative Group on Hormonal Factors in Breast Cancer, 1997	NA	Prospective case-control & cohort	52705 cases 108411 controls from 51 epidemiol. studies	HRT	<ul style="list-style-type: none"> - RR of breast cancer increases with increasing duration of use for current or recent users. - Risk reduced after use ceases.
Collaborative Group on Hormonal Factors in Breast Cancer, 2001	NA	Prospective case-control & cohort	58209cases 101986 controls	Familial breast cancer	<ul style="list-style-type: none"> - Lifetime incidence of breast cancer is 5.5% for women with 1 affected 1st degree relative - 13.3% for women with 2 affected 1st degree relatives

Authors	Country	Type of study	Number of participants	Main risk factors studied	Conclusive remarks
Collaborative Group on Hormonal Factors in Breast Cancer, 2004	NA	Prospective & retrospective	83000cases from 53 epidemiol. studies	Abortion	- No adverse effect on subsequent risk of breast cancer
Collaborative Group on Hormonal Factors in Breast Cancer, 2002b	NA	Prospective case-control & cohort	58515 cases 95067 controls from 53 epidemiol. studies	Alcohol and tobacco	- RR increased with increasing intake of alcohol. - Relationship between smoking and breast cancer confounded by alcohol consumption.

Key:

BMI: Body mass index; BBD: benign breast disease; pre-mp: premenopausal; post-mp: postmenopausal; SES: socioeconomic status; oral cont: oral contraceptives; assoc: associated/ association; RR: relative risk; NA: not applicable; ERT: oestrogen replacement therapy; HRT: hormone replacement therapy; epidemiol: epidemiological; b.feeding: breastfeeding.

Table 6, Newly diagnosed breast cancer incidence figures by age group for women in Victoria, Australia in 1996.

Risk Age Groups (years)	Total Number	Age Specific Rate per 100, 000
0 - 24	1	0.5
25 - 29	14	7.8
30 - 34	55	29.6
35 - 39	91	52.6
40 - 44	208	127.7
45 - 49	299	219.5
50 - 54	305	280.1
55 - 59	310	324.6
60 - 64	279	293.8
65 - 69	300	323.9
70 - 74	267	354.2
75 - 79	171	286.0
80 - 84	149	370.5
85 +	108	339.2

Adapted from (Giles *et al.* (eds), 1999).

Over time, the incidence of breast cancer among Australian women has increased by approximately 1.5 percent annually (Kricker and Jelfs, 1996). Figures produced by a joint collaboration between the National Breast Cancer Centre and the Australian Institute of Health and Welfare estimate that the incidence of breast cancer increased from 57 per 100 000 woman-years in 1982 to 67 per 100 000 woman-years in 1992. Whilst the incidence of breast cancer has tended to increase over time, figures for mortality from breast cancer have tended to remain steady at 19 to 20 per 100 000 woman-years over time (Kricker and Jelfs, 1996). The increase in incidence over time may be the result of improvement of screening techniques, and hence the better detection of early stage breast cancers. Research by Baines and To (1990) investigate breast self examination (BSE) practices over five years of women aged 40 to 49 years,

and 50 to 59 years on entry into the study. Their research found that there was a general improvement of BSE frequency and techniques, with minimal influence by age, ethnic origin, smoking history and educational status.

1.3.2 Reproductive Factors

It is well documented that the breast undergoes a tightly regulated pattern of growth and differentiation, which is under direct control from sex steroid hormones and growth factors (Spicer *et al.*, 1995; Davis and Sieber, 1997). Growth and differentiation of the breast differs depending on the age of the woman. It is only fitting therefore that each stage of a woman's reproductive life be investigated in turn, especially in relation to contributing to breast cancer risk.

1.3.2.1 Menarche.

At menarche a woman's body undergoes change in order to accommodate the monthly cycling of sex steroid hormones, and to prepare the body for childbearing. The monthly cycling of hormones is accompanied by changes of the terminal duct lobular unit (TDLU) within the breast. The follicular phase of the menstrual cycle (the first fourteen days) sees a gradual increase in oestrogen concentrations, which stimulates the proliferation of ductal and lobule tissue (Spicer *et al.*, 1995). Following ovulation, the corpus luteum stimulates the secretion of progesterone, which contributes to the changes induced by oestrogen, as well as transforming cells into secretory cells (Spicer *et al.*, 1995). If fertilisation does not occur, the progesterone concentrations decrease, which subsequently results in a decrease of breast cell proliferation and a wave of cell death by apoptosis (Spicer *et al.*, 1995).

Some researchers have suggested that certain characteristics of the menstrual cycle, such as the time it takes for regular menstrual cycles to establish, the length of menstrual cycles and the age at which these cycles begin, may increase one's likelihood of developing breast cancer (Henderson *et al.*, 1981; Olsson *et al.*, 1983; Henderson *et al.*, 1985; Whelan *et al.*, 1994; Butler *et al.*, 2000).

The establishment of regular menstrual cycles within one year of the first menstrual cycle has been found to double the risk of breast cancer, compared to a situation where menstrual cycles establish regularity in five or more years (Henderson *et al.*, 1981; Butler *et al.*, 2000). When combined with an early age at menarche, a rapid establishment of regularity in menstrual cycles increased the risk of developing breast cancer four-fold (Henderson *et al.*, 1981). LaVecchia and colleagues (1985) suggested that women with regular menstrual cycles, established in the first few years after menarche, have higher levels of luteal-phase progesterone (and this possibly correlates with high luteal-phase oestrogen) than women with irregular menstrual cycles. Research by Ferguson and Anderson (1981) suggested that a regular menstrual cycle of 28 days is not only associated with the regular cycling of oestrogen and progesterone but also the cycle of cell multiplication (mitosis) and cell death (apoptosis). This research also suggests that variations in hormone levels have been found to influence the rates of apoptosis, and unequal cell turnover is likely to lead to the progressive increase of cell multiplication in epithelial cells of the breast (Ferguson and Anderson, 1981). Research by Paradiso and colleagues (2001) found that breast biology, in particular oestrogen receptors, progesterone receptors, proliferative activity as measured by thymidine labelling index, and tumour size, were influenced by menstrual cycle and seasonal variations.

The length of the menstrual cycle and the duration of menstrual activity have been positively correlated with breast cancer risk. Research by Henderson and colleagues (1985), and Whelan and colleagues (1994) have found that a short menstrual cycle of less than 28 days conferred a greater risk of breast cancer than longer cycles of greater than 28 days. Their reasoning behind this theory is that women who have short menstrual cycles, would have more cycles throughout a year, and would thus have more time spent in the luteal phase of the menstrual cycle (Whelan *et al.*, 1994). More time in the luteal phase of the menstrual cycle would mean an increase in time spent on cell proliferation in TDLU (since it has been shown that mitotic activity of the breast epithelium is maximal in the luteal phase of the cycle). Moreover, if fertilisation does not occur, there would also be an effect on the frequency of apoptosis (Anderson *et al.*, 1982). These findings are also supported by the research of Olsson and colleagues (1983), who found that women who had breast cancer, had short cycles of 26.4 days on

average compared to a control group of women who had 28.6 day menstrual cycles on average. These researchers have suggested that a woman with short menstrual cycles was more likely to have high plasma 17β -oestradiol concentrations early in the cycle and low plasma luteinising hormone concentrations, whilst a woman with long cycles would have low plasma 17β -oestradiol and high luteinising hormone concentrations (Olsson *et al.*, 1983). These findings support the strong connection between oestrogen stimulation and the risk of developing breast cancer (Zumoff *et al.*, 1975; MacMahon *et al.*, 1982; Olsson *et al.*, 1983; LaVecchia *et al.*, 1985).

1.3.2.2 Parity.

Pregnancy and related factors, such as the age at first full term pregnancy, the number of full term births, interruptions in pregnancy (such as abortions) and lactation regimes, have opposing influences on the risk of developing breast cancer.

The age at first full term pregnancy is related to breast cancer risk. Data from a Boston study found that women with breast cancer were more likely to have had their first child at a later age than women in the control group (27.1 years and 25.5 years respectively) (MacMahon *et al.*, 1970). The same researchers also found that if a woman gave birth to her first child under the age of eighteen, that she would only have 40 percent of the breast cancer risk of a nulliparous woman (MacMahon *et al.*, 1970). This decrease in risk, (compared to the risk for a woman with a first full term birth after the age of thirty years), is widely supported throughout the literature, even though the strength of the association varies from one study to another (MacMahon *et al.*, 1970; Kelsey *et al.*, 1993). Research from the Nurse's Health Study Cohort, however, suggests that the risk of breast cancer is 50% higher in women with a family history of the disease and who have their first birth at an early age (Colditz *et al.*, 1996). The association found in the Colditz and colleagues (1996) study is most likely due to the effect of family history, rather than the age at first full term birth, on breast cancer risk. The reasons for the correlation between age at first birth and the risk of developing breast cancer are not well understood, but it is speculated that as pregnancy induces change in the hormonal profile of a women, that this change would result in change in the tissues that are under hormonal control. It is thought that pregnancy induces structural changes within the breast, through differentiation of stem cells, to accommodate for the increase in the

production of milk (MacMahon *et al.*, 1970; Russo *et al.*, 1982; Bernstein *et al.*, 1986; Spicer *et al.*, 1995). It seems that, by an undetermined mechanism, that this differentiation of breast cells renders the breast tissue less susceptible to carcinogenic stimuli and thus brings about protection from cancer induction (MacMahon *et al.*, 1970; Miller, 1993; Lambe *et al.*, 1994).

Research by MacMahon and colleagues (1982) has also found that high parity (or a high number of births) provides additional protection against the risk of developing breast cancer, independent of the age at first birth. However the extent of this protection is determined by the woman's age at the time of her subsequent births (MacMahon *et al.*, 1982). If a woman's second birth occurred under the age of 25 years, the risk of developing breast cancer was only one third of that of a woman who had only one birth under the same age (MacMahon *et al.*, 1982).

There appears to be a general consensus from epidemiological studies that pregnancy, and its occurrence early in life, is a protective factor against the risk of developing breast cancer. Some researchers have investigated the link between incomplete pregnancies, arising from spontaneous or induced abortions, and breast cancer. Their research is based on the assumption that the risk of breast cancer may be increased because the birth does not go to term, and would no longer have a protective effect (Pike *et al.*, 1981; Hadjimichael *et al.*, 1986; Parazzini *et al.*, 1991). Work by Parazzini and colleagues (1991) found that women who had at least one abortion before their first full term pregnancy, had approximately 20% greater risk of developing breast cancer, than women who did not have any incomplete pregnancies. They also found that there was no increase in breast cancer risk if the incomplete pregnancy occurred after a woman's first full term pregnancy (Parazzini *et al.*, 1991). However other researchers have found an inverse relationship or no correlation at all (Salber *et al.*, 1969; Paffenbarger *et al.*, 1980; Vessey *et al.*, 1982; Helmrich *et al.*, 1983). Research bringing together the results of 53 studies, also suggests that the risk of breast cancer is not adversely affected if a woman has pregnancies ending in spontaneous or induced abortions (Collaborative Group on Hormonal Factors in Breast Cancer, 2004). It is difficult to arrive at a definitive conclusion in regard to this subject because of the inconsistencies with the research findings. However, research in experimental animals suggests that with an

incomplete pregnancy there is an increased susceptibility of breast carcinogenesis due to the incomplete differentiation of terminal end buds to alveolar buds and lobules (Russo and Russo, 1980). In women the associated risk with incomplete pregnancy and breast cancer is not well known but the similarities between the experimental animal model and the human situation are close. During pregnancy there is the interplay between prolactin, oestrogen and progesterone which all act to promote breast growth and differentiation. If the pregnancy was interrupted, the growth and differentiation would also be incomplete, and like the animal model, the undifferentiated structures would render the breast susceptible to carcinogenesis (Russo and Russo, 1980; Russo *et al.*, 1982; Hadjimichael *et al.*, 1986).

1.3.2.3 Lactation.

The links between breast cancer and reproductive-type factors (such as parity, age at menarche and menstrual cycle patterns, age at menopause) are well established, which raises the possibility that there is an association between breastfeeding and breast cancer. The possibility of an association between breastfeeding and breast cancer was raised approximately seventy years ago, where the researchers found that women who had breast cancer, had more term pregnancies not followed by breastfeeding, than controls (Wainwright, 1931). The fact that these women did not breastfeed when their breasts had developed for nursing, resulted in a greater likelihood of breast cancer development (Wainwright, 1931). A large case-control study performed in 1970 reported that there appeared to be no association between breast cancer development and breastfeeding (MacMahon *et al.*, 1970). However research conducted since then has produced conflicting results. Some studies have suggested that there is a reduction in breast cancer risk with longer periods of lactation, whilst others have reported a weak association or no association at all (McTiernan and Thomas, 1986; Siskind *et al.*, 1989; London *et al.*, 1990; United Kingdom Case Control Study Group, 1993; Michels *et al.*, 1996). A large re-analysis of data (consisting of 50302 breast cancer cases and 96973 controls) collected world-wide from women with and without breast cancer suggests that the longer women breastfeed, the more they are protected against breast cancer development (Collaborative Group on Hormonal Factors in Breast Cancer, 2002a). This study also found that there is an approximate 4.3% (95%CI, 2.9-5.8) reduced risk of breast cancer for every year that a woman breastfed, in addition to a 7% (95% CI, 5.0-

9.0) reduction in risk for each birth. The inconsistencies between these findings may be due to a number of factors. One group of researchers has suggested that the discrepancies may lie with differences in breastfeeding patterns over time (London, 1994). In Victoria, Australia, in the 1950's, approximately 48 percent of newborns were fully breast-fed at three months of age, but the frequency of breastfeeding decreased beyond six months (approximately 39 percent) (Nursing Mothers Association of Australia, pers. commun.). Over the following decades the frequency of breastfeeding mothers again declined to a low of 21 percent for newborns at three months of age, during the 1970's (Nursing Mothers Association of Australia, pers. commun.). The same trends in breastfeeding patterns over time were witnessed in the United States although there may be underlying reasons for these trends (Hendershot, 1984). These trends are most likely due to the fact that in the first half of the century nursing mothers were encouraged to adhere to a schedule of breastfeeding five times per day which was the procedure followed by the women considered as postmenopausal in the U.S study (Newcomb *et al.*, 1994; London, 1994). However in the case of premenopausal women, who breastfed at a later date, their infants were most likely fed "on demand" when the infant was hungry, which is thought to offer greater protection, as the infant was nursed more frequently (Newcomb *et al.*, 1994; London, 1994). It has been reported that some women have difficulty in lactating, which may indicate some hormonal imbalance, possibly resulting in their breast tissue being less-differentiated than their lactating counterparts, which may increase their predisposition to breast cancer (Byers *et al.*, 1985; Michels *et al.*, 1996).

Inconsistency in reported results also applies to the confounding effects of parity, as women who have a high parity tend to have more opportunity to breast-feed than those who have only one child or are nulliparous (Byers *et al.*, 1985). Women who have a high parity also tend to breastfeed for longer periods of time per child, which conferred an even greater protection from developing breast cancer (Byers *et al.*, 1985).

In those studies that have found a protective effect of breastfeeding on the development of breast cancer, the effect was mainly confined to premenopausal breast cancer (Byers *et al.*, 1985; United Kingdom National Case Control Study Group, 1993). The mechanism for this protective effect of lactation is not well understood. It is suggested

that as lactation interrupts ovulation, that there may also be changes to the breast ductal epithelial cells resulting in an inhibition of breast tumour growth (Hendershot, 1984; McTiernan and Thomas, 1986; Newcomb *et al.*, 1994; Michels *et al.*, 1996). Research from experimental animals suggests that there may also be increased elimination, or "flushing" of carcinogens from the secretory cells of the mammary gland during lactation (McTiernan and Thomas, 1986; Dao *et al.*, 1960). The rate of this increased elimination of carcinogens from the mammary gland via breast milk is most likely under the influence of the hormones that play a role in pregnancy (Dao *et al.*, 1960).

1.3.2.4 Menopause.

Menopause is the final stage of a woman's reproductive life during which time all glandular elements of the breast regress, whilst adipose tissue increases. Research by Spicer and colleagues (1995) has found that cellular proliferation in the breasts of postmenopausal women tends to be less than that of premenopausal women. This lower rate of cell proliferation may be due to the decline of plasma oestrogen concentrations during the menstrual cycle (Spicer *et al.*, 1995). Key and Pike (1988) have suggested that this is likely to be the case since when they looked at the effect of menopause on the age-incidence curve for breast cancer, they found that risk increased with age although there was a much steeper increase during the premenopausal period than the postmenopausal period. The increase in incidence of breast cancer for premenopausal women was most likely due to the influence of high levels of cyclic oestrogen and progesterone compared to the constant low levels of oestrogen and progesterone in postmenopausal women (Key and Pike, 1988). Research reported by Weiss (2001) suggests that perimenopausal women (women who begin irregular menstrual cycles until menopause) have higher oestrogen secretion and lower progesterone secretion than younger women in both follicular and luteal phases. Clark and colleagues (1984) suggested that because of the cycling of hormones in the premenopausal group, there may be no free hormone receptors available for routine assay, as required for hormone receptor tests. Barrett-Lee and colleagues (1987) suggested that oestrogen receptor positivity tends to be associated with increasing age rather than menopausal status. This in turn may lead to oestrogen deficiency, and thus an increase in hormone receptor levels. Therefore, premenopausal women diagnosed with breast cancer, are more likely to have low levels of oestrogen receptors associated with their tumours and therefore

would be less responsive to hormone treatment, resulting in a less favourable prognosis. Research by Hall and colleagues (1990) suggests that early-onset breast disease, like that associated with premenopausal women, tends to occur as a result of genetic alterations in chromosome 17q21.

The age at which menopause occurs influences breast cancer risk. Data from a study of women recruited through the Connecticut Cancer Registry, has found that the relative risk of breast cancer is increased with increasing age at natural menopause (Table 7) (Trichopoulos *et al.*, 1972). This trend has been widely supported by a number of studies (Stavraky and Emmons, 1974; Henderson *et al.*, 1975; Kelsey *et al.*, 1993).

Table 7, Relative risk of breast cancer, with increasing age at natural menopause.

Age at natural menopause (years)	Odds ratio
< 45	0.73
45 - 49	0.93
50 - 54	1.07
55 +	1.48

(Adapted from Trichopoulos *et al.*, 1972)

In comparison, women who undergo surgical menopause have a reduced risk of breast cancer. A bilateral oophorectomy before the age of 40 years is associated with a 50% reduction in risk, while hysterectomy alone does not appear to affect breast cancer risk (Brinton *et al.*, 1988; Irwin *et al.*, 1988). The reduction in risk of breast cancer was greatest for women who underwent surgical menopause before the age of 35 (one-third the risk of women undergoing natural menopause) (Trichopoulos *et al.*, 1972). Research by the Collaborative Group on Hormonal Factors in Breast Cancer (1997) suggest however that once a women's age at menopause has been taken into consideration, that there is no difference in relative risk of breast cancer between women who have undergone surgical menopause compared to women who have attained natural menopause (2.3% versus 2.8%, $p=0.24$). It was speculated that the reduction in risk

through the occurrence of an early menopause is associated with the decline in ovarian hormone stimulation (Henderson *et al.*, 1975; Key and Pike, 1988). This decline in ovarian hormone stimulation results in a reduction of oestradiol concentration in the body, which in turn would lead to a decreased rate of cellular division (Key and Pike, 1988).

A combination of early age at menarche and a late age at menopause would therefore prolong the time of the menstrual cycling of sex hormones, and thus would substantially increase a woman's risk of breast cancer development (Henderson *et al.*, 1985; Rosner *et al.*, 1994).

1.3.3 Family History

Epidemiological studies have indicated that a woman's risk of breast cancer may be increased if there is a family history of the disease. Data from the Nurse's Health Study, which began in 1976 and recruited biannually until 1990, suggested that the degree of kinship and the number of closely affected relatives forms a strong relationship with breast cancer development (Colditz *et al.*, 1996). This study showed that 4.6% of the women reported having a mother diagnosed with breast cancer; 1.5% reported that they had at least one sister affected and 0.1% reported having an affected mother and sister (Colditz *et al.*, 1996). Although the percentage of affected relatives was low, the authors have pointed out that there is still a major concern for women with a number of closely affected relatives (Colditz *et al.*, 1993; Colditz *et al.*, 1996). The study of Andrieu and colleagues (1991) is in agreement with the statements made from the Nurse's Health study, however, they have also included affected family members from other generations. For example, a first degree relative was classified as one's mother, father or sibship, which is the category that would encompass the figures given by the previously mentioned study, a second degree relative would be one's aunt or grandmother (Andrieu *et al.*, 1991). The work of Sattin and colleagues (1985) found that there was a 2.3 fold higher risk (relative risk; 1.9 - 2.7, 95% confidence intervals) of developing breast cancer if the participant had a first degree relative affected, as compared to women with no family history of the disease. Conversely, the relative risk of breast cancer was lower

than for a first degree relative if a second degree relative was diagnosed (relative risk; 1.4 - 1.8, 95% confidence intervals) (Sattin *et al.*, 1985). A re-analysis of data from 52 epidemiological studies world-wide conducted by the Collaborative Group on Hormonal Factors in Breast Cancer (2001) is in agreement with the notion of an increased risk of breast cancer with a first degree relatives affected. As this study represents approximately 80% of the published epidemiological studies in this area of research, it gives strong support of family history increasing the risk of breast cancer. The Collaborative Groups research also suggests that for a woman who has one or more first degree relatives diagnosed with breast cancer that her risk is dependent on her age and the age at which her relatives were diagnosed. The relative risk of breast cancer is higher for younger women and for a fixed age proband, but is also higher if the affected first degree relatives are diagnosed at a younger age (Collaborative Group on Hormonal Factors in Breast Cancer, 2001). The mechanisms underlying this relationship are strongly debated although most researchers are in agreement that family history is an established risk factor for breast cancer development (Bain *et al.*, 1980; Sattin *et al.*, 1985; Andrieu *et al.*, 1991; Colditz *et al.*, 1993; Colditz *et al.*, 1996;). A review of medical records in 1969 unveiled familial clustering of childhood and adult neoplasms (Malkin *et al.*, 1990). Segregation analysis demonstrated that these clusterings were the result of a mutation in the p53 tumour suppressor gene and are the underlying defects in patients with cancer associated with Li-Fraumeni Syndrome (Malkin *et al.*, 1990; Weber *et al.*, 1994).

Some researchers have suggested that the relationship between family history and breast cancer should be investigated in light of other known factors, such as the factors that are hormonally influenced. Bain and colleagues (1980) have studied the effects of family history on the risk of breast cancer and have found that even after all the established risk factors (such as menopausal status and age) have been controlled for, there is still an increase in risk that remains unexplained. It has been reported in other studies that the effects of family history on breast cancer may be confounded by age at onset of the disease, the hormonal mechanisms involved and evidence of bilateral or unilateral disease (Bain *et al.*, 1980; Brinton *et al.*, 1982; Sattin *et al.*, 1985; Ottman *et al.*, 1986). Most researchers are in agreement that the effects of family history on breast cancer development are strongest for women who are themselves or have relatives diagnosed at

40 years of age or younger (Brinton *et al.*, 1982; Ottman *et al.*, 1986). This effect has also encompassed the effects of a premenopausal versus a postmenopausal diagnosis. Colditz and colleagues (1993) found that there was a higher risk of breast cancer among premenopausal women, whose mother was diagnosed before the age of 40 years, than postmenopausal women (relative risk; 2.6, 1.6 at 95% confidence intervals, respectively). These results are consistent with the findings of Bain and colleagues (1980) and Brinton and colleagues (1982).

A family history of breast cancer may influence the number of breasts affected by cancer. Sattin and colleagues (1985) found that breast cancer risk was greater if a first-degree relative was diagnosed with unilateral breast disease rather than bilateral disease. However research by Ottman and colleagues (1986) found that there was a higher risk associated with a bilateral diagnosis than a unilateral one. Both of these studies were population based, so the data from them holds true to the general population. However, it would be expected that a bilateral diagnosis would indicate an invasive cancer, and thus would have more probability of being inherited than a unilateral cancer, especially if the outcome was diagnosed at an early age (RR 10.5, 95% CI for bilateral disease under 40 years of age; RR 2.4, 95% CI for unilateral disease) (Ottman *et al.*, 1986).

When all the data regarding the age at onset of disease, bilaterality versus unilaterality and menopausal status, are combined it seems that a common trend emerges that implicates hormonal mechanisms for breast cancer development. It has already been discussed that breast cancer growth is mediated through the interplay of hormones throughout a woman's reproductive life. The effect of family history on breast cancer risk is most likely mediated through the same pathway, whether there is a genetic defect along this pathway or some other explanation is, however, a widely contested issue.

Slattery and Kerber (1993) have suggested that family history may represent shared genetic and environmental components. The genetic aspects of family history may be acquired through the inheritance of genes with defects, that predispose a woman to breast cancer. The cancerous phenotype may be the result of a number of genetic or biochemical mechanisms that have been altered; or may imply that these sorts of alterations have been caused by environmental factors (Colditz *et al.*, 1993; Slattery and

Kerber, 1993). The completion of the mapping of the human genome will provide valuable insight into the effects of altered genetic patterns on disease causation. In the case of breast cancer individuals with inherited susceptibility (even though the number of affected women in this category is low) would remain asymptomatic for decades before the onset of disease (Hall *et al.*, 1990; Evans *et al.*, 1994).

Families with multiple cases of breast cancer would derive considerable benefit from genetic testing, which investigates the candidate genes that would predispose them to a cancerous phenotype. These patients could be kept under close monitoring in order to detect early stages of disease (Evans *et al.*, 1994). The main genes that are thought to be involved in breast carcinogenesis fall into two broad categories; oncogenes and tumour suppressor genes. Oncogenes are positive control genes that regulate the normal growth pathways of the cell. Mutations in oncogenes alter the normal activity of the cell, or increase the expression of gene products, which then drive the cell towards malignancy (Cawkwell and Quirke, 1994; Bland *et al.*, 1995). Examples of oncogenes that are thought to be involved in breast cancer development include *ras*, *c-erbB-2* and *myc* (Kumar *et al.*, 1990; Barnes, 1993; Bland *et al.*, 1995). *Ras* has been implicated in breast carcinogenesis, as like many other types of neoplasms, it is thought to be an initiator in the early stages of cancer development (Kumar *et al.*, 1990). However its involvement in breast carcinogenesis is likely to account for a small percentage of cases (Phillips, personal communication; Liu, 1993). The expression of the *c-erbB-2* gene, which codes for a growth factor similar to the epidermal growth factor receptor, has been shown to correlate with breast cancer prognosis (Todd *et al.*, 1992; Barnes, 1993; Willsher *et al.*, 1996). *c-erbB-2* which is located on band q21 of chromosome 17, has been found to be associated with node positive breast cancers and its amplification may be a significant predictor of tumour aggressiveness and thus give an overall indicator of survival and relapse (Slamon *et al.*, 1987; Barnes, 1993; Willsher *et al.*, 1996). It has been shown that *myc*, which encodes a protein (p62), is required for cell proliferation and differentiation and is possibly involved in DNA replication (Cawkwell and Quirke, 1994). Mutations in *myc* have been suggested to occur early in carcinogenesis and are implicated in a more aggressive phenotype of breast cancer. These often translate to a poorer prognosis (Bland *et al.*, 1995).

Tumour suppressor genes are negative control genes of cell proliferation and differentiation. The loss of these genes, through point mutations, insertions and deletions, is associated with a greater tumourigenic potential because of the loss of normal regulation (Cawkwell and Quirke, 1994). Inherited mutations in BRCA1, BRCA2 and p53 are known to predispose a woman to a higher breast cancer risk. BRCA1, a breast cancer susceptibility gene, has been mapped to chromosome 17q by linkage analysis of families with multiple cases of breast cancer (Hall *et al.*, 1990; Nagai *et al.*, 1995). It encodes a genomic region of approximately 100 kilobases, which consists of the code for 1863 amino acids and contains a zinc-binding domain near the amino terminus, which is thought to regulate transcription (Miki *et al.*, 1994; Langston *et al.*, 1996). Genetic susceptibility arises from the inactivation of the BRCA1 alleles, which would result in the breast tissue being "unprotected" by the tumour suppressor properties of the gene (King, 1992; Miki *et al.*, 1994; FitzGerald *et al.*, 1996). This inactivation of tumour suppression resulting from mutation in the BRCA1 gene, involves all classes of mutations (missense, nonsense, frameshift, deletions, insertions, and intronic) (Collins, 1996; FitzGerald *et al.*, 1996; Langston *et al.*, 1996). However, of these mutations, only a small number are reported frequently in the literature. Of particular interest is the frameshift mutation at position 185 on exon 2, involving the deletion of adenine and guanine (denoted 185delAG) (Collins, 1996; FitzGerald *et al.*, 1996). The 185delAG mutation has a one percent prevalence in the Jewish population, however, it is identified frequently in young Jewish women with breast cancer diagnosed at or before the age of 40 years (21% prevalence) (FitzGerald *et al.*, 1996). There also appears to be a strong genetic predisposition for breast cancer in young women of the general population. Several studies have reported that there is a 1-5% chance of breast cancer diagnosis in women up to the age of 80 years or older that is accounted for by the inheritance of a mutant BRCA1 allele (Claus *et al.*, 1991; King, 1992; Miki *et al.*, 1994). However in younger women (younger than 30 years), up to 40% to 50% of those diagnosed with breast cancer have a genetic predisposition (Claus *et al.*, 1991; King, 1992; Miki *et al.*, 1994).

BRCA2, breast cancer susceptibility gene number 2, has been mapped to chromosome 13q, and confers a proportion of the risk for early onset breast disease equivalent to that of BRCA1 (Miki *et al.*, 1994; Wooster *et al.*, 1994). BRCA mutations are not limited to

women with strong family histories of breast cancer. There may be coincidental cases carrying a mutant BRCA gene, within a family with multiple cases of breast cancer, which would reflect the high incidence of sporadic cases (FitzGerald *et al.*, 1996; Langston *et al.*, 1996). Also in families where there are a small number of breast cancer cases, there may still be carriers of BRCA mutants, who never produce the symptoms of disease (King, 1992; Miki *et al.*, 1994; Langston *et al.*, 1996).

A third breast cancer susceptibility gene, BRCA3, has recently been suspected of contributing to familial aggregation of breast cancer risk. BRCA3 is located on chromosome 13q21 and is suggested to explain the remainder of familial breast cancer risk that cannot be attributed to BRCA1, BRCA2 or p53 (Antoniou *et al.*, 2001; Thompson *et al.*, 2002). The research of Thompson and colleagues (2002), of high-risk breast cancer families of Western Europe, however, found no evidence of linkage between BRCA3 and breast cancer risk. Models derived by Antoniou and colleagues (2001) however, found that a number of low penetrance genes (possibly including BRCA3) with additive effects may account for the residual familial aggregation of breast cancer, not explained by BRCA1 and BRCA2.

p53 is a protein that codes for the TP53 gene, located on chromosome 17p (Malkin *et al.*, 1990; Evans *et al.*, 1994; Miki *et al.*, 1994). The protein is composed of eleven exons and ten interspersed introns; exon 1 is a non-coding exon; exons 2 to 4 encode the amino terminal of the protein, which is thought to be involved as a transcriptional activator; exons 5 to 9 are a highly conserved region of p53 which binds to simian virus 40 T antigen and because of this association it acts as a tumour antigen; and exons 10 and 11 encode the carboxy terminal of the protein and function for DNA binding, nuclear localisation and oligomerisation (Jenkins *et al.*, 1988; Fields and Jang, 1990; Raycroft *et al.*, 1990; Shaulsky *et al.*, 1990; Hupp *et al.*, 1992; Sturzbecher *et al.*, 1992; Malkin, 1994; Furihata *et al.*, 1995). Greater than 95 % of mutations detected in the p53 protein are found in the evolutionary conserved region (exons 5 to 9, and their intervening introns), but the distribution of mutations is highly dependent on the type of cancer and on tissue specificity (Levine *et al.*, 1991; Furihata *et al.*, 1995). Research in the last few years has detected mutations outside of this conserved region, however as more than 70% of these mutations generate stop codons or are frameshift mutations,

they cannot be detected by some methods of mutation screening, such as immunohistochemistry (Coles *et al.*, 1992; Greenblatt *et al.*, 1994).

Since its discovery in 1979, much research on p53 has been undertaken to determine its function and possible involvement in cancer progression (Culotta and Koshland, 1993; Furihata *et al.*, 1995; Kovach *et al.*, 1996). Several researchers have suggested that p53 is involved in a variety of functions, such as initiating apoptosis, blocking DNA replication and controlling DNA repair before synthesis, and regulation of transcription (Levine *et al.*, 1991; Culotta and Koshland, 1993; Cawkwell and Quirke, 1994; Malkin, 1994; Furihata *et al.*, 1995). Also there has been some debate about classifying p53 as a tumour suppressor gene or an oncogene. It has been discovered that p53 can function in both roles. Under normal conditions, wildtype p53 is located in the nucleus and acts to suppress tumour growth by initiating apoptosis and blocking cell division when DNA breaks are detected (Levine *et al.*, 1991; Culotta and Koshland, 1993; Malkin, 1994; Furihata *et al.*, 1995). However, if there is a mutation introduced into the p53 protein, the tumour suppressor properties are inactivated, and the cell can proliferate uncontrollably, thereby increasing the probability of neoplastic transformation, as apoptosis is inhibited (Harris, 1993; Elledge and Allred, 1994; Malkin, 1994; Furihata *et al.*, 1995). The introduction of a mutation into p53, therefore transforms its tumour suppressor effects into potential oncogenic activity (Coles *et al.*, 1992; Culotta and Koshland, 1993; Miki *et al.*, 1994).

In respect to human cancer, p53 is considered to be the most commonly mutated gene, however the degree of mutations arising in this gene is largely dependent on the tissue type and the factors governing differentiation in these tissues (Hollstein *et al.*, 1991; Elledge and Allred, 1994). In breast cancer, the rate of p53 mutation varies from 15 to 50% depending on the stage of disease and the method used for screening of mutations (Coles *et al.*, 1992; Elledge and Allred, 1994). Research by Elledge and Allred (1994) suggests that non-invasive breast cancers have a low incidence of p53 alterations, whilst 15% of in situ cancers have p53 mutations and the most invasive cancers have 2 to 3 times a higher incidence of altered p53. These data suggest that p53 is most likely involved late in the transformation process, a point that is supported by the work of Caleffi and colleagues (1994). Caleffi and colleagues (1994) found that p53 mutations

relate to the severity of disease and other prognostic factors for disease, such that wildtype p53 tends to be associated with oestrogen receptor positivity and mutant p53 tends to be associated with oestrogen receptor negativity. Other researchers are in agreement that p53 mutations are associated with a significantly worse prognosis of breast cancer and increased metastatic potential (Elledge and Allred, 1994; Furihata *et al.*, 1995; Kovach *et al.*, 1996).

1.3.4 Hormone Therapies

Hormone therapies have been, and still are, used throughout a woman's reproductive life and decline of reproductive years, to combat a variety of ailments. In the past pregnant women have exposed themselves, as well as their developing foetus in utero, to synthetic oestrogens, in order to prevent miscarriage (Thomas, 1978). This practice is no longer conducted today. Many young women use oral contraceptives throughout their reproductive years, not just as a contraceptive, but also to alleviate the symptoms of menstruation (Ory, 1982). Perimenopausal and postmenopausal women receive hormones at the end of their reproductive life for the treatment of menopausal symptoms (Thomas, 1978; Pritchard and Sazuka, 1995; Van Winter and Bernard, 1998). The use of exogenous hormones has come under scrutiny, especially since a number of reports have suggested that hormone use may increase a woman's likelihood of some cancers (Whitcroft and Stevenson, 1992).

1.3.4.1 Oral contraceptives.

Oral contraceptives have achieved large scale use since the early 1960's, where they were seen as a means of improving women's health (Pike *et al.*, 1981; Sulak, 1999). Not only are oral contraceptives used as a contraceptive, as their name suggests, but different doses of oestrogens and progestogens in combination are used to alleviate the symptoms that accompany the monthly changes throughout the menstrual cycle. Some of these changes include mood swings, facial acne, menorrhagia (abnormal heavy menstrual flow), dysmenorrhoea (difficult or painful menstruation), amenorrhoea (abnormal suppression of menstruation), and menstrual irregularity (Sulak, 1999). But these symptoms may point to a deeper problem that may lead to difficulties with conceiving

children, and even more serious, cyst formation (Thomas, 1978; Sulak, 1999). The use of oral contraceptives may actually reduce these symptoms, and as one researcher has suggested, their use may decrease the risk of benign breast disease (Thomas, 1978; Ory, 1982). Thomas (1978) have proposed from their review of case-control and cohort studies, that benign breast disease is inversely related to the amount of progestogen in the oral contraceptive preparation, and this association may help to explain how oral contraceptive use has a protective effect. However other researchers have found that there is no altered risk of benign breast disease in women who use oral contraceptives compared to non-users (Sartwell *et al.*, 1973; Yu *et al.*, 1992; Goehring and Morabia, 1997). The disparity in the conclusions from these studies may be explained by the fact that the first oral contraceptives contained high doses of oestrogens, whereas the oral contraceptive preparations in use today contain substantially lower oestrogen doses, in combination with synthetic progestogens (Santow, 1991). The main problem here is that some forms of benign breast disease can increase the risk of breast cancer while others do not proceed to malignant transformation. The researchers who have suggested there is a protective effect associated with oral contraceptive use have not emphasised which histological types of benign breast disease are implicated, and thus, which lesions would predispose to breast cancer development (Thomas, 1978; Plu-Bureau *et al.*, 1994). A cohort study of French premenopausal women with benign breast disease found that there was no association between duration of use of oral contraceptives and increased breast cancer risk (Plu-Bureau *et al.*, 1994). The case-control study of Tzingounis and colleagues (1996) also suggests that there is no increase in risk of breast cancer with oral contraceptive use, rather oral contraceptive use decreases the incidence of benign breast disease.

For those women who use oral contraceptives, the dose of oestrogen and progestogen, and the duration for which they are taken, are thought to lead to an increase in the risk of breast cancer. Research by the United Kingdom National Case Control Study Group (1989) found a highly significant risk of breast cancer, with increasing duration of use of oral contraceptives (Table 8).

Table 8, Relative risk estimates for breast cancer of women using oral contraceptives compared to women not using oral contraceptives.

Time period of use (months)	Relative Risk (range)
1 – 48	0.95
49 – 96	1.43 (0.97 – 2.12)
> 97	1.74 (1.15 – 2.62)

(Data adapted from UK National Case Control Study Group, 1989)

These results are supported by the work of Pike and colleagues (1981), Meirik and colleagues (1986), and White and colleagues (1994), who showed that in cases where women use oral contraceptives for more than 8 years, the relative risk of breast cancer is substantially higher than if she was a non-user. Whilst research by the Collaborative Group on Hormonal Factors in Breast Cancer (1996) suggests that women who are currently using combined oral contraceptives or have used them in the past 10 years are only at a slightly increased risk of breast cancer. They found no evidence of an increased risk of breast cancer for women who have ceased use of oral contraceptives for 10 or more years. In some studies this trend was also apparent if there was long-term use of oral contraceptives before first full term pregnancy (Pike *et al.*, 1981; McPherson *et al.*, 1987). However, other studies found there was no difference in relative risk with oral contraceptive use before or after full term pregnancy or that there was no association at all (UK National Case Control Study Group, 1989; Pike *et al.*, 1993; White *et al.*, 1994). For some of these studies however, the long-term effects of oral contraceptive use cannot be ascertained because wide scale use of these preparations only began since the 1970s. A study by the World Health Organisation (1990) found that there was a small relative risk but no trend of increasing risk of breast cancer with oral contraceptive use before a first full term pregnancy. However, when the data was explored further, the researchers found that the increase in risk was mainly confined to women who had a "non-viable" pregnancy before a term pregnancy and women who used oral contraceptives for more than two years before their first term pregnancy (WHO, 1990). The very long use of oral contraceptives by young women before first full term pregnancy may be a very important factor that puts them at great risk of breast cancer. But this effect is most likely due to the lack of an early pregnancy (therefore the early

protective effect) rather than the long duration of use of oral contraceptives. During puberty, mammary epithelial cells undergo increased replication and these cells only become differentiated after first term pregnancy (White *et al.*, 1994). The time period from menarche to first full term pregnancy would be a critical period for genetic damage, and thus the cells would be susceptible to the effects of oral contraceptives (Pike *et al.*, 1993; White *et al.*, 1994). Work reported by Williams and colleagues (1991) also suggested that oral contraceptive use reduces oestrogen receptor expression and increases the proliferative state of the breast tissue. Oestrogen receptor expression decreases during the natural menstrual cycle, but in oral contraceptive users, the down-regulation occurs at an earlier time point, following the start of hormone ingestion on approximately day 5-7 of the menstrual cycle (Williams *et al.*, 1991). The extent of breast cell proliferation amongst oral contraceptive users would rely largely on the doses of oestrogen and progestogen in the oral contraceptive preparation (Pike *et al.*, 1993). The dosage of hormones in the oral contraceptive preparation is also thought to have an influence on breast carcinogenesis. The long duration of the use of oral contraceptive pills containing high doses of oestrogen is reported to result in a highly significant increase in risk of breast cancer; however the use of progestogen only pills for greater than 12 months has been shown to have a protective effect (UK National Case Control Study Group, 1989). For a pill containing a combination of progestogen and greater than 50µg of oestrogen, the relative risk of breast cancer is lower than if the combination pill contains less than 50µg of oestrogen (UK National Case Control Study Group, 1989).

1.3.4.2 Hormone replacement therapy.

Hormone replacement therapy (HRT) is routinely prescribed for perimenopausal or postmenopausal women to alleviate the symptoms of menopause, such as hot flushes (or vasomotor instability), vaginal dryness and urinary complications (Speroff *et al.*, 1994; Pritchard and Sazuka, 1995). Not only does HRT alleviate the symptoms associated with menopause, but HRT slows bone loss which is associated with postmenopausal osteoporosis, thereby preventing morbidity and mortality attributable to fractures (Lindsay *et al.*, 1980; Weiss *et al.*, 1980; Pritchard and Sazuka, 1995). Studies by the Women's Health Initiative Steering Committee (2004) on the effects of conjugated equine oestrogen in postmenopausal women suggests that the use of oestrogen alone reduces the risk of hip, clinical vertebral and other fractures, to a similar magnitude of

those observed in users of formulations containing oestrogen plus progestin. However, they also found that the risk of total cardiovascular events were 12% higher in women taking conjugated equine oestrogen. This is in contrast with earlier work by Barrett-Connor and Bush (1991), which suggests that hormone replacement therapy reduces total blood cholesterol by 12% and reduces low density lipoprotein (LDL) by 20%, which leads to an increase in high density lipoprotein (HDL), thereby decreasing the risk of coronary heart disease. A previous study by the Women's Health Initiative (2002) also found reductions in LDL (12.7%) and increases in HDL (7.3%) and triglycerides (6.9%) with the use of oestrogen plus progestin formulations relative to those using placebos, however total cardiovascular disease events were increased by 22% in women using the combined hormone formulations. These discrepancies could be due to the type of study used and the characteristics of their participants. The 2002 study of the Women's Health Initiative was an observational study of healthy postmenopausal women (natural and surgical menopausal women included), whilst the 2004 study was a randomised study of women who had undergone a hysterectomy. All these conditions, which are alleviated by HRT, are highly ranked as the causes of deaths among women 55 years or older (Pritchard and Sazuka, 1995). In summary it seems that the recognition of some of the benefits of hormone therapy has not only led to it being used widely amongst postmenopausal women, but its use has also led to the improvement in many women's quality of life (Steinberg *et al.*, 1990; DiSaia, 1993; Pritchard and Sazuka, 1995). The use of HRT is an individual choice and the risks of breast cancer need to be weighed up against the benefits of reduced menopausal symptoms and reduced risk of fractures attributable to bone loss.

Other studies have suggested that HRT use may increase the risk of some cancers, especially endometrial, ovarian, cervical, and breast cancers (Whitcroft and Stevenson, 1992). It is widely accepted that oestrogens promote the growth of mammary tumours, although it has been suggested that taking exogenous oestrogens further increases the risk, especially if they are taken for long periods of time (Steinberg *et al.*, 1990; Colditz *et al.*, 1995; Newcomb *et al.*, 1995). The Collaborative Group on Hormonal Factors in Breast Cancer (1997) suggest that breast cancer risk increases with increasing duration of use of HRT amongst women who have ever used HRT ($p=0.003$, X^2 for trend across categories of duration =8.7). Research by Million Women Study Collaborators (2003) is

in agreement with his trend (RR 1.66, 95%CI 1.58-1.75, $p < 0.0001$). Early research by Colditz and colleagues (1990) have suggested an alternative view, there is no apparent effect of duration of use of oestrogens, but that the effect is due to the type of hormone therapy. In the prospective cohort study, Colditz and colleagues (1995) found that if the hormone therapy consists of a combination of oestrogens and progestin, the relative risk of breast cancer is significantly higher than if oestrogen alone is used (RR 1.41, 95% CI= 1.15-1.74; RR 1.32, 95% CI= 1.14-1.54 respectively) (Colditz *et al.*, 1995). Research by the Million Women Study Collaborators (2003) also suggests that there is a difference in breast cancer risk with the use of oestrogen only and oestrogen-progestin formulations, however these results are not affected by the type of oestrogen (equine oestrogen or oestradiol) or progesterone (medroxyprogesterone acetate, norgestrel, or norethisterone) in the formulation. The Women's Health Initiative (2002) are in agreement with these findings, but have also found that invasive breast cancer risk was increased by 26% in users of oestrogen plus progestin formulations whereas women using conjugated equine oestrogen had a 23% lower risk than those using placebos. Historically, progestin was added to hormone preparations to counteract the adverse effects of oestrogen (Colditz *et al.*, 1995). Colditz and colleagues (1995) believe that the addition of progestins does not reduce risk of breast cancer but rather progestins may enhance the proliferation of epithelial cells in the breast. If this is the case, then hormone preparations containing progestin may also increase the risk of benign proliferative epithelial disorders. Research examining the link between HRT and benign breast disease is fairly inconsistent to date, but this may be due to the assessment of all histological types of benign breast disease. It is accepted theory that some benign proliferative diseases have a greater predisposition to breast cancer development than other histological types. So when exploring the risks attributable by HRT, for example, relationships should be assessed according to the degree of atypia (Rohan and Miller, 1999). Having stated this, there is no evidence that HRT use increases the incidence of benign breast abnormalities, however routine breast examination of HRT users may increase the incidence of detection, and for some women the benefits of HRT far outweigh the risks (Colditz *et al.*, 1990; Tzingounis *et al.*, 1996). In contrast to this view, mammographic specificity has been found to differ according to when the mammogram was taken during the menstrual cycle and whether exogenous hormones were used. Research by Ursin and colleagues (2001) suggests that for women not using

exogenous hormones, the mammogram was more sensitive during the follicular phase. Women taking hormone replacement therapy however, tended to assume the hormone patterns of premenopausal women and thus have dense breasts during the first half of the month taking HRT. A study by Harvey and colleagues (1997) found that any breast masses found in the breast of women taking HRT had reduced in density with short-term cessation of HRT.

1.3.5 Lifestyle

The way in which we live may have some influence on the types of ailments we may expect to face and die of. Where we live may govern the types of exposures we may have to chemicals, or even access to certain dietary requirements and access to health care services. Lifestyle factors includes the geographical location in which we live, the types and amount of dietary requirements that are consumed, and body mass. The following factors presented are much debated factors for influencing the risk of breast cancer. As the focus of the current work is on determining the potential for organochlorine pesticides to contribute to breast cancer risk, these lifestyle factors need to be addressed in order to determine how these pesticides could impose risk.

1.3.5.1 Geographical location.

There are striking variations in breast cancer incidence and mortality rates throughout the world. Breast cancer appears to be a condition experienced by women of Westernised countries (such as North America, Northern Europe, and to a lesser extent, Australia) but the incidence and mortality data are either incomplete or are low for women in parts of Asia (Table 9) (Waterhouse *et al.*, 1982; Kricke and Jelfs, 1996).

Table 9, Age standardised incidence and mortality rates of breast cancer per 100,000 woman-years from women in Australia between 1986-1995 by country of birth.

Country of birth	Age standardised rate rate of incidence*	Age standardised rate of mortality*
Egypt	82.0	22.9
United States	78.0	26.5

South Africa	77.2	21.9
Australia	71.9	20.9
Ireland	70.9	13.4
Scotland	69.9	21.3
Netherlands	66.2	8.8
Lebanon	63.2	18.0
Italy	56.7	18.1
Malta	53.4	7.7
Greece	52.8	15.6
Vietnam	49.0	8.0
Turkey	47.0	19.4
China	39.2	10.2

*Age standardised to the World Standard Population

(Adapted from Kricker and Jelfs, 1996)

The variations in mortality and incidence rates cannot be explained by one factor alone, rather, international differences are most likely the result of the interplay of many factors, of which may include sociocultural differences, biological and environmental factors (Mayberry and Stoddard-Wright, 1992; Weiss *et al.*, 1995). Many studies have investigated these international differences by either examining the incidence and mortality rates of indigenous populations compared to white women, or by looking at the factors associated with different generations of migrants from low risk countries to high risk countries (Haenszel and Kurihara, 1968; McMichael and Giles, 1988; Mayberry and Stoddard-Wright, 1992; Weiss *et al.*, 1995). A report by Weiss and colleagues (1995) investigated the differences in breast cancer mortality and the factors that contribute to these differences among African American women, white American women and Hispanic women. They found that the African American women tended to have a poorer prognosis, partly because they presented with symptoms of a later stage of breast disease, which also meant that these women had a higher mortality from breast cancer than the other women studied (Axtell and Myers, 1978; Chen *et al.*, 1994). The poor prognostic indicators included a more advanced disease, a greater tumour size, a greater involvement of lymph nodes and a greater likelihood of oestrogen receptor negativity (Table 10) (Weiss *et al.*, 1995).

Table 10, Comparison of prognostic indicators for breast cancer between African American women, white Americans, and Hispanic women.

Prognostic Indicator	African Americans	Whites	Hispanics
Stage of disease			
Stage II or greater	72%	43%	42%
Tumour size	2.4 cm	1.8 cm	1.7 cm
Positive lymph nodes	44%	33%	29%
Body weight (kg)	82.7	65.9	67.7

(Adapted from Weiss *et al.*, 1995)

However, a higher mortality from breast cancer among the African American women may be the result of higher levels of obesity, a lower socioeconomic status (as dictated by income and level of education attained) or even the lack of access to health care, such as lack of breast mammography use compared with white women (Lipworth *et al.*, 1970; Farley and Flannery, 1989; Cella *et al.*, 1991; Wells and Horn, 1992; Hunter *et al.*, 1993; Weiss *et al.*, 1995). The same sorts of parallels can be drawn with the indigenous Australian women compared with white Australians. However the data for breast cancer incidence and mortality amongst indigenous Australian women is fairly incomplete, as data have only been collected from the Northern Territory, Western Australia and South Australia (Kricker and Jelfs, 1996). A recent report of Australia's Health (2000) however, suggests that the data collected from these areas is of reasonable quality even though the estimations of size and composition of indigenous populations is not available. The report also suggests that there are marked differences between the health of indigenous and non-indigenous populations across a broad range of health indicators (AIHW, 2000).

In Australia, the large influx of migrants between 1950 and 1975, and their respective mortality rates from breast cancer, gives important evidence that environmental factors may be involved in the later stages as a promoter of breast carcinogenesis (McMichael and Giles, 1988). The work of McMichael and Giles (1988) reported that the mortality rates of women migrating to Australia from Southern Europe (Italy, Greece, Yugoslavia

and Malta) converged upon the rates of Australian-born women with a longer duration of residence in Australia. The increase in the rate of carcinogenesis following migration to Australia may be influenced by the changes in environment, hormonal factors and/ or modifications to diet in the host country (McMichael and Giles, 1988). In a more classic experiment, the death rates from breast cancer among women migrating from Japan to the United States, where the risk of breast cancer is high, were far greater than women remaining in their birth country (Haenszel and Kurihara, 1968). Of interest is the finding that the mortality rate of the US Japanese women still remained far below that of the white American women (Haenszel and Kurihara, 1968).

The incidence and mortality trends of breast cancer vary internationally as a result of a number of factors. In Australia the percentage of women having regular mammograms has increased between 1988 and 1994 (Smith *et al.*, 1998). This increase in the use of screening did not translate to an increase in mortality, rather, it reflected early diagnosis of a cancer or the detection of lesions which otherwise would not have been detected (Smith *et al.*, 1998). The frequent detection of less aggressive cancers with little nodal involvement, coupled with improved treatments, suggests that the mortality rates from breast cancer would be reduced, although this is still an issue of debate for remote communities of indigenous women in Australia and other parts of the world (Smith *et al.*, 1998).

Women migrating from a low risk country to a high risk country are likely to be affected by changes in the environment and possibly lifestyle, which could see them adopting the breast cancer risk of the host country. However research suggests that most first generation migrants maintain the cuisine of their country of birth, but this may not be the case for second and third generation migrants who may adopt some of the lifestyle of the host country (Haenszel and Kurihara, 1968).

1.3.5.2 Weight and Height.

Research suggests that weight, height and other body mass indicators are related to breast cancer risk. In women there are three main lifetime periods in which substantial weight gain occurs; during menarche fatty deposits accumulate in the hips and buttocks; and during pregnancy and menopause there is an increase in body fat distribution

centrally and in the breasts (LeMarchand *et al.*, 1988; Ley *et al.*, 1992; Smith *et al.*, 1994). Some researchers suspect that a greater weight gain during adolescence, accompanied by little physical activity, is related to a greater risk of breast cancer. However this effect may be the result of diet itself rather than adiposity (LeMarchand *et al.*, 1988). Conversely a low weight such as that attained by athletes, ballet dancers and some immigrant populations relates to an altered regulation of hormone secretion, thus lowering the levels of circulating oestrogen and lowering the risk of breast cancer (Warren, 1980; Frisch *et al.*, 1987). The same sort of trend is also apparent for women near the onset of menopause, where excess weight translates to a greater potential for breast cancer development (denTonkelaar *et al.*, 1994; Kumar *et al.*, 1995). However the work of Lubin and colleagues (1985) suggest that overweight women with an early onset of menopause are at no greater risk compared with overweight women with a late age at menopause. However if the women are postmenopausal and are gaining weight (regardless of whether they are overweight or in the ideal weight range) there is a positive correlation with breast cancer risk (Lubin *et al.*, 1985).

The association between weight and risk of breast cancer in pregnant women and postmenopausal is very similar to the trends of postmenopausal women. Accumulation of body fat increases during pregnancy, however some research suggests that the degree of weight gain, as influenced by the level of exercise during this period, may be a better predictor of breast cancer risk (Bernstein *et al.*, 1994; Smith *et al.*, 1994). Kumar and colleague's (1995) research has found that women with low levels of activity during their reproductive years, were more likely to have a higher body fat percentage and low metabolic rates, thereby increasing the likelihood of the gradual gaining of weight and a higher breast cancer risk. The study of Kumar and colleagues (1995) also reported the mean weight of women with breast cancer compared to control women, over different life-stages. Over all age ranges tested the women with cancer recorded higher mean body weights than the controls, which also translated to a significant ($P < 0.001$) difference in percentage body fat (Kumar *et al.*, 1995). Similar trends were seen for women less than 60 years of age at diagnosis in a Dutch study (denTonkelaar *et al.*, 1994).

There are several speculated mechanisms, which may explain how body mass indicators increase the likelihood of breast cancer. The most widely accepted of these theories is the fact that higher body mass relates to high percentage of fatty deposits. These increases in body fat affect hypothalamic function and can initiate the extra production of oestrogens, either by increasing the availability of biologically active oestradiol and/or by increasing the concentrations of oestrogen receptors (Lubin *et al.*, 1985). These changes in the proportions of oestrogens could possibly affect hormone related cycles, such as menstruation, pregnancy, lactation and menopause, and thus be associated with increased breast cancer risk (Zumoff, 1982; Lubin *et al.*, 1985; Kumar *et al.*, 1995).

Research has shown that a high body mass is related to low levels of serum sex hormone binding globulin (SHBG), high levels of free testosterone, as well as increased levels of oestrogen (Schapira *et al.*, 1991; Kaye *et al.*, 1991; Stoll and Secreto, 1992). The level of SHBG will determine the amount of available oestrogen that can interact with hormone responsive tissues, such as the breast (Schapira *et al.*, 1991; Schapira *et al.*, 1994). Results of Schapira and colleagues work (1991) suggest that the levels of SHBG were lower in women with breast cancer, and possibly resulting in higher levels of oestrogen, compared to control women. The levels of SHBG were reduced with increasing amounts of fatty deposits localised to the breast, however SHBG levels were increased if there was substantial weight loss at any time (Schapira *et al.*, 1994; Enriori *et al.*, 1986).

Obesity is most likely a marker of the excessive intake of dietary fat and is thus a potentially modifiable risk factor for breast cancer and other dietary related diseases (Kumar *et al.*, 1995; Zhang *et al.*, 1995). Women, who are overweight and thus may be at increased risk of breast cancer, may benefit from modifying their diet and increasing their levels of physical exercise, to reduce their risk. As body mass is related to diet, there is the potential for public health intervention programs to reduce the risk of breast cancer and other diseases that can benefit from diet modifications.

Body mass is directly related to diet and levels of activity. Animal experimentation and some human epidemiological studies suggest that nutrition may play an important role in breast carcinogenesis (Graham *et al.*, 1982; Rohan *et al.*, 1988; Howe *et al.*, 1990;

Ingram *et al.*, 1991). In studies that investigated the links between dietary intake and breast cancer, particular emphasis was focused on the intake of dietary fat, especially the types and amounts consumed. Although the research surrounding the hypothesis that a high fat intake increases the risk of breast cancer is controversial and unsubstantiated, the current research needs to address this hypothesis as it may provide evidence for how organochlorine pesticides may be introduced into the body. Research by Howe and colleagues (1990) suggested that the risk of breast cancer attributable to a high intake of dietary fat increased, especially amongst postmenopausal women. Among postmenopausal women there was a highly significant relationship between total fat, saturated fat and mono-unsaturated fat consumption and breast cancer risk (Howe *et al.*, 1990). There was no increase in risk with the consumption of poly-unsaturated fats, nor was there any relationship evident for premenopausal women ($p = 0.21$) (Table 11).

Table 11, Relative risk estimates for the dietary fat intake of postmenopausal women, and the risk of breast cancer.

Type of fat	Relative risk	95% CI	Significance
Total fat	1.48	-	$p = 0.0002$
Saturated	1.46	1.23 - 1.72	$p < 0.0001$
Mono-unsaturated	1.41	1.19 - 1.67	$p < 0.0001$
Poly-unsaturated	1.25	0.91 - 1.71	$p = 0.16$

(Adapted from Howe *et al.*, 1990)

The work by Richardson and colleagues (1991), however, found that premenopausal women had a significantly increased risk of breast cancer if they consumed large amounts of mono-unsaturated fats ($p = 0.025$), whilst postmenopausal women followed the same trends as those already discussed. Some other studies have shown no correlation with menopausal status, dietary fat consumption and breast cancer risk (Graham *et al.*, 1982; Willett *et al.*, 1992; Willet and Hunter, 1994).

Dietary intake is a major factor influencing international differences in breast cancer incidence. In particular, studies have focused on women migrating from a low incidence country (Japan) to a high incidence country (United States). Research by Carroll and

Hopkins (1979) investigated the dietary fat consumption patterns of Japanese women and US women. They found that Japanese women consumed more unsaturated fats than the Americans, who had a higher intake of saturated fats. Also, the Japanese, as well as Eskimo populations, tended to consume more dietary lipids from marine sources, which have a high percentage of omega-3 fatty acids (Karmali *et al.*, 1984; Rose and Connolly, 1993). It is suspected that foods rich in omega-3 fatty acids, such as fish and brown seaweed, suppress mammary tumour growth by blocking the tumour promoting properties of carcinogens or by inhibiting prostaglandin synthesis (Rao and Abraham, 1976; Hillyard and Abraham, 1979; Karmali *et al.*, 1984; Carroll and Braden, 1985; O'Connor *et al.*, 1985; Gabor and Abraham, 1986; Karmali, 1987; Kort *et al.*, 1987). Conversely, foods rich in omega-6 fatty acids, such as meat products, which are consumed at high levels amongst American women, are thought to stimulate mammary tumour growth, and may account for the high breast cancer incidence rates in the US (Rose and Connolly, 1993).

A major source of fat in some diets is derived from dairy and meat products and may contribute to international differences in breast cancer mortality rates (Armstrong and Doll, 1975). Milk and milk products contain fat, calcium, vitamin A and riboflavin, however their contribution to the diet and breast cancer risk has resulted in conflicting reports (Ursin *et al.*, 1990). Research by Ursin and colleagues (1990) found that there was a weak positive association with milk consumption and cancers of the reproductive organs, including the breast (Odds ratio, 1.5, 95% CI; 0.9-3.2). However a report from Knekt and colleagues (1996) found that the total amount of milk fat in the diet was not significantly correlated with breast cancer risk. If anything, Knekt and colleagues (1996) found a weak inverse association between milk consumption and breast cancer. A report by van't Veer and colleagues (1989) also confirmed this relationship. Possible mechanisms by which milk consumption is thought to reduce breast cancer risk have been proposed. Newmark and colleagues (1984) believe that calcium ions derived from milk provide protection against breast cancer by binding fatty acids and bile acids in insoluble compounds, and along with lactose, may inhibit hormonal function. Van't Veer and colleagues (1989) believe that the lactic acid producing bacteria used in the fermentation of milk may interfere with other gut flora, thereby altering the metabolism of oestrogen and bile acids, and thus having a favourable impact on the immune system

and breast cancer risk. Other researchers are in agreement (Shahani and Ayebo, 1980; Miller *et al.*, 1983). The findings of a New York study suggest that the effect of dietary fat consumption on breast cancer risk cannot be evaluated without the consideration of cholesterol intake, synthesis and feedback mechanisms, since steroid oestrogens can be derived from cholesterol and bile acids (Papatestas *et al.*, 1982). This research and others, also suggest that obesity may influence the level of faecal steroids, as it is associated with increased endogenous cholesterol synthesis, and in older women has been attributed to increased oestrogens from the conversion of androstenedione in adipose tissue (Brown and Strong, 1965; Papatestas *et al.*, 1982; Miller *et al.*, 1983). Reddy and colleagues (1975) suggest however, that high fat diets affect the composition of intestinal flora, thereby altering their propensity to neutralise sterols and bile acids for faecal excretion. Research by Le and colleagues (1986) has suggested that the degree of breast cancer risk is dependent on the amount of cream in the milk and the frequency of milk consumption. Consumption of milk and dairy produce is also higher in agricultural areas and its consumption is dependent on occupation (Rohan and Bain, 1987; Knekt *et al.*, 1996).

There are other mechanisms by which dietary fat consumption is thought to increase tumourigenic potential. Both saturated and unsaturated fats are thought to act during the promotional stages of carcinogenesis and this promotion is largely dependent on the amounts and sources of fat in the diet (Carroll and Hopkins, 1979; Carroll, 1986; Karmali, 1987). Poly-unsaturated fats rich in linoleic acids are thought to influence the metabolism of some chemical carcinogens; by either stimulating direct carcinogenesis; by concentrating the carcinogen in a particular area where it can illicit a particular response; and / or by total removal of the carcinogen (Tannenbaum, 1942; Hill *et al.*, 1971). Also oestrogens may be synthesised from steroids found in adipose tissue, as a consequence of a high fat diet, which would affect the regulation of hormonal events and immunological responses (Hill *et al.*, 1971; Mertin and Hunt, 1976; Vitale and Broitman, 1981; Carroll, 1986; Richardson *et al.*, 1991). The composition of the gut flora is also dependent on the nature and amounts of dietary fat consumed. People on high fat diets tend to have intestinal flora that may produce carcinogens and oestrogens from faecal biliary steroids, thereby altering the entero-hepatic cycling of steroid

hormones and increasing the likelihood of carcinogenesis (Hill *et al.*, 1971; Le *et al.*, 1986).

Other studies have also investigated the links between dietary fibre, alcohol consumption and cigarette smoking, and the risk of breast cancer. Research has consistently suggested that there is an inverse relationship between dietary fibre consumption and breast cancer risk (Howe *et al.*, 1990; Baghurst and Rohan, 1994; Baghurst and Rohan, 1995). Research by Howe and colleagues (1990) found that the relationship between dietary fibre intake and breast cancer was most predominant among postmenopausal women ($p = 0.002$), although the difference between pre- and post-menopausal women was not statistically significant ($p > 0.05$).

It is suspected that fibre rich foods interfere with the entero-hepatic cycling of oestrogens by encouraging the development of bowel microflora. This in turn would enhance the binding of unconjugated oestrogens, thereby reducing the oestrogen's bioactivity and encouraging their elimination as faecal waste (Goldin *et al.*, 1982; Shultz and Howie, 1986; Rose, 1990). Research, which reported on the diets of vegetarian and omnivorous women found that vegetarian women consumed higher quantities of dietary fibre which in turn prevented the conjugation and reabsorption of oestrogens (Goldin *et al.*, 1982). Research by Adlercreutz and colleagues (1986) has also found that a high fibre diet (containing foods rich in isoflavonoids and lignans) stimulates the synthesis of sex hormone binding globulin, which in turn reduces the bioactivity of oestrogen and the availability of oestrogen binding sites. Thus, isoflavonoids and lignans act as anti-oestrogens.

Alcohol consumption is one of the only established dietary factors that has been associated with breast cancer risk. Research by Longnecker and colleagues (1988) found that light alcohol intake was associated with a weak to modest correlation with breast cancer risk, whilst intakes of 24 grams per day or more, was associated with a significant increased risk. The work of Rohan and Bain (1987) and Talamini and colleagues (1984) also found an increased risk of breast cancer amongst drinkers compared with non-drinkers. The risk was also elevated among women who drank most frequently (greater than one litre per day) and who drank more than one type of

alcoholic beverage (multivariate relative risk; 7.6 at 95% confidence intervals: 3.8-15.2) (Talamini *et al.*, 1984). However Rohan and Bain (1987) did not control for other lifestyle factors such as diet, socioeconomic status, frequency of cigarette smoking and other dietary and body mass indicators, which are thought to confound the affects associated with alcohol consumption. Research by Wynder and colleagues (1977) suggested that excessive alcohol and tobacco consumption in combination are associated with a powerful carcinogenic effect on the digestive tract. Research by the Collaborative Group on Hormonal Factors in Breast Cancer (2002) suggest that the relative risk of breast cancer was increased with increasing consumption of alcohol, both in never- and ever-smokers and the magnitude of the increase was the same in each group (7.1% increase in relative risk of breast cancer for each additional 10g of alcohol consumed per day; 95%CI 5.5-8.7%, $p < 0.00001$). Early research by Wynder and colleagues (1977) suggest that the risk associated with this consumption of alcohol depends on the type of alcohol consumed and the duration of smoking. People consuming hard liquors such as whisky, in excess, and heavily smoking were at the highest risk of disease, however this risk decreased with lower levels of consumption (Wynder *et al.*, 1977). Alcohol has been correlated with higher levels of serum high-density lipoproteins, and thus in moderation, alcohol may be beneficial for lowering heart disease (Gordon and Kannel, 1983; Colditz *et al.*, 1985). The exact mechanism in which alcohol exerts its effect on breast cancer development is not well understood. Talamini and colleagues (1984) have suggested that alcohol may induce changes in the liver, which in turn may affect oestrogen metabolism or may affect the level of steroid binding globulins. Williams (1976) suggests however, that the association between alcohol consumption and breast cancer may be related to the increased secretion of pituitary stimulated hormones, such as prolactin and thyroid stimulating hormone, which would increase mitotic activity in target tissues, and hence lead to an increased susceptibility to malignancy. A small randomised trial by Dorgan and colleagues (2001) suggest that sex hormone levels may be increased after consumption of 30g of alcohol per day for 8 weeks, which are levels consistent with a clear excess risk of breast cancer.

There are several problems associated with the information obtained from dietary questionnaires that must be taken into account, in order to test for variations between study groups. Questionnaires that do not provide specific data on portion sizes,

dramatically reduce any variability that may be apparent between test groups, in estimated consumption (Graham *et al.*, 1982; Lubin *et al.*, 1989). Subsequently there is also the problem with overestimation and underestimation of amounts and frequency of consumption of particular foods (Graham *et al.*, 1982; Howe *et al.*, 1990). Research by Rohan and colleagues (1988) suggests that if the study subjects have been diagnosed with cancer and have a detailed knowledge of the effects of certain foods on disease processes, such as dietary fats on breast cancer, they are more likely to underestimate their consumption. Conversely, there is the perception that if the food is high in nutrients and will prevent disease, some people are likely to overestimate their consumption. It is likely that dietary consumption during childhood and adolescence may affect breast cancer risk decades later, which poses a problem with long term dietary recall (Graham *et al.*, 1982; Rohan *et al.*, 1988; Lubin *et al.*, 1989; Willett and Hunter, 1994). Recalling past dietary patterns is difficult for a person who has changed their diet as a result of diagnosis of disease. However, research by Graham and colleagues (1982) suggests that there is some consistency in diet throughout life.

1.4 ORGANOCHLORINE PESTICIDES

As alluded to in the previous part of this thesis, there is still a percentage of breast cancer cases that cannot be explained by the known risk factors. There is the possibility that the unaccountable risk may be explained by the presence of environmental contaminants. Many researchers have found that organochlorine pesticides are more abundant in the adipose tissue and blood of breast cancer patients, than in controls, and thus may account for some of the unexplained risk factors. Other findings however have not found any association between blood or tissue organochlorine levels and breast cancer. This section of the thesis will provide a historical review on the use of these pesticides and their implications on wildlife and human health.

Organochlorines is the broad term given to a group of chemicals with "pesticidal" activity, which, within their chemical structures contain one or more carbocyclic rings surrounded by chlorine atoms (Figures 2, 3, and 4). It is the nature of the bonds between

carbon, hydrogen and chlorine that makes these pesticides chemically stable and thus very persistent in the environment, and in wildlife and humans. When these chemicals were marketed for use as insecticides they were thought as being cheap to manufacture, to be effective against numerous insect pests and "apparently safe" to man and other warm-blooded animals (Hassell, 1990). Falling under this umbrella term, are three main families of pesticides; (I) the DDT family (I,I,I-trichloro-2,2-bis(4chlorophenyl)ethane), (II) the HCH family (hexachlorocyclohexane), and (III) the cyclodiene family (Hassell, 1990). The following is a description and history of use for each family of pesticides.

1.4.1 DDT family

Over one hundred years ago, the first molecules of DDT were synthesised by a German chemist by the name of Othmar Zeidler (Carson, 1962; Metcalf, 1973). Othmar Ziedler's work on DDT formed an important part of his doctoral thesis, however it was not until some 65 years later that the insecticidal properties of DDT were recognised (Metcalf, 1973). The magnitude of this discovery won the discoverer, Dr Paul Muller, the Nobel Prize in 1948 (Carson, 1962; Metcalf, 1973). There was an immediate interest in a chemical which was cheap to manufacture, which was apparently safe to administer to skin on humans and other mammals, and was persistent against different arthropods for months after application (Metcalf, 1973; Hassell, 1990).

For this reason DDT proved to be an important control mechanism for vector-borne diseases such as typhus, river blindness, yellow fever and for the eradication of malaria (Carson, 1962; Metcalf, 1973; Hassell, 1990). The World Health Assembly, in 1955, implemented a proposal to eradicate malaria by house spraying with DDT, and some 20 years later malaria was pronounced eradicated in 37 countries of the world (WHO, 1972). Also from this program, approximately 50 million human lives were saved and approximately one billion illnesses were prevented by the household spraying with DDT (Knipling, 1953). DDT was also used in the Second World War to dust thousands of soldiers, refugees and prisoners to combat lice (Carson, 1962; Metcalf, 1973). Apart from these control measures of vector-borne diseases, DDT was also extensively used for agricultural and other household purposes. It became the standard remedy against

many destructive pests in banana and cotton plantations, and in apple and pear orchards, and was the standard agent for moth-proofing households (Standards Branch, Queensland Department of Primary Industries, 1980).

The factors that make DDT effective as an insecticide have also lead it to become a hazard to wildlife and possibly to man. The potential hazards of DDT led to its demise and total prohibition of use. In Australia DDT was prohibited from all uses in 1987. DDT is soluble in many organic solvents including natural oils and body lipids, it has very low water solubility, low vapour pressure and is very stable to photo-oxidation.

The three main ways DDT can be degraded is by dehydrochlorination to form DDE, reductive dechlorination to form DDD or by oxidation to form dicofol (Hassell, 1990). The breakdown products, in part, can determine the persistence and the efficacy of DDT in the environment. Studies have shown that DDE is the major residue of the original DDT molecule found in animal tissue. Research has found that insect resistance to DDT was correlated with the insect's ability to degrade DDT to the less insecticidal metabolite DDE (Knipling, 1953; Hassell, 1990). Examples of resistant mosquitoes and body louse were found in Korea, which had potentially serious ramifications for the transmission of typhus, relapsing fever and other vector-borne diseases (Knipling, 1953). A metabolite of DDT, dicofol, is able to exert insecticidal properties, and was commonly used as an acaricide (Standards Branch, Queensland Department of Primary Industries, 1972; Standards Branch, Queensland Department of Primary Industries, 1980).

Table 12; Comparison of organochlorine pesticide concentrations in blood and adipose tissue between breast cancer and control groups from various countries.

Authors	Country	Number of participants	Type of medium analysed	Pesticides measured	Conclusive remarks
Unger <i>et al.</i> , 1984	Denmark	2 data sets; deceased: 18 cancers, 35 non-cancers new diagnoses: 14 cancers, 21 non-cancers	Breast adipose tissue	DDE, PCBs	- Higher PCB levels in deceased cancers versus controls - No significant difference between data sets for DDE and PCB content
Mussalo-Rauhamaa <i>et al.</i> , 1990	Finland	44 cancers, 33 postmortem or accident fatality controls	Breast adipose tissue	DDTs, HCB, PCBs, HE, β HCH	- No significant differences in levels of DDTs, PCBs, HCB, or HE between cancers and controls - Significant difference in β HCH levels between cancers and controls ($p=0.026$)
Falck <i>et al.</i> , 1992	USA	20 cancers, 20 benign breast disease controls	Breast adipose tissue	HCB, HE, DDTs, OXC, PCBs, TNON	- DDTs and PCBs significantly different between cancers and controls - No differences with other pesticides measured
Wolff <i>et al.</i> , 1993	USA	58 cancers, 171 matched controls	Serum	DDE, PCBs	- Mean levels of DDE higher in cancers than controls ($p=0.03$) - PCB levels not significantly different

Authors	Country	Number of participants	Type of medium analysed	Pesticides measured	Conclusive remarks
Dewailly <i>et al.</i> , 1994	Canada	20 invasive adenocarcinomas, 17 benign breast disease controls	Breast adipose tissue	DDE, PCBs, β HCH, HCB, chlordanes	<ul style="list-style-type: none"> - Significant difference in DDE levels between ER+ cancers and controls - No differences with other pesticides measured
Krieger <i>et al.</i> , 1994	USA	150 cancers, 150 matched controls	Serum	DDE, PCBs	<ul style="list-style-type: none"> - No differences in DDE or PCB levels between cancers and controls - Significantly higher levels among blacks and Asians versus whites
Hunter <i>et al.</i> , 1997	USA	236 cancers, 236 controls	Plasma	DDE, PCBs	<ul style="list-style-type: none"> - Non-significantly lower levels of DDE and PCBs in cancers than controls
Schechter <i>et al.</i> , 1997	North Vietnam	21 invasive adenocarcinomas, 21 benign non-hyperplastic controls	Serum	DDTs	<ul style="list-style-type: none"> - No increase in relative risk of cancer with increasing concentrations of pesticide
Van't Veer <i>et al.</i> , 1997	Europe *	265 post-menopausal cancers, 341 matched controls	Buttock adipose tissue	DDTs	<ul style="list-style-type: none"> - Lower DDE levels in cancers compared to controls

Authors	Country	Number of participants	Type of medium analysed	Pesticides measured	Conclusive remarks
Gutties <i>et al.</i> , 1998	Germany	45 cancers, 20 benign breast disease controls	Breast adipose tissue	DDTs, HCB, PCBs, β HCH	- Significant difference in DDE and some PCB levels between cancers and controls ($p=0.017$) Levels of other pesticides measured lower in cancers than controls
Liljegren <i>et al.</i> , 1998	Sweden	43 cancers, 35 controls	Breast adipose tissue	PCBs, DDE, HCB	- No significant differences in DDE and PCB levels between cancers and controls Increased OR for post-menopausal women with ER+ tumours and HCB levels (OR, 7.1; 95% CI: 1.1-45)
Moysich <i>et al.</i> , 1998	USA	154 post-menopausal cancers, 192 community controls	Serum	DDE, HCB, PCBs	- No difference in DDE or HCB levels between cancers and controls
Bagga <i>et al.</i> , 2000	USA	73 cancers, 73 reduction mammoplasties	Breast adipose tissue	DDTs	- DDE levels significantly higher in cancers than controls ($p=0.006$) - Total DDT (DDT, DDE, DDD) weakly significant ($p=0.04$)

Key:

* Europe: Germany, Netherlands, Northern Ireland, Switzerland and Spain; DDE: dichlorodiphenyldichloroethylene; DDTs: total DDT (DDE, DDD, DDT); PCB: polychlorinated biphenyls; HCB: hexachlorobenzene; HE: heptachlor epoxide; β HCH: beta isomer of hexachlorocyclohexane; OXC: oxychlordane; TNON: trans nonachlor; chlordanes: combined isomers of chlordanes; ER+: oestrogen receptor positive; OR: odds ratio.

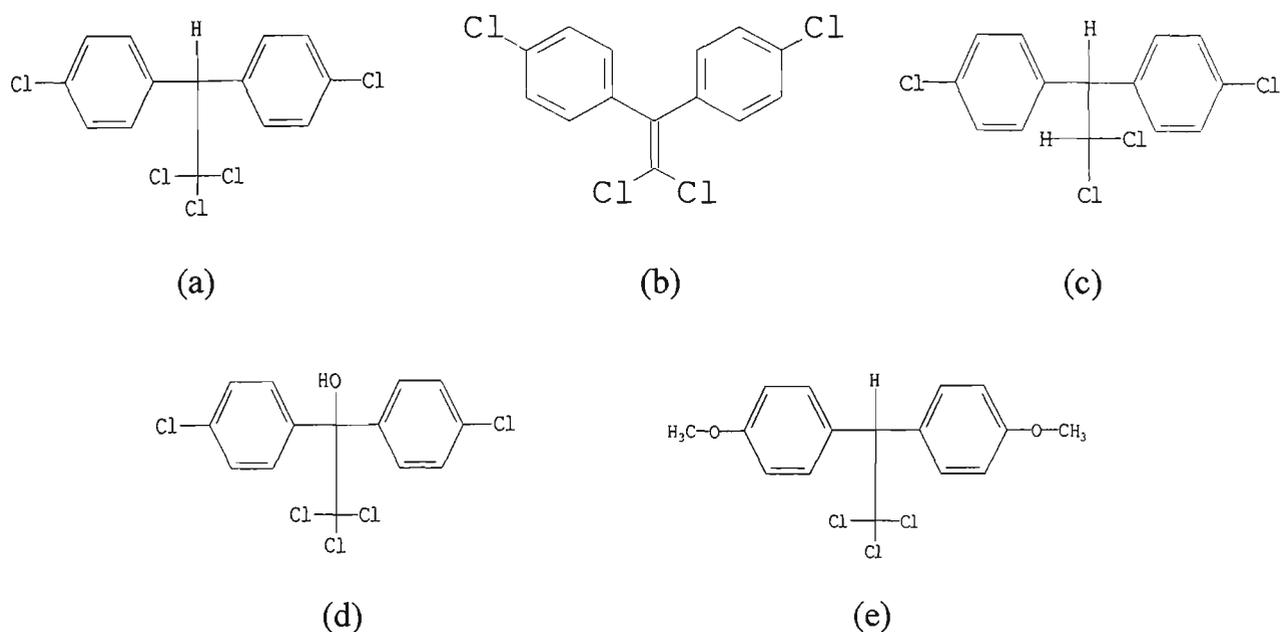


Figure 2, Chemical structures of pesticides belonging to the DDT family. (a) DDT, (b) DDE, (c) DDD, (d) dicofol, (e) methoxychlor

However, the fact that these pesticides are highly persistent and relatively non-specific, has led to the accumulation of toxic effects in higher animals. Research by Woodwell and colleagues (1967) found that the levels of DDT and its metabolites, increased with increasing trophic level; from 0.04 parts per million in plankton to 75 parts per million in ring billed gulls; thus illustrating the propensity of persistent chemicals to accumulate in non-target species. Lundholm (1988) explored the idea that pesticides such as DDT and its metabolites may induce toxic effects in birds; resulting in infertility or altered egg formation, a reduction in eggshell thickness and thereby a reduced hatchability and survival of nestlings. By administering isomers of DDT and DDE to two varieties of ducks, Lundholm (1988) found that the isomers of DDE were able to inhibit calmodulin-dependent events of the shell gland mucosa. From the same study, it was found that DDE and DDT were able to inhibit the specific binding of progesterone to its receptor in a dose-response manner, thereby resulting in a major reproductive disturbance. Similar effects have been described by other researchers (Bitman *et al.*, 1968; Wurster and Wingate, 1968; Olsen and Olsen, 1979). Consistent with these observations is the suggestion that female birds may be more resistant to the effects of DDT poisoning because of the calcium-mobilising action of oestrogenic hormones (Wurster and Wingate,

1968). However with extreme exposure, DDT interferes with the normal calcification processes, producing symptoms similar to those resulting from calcium deficiency (Wurster and Wingate, 1968).

In mammalian species reproductive disturbances have also been reported and linked with pesticide exposure. Various studies on marine mammals, have found that certain populations have reduced as a result of environmental contamination with organochlorines (Jensen *et al.*, 1979; Bergman and Olsson; 1985). Studies on the effects of DDT on seal populations in the Baltic Sea have found that the numbers of seals fell from greater than 3000 to less than 500, during a period when there was frequent use of pesticides (Jensen *et al.*, 1979). This decline in population was accompanied by a decline in pup production (Jensen *et al.*, 1979). Bergman and Olsson (1985) found that the detection of DDT in the tissues of female seals was associated with a variety of reproductive problems, and damage to the endocrine, genital and urinary systems. Similar studies in Beluga whales (*Delphinapterus leucas*) found that whales exposed to organochlorine pesticides exhibited hermaphroditic characteristics, had little ongoing ovarian function and were immunosuppressed.

It was only when these types of studies were brought to the attention of the public, by the publication of *Silent Spring* in 1962, that the potential dangers of DDT and other chemicals to human health were recognised (Carson, 1962). Some of the first studies to investigate the links between DDT and human health were conducted using occupationally exposed people, such as those people living in agricultural areas or pesticide applicators. Research by Guardino and colleagues (1996) suggests that the mean levels of DDT and its analogues were significantly higher in occupationally exposed workers (mean level of 10.4 µg/ L for total DDT) than those found in controls (mean level of 4.1 µg/ L for total DDT). Research by Laws and colleagues (1967), also found concentrations of DDT in the tissues of males working in DDT manufacturing plants, however could not attribute any ill-effects on health to the exposure. Conversely, several studies have investigated the links between DDT exposure and the health of males. DDT has been implicated in increasing the risk of several cancers, Hodgkin's disease, Parkinson's disease syndrome, affecting neurological and behavioural function, optic

atrophy, hyperestrogenism and reduced sperm counts (Wiklund *et al.*, 1989; Baker and Wilkinson, 1990; Sharpe and Skakkebaek, 1993; Fleming *et al.*, 1994). Studies reported by Sharpe and Skakkebaek (1993) suggest that sons of women exposed to DES (diethylstilbestrol) and other exogenous oestrogens during pregnancy, may have an increased risk of reproductive disorders. Exogenous oestrogens can compromise the production of testosterone, thereby resulting in impaired masculinisation of the reproductive tract and genitalia (Sharpe and Skakkebaek, 1993). Exogenous oestrogen exposure during foetal and early neonatal life has also resulted in smaller testes in adults, which subsequently resulted in the reduced number of Sertoli cells and reduced the capacity to support spermatozoa (Sharpe, 1993). Research by Figa-Talamanca and colleagues (1993) also suggests that there is an increased risk of liver cancer (standard mortality rate = 571, 95% CI; 154-1463) coinciding with the peak use of organochlorine pesticides, especially DDT.

Research conducted on the effects of occupational pesticide exposure to women is fairly scarce. There is a commonly held perception that women would not have significant exposure to pesticides through farming and agricultural ventures (McDuffie, 1994). However, this is generally not the case. Internationally, a large proportion of those engaged in agriculture are women, and although they may not directly handle the pesticides, their exposure would be quite considerable (McDuffie, 1994). Women living or working in an agricultural setting would be exposed to pesticides by handling contaminated clothing, by handling or the ingestion of contaminated food or drinking water, or through being a bystander while pesticides are being applied (McDuffie, 1994). Studies by Stubbs and colleagues (1984) and Blair and colleagues (1993), and research reported by Baker and Wilkinson (1990), have investigated the links between the health of female agricultural workers and pesticide exposure. These studies have uncovered similar findings; marked increases in risk of several cancers including cervical, lymphatic, digestive, multiple myeloma and leukaemia. A study of agriculturally exposed women (where the women were living or working on a farm where insecticides or herbicides were used) to pesticides found a two-fold increased risk of multiple myeloma (Zahm *et al.*, 1992). Research by Eriksson and Karlsson (1992) found there was elevated relative risk of multiple myeloma with chronic exposure to DDT. An Italian study has also found an

elevated risk of ovarian mesothelial cancer (odds ratio = 4.4) attributable to herbicide exposure (Donna *et al.*, 1984). Research by Zahm and colleagues (1993) suggested that there is a statistically non-significant increase in risk of Non-Hodgkin's Lymphoma (NHL) attributable to the handling of pesticides (relative risk 1.2; 95% CI: 0.3-4.2). This trend was greatly marked with the use of chlorinated pesticides such as DDT (relative risk 1.7) (Zahm *et al.*, 1993). Zahm and colleagues (1993) also found that the relative risk of NHL varied depending on how, and for what purpose, the chlorinated pesticides were used, such that pesticides used on dairy cattle resulted in an odds ratio of 3.0 (95% CI: 0.9-9.3), compared with an odds ratio of 1.6 (95% CI: 0.5-4.3) for the use of pesticides on farm buildings or lots.

The most significant research efforts have investigated the effects of pesticide exposure on reproductive tissues. Research has suggested that chlorinated pesticides, in particular DDT, may have oestrogenic activity, as the chemical structures of DDT and 17β -oestradiol are very similar (Baker and Wilkinson, 1990). These similarities would enable DDT to interact with the oestrogen receptor or associated enzymes, with high affinity, and thus, potentially interfere with the hormone-responsive mechanisms of the reproductive system (Baker and Wilkinson, 1990). Levels of DDT and its analogues have been detected in the serum, adipose and breast milk of women (owing to its lipophilic nature), and have been implicated in increasing the risk of cancers of the reproductive system, especially breast cancer.

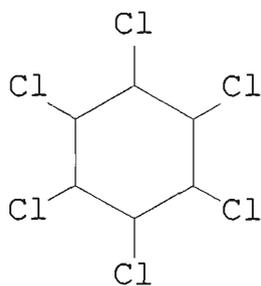
Overseas research, which has investigated the possible links between organochlorine exposure and breast cancer, has yielded conflicting results. Most research in this area has focused on the levels of DDT and its metabolites in breast milk, adipose tissue and blood, owing to its widespread use around the world. In five studies, reported in Table 12, there were significantly higher levels of DDT or its metabolites detected in cancer cases compared to control groups. However in seven studies no association was found between DDT levels detected and subsequent risk of breast cancer (Table 12). In an Australian context however, concentrations of these pesticides have been detected in various tissues but no links have been drawn with breast cancer risk or risk of other health problems. Research over time, suggests that the levels of DDT and its metabolites measured in

breast milk and adipose tissue have declined. A study by Wasserman and colleagues (1968) in Western Australia found average DDT levels of 9.3µg/mL in adipose tissue compared to a later study in New South Wales by Ahmad and colleagues (1988), where 3.72µg/mL levels were detected. Levels of DDT in breast milk followed the same trend over time. The concentration of DDT and its metabolites in breast milk were substantially lower in a 1990 study at 46ng/ml compared to 139ng/mL in a 1972 study both conducted in Victoria, Australia (Newton and Greene, 1972; Monheit and Luke, 1990). In lactating women, breast milk may be a major route for the elimination of lipophilic contaminants, thereby decreasing the body burden of contamination in these women. The detection of pesticide contaminants in human breast milk raised the awareness for the potential transfer of contaminants to breast-fed children. Research by Monheit and Luke (1990) indicated that, on the basis of pesticide levels detected in breast milk, the calculated daily intake of some pesticides by infants exceeded the World Health Organisation Acceptable Daily Intakes (WHO ADI). Stevens and colleagues (1993) also found that the intakes of some pesticides by some infants exceeded the ADI set by the World Health Organisation. However, Jensen (1983) suggested that although the intake of pesticides may be exceeded, the significance of this in terms of toxicity to the infant is relatively unclear. The ADIs are a measure of intake for adults and account for exposure over a lifetime, however infants only consume breast milk for a limited period of time, so ADIs may not be an appropriate measure of exposure (Jensen, 1983; Monheit and Luke, 1990). To date the only health effects reported in infants are those resulting from chronic exposures and include reduced body weight, slower psychomotor development and vitamin K deficiency causing haemorrhagic disease (Gladden *et al.*, 1988; Koppe *et al.*, 1989; Jacobson *et al.*, 1990). Of the levels of pesticides generally found in human breast milk in most studies, there is no evidence of deleterious effects on infants, and the potential benefits of breastfeeding far outweigh the risks. Another route of exposure for infants to contaminants is through the placenta before delivery, however the levels detected in placental tissue and cord blood have been reported as being substantially lower than that found in the mother's tissues (Eyster *et al.*, 1983; Sim and McNeil, 1992). Studies reporting the levels of contaminants in breast milk should therefore take into account the other routes of potential exposure to infants.

As presented earlier, a number of studies have determined the levels of pesticides in serum exclusively, whereas others have used adipose tissue. Significant relationships between DDE and breast cancer have been reported from research using blood components and adipose tissue, and the lack of association has also been reported for both blood and adipose tissue. A number of studies have tried to gauge which biological medium is the most reliable, in regards to determining overall body burden, and thus can be used as a biomarker for pesticide exposure in the future. Research by Eyster and colleagues (1983) measured the concentrations of pesticides in a variety of human tissues; serum, adipose tissue, breast milk, placenta, cord blood, biliary fluid and faeces; in an attempt to ascertain which tissue(s) reflect overall body burden. They found that polybrominated biphenyls (PBBs) were primarily stored in adipose tissue, even though levels were detected at lower concentrations in serum and the other tissues (Eyster *et al.*, 1983). Eyster and colleagues (1983) also suggest that levels detected in the blood are most likely in transit to an area in which there is high fat content. Other studies found levels of organochlorines in adipose tissue but levels were not detected in serum or were below the limit of detection (Wolff *et al.*, 1982; Archibeque-Engle *et al.*, 1997). Archibeque-Engle and colleagues (1997) suggest that organochlorines may not be detected in serum as levels in serum would indicate that there is recent exposure or acute exposure to the pesticide, which is unlikely in most industrialised countries owing to the restricted use. Organochlorines measured in adipose tissue are more likely to produce a true representation of body burden and are better indicator of long-term exposure (Archibeque-Engle *et al.*, 1997). As already stated, organochlorine pesticides, such as DDT, are stored primarily in adipose tissue and can be found circulating in the blood, bound to fatty components. The breast consists of blood vessels, which may carry blood tainted with organochlorine pesticides, and the degree of the contamination can influence the effect of pesticide on the tissue and may depend on the amount of lipid in the blood and the physiology of the surrounding tissue (Brown and Lawton, 1984). If a breast tumour involves the oestrogen receptor, then there is the likelihood that the levels of pesticide may affect the amount of hormone able to bind to receptors. Subsequently, however, the tumour itself may determine the involvement of surrounding tissue in the disease process and thus may potentially determine the levels of pesticide that are able to partition between blood and adipose tissue (Eyster *et al.*, 1983; Haagensen, 1986).

1.4.2 HCH family

The hexachlorocyclohexane family (or historically and inappropriately referred to as benzene hexachloride) is comprised of five isomers; alpha, beta, gamma, delta and others. The gamma isomer (also referred to as lindane) is the most insecticidally effective isomer and was first discovered in 1944 (Figure 3) (IARC, 1979). It is relatively unknown as to why only one isomer of those mentioned is highly insecticidal and the others are fairly inert. The beta isomer of HCH constitutes approximately 3 to 14 percent of a technical preparation and is regarded as one of the inactive isomers of HCH, but is also the most bioaccumulative (VanVelson *et al.*, 1986).



(a)

Figure 3, The chemical structure of the only member of the hexachlorocyclohexane family (a) lindane.

Lindane is slightly soluble in water, moderately soluble in kerosene and highly soluble in organic solvents, such as acetone (Hassell, 1990). For these reasons, lindane is considered to be least bioaccumulative and persistent of all organochlorines. Studies by Stewart and Fox (1971) found that applications of lindane to grasslands for the control of wireworm (*Agriotes* spp.) were rendered ineffective after two years, in comparison to other organochlorines which were still up to 100% effective after 17 to 20 years (Johnson *et al.*, 1971).

The effect of lindane on animal health has been investigated extensively. In a study by VanVelson and colleagues (1986) male and female rats were fed a diet containing various concentrations of β HCH for 13 weeks. They found that in female rats there were significant hyperplastic and metaplastic changes of the uterus and an absence of corpus

lutea in the ovaries at chronic exposure levels (VanVelson *et al.*, 1986). Research by Criswell and Loch-Caruso (1999) suggests that lindane may inhibit the force of uterine contractions in pregnant rats by eliminating gap junction communication between smooth muscle cells. Their research also found that the beta isomer of HCH had no effect on gap junctions but was able to increase contraction frequency (Criswell and Loch-Caruso, 1999). Research by Walsh and Stocco (2000) found that lindane was able to inhibit steroid production in laboratory animals by reducing protein-mediated cholesterol transfer. Other reported toxic effects on the reproductive system include testicular atrophy, increased oestrone metabolism by liver microsomal enzymes, increased uterine weight and altered oestrous cycles and steroid hormone concentrations (Cooper *et al.*, 1989; Beard and Rawlings, 1999; Welch *et al.*, 1971). The most notable effect reported of lindane exposure on animals is that on the central nervous system. Research suggests that exposure to lindane is able to induce convulsions and exert neurotoxic effects in mice and rats (Cooper *et al.*, 1989; Damgaard *et al.*, 1999). The ability of lindane to induce convulsions is most likely due to its interaction with the γ -aminobutyric acid (GABA) receptor, which leads to the inhibition of chloride flux through the receptor, and thus blocks neurotransmission (Joy and Albertson, 1987; Damgaard *et al.*, 1999). Other effects of lindane exposure on animal health that have been reported include depression, lower concentrations of erythrocytes and haemoglobin which resulted in anaemia, and higher liver weights as a result of microsomal enzyme induction (VanVelson *et al.*, 1986).

Hexachlorocyclohexanes have also been detected in human tissue, however their presence has not been consistently linked with effects to human health. For example, a National Human Tissue Survey by the Environmental Protection Agency from 1970 to 1983, detected β HCH levels in 98.7% of the population; in females alone the isomers were detected in almost the entire population sampled (99%) (Robinson *et al.*, 1990). However, there were no reports of ill-health from the pesticide exposure, the study was only ascertaining types and quantities of pesticides.

Research investigating the links between HCHs and breast cancer is fairly inconsistent. Mussalo-Rauhamaa and colleagues (1990) found statistically elevated levels of β HCH in breast cancer patients as compared to controls. They also found that when the β HCH

concentrations were adjusted for age and parity in a stepwise logistic regression, the Odds Ratio was 10.51 for women containing greater than 0.1 mg/kg (95% CI; 2.00 – 55.26). No other studies have found any statistically significant association. Research by Dewailly and colleagues (1994) reported no difference in the levels between controls, oestrogen receptor positive and oestrogen receptor negative tumours (39.7 µg/kg, 39.7 µg/kg, and 34.7 µg/kg respectively).

1.4.3 Cyclodiene family

The cyclodiene family of pesticides consist of aldrin, dieldrin, chlordane, heptachlor, endrin and endosulfan (Figure 4). Production of these chemicals for use as insecticides began with the synthesis of aldrin and dieldrin in 1948 (Shell Chemical Co. *et al.*, 1974). Some ten to fifteen years later the wide-scale production of chlordane, heptachlor and endrin commenced. These pesticides were primarily used to prevent crop destruction from soil-borne insects, to dress seed grain to prevent insect infestation and for topical application to animals to control fleas mites and lice (Standards Branch, Queensland Department of Primary Industries, 1972; DeJong, 1991; NHMRC, 1993). But their most effective use was as a termite control agent and for the treatment of cotton, because they were highly effective and inexpensive preventative agents. Aldrin remained the pesticide of choice during the 1950s, until it was realised that dieldrin required fewer applications because of its residual effectiveness (Shell Chemical Co. *et al.*, 1974). However, in the late 1950s the insects in which these agents were targeting (including the boll weevil on cotton crops) became resistant to all organochlorine pesticides (Shell Chemical Co. *et al.*, 1974).

Cyclodienes were commonly used to prevent infestations of crickets, ants and grasshoppers in lawns and for moth-proofing around the house. Cyclodienes such as dieldrin were important agents for public health measures, specifically, for the control of the tsetse fly, which is the vector for sleeping sickness and can render large areas of land uninhabitable (NHMRC, 1993).

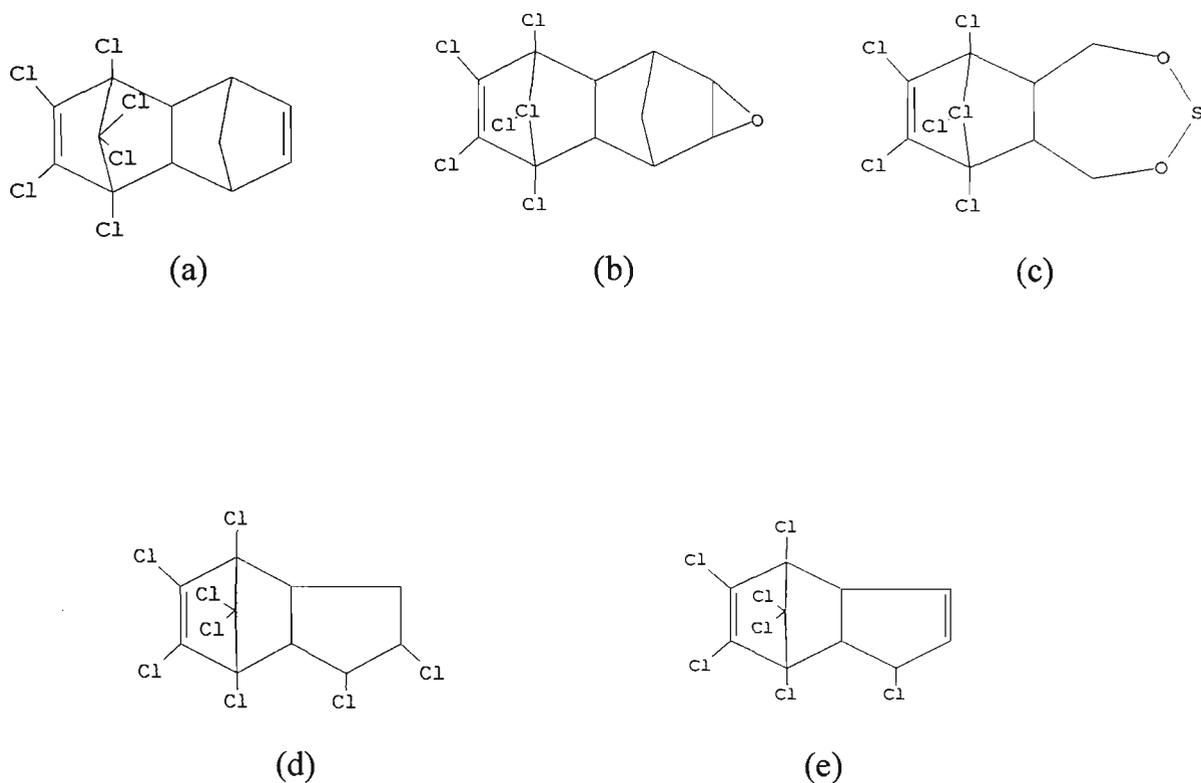


Figure 4, Chemical structures of pesticides belonging to the cyclodiene family of chemicals. (a) aldrin, (b) dieldrin, (c) endosulfan, (d) chlordane, (e) heptachlor

Many members of the cyclodiene family are stereochemically related. Aldrin is rapidly metabolised to dieldrin by NADPH-dependent microsomal enzymes (Hassall, 1990). Heptachlor is a constituent of chlordane, but rapidly converts to a persistent and highly toxic epoxide for storage in soil, and in tissues in plants and animals. Chlordane, in its purest form, comprises two isomers: *cis* and *trans*, with the *cis* isomer being the most toxic of the two mentioned (Hassall, 1990). Endosulfan consists of alpha and beta isomers, and after deposition, is metabolised to its most toxic form, endosulfan sulfate. Cyclodienes have the same detrimental effects on wildlife as DDT and its analogues. The characteristics that have made them a popular domestic and agricultural chemical, such as high toxicity and non-specific biocidal properties, have led to them being responsible for various wildlife deaths. For example, in 1955, two thousand acres of the Florida Salt-Marshes were sprayed with dieldrin to control sandfly larvae. Not only was dieldrin successful at controlling the larvae, but due to its high toxicity and persistence, it substantially reduced the fish living in the saltmarshes and completely "wiped out" the entire crab population (NHMRC, 1993). Laboratory research by Haake and colleagues

(1987) found that injections of dieldrin, heptachlor and chlordane into three week-old rats induced hepatic microsomal cytochrome P450 activity and induced the monooxygenases required to hydroxylate testosterone, thereby altering the capacity to metabolise other endogenous and exogenous chemicals. Cranmer and colleagues (1984) also report of altered plasma concentrations of corticosterone in prenatally exposed mice fed a diet containing chlordane. Previous research by Welch and colleagues (1971) also report the ability of chlordane to enhance hepatic metabolism of oestrogens and reduce the effects of oestrogens on the uterus, thereby potentially affecting reproductive success. Research by Bondy and colleagues (2000) suggests that male and female rats treated with various chemical forms of chlordane exhibited significant hepatocellular hypertrophy as well as increased induction of hepatic drug metabolising enzymes.

Like other organochlorine pesticides, the first signs of potential effects of cyclodiene exposure on human health were recognised in studies of occupationally exposed workers. The use of chemicals such as dieldrin, chlordane and heptachlor for termite control, creates a real potential for substantial exposure of pest control operators and the inhabitants of the treated buildings (NHMRC, 1993). However, to date, there is no strong evidence to indicate that there are increases in risks of cancers in occupationally exposed workers to chlordane or heptachlor (NHMRC, 1993). A 1979 report of the International Agency for Research on Cancer (IARC) suggests that there is limited evidence of carcinogenicity in animals attributable to chlordane and heptachlor exposure. From the same report, the evidence in humans was inadequate to permit a conclusion regarding any causal association (IARC, 1979). In a reviewed report of the IARC (1991) sufficient evidence was provided to state that chlordane and heptachlor were carcinogenic to animals, however there was still insufficient data for any causal relationships in humans. Research by Blair and colleagues (1983) has found that there is an increased risk of lung cancer amongst termite control applicators (Standard Mortality Rate 142). The authors have also stated that this group of exposed workers were not exclusively involved in termite control and may have had exposures to a range of different agents, so therefore their risk cannot be directly attributed to cyclodiene exposure (Blair *et al.*, 1983). Conversely, research by Woods and colleagues (1987) suggests that there may be a slight increase in relative risk for non-Hodgkin's disease (RR 1.61, 95%CI: 0.7-3.8) in men

exposed to chlordane, although this relationship was not statistically significant. A study by Zahm and colleagues (1993) is in agreement with an increased relative risk. Research by Schindell and Ulrich (1986), on production and non-production workers in the manufacture of chlordane, suggests that even though production workers had higher levels of chlordane in their blood, this was not correlated with an increased risk of cancer. In fact, they suggest that there was an inverse relationship with cancer mortality to length of employment.

Research of aldrin and dieldrin formulation workers by DeJong (1991), found that in 20.5% of the study group abnormal electroencephalograms (EEG) were recorded, in comparison to 9% of the controls. With the withdrawal of aldrin and dieldrin exposure, the workers had a marked reversal of EEG recordings (DeJong, 1991). Research by Ribbens (1985) however, revealed no indications that aldrin, dieldrin or endrin exposure in manufacturing plants was associated with any increased risk of mortality from cancer. Other human health effects that have been reported from cyclodiene exposure include, convulsions as a result of neuronal cell loss, effects on the peripheral nervous system manifesting as Parkinson's disease syndrome, and aggressive behaviour due to the inhibition of neurotransmitter mediated responses in the brain (Baker and Wilkinson, 1990).

Cyclodienes, and the other organochlorine chemicals mentioned, are lipophilic chemicals. It is this property that favours their accumulation in human tissue. Human exposure to cyclodienes may have occurred through all portals of entry; inhalation of contaminated ambient air; through direct skin contact of contaminated substances; or through ingestion of foodstuffs containing contaminants. However exposure may also occur in countries where pesticides have not been used, through long-range atmospheric and oceanic transfer (Barrie *et al.*, 1992). Inuit communities from Nunavik (Arctic Quebec, Canada) rely on sea mammals for subsistence, and it is this consumption of seals and beluga that leads to elevated body burdens of organochlorine pesticides. Research by Dewailly and colleagues (2000) suggests that Inuit infants from Nunavik, exposed prenatally to dieldrin and other organochlorine pesticides may be at increased risk of infectious diseases such as meningitis, and middle ear infections. The potential links between cyclodiene exposure

and women's health have not been elucidated. Falck and colleagues (1992) found that the combined levels of heptachlor epoxide and oxychlorodane were higher in breast cancer cases than controls, however the difference was not statistically significant ($p>0.05$). Research by Dewailly and colleagues (1994) also found that there was a non-significant association between cyclodiene levels and breast cancer ($p>0.05$).

1.5 TOXICOLOGICAL TESTS

Research of agriculturally or occupationally exposed workers to pesticides has found a magnitude of adverse hormone-mediated effects. The research investigating the occupational exposures to pesticides has provided some evidence that some exogenous chemicals may exhibit oestrogenic activity. In addition to these claims, abnormal development of the reproductive system and therefore reduced reproductive success have been reported in exposed wildlife. The levels of xenobiotics have been measured in several body tissues and have been linked to increased risk of disease, whilst some researchers would argue that there is no causal association. But the mere presence of these compounds in the body is not evidence enough to state that they cause cancer or other forms of disease. An understanding of what processes or subtle changes these organochlorine chemicals can elicit is fundamental to determining whether they are involved in the disease process. However, to deliberately test xenobiotics and their effects on disease processes in humans is unethical. Animal models and in vitro models (such as cell and organ cultures) are the only feasible options for these types of tests. Many researchers, however, would argue that these models do not provide accurate predictions for potential risks in humans, due to the fact that an animal's propensity to react to xenobiotic chemicals may be different to humans. Also, some of the observed outcomes in animals due to chemical exposure can only be used to predict what might occur in some humans and only some of the time (Weisburger and Rall, 1972). However, bearing this in mind, animal models tend to be more homogenous than humans, especially the inbred strains, and therefore can be exposed to agents under strictly controlled conditions (Weisburger and Rall, 1972). To negate the effects of animal specific responses to

chemical exposure, cell and organ cultures of human origin have been used, which in terms of carcinogenic processes, are cheaper and quicker to establish.

1.5.1 Animal models and pesticides

The effect of pesticides on animals in the wild has revealed that many chemicals that are accidentally released into the environment can disrupt reproductive development. Controlled experiments in the laboratory, using animal models, such as rodents, provide an ideal *in vivo* model to test the effects of chemical agents on the bodily systems of animals and thus apply that knowledge to the human body. Presented here are a few examples of *in vivo* tests involving organochlorins and their effects on the reproductive system.

The early work of Robison and colleagues (1985) studied the effects of DDT on tumour cells in Wistar-Furth rats and the ability of the pesticide to support tumour growth. They found that by allowing a tumour to develop in the presence of naturally occurring oestradiol, that the administration of DDT was able to further support the tumour growth in a dose-dependent fashion (Robison *et al.*, 1985). However, the fact that the tumour was allowed to develop before DDT was applied, cannot rule out the possible effect of another chemical supporting the tumour growth. Other reports, however, support the claim that DDT possesses promotion qualities in terms of the carcinogenesis model (Blair *et al.*, 1990).

In a study involving injected Sprague-Dawley rats, Oduma and colleagues (1995) found that heptachlor significantly inhibited oestradiol and progesterone levels in plasma, in a dose-dependent manner, an effect that was also dependent on the stage of oestrous cycle. Similar effects on reproductive organs and cycles were witnessed with lindane and β -HCH. Cooper and colleagues (1989) found that lindane delayed the appearance of regular vaginal cycles in Fisher-344 rats.

Research by Nesaretnam and colleagues (1998) suggests that a PCB congener (another type of fat soluble chlorinated chemical) may be able to influence mammary carcinogenesis in a rat system. These researchers found that 3,3',4,4'-tetrachlorobiphenyl (TCB) enhanced the development of DMBA-induced mammary tumours in young female rats (Nesaretnam *et al.*, 1998). They also found that given a high fat diet, as well as TCB, the tumours grew at a rapid pace and were predominantly in situ ductal carcinoma of the breast, but possessed the potential to become invasive (Nesaretnam *et al.*, 1998).

1.5.2 Cell cultures and pesticides

Assays that utilise cell cultures to express specific proteins or receptors in comparison to cultures that do not, have been important in defining the biochemical, cellular and genetic effects of exogenous chemicals on the development of human disease. The MCF-7 cells, which were derived from a pleural effusion of a patient with metastatic mammary carcinoma, were established in 1973 (Soule *et al.*, 1973). These cells provide an ideal model to study the effects of oestrogenic chemicals on breast cancer development, owing to the fact that the cells express oestrogen receptors (Wiese *et al.*, 1992). Assays using the MCF-7 cells as a model system are diverse because of their ability to define multiple endpoints, such as cell proliferation, gene and protein expression, enzyme activity and reporter gene activity (Safe *et al.*, 1998). The proliferative effect of oestradiol on MCF-7 cells was first recognised in 1976 and since then the effects of exogenous oestrogens has received considerable attention. The discovery that oestrogen was able to promote cell growth led many researchers to believe that there must be some interaction between oestrogen and a receptor in order for the hormone to elicit a response.

The specific mechanisms by which exogenous chemicals are able to inhibit binding of oestradiol to the receptor and affect cellular proliferation on oestrogen target cells is not clearly defined. Research by Ballare and colleagues (1989) suggests that in oestrogen-receptor positive tumours, the majority of proliferative cells lack oestrogen receptors. The expression of oestrogen receptors in these tumours is most likely associated with cellular differentiation and thus are more likely to play a role in activating the genes or peptides

that are required for the control of proliferative activity (Ballare *et al.*, 1989; Fanelli *et al.*, 1996). Other researchers suggest that oestrogenic chemicals may induce expression of regulatory genes involved in the cell cycle (Dees *et al.*, 1997a; Shekhar *et al.*, 1997). Researchers have shown that oestrogens are capable of inducing G1 to S phase transition of the cell cycle in oestrogen responsive breast cancer cells, in the absence of mitogenic stimulation (Foster and Wimalasena, 1996; Dees *et al.*, 1997a). Oestrogens have been shown to modulate transcription of numerous genes and the progression of the cell cycle through the stimulation of cyclin synthesis and the activation of cyclin-dependent kinase (Cdk) (Foster and Wimalasena, 1996; Beato, 1989; Dees *et al.*, 1997a).

Exogenous chemicals, such as organochlorine pesticides, that can interact with the cytosolic receptors directly or in some capacity, have the potential to elicit response at extremely low concentrations. Therefore when investigating the oestrogenic potency of environmental chemicals it is important to determine whether the responses occur through the receptor pathway or by some other means (Gaido *et al.*, 1997). Organochlorine pesticides have been described as having similar chemical structures to the endogenous 17β -oestradiol, and thus said to exhibit oestrogenic activity. By acting as oestrogen mimics, antagonising the effects of endogenous oestrogen, altering the pattern of synthesis and metabolism of endogenous hormones or by modifying receptor levels, pesticides could potentially disrupt normal endocrine function, leading to reproductive failure and possibly cancer of oestrogen-responsive tissues. Research by Kupfer and Bulger (1976) and Gaido and colleagues (1997) suggests that isomers of DDT directly interact with the oestrogen receptor and are androgen receptor antagonists. Kupfer and Bulger (1976) also found that *o,p'*-DDT was able to bind specifically to the 8S moiety in uterine cytosol, thereby suppressing the binding of oestradiol in this region, whilst in turn, increasing the oestradiol binding in the 3-4S region. Soto and colleagues (1995) have devised a bioassay, using oestrogen-receptor positive MCF-7 breast cancer cells grown in culture, to determine the oestrogenic potency of environmental chemicals. They found that as a consequence of DDT exposure, there was significant oestrogenic activity induced in MCF-7 cells (Soto *et al.*, 1995). However the extent of activity varied depending on the different DDT isomers; such that *o,p'*-DDT exhibited activity at $1\mu\text{M}$, whilst *p,p'*-DDT showed oestrogenic activity at $10\mu\text{M}$ (Soto *et al.*, 1995). Also, these researchers found

that o,p'-DDT was able to competitively bind with oestradiol to the oestrogen receptor. o,p'-DDT inhibited binding of oestradiol but did not alter the number of binding sites of the receptor protein, instead increased the ratio of unbound to bound oestradiol, which results in reduced affinity of oestradiol for the receptor (Kupfer and Bulger, 1976). Research by Dees and colleagues (1997a) found that DDT was able to stimulate Cdk2 activity (which is responsible for the progression of the cell cycle) in MCF-7 cells, but was unable to induce such activity in oestrogen receptor negative cells. The Cdk2 activity stimulated in oestrogen receptor positive cells was inhibited by the addition of the anti-oestrogen ICI 182,780, suggesting that DDT mediates an effect on cells through the oestrogen receptor (Dees *et al.*, 1997a). As Cdk2 activation correlates closely with the transition from G1 to S phase of the cell cycle, it can be stated that DDT is capable of inducing cell cycle progression in oestrogen receptor positive cells. DDT was also able to mimic the activity of oestradiol by increasing cyclin D1 protein synthesis (Dees *et al.*, 1997a). Cyclin D1 is responsible for the development and proliferation of normal breast epithelium, and its overexpression may be used as a prognostic indicator for breast cancer invasiveness (Weinstat-Saslow *et al.*, 1995). Research by Weinstat-Saslow and colleagues (1995) suggests that with the progression of breast disease, from a benign state through to invasive breast cancer, there is an increase in cyclin D1 mRNA overexpression. Endosulfan and dieldrin were also able to exhibit oestrogenic activity at various concentrations, but were also able to slightly elevate oestrogen receptor levels in MCF-7 cells. Research by Arnold and colleagues (1996) found that dieldrin and endosulfan alone, could only weakly inhibit the binding of oestradiol to the oestrogen receptor, but in combination exhibited a greater inhibition.

Given the competitive affinity for the oestrogen receptor by 17β -oestradiol and organochlorines, some researchers have investigated the possible effects of environmental chemicals on apoptosis. Apoptosis is a normal physiological process consisting of a series of programmed events, which lead to cell death (Burow *et al.*, 1999). In hormone responsive tissues, apoptosis is controlled by oestrogens that regulate apoptosis through the expression of apoptosis regulatory proteins, such as Bcl2 (Jaattela *et al.*, 1995). Research by Burow and colleagues (1999) investigated the expression of Bcl2 in MCF-7 cells that were treated with 17β -oestradiol, DDT and alachlor. Their research suggests that

the exposure to all three compounds resulted in an overexpression of Bcl2, but subsequent administration of the antioestrogen ICI 182,780, caused the expression to be inhibited (Burow *et al.*, 1999). Overexpression of Bcl2 in MCF-7 cells has also been linked to a resistance to tumour necrosis factor (TNF) induced apoptosis (Jaattela *et al.*, 1995). Therefore the treatment of oestrogen receptor positive cells with organochlorine enhances apoptosis, but this effect can be reduced with anti-oestrogenic treatment.

1.5.3 Molecular basis of cancer

The induction of cancer is speculated to involve a number of key events; (a) the interaction between carcinogens and molecular and cellular receptors; (b) the activation of biochemical pathways by carcinogens; (c) interactions of carcinogens with factors that control cellular growth and development; and (d) cytogenetic effects by carcinogens of growth and multiplication of the modified cells (DeRisi *et al.*, 1996; Carulli *et al.*, 1998; Bubendorf *et al.*, 1999).

Cancers are a group of cells exhibiting an unrestrained proliferative phenotype, which develops as a result of a series of molecular changes occurring within the cell (Wang *et al.*, 1999). These cellular changes are thought to lead to expression changes of numerous genes accompanied by the histological or clinical classification of abnormal cell growth (Wang *et al.*, 1999). DNA array technology allows a researcher to study thousands of genes simultaneously in a single experiment, in an effort to identify genes and monitor changes in their activity within a disease state (Heller *et al.*, 1997). An array consists of a series of DNA or protein elements in an organised sequence, attached to either a filter or a glass slide (Rockett and Dix, 1999).

The potential applications of arrays in medical research as reported by Carulli and colleagues (1998) and Rockett and Dix (1999) are far reaching;

- (i) identify genes critical for a developmental process;
- (ii) identify genes that mediate cellular responses to chemical or physical stimuli;

- (iii) understand molecular events effected by mutation in a gene of interest;
- (iv) identify molecular markers for disease processes;
- (v) identify potential drug therapy targets; and
- (vi) identify molecular events associated with drug treatment.

Research by Moch and colleagues (1999) have utilised an array system to identify genes involved in renal cell carcinoma. It is thought that renal cell carcinoma involves the accumulation of genetic alterations in numerous different genes, some of which have been identified and others that are still unknown (Moch *et al.*, 1999). The array technology was used in this instance to provide information on the differential gene expression between renal cell carcinomas and normal kidney tissue, in an effort to facilitate treatment outcomes (Moch *et al.*, 1999). A study by Heller and colleagues (1997) utilised an array to investigate the different cell types that contribute to the development of rheumatoid arthritis. From their research findings, they were able to determine that the major gene involved in the early stages of rheumatoid arthritis was tumour necrosis factor, and that its expression preceded the expression of other gene products, thereby establishing a hierarchy of signalling events (Heller *et al.*, 1997).

In terms of researching breast cancer with array technology, most efforts have focused on comparing familial inheritance of mutant alleles of BRCA1 and BRCA2, or on identifying genes that can be correlated with specific treatment regimes. Research by Duggan and colleagues (1999) presented at the Microarray Meeting in Arizona, has investigated the genes up or down-regulated in familial BRCA1, BRCA2 and sporadic breast cancers. They identified cyclin D1 as being up-regulated in tumours that were known to be associated with BRCA2, however cyclin D1 expression remained unchanged in BRCA1 tumours and sporadic tumours (Duggan *et al.*, 1999). Cyclin D1 has not been linked with BRCA mutations in previous research but has been correlated with oestrogen receptor status (Dees *et al.*, 1997a). In the study group of Duggan and colleagues (1999) the tumours associated with BRCA2 mutations were all oestrogen receptor positive, and the BRCA1 tumours were all oestrogen receptor negative.

An array can be used as a toxicological tool for identifying genes, which may be effected by mutation as a result of chemical stimuli. Arrays have been developed for the Environmental Protection Agency (EPA) to identify the carcinogenicity or toxicity of chemical compounds on toxicologically important genes, and to elucidate their mode of action through gene expression pathways (Rockett and Dix, 1999). Research by Holden and Pennie (1999) have also utilised arrays in much the same manner as the EPA, but have specifically targeted toxicity endpoints (hepatotoxicity, endocrine disruption and carcinogenicity) in investigating the transcriptional activation of genes in response to toxic compound exposure.

1.6 CONCLUSIONS

A significant amount of research effort has focused on identifying the factors that are thought to put women at higher risk of developing breast cancer. These factors have been discussed intensely throughout this chapter. However, there is still a percentage of women presenting with breast cancer, that have none of the traditional risk factors. There is the potential for exposure to environmental chemicals to increase breast cancer risk as some researchers suggest. But there are conflicting studies investigating the relationships that may occur between breast cancer and pesticides. Therefore there is a need to further investigate and identify whether breast cancer and organochlorine pesticides are associated, by taking into account the established risk factors. Chapter 2 will investigate the risk factors for the continuum of breast disease events. Chapter 3 will explore the relationships that may exist between breast cancer and organochlorine pesticides. If it is postulated that there is a relationship between organochlorine pesticides and breast cancer, possibly acting through growth factor pathways (or oestrogen receptor pathways) then exposure of breast cells to pesticides may result in changes to gene expression, as detected by gene array. To date there is little information linking the investigation of breast cancer aetiology and pesticide exposure to the application of new gene array technology. Chapter 4 will attempt to determine any mechanisms or pathways that organochlorine pesticides may act through to bring about cellular change, by using cell culture assays and microarray.

1.6.1 Aims

To identify whether there is a relationship between breast cancer and organochlorine pesticides, with specific reference to the commonly used organochlorine pesticides in Australia, in the past.

To investigate the cytotoxicity (by proliferation and cell survival studies) of the organochlorines found to be associated (from the first aim) with breast cancer, by use of breast cell cultures.

To determine the possible pathways by which specified organochlorine pesticides act on breast cells to alter regulation of growth, with particular reference to the oestrogen receptor pathway.

CHAPTER 2
EPIDEMIOLOGY AND CLINICAL PATHOLOGY IN
A POPULATION BASED ON A CONTINUUM OF
BREAST DISEASE EVENTS.

2.1 INTRODUCTION

Epidemiology is the study of the occurrence and distribution of disease and one of its purposes is to identify a range of causal and risk factors that may affect health in the population. It is important therefore when looking at potential factors that may affect health to be retrospective and review the factors that are already known or speculated to influence disease development.

The cause of breast cancer is still relatively unknown, although researchers have accumulated a considerable amount of information on the factors, which may increase one's risk of developing the disease. Breast cancer is predominantly a disease that develops in women but in rare circumstances it can develop in men. Some of the factors that are thought to increase the likelihood of breast cancer development as documented earlier include;

- characteristics of the menstrual cycle, such as the time it takes to establish regular menstrual cycles, the length of a menstrual cycle and the age at which these cycles begin;
- pregnancy and related factors, such as age at first full term pregnancy, number of full term pregnancies, interruptions in a full term pregnancy (abortions) and lactation regimes;
- menopause factors, such as age at menopause and type of menopause (natural or induced);
- family history, such as the degree of kinship for family history of breast cancer and the inheritance of mutations in tumour suppressor genes or oncogenes;
- hormone therapies, such as oral contraceptive use, hormone replacement therapy use, the length of time for use of hormone therapies and the types and amounts of hormones in the hormone preparations;
- lifestyle factors, such as geographical location, country of birth and any migration patterns, socioeconomic status, diet, weight, height and other body mass indicators; and
- other factors which are still being researched, such as exposure to environmental contaminants.

Therefore in an attempt to determine the relationship between breast cancer and the proposed risk factor, organochlorine pesticides, one must ensure that the established risk factors are identified, to adjust for any confounding. This chapter will investigate whether organochlorine pesticides are related to any of the established risk factors for breast cancer, with respect to different breast disease states.

Two different approaches were undertaken to assess organochlorine pesticide exposure (1) a qualitative assessment using a questionnaire to determine occupational and private exposure to a range of chemicals including the pesticides under investigation in this study, (2) a quantitative assessment of chemical exposure (with specific reference to organochlorines) by extraction and gas chromatographic analysis of breast biopsy specimens.

2.1.1 Aims

To determine whether there is an association between the concentrations of organochlorines in the breast adipose tissue of women and the degree of severity of breast disease according to different classifications of breast disease.

To identify any occupational or non-occupational sources of exposure to organochlorine pesticides of women presenting with breast complaints.

2.2 METHODS

Various steps were undertaken to assess organochlorine pesticide exposure and to relate this exposure to breast cancer development. The steps were as follows and will be discussed in order:

- Development of a questionnaire, to obtain demographic details, clinical history, chemical exposure and dietary exposure relevant to breast cancer risk.

- Approval of Human Research Ethics proposals and consent for participation in the study.
- Recruitment of participants into the study, by identifying the setting and inclusion criteria for the subjects.
- The administration of the questionnaire, by interview.
- The collection of breast adipose tissue from breast biopsies performed on participants as a routine part of their medical diagnosis and assessment by pathologists.
- Refinement of a method for the extraction of pesticides from the breast adipose tissues.
- Solvent extraction of pesticides from breast adipose tissue.
- Analysis of organochlorine pesticide levels in the extracts of breast adipose via gas chromatography.
- Results processing and statistical analyses of pesticide levels, in conjunction with clinical and epidemiological data.

2.2.1 Questionnaire development

A questionnaire, developed initially by Ms Ann Alder (Lecturer, Health Sciences, VUT) to determine the risk factors for breast cancer that may be important for consideration in relation to organochlorine pesticide exposure. The questionnaire contained details about demographic location, the presence or absence of known risk factors for breast cancer (such as reproductive information), as well as dietary information and data about pesticide exposure (Appendix A). The questionnaire was later modified to include components of a short fat questionnaire from Dobson and colleagues (1993) with a view to determine the number of fat intake related events in the women's diets. This additional component was included in the questionnaire because it provided a broad measure to highlight aspects of their diet that might be linked to increased susceptibility to cancer and potential exposure to organochlorine pesticides (Dobson *et al.*, 1993). A small pilot study was conducted in the year of 1995 as part of a 1year Honours project using this questionnaire and modifications were made before use for the present study.

The questionnaire consisted of 116 questions and due to the large number of questions and the relatively small study group, the responses to some questions were combined (refer to section 2.2.10). All completed questionnaires were coded with a BC number and names omitted from copied questionnaires to maintain confidentiality. The Data Manager at St Vincent's Hospital kept original copies of the questionnaires under lock and key. The date of birth and the date of entry into the study were recorded for each participant, to identify the patient's age and for future monitoring of the recruitment process. Age at entry into the study, as deduced from the points above, was used as a criterion rather than age at diagnosis.

The patients in most instances recalled weight and height, however in some instances estimates were taken or the medical records were perused to gather this information. A body mass index (BMI) was calculated from the recorded weight (in kilograms) and height (in metres squared). A BMI below 20 is considered as underweight, 20 to 25 is the ideal BMI range, greater than 25 and up to 30 is overweight, and greater than 30 is obese. According to the National Health and Medical Research Council (1992), the acceptable or ideal range is based upon a number of studies that indicate that BMI estimates in this range are associated with the lowest mortality rate. However BMI estimates can be misleading, as they are correlated with body fat and do not account for heavily muscular people, who may have a high BMI and low body fat.

The postcode of the patient's residence was recorded, to ascertain if the residence for each subject was urban or rural and might increase the likelihood of chemical exposure. A protocol which is used by Australia Post for assigning postcodes to metropolitan and regional areas was followed for this study. No account was made as to whether the women residing in regional areas were from large regional cities, such as Bendigo, or from smaller regional or remote rural areas.

The family history of breast cancer (if any) was recorded for each participant. If a family history was recorded, the degree of kinship was noted (for example, maternal or paternal grandmother or aunt, sister, mother, daughter etc.).

Previous liver disease was noted to ascertain whether there was any impairment of liver function, which may be important for consideration in pesticide accumulation or altered oestrogen metabolism. If liver disease had occurred, the type of condition diagnosed, when it occurred and the types of treatment were recorded. The same sort of data was collected for women who may have had previous breast diseases.

Menopausal status was documented for each participant. The time since the last menstrual period was also recorded to ascertain women who may be perimenopausal. As most of the study group were unquestionably classified as postmenopausal, those who were still menstruating (even if it was irregularly) were classified as premenopausal. No account was made as to the number of perimenopausal women involved in the study.

All participating women were asked if they were currently taking or had taken oral contraceptives or hormone replacement therapy (HRT) in the past, and if so, what brands and for how long. The types of hormone therapy (for example oestrogen alone, combination of oestrogen and progestagen etc.) were also documented, where possible.

The patients were asked if they had an oophorectomy or a hysterectomy performed and the circumstances as to why they had these procedures (if applicable). The number of children born live to each participant was recorded, as well as the age at which the first full term pregnancy occurred.

Cigarette smoking has been linked to the development of many cancers. The patients were asked if they had ever (in the past and currently) smoked cigarettes, for how long and a rough estimate of the number of cigarettes they would smoke each day. Alcohol consumption was recorded and the results presented are a reflection of the combination of women who drink on social occasions as well as women who drink on a more frequent basis.

The questionnaire also included questions aimed at assessing the patient's exposure to chemicals around their residences and in occupational settings. The patients were asked to record the types of industries (if any) that were close to their place of residence. An industry was assessed as being close if it fell within a five kilometre radius from the

residence. The patients were prompted to recall the industries with a list suggesting various industries that may be near their residence, or were given the option to list industries that were not specified (refer to Appendix A, question 9).

The patient's occupation was recorded to ascertain those women who may be at high risk of occupational exposure to organochlorines, as well as to determine the socioeconomic status of the study group. Women were recorded as having professional careers, being retired, unemployed or occupied by home and family duties, employed in administration, clerical duties or the sales areas, or employed in trades and labour areas. The type of industry in which the women were employed was also recorded.

The occupation of the patient's partner (if applicable) was also recorded, to ascertain whether the patient might have had an indirect exposure to organochlorines. The type of industry in which the patient's partner worked was also recorded, if appropriate.

Patients were asked to record the use of herbicides and pesticides around the home to assess potential chemical exposure. The patients were also asked to recall the brand of chemical that was used (if applicable), how often the chemicals were used and whether any protective equipment was used to minimise their exposure. The brands of chemicals used as herbicides and pesticides were documented.

The participants were asked to recall if they had ever lived or worked on a farm where chemicals may have been used for treating animals or crops. If applicable, the women were also asked for how long they had lived or worked on these farms, if they had handled any of the chemicals and if not if they may have washed contaminated clothing, whether protective equipment was used and the types of chemicals (if known) that may have been used.

The patients were asked to recall if they had ever lived in a house that had been treated with pesticides, for what reason the house was treated and if known, the brands that may have been used. The patients were also asked to recall how often their local council or shire sprays herbicide or pesticide in their area.

2.2.2 Human research ethics and consent

The research presented was developed in conjunction with the Breast Clinic staff at St Vincent's Hospital, as this was the intended venue for subject recruitment. A Human Research Ethics application was submitted to St Vincent's Hospital, Victoria University and also the Mercy Hospital For Women. Human Research Ethics applications were approved from Victoria University (HRETH 41/96), St Vincent's Hospital (27/94) and Mercy Hospital for Women (project number 96/5) (see Appendices B, C, and D for approval letters respectively). Approval was granted for the recruitment of patients with various breast complaints, the collection of information via a questionnaire, the collection and analysis of human breast tissue and for the collation of questionnaire and analytical data. An application for Human Research Ethics was not submitted to Peter MacCallum Cancer Institute as the patients recruited from this hospital were under the private care of one of the collaborators operating from St Vincent's Hospital.

Consent for each patient's involvement in the study was obtained by completion of a patient declaration form (Appendix E). Completion of the form was achieved either as the patient presented to the Outpatient's Breast Clinic at St Vincent's Hospital, or by mail, with a signed letter from the doctor concerned, explaining what was required by them if they wished to be involved in the study (Appendix F).

2.2.3 Interviews

The questionnaire was administered by Victoria University researchers to consenting patients, whereby the patients were asked the questions in sequence and their answers noted. The interviews conducted in the first year of recruitment (1995) were undertaken by one person (Ms Sunn). Ms Sunn has a background in exercise/ sports physiology with experience in recruiting and interviewing athletes with respect to physiological and lifestyle factors. Ms Sunn was employed in the first year to conduct these interviews (approximately 15). In the second and subsequent years, the interviews were conducted by the current PhD candidate (Ms Taylor) following the same format as the first, with

patients recruited from St Vincent's Hospital. Ms Sunn trained the second person in the interviewing process and once Ms Sunn was satisfied that the interviewing protocol was conducted at the same standard, she no longer undertook this task. It is envisaged that the inter-interviewer bias would be low as the same interviewer interviewed most of the patients recruited for this study. Patients consenting to be involved in the present study and recruited from either the Mercy Hospital Inc. or Peter MacCallum Cancer Institute were interviewed over the telephone and the answers noted once consent had been received by mail. Some patients recruited from the Mercy Hospital Inc. were unable to be interviewed at the time the interviewer called, so a questionnaire was sent to them for completion with a reply envelope provided. Neither of the interviewers was informed of the patient's condition before the interview, however the pathologic diagnosis may have emerged during the interview.

2.2.4 Setting

The subjects used in this study were women attending three hospitals in metropolitan Melbourne who presented to their local doctor for suspected breast tumours. In the early years of the study (1995 and 1996) women were recruited from St Vincent's Hospital. St Vincent's Hospital is an institution covering the needs of public and private patients under the care of breast surgeons, in the most part, for the management of benign and malignant breast conditions. Women attending St Vincent's are drawn from metropolitan and regional areas of Victoria and bordering towns of New South Wales.

From 1997, women attending two other hospitals, Mercy Hospital Inc. and Peter MacCallum Cancer Institute, for breast complaints were included in the study. The Mercy Hospital Inc. is a hospital servicing the needs of women under the care of breast surgeons and plastic surgeons operating from two campuses (Mercy Hospital For Women and the Werribee Mercy Hospital). In the most part, women attending the Mercy Hospital Inc. were women undergoing breast reduction procedures or for the management of benign breast conditions. Peter MacCallum Cancer Institute, as its name suggests, is committed to the care of patients with cancer. Women under the care of one breast surgeon for the management of breast cancer were recruited from Peter MacCallum Cancer Institute.

2.2.5 Subjects of the study

Adult women (over the age of 18 years) presenting to the Outpatients Breast Clinic at St Vincent's Hospital or to private consultation clinics at St Vincent's Hospital, Mercy Hospitals Inc. or Peter MacCallum Cancer Institute for breast complaints were recruited for this study. These were women undergoing routine biopsy of breast tissue for diagnostic tests and for whom there was sufficient superfluous tissue (approximately 1cm³) available for pesticide residue analysis. The superfluous breast tissue was placed in the -70°C freezer by pathology staff and the patients details noted for follow up to obtain consent from the patients for inclusion in the study. This generated a list of patients that were potentially suitable for the study, which was cross-checked against a list of patients attending the Outpatients Breast Clinic at St Vincent's Hospital. In the case of patients who may have received private treatment for their breast conditions, the consulting doctors were contacted to obtain approval to approach the patients for consent to be involved in the study. The consulting doctors always made the final assessment of the appropriateness of patients to be included in the study prior to the interviewer approaching the patient for consent and for interviewing. In the case of patients who did not provide consent for participation in the study, the breast tissue samples that were not required for diagnostic purposes were destroyed.

The subjects recruited were adult women of varying ages presenting for breast complaints. Based on the Victorian incidence rates for breast cancer, 90% of women presenting with breast cancer would be aged 40 years or older and this was the case in the present study. Healthy women presenting for other breast complaints were also included in the study. Between September 1995 and February- March 1999, 203 samples of human adipose tissue from the breast, were collected from women under the care of breast surgeons and plastic surgeons, operating from the three collaborating hospitals.

A number of women were excluded from the study and the reasons for this are given as follows. Eighteen women, who consented to be involved in the study, were excluded due to the lack of extractable adipose material for pesticide residue analysis. Five other women were also excluded because they had very advanced breast disease, were diagnosed with dementia or were diagnosed with other complicating conditions, and were

deemed unsuitable for the study by the consulting surgeon. Two women did not provide a completed consent form for inclusion in the study, even though their interest in the study was noted. Difficulties in obtaining skilled interpreters at the time of consultation resulted in the exclusion of two women. Nineteen women were lost to follow up; through their unavailability for interview at the time of consultation, through the completion of treatment and hence their non-attendance at breast clinics or by lack of response to correspondence by mail. When all exclusions were taken into account, 157 women met the criteria for inclusion in the study. There is no indication that the women excluded from the study were different from the women participating in the study.

Of these 157 women included in the study most were recruited from St Vincent's for various breast abnormalities however only 9% of these women under the care of one consulting doctor (MAH) are represented in this study. The women recruited from the Mercy Hospital Inc. and Peter MacCallum Cancer Institute account for less than 9% respectively of the women attending both hospitals for various breast conditions. Of these 157 women, 11 were recruited in 1995, and 31, 93 (48 subjects from St Vincent's Hospital, 30 from Peter MacCallum and 15 from Mercy Hospital) and 22 (8 from St Vincent's, 10 from Peter MacCallum and 4 from Mercy Hospital) were recruited in 1996, 1997 and 1998 respectively. Because of the uneven spread of numbers of participants per year of recruitment, the analyses to follow were not stratified on the basis of year of entry into the study, nor was it taken into account a comparison of participants obtained from the different hospitals.

2.2.6 Collection of breast tissue specimens and pathology data

Breast adipose tissue was collected by breast surgeons, using standard protocols, at St Vincent's Hospital, Peter MacCallum Cancer Institute and Mercy Hospital For Women. The biopsy tissue was obtained from women who underwent routine biopsy for breast complaints, whether they were for reductions in breast volume, or suspected benign or malignant conditions. The patient details were recorded from pathology records for all breast adipose samples subsequent to obtaining consent. In the instances where consent was not obtained from the patient, or if the consulting doctor decided that the patient was

no longer appropriate for the study, the tissues and any records of patient details were destroyed. The breast tissue specimens with corresponding consent were then processed in two ways; (1) biopsy specimens were processed by the pathologists at each collaborating hospital using standard protocols set by the NHMRC for reporting breast tumours (Jeremy Parry, personal communication); and (2) adipose tissue (approximately 1cm³ if available) was excised from breast tissue for pesticide analysis.

2.2.6.1 Pathology data

Pathology data was generated from routine diagnostic tests by the pathologists operating at the three collaborating hospitals. The tests performed on all breast biopsies included an assessment of malignancy. Breast biopsy tissue obtained from reduction mammoplasties or that were found to be “benign” in nature were not assessed any further. Breast tissues that were deemed malignant were subsequently graded to determine the degree of differentiation, the tumour size and the involvement of axillary nodes. Malignant breast tissues were also assayed for oestrogen and progesterone receptor status. All pathology data was collected for use in the present study from St Vincent’s Hospital, Mercy Hospital and Peter MacCallum Cancer Institute, by giving a list of the patients, for whom the data was required, to the Data Manager, the Director of Pathology and the Pathologist’s Secretary respectively.

In the first instance the 157 consenting women from the present study were divided into two groups, benign (n = 43) or cancer (n = 114). The results from the questionnaire and the pesticide levels from each patient were compared between the two groups.

It became apparent through the collection of pathology data, that the pathology of some tumours could be classified further into a number of different categories. According to Dupont and Page (1985) and Bodian (1993) benign breast disease can be further classified according to the degree of epithelial proliferation, to produce more descriptive groups of benign breast disease. Dupont and Page (1985) also suggest that there is a continuum of events from a truly benign state through to an invasive cancer with a poor prognosis. On this basis all 157 tumours were reclassified with the assistance of one of the collaborators of the study (M.A.H), into the categories listed below and the questionnaire and pesticide levels compared between the groups.

1. Normal (n = 6), as determined from breast mammoplasty
2. Non-Proliferative Benign (n = 19), included women diagnosed with fibroadenoma, cysts, apocrine metaplasia and lesions with mild atypia
3. Proliferative Benign (n = 13), included women diagnosed with moderate or florid hyperplasia, intraductal papilloma and sclerosing adenosis
4. Atypical Hyperplasia (n = 5), including both ductal and lobular types
5. In situ Carcinoma (n = 12), including both ductal and lobular types
6. Grade 1 Carcinoma (n = 32)
7. Grade 2 Carcinoma (n = 42)
8. Grade 3 Carcinoma (n = 27)

Given that some of the above categories contained a small number (less than 10) of patients, and that some of these categories are precursors to breast cancer development, the subjects were further classified as truly benign or truly cancerous:

1. True Benign (combined groups 1 and 2 from second round) (n = 25)
2. Pre-Cancer (combined groups 3, 4 and 5 from above) (n = 30)
3. True Cancer (combined groups 6, 7 and 8 from above) (n = 102)

Samples that were included in the pre-cancer group were excluded from the analyses of true benign versus true cancer with respect to organochlorine pesticide concentrations, but were included in the analyses of crude benign versus cancer and the spectrum of breast diseases. Breakdowns of the patient pathology categories, which are utilised in the statistical analyses, against the variables collected from the questionnaire and pesticide levels in the breast tissues, are presented in Table 13.

Table 13, Disease status data of the study population collected from Victoria, Australia between 1995 and 1999.

Variable	Frequency of the study population	Frequency of the study population	Frequency of the study population
Normal Tissue		6	

Non-Proliferative Benign	43 benign	19	25 true benign
Proliferative Benign		13	
Atypical Hyperplasia		5	30 pre-cancer*
In Situ Carcinoma		12	
Grade 1 Carcinoma	115 cancer	32	
Grade 2 Carcinoma		42	102 true cancer
Grade 3 Carcinoma		27	

Key; * excluded from the statistical analyses as this category is suspected to be a predisposing condition for breast cancer development.

2.2.6.2 Processing of breast tissue for pesticide residue extraction

Samples of breast tissue (approximately 1cm³ or greater) were wrapped in aluminium foil by pathology staff, stored in labelled specimen containers and kept at -70°C until collection by Victoria University researchers. The labels on the specimen jars contained information regarding the patients hospital UR number and surname, which was used to obtain pathology details, but upon collection the samples were assigned a BC coded number. The samples were transported on ice to Victoria University, where the patient details were recorded and the samples stored at -70°C until extraction and analysis.

Each piece of breast tissue was aseptically transferred to a sterile vial. The vials were placed in an incubator at 65°C for 48 hours. The liquefied breast adipose extract was aseptically transferred to another vial using a sterile pasteur pipette. Approximately 2 to 3mL of hexane were added to the remaining breast tissue and the vial vortexed for 1-2minutes, in order to extract any residual fat from the sample. The hexane/fat solution was combined with the liquefied breast adipose extract, and a stream of nitrogen was applied to the vial, to remove any hexane. The extract was stored in a refrigerator at 4°C, until extraction for organochlorine pesticides and gas chromatography analysis.

2.2.7 Refinement of method for the extraction of pesticides from breast adipose

Several methods exist for the extraction and identification of organochlorine pesticides in various types of environmental and biological samples. An Australian company

(Scientific Glass Engineering, SGE) has developed a Universal Trace Residue Extractor (Unitrex) which allows the simultaneous extraction of organochlorine pesticides from 10 adipose samples in a thirty minute turn-around time. Briefly, the Unitrex extracts organochlorine pesticides from lipid samples by distilling the pesticide out of the lipid, under the influence of a stream of nitrogen (mobile gaseous phase) and high temperature (235°C), whilst the fat remains bound to glass beads contained in fractionation tubes. The organochlorine pesticides are swept by the nitrogen into glass traps containing Florisil (magnesium silicate) that acts as a solid phase for absorbing the pesticides, which are then eluted from the traps by the addition of solvents. Before processing samples of breast adipose, the Unitrex extraction method was refined to optimise sample recovery, using fortified samples of ghee.

Fortified ghee samples were prepared to determine the efficiency and the recovery rates of the Unitrex system being used. A 25gram sample of ghee was melted in a waterbath at between 50 and 60°C. The samples were then spiked by adding 1 ml of a 50µg/ml endosulfan sulfate and dieldrin mix standard to the ghee. The fortified ghee was placed on a hotplate and stirred for 20 minutes and aliquoted into 5ml volumes. The aliquots were stored at 4°C until needed. Aliquots of unadulterated ghee were also stored for extraction, to act as negative controls, and to make up sample volumes to 1.14 ml for injection into the Unitrex. A fortified sample was extracted at each of the Unitrex ports, and the extracts were concentrated under a stream of nitrogen and analysed by gas chromatography.

The particular sections of the Unitrex extraction method that needed refinement included, florisil preparation, packing of traps and the calibration of the operating procedures for the Unitrex. These will be discussed in sequence.

2.2.7.1 Florisil preparation

Before use the florisil needed to be partially deactivated to maximise the number of available pesticide binding sites. Deactivation was achieved by adding a measured volume of water to a pre-weighed amount of Florisil. The florisil was washed with deionised water to remove any impurities, drained and allowed to dry in the oven at 100°C overnight. The florisil was then activated in a muffle furnace at 600°C for 2 hours. Transfer of the florisil after 2 hours activation, to an oven at 100°C for 30 minutes,

allowed it to cool. The florisil was transferred to a sealed container, placed in a dessicator under vacuum and allowed to equilibrate for 48 hours. The activated florisil can be kept for a period of 1 month if stored in the dessicator.

On the day before the assay, partial deactivation was performed by adding a measured amount of distilled water to a weighed quantity of activated florisil. Initially different levels of deactivated florisil were made to determine the optimum level which allowed for a 95-100% recovery of pesticide from a fortified sample. The different levels of percentage deactivation were 1, 2, 3, 4, and 5%. For example a 1% deactivation was achieved by weighing out 24.75grams of Florisil and adding 0.25millilitres of sterile distilled water.

2.2.7.2 Packing of the glass traps

The cone end of the glass trap was plugged with silanised glass wool. A measured volume (0.8 ± 0.05 grams) of anhydrous sodium sulfate was then placed into the trap, followed by a measured amount of deactivated florisil (1.1-1.2 grams). The remainder of the trap was filled with glass wool to provide solid packing.

2.2.7.3 Calibration of the Unitrex

The Unitrex was turned on and the nitrogen regulator opened. The temperature setting was adjusted to 235°C (as suggested by the installation and operation manual) and allowed to equilibrate. A manual flow meter was connected to the outlet port of each fractionation tube and used to determine the flowrate of gas through the Unitrex at a given pressure. A flowrate of between 180 and 240ml/min was required for the operation of the Unitrex, and the pressure controls were altered until this flowrate was achieved. The flowrate of gas through the Unitrex was monitored each time the Unitrex was in use.

2.2.7.4 Refinement results

The Unitrex operating manual suggests that dieldrin be used to determine the optimum deactivation rate for florisil, therefore the present study only tested dieldrin for recovery. It was found that all levels of deactivated florisil were satisfactory at obtaining a suitable recovery, between 50 and 100%. A 4-5% level of deactivation was used for subsequent analyses, and was the level used at the State Chemistry Laboratory (Ron Walsh, personal

communication). Recovery rates of pesticide from the fortified sample were calculated by dividing the concentration recovered by the concentration of the standard injected. The recovery rates of pesticide were between 60% and 98%.

It was found that the flowrate between the different fractionation tubes varied, with consistently low flowrates of gas through one particular tube, at each time the Unitrex was operating. Therefore at each time of operation, the fractionation tube with the low flowrate was not used for the separation of pesticides from breast adipose tissue and another tube with a consistent flowrate was used for quality control (with the fortified sample being extracted). This variation in flowrate may have been the result of non-uniform packing of the fractionation tubes with the glass beads and may have resulted in inconsistent recovery rates of fortified samples. Armishaw and Millar (1993) suggest that the sweeping rate of pesticides, and thus recovery may be improved by removing the glass beads, which was not performed in the present study prior to breast biopsy extraction. To determine the pesticide concentrations in the breast biopsies however, the varied recovery rates need to be taken into consideration. This was achieved by the following calculation. For example, if a breast adipose sample was found to have 4µg/ml of dieldrin and the fortified sample run at the same time of extraction achieved a 52% recovery, then the amount of dieldrin in the sample would be 7.69µg/ml (4 multiplied by 100, divided by 52).

2.2.8 Extraction of pesticides from breast adipose tissue.

The power switch of the Unitrex was turned on and the temperature was set at 235°C. The nitrogen was regulated until a flowrate of 180 to 240ml/min was achieved in 80% of the fractionation tubes, and the Unitrex was allowed to equilibrate. While the Unitrex was stabilising at the required conditions, the traps were packed, as described in section 2.2.7.2.

Samples of fortified ghee or breast adipose tissue were placed in a preheated waterbath at 50°C until molten. If the breast adipose sample was below the required injection volume, the remainder of the volume was made up with unadulterated ghee. The sample to be

extracted (1.14ml) was taken up into a prewarmed syringe and injected through a pre-punctured septum into the fractionation tube. An insulation wedge was installed after each injection to help maintain thermal equilibrium of the system and to act as a visual indicator as to which fractionation tubes had been injected with the sample.

A fractionation period of 30 minutes was allowed, to ensure transfer of the pesticides from the fat to the traps. After the fractionation period, the silicon rubber connector was disconnected from the stainless steel purge gas tube on the manifold assembly. This allowed the fractionation tube, with the florisil trap to be removed from the heating block. The fractionation tube was put aside for cleaning, whilst the florisil trap was connected to a reservoir. The pesticides were eluted from the traps into graduated tubes, by adding 12 ml of diethyl ether/hexane solution (20:80) to each reservoir and collecting the eluate. Prior to the concentration of the samples under nitrogen, 20 μ L of 100 μ g/ml endosulfan sulfate (internal standard) was added.

The collected fractions were adjusted to 1 ml under the influence of a stream of nitrogen. The pesticides in the fractions were separated, detected and identified by gas chromatography.

2.2.9 Analysis of organochlorine pesticides using gas chromatography

The pesticides, hexachlorobenzene, beta isomer of hexachlorocyclohexane, heptachlor epoxide, cis and trans isomers of chlordane, DDD, dieldrin DDE and an endosulfan sulphate internal standard, in the breast adipose tissue were analysed by gas chromatography. These pesticides or metabolites of pesticides were used for the screening process because they were used extensively in Australia for the treatment of pest infestations or in the manufacture of machinery used for various industries. DDT, although a commonly used chemical, was not analysed because it is not stable at the temperatures used for the gas chromatographic analysis, and would be degraded to a more stable form, such as DDE. Also, DDT has been banned for some time in Australia, and it would not be expected to be detected in human tissue today, owing to its instability.

A number of standards were purchased, to identify peaks of interest resulting from gas chromatographic analysis of breast adipose. The standards of β BHC (1.2.3.4.5.6-Hexachlorocyclohexane (beta isomer)), endosulfan sulfate, dieldrin (hexachloro-epoxy-octahydro-endo-exodimethanonaphthalene), heptachlor epoxide (1.4.5.6.7.8.8-heptachloro-2.3-epoxy-3a.4.7.7a-tetrahydro-4.7), 4.4'-DDT (1.1.1-trichloro-2.2-bis(p-chlorophenyl)ethane), 4.4'-DDE (1.1-dichloro-2.2-bis(p-chlorophenyl)ethylene), cis-chlordane (alpha-chlordane), trans-chlordane (beta chlordane/gamma chlordane), 4.4'-DDD (1.1-dichloro-2.2-bis(chlorophenyl)ethane/ p.p'-TDE), and hexachlorobenzene (perchlorobenzene) were purchased from Alltech Associates Pty Ltd, Australia.

Endosulfan sulfate was used as an internal standard for the determination of the concentration of individual pesticides in the breast adipose samples. Each standard that was being assayed in the present study (as listed previously) was made up in a series of concentrations ranging from 1ng/ml to 10 μ g/ml. To each concentration of standard, the internal standard was added, such that in 1ml of standard there was 2 μ g/ml of endosulfan sulfate. Each standard was then run on the gas chromatograph and a peak area ratio of the standard to the endosulfan sulfate was calculated. This was calculated by taking the area under the peak for the particular standard of interest as generated by the Star chromatography software, divided by the peak area of endosulfan sulfate. The calibration curve for each pesticide was plotted and consists of concentration in μ g/ml of each particular standard versus the peak area ratio. A representative calibration curve is presented in Figure 5 (also see Appendix G, for the other pesticide calibration curves). An equation of the linear relationship between peak area ratio and concentration was calculated for each calibration curve and subsequently used for the determination of pesticide levels in each breast biopsy.

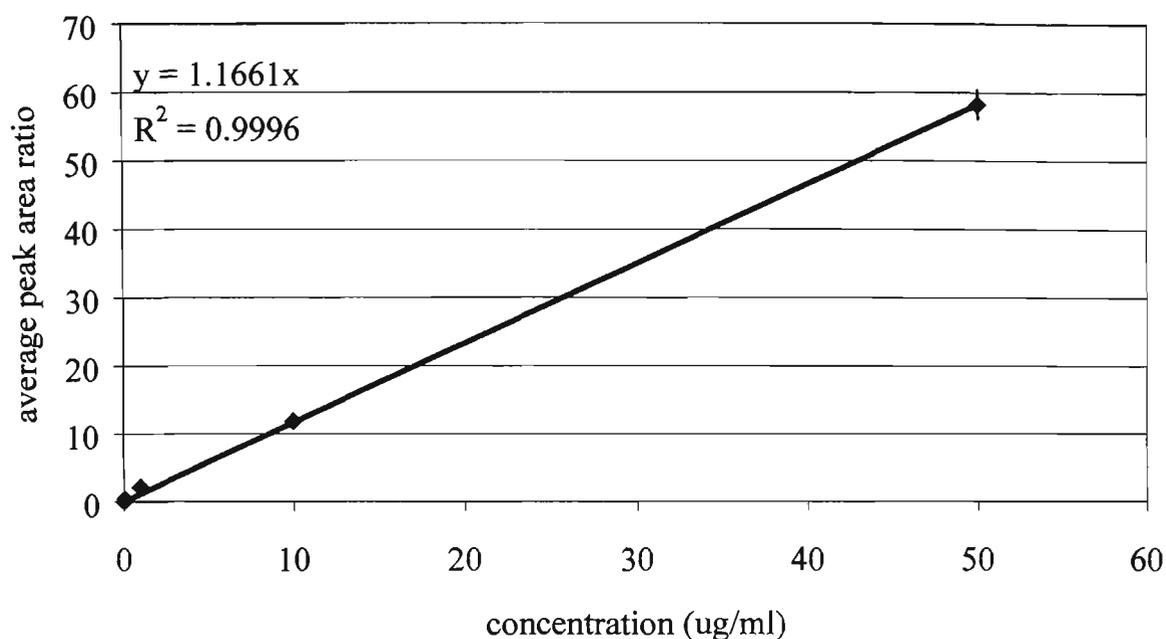


Figure 5, Calibration curve of hexachlorobenzene, using endosulfan sulphate as the internal standard, to calculate the peak area ratio.

The internal standard method for determining pesticide concentrations in the breast biopsies was used for the identification of pesticides by using the retention time of endosulfan sulfate in relation to the retention times of the other pesticides. In addition, because the volumes of internal standard are uniform in the standards and the breast samples, the internal standard allowed for variation that may occur within the gas chromatograph from run to run.

The first run of each day consisted of a hexane injection, to clean out any residues that may have existed on the column or in the injection liner. Extracts of breast adipose tissue also containing $2\mu\text{g/ml}$ of endosulfan sulfate were run in sequence and individual peaks were identified by comparison with retention times in a standard mix containing each of the pesticides under investigation. The standard mix was injected into the gas chromatograph at the end of each series of runs (Figure 6).

Once the peaks were identified in the breast adipose extracts, they were quantified using the equations generated for the calibration curves of each corresponding pesticide. The concentrations of pesticides in the breast adipose extracts determined from the calibration curves was expressed in $\mu\text{g/ml}$. As the initial material that was extracted was measured in

grams and varied from patient to patient, the amounts of pesticides expressed in $\mu\text{g/ml}$ were subsequently corrected for the amount of adipose tissue that was extracted by the Unitrex and were expressed in ng/g .

2.2.9.1 Gas chromatography parameters

A Varian 3400 gas chromatograph with Star Work Station software (Varian P.L, Walnut Creek, CA) was used for the pesticide residue analysis. Separation of the organochlorine pesticides was achieved with a DB1701 capillary column (length = 30m, Internal diameter = 0.25mm, film thickness = 0.5 μm ; J and W Scientific) using a temperature program. A DB5 capillary column (length = 30m, Internal diameter = 0.25mm, film thickness = 0.25 μm ; J and W Scientific) was used as a confirmation column. The injector was held at 250°C, the detector temperature was 300°C and the column was linearly heated from an initial temperature of 190°C to a final temperature of 270°C, at a rate of 2°C per minute.

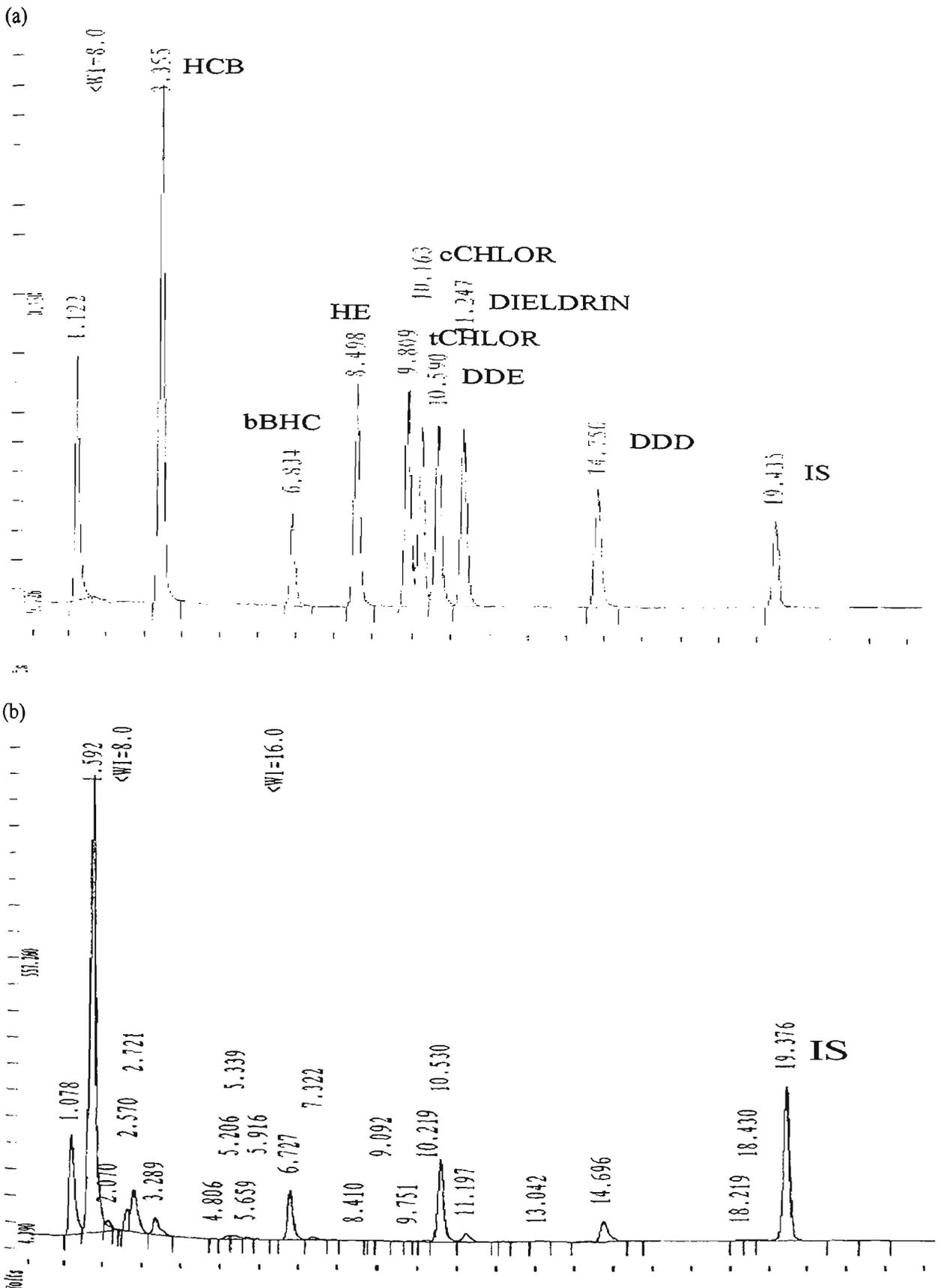


Figure 6. Representative chromatograms of the organochlorine pesticides assayed in the present study (a) a standard mix, (b) a representative chromatogram for one of the breast biopsy samples. The chromatograms represent the voltage (thus peak height) versus time of elution. Key: HCB = hexachlorobenzene, bBHC = beta isomer of benzene hexachloride, HE = heptachlor epoxide, tCHLOR = trans isomer of chlordane, cCHLOR = cis isomer of chlordane, DDE = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene, DDD = 1,1-dichloro-2,2-bis(chlorophenyl)ethane, IS = internal standard, endosulfan sulfate.

2.2.10 Questionnaire processing for statistical analyses

The questionnaire consisted of 116 questions in total and due to the large number of questions and the relatively small study group (157 consenting patients) some categories were collapsed and the responses combined prior to coding. Prior to statistical analyses the data obtained from the questionnaire was also processed to account for missing values. All completed questionnaires were coded with a BC number for identification purposes and the data was entered into a spreadsheet. A series of codes were developed for the questions that may have had multiple responses, and these codes were entered into the spreadsheet (refer to Appendix H). For example, if a patient recorded being born in Australia, the answer was coded 1, whereas women born elsewhere in the world were coded 2.

2.2.10.1 Reproductive factors

The number of children born live to each participant was recorded, as well as the age at which the first full term pregnancy occurred. Due to the small sample size the women were only classified as being nulliparous or parous in the results. The age at first full term pregnancy was subtracted from the age at entry into the study to produce the total number of years devoted to being a mother (or "mum years").

Menopausal status was documented for each participant. As most of the study group were unquestionably classified as postmenopausal, those who were still menstruating (even if it was irregularly) were classified as premenopausal. No account was made as to the number of perimenopausal women recruited for this study, however as the total number of women classified as premenopausal totals 23%, the number is likely to be small.

The patients recorded whether they had an oophorectomy or a hysterectomy performed and the circumstances as to why they had these procedures. As less than 23% of the women involved had either of these procedures, the results only present whether the women had an oophorectomy and hysterectomy (coded 1) or not (coded 2).

2.2.10.2 Family history

The family history of breast cancer (if any) was recorded for each participant. If a family history was recorded, the degree of kinship was also noted as first degree (eg, mother, daughter or sister) or second degree (eg, grandmother or aunt). Approximately 70% of the women involved in this study did not report of any family history, and due to this and the small sample size, the final category used only reflects whether there is a breast cancer history of some degree or no history at all.

2.2.10.3 Hormone therapies

The women recruited for the study were asked if they were currently taking or had taken oral contraceptives or hormone replacement therapy (HRT) in the past, and if so, what brands and for how long for each of oral contraceptives and HRT. Present and past use were combined (ever use coded as 1, and never used coded as 2), as some women alternated between periods of use and non-use. The brands of hormone therapy prescribed for use were noted, to ascertain the types of hormones (for example oestrogen, progestagen or combinations of both) being consumed. However as there are various formulations of hormones in oral contraceptive and replacement preparations, and due to the small sample size, these results are not included.

2.2.10.4 Lifestyle

Previous liver disease was noted to ascertain whether there might have been some impairment of the detoxification properties of the liver. However, because only 5% of the study group had been diagnosed with liver disease in the past, the result section only documents the presence or absence of liver disease, and not the type of condition, when it occurred nor the treatment used. The same types of data were collected for women who may have had previous breast disease. Only 19% of the participants had reported previous breast diseases, so the results only reflect the presence or absence of breast disease.

Patients were asked if they had ever smoked cigarettes (in the past and currently), for how long (in years) and a rough estimate of how many cigarettes smoked per day. Women who recorded smoking either in the past or currently were classified as ever smoking (coded 1) and women who had never smoked cigarettes were coded as 2. The time spent smoking was combined to give a mean number of years, accounting for the past and the

present, and the number of cigarettes per day that were smoked was also combined to account for the mean number in the past and the present.

2.2.10.5 Organochlorine pesticides

The questionnaire also included questions aimed at assessing the patient's exposure to chemicals around their homes and occupationally. The patients were asked to record the types of industries, from a list provided, that might fall in a five kilometre radius from their home. For the purposes of statistical analyses, the list of industries was further classified into three broad categories and coded accordingly; (1) residential only, (2) potential low exposure to organochlorine pesticides, and (3) potential high exposure to organochlorine pesticides. Patients who were identified as having a potentially low exposure to organochlorines from industries near their residence had the following types of businesses in their area; business, commercial, transport, clothing manufacture, metalwork and engineering, health and welfare, educational institutions and public service businesses. Conversely, patients classified as having a potentially high exposure to organochlorines from industries near their residence, had the following businesses in a 5km radius; agriculture, chemical manufacture, cleaning and dry cleaning, wood production, food processing, electrical components and textile production.

To assess occupational exposure to chemicals, the patients were asked to record the industries in which they were employed. The industries in which the patients worked was classified on the basis of potential low exposure to organochlorine pesticides (coded 1) and potential high exposure to organochlorine pesticides (coded 2). Particular industries in which the women worked were classified into the two categories above, using the same criteria as described for the industries near the place of residence.

The occupation of the patient's partner (if applicable) and the industry in which the partner was employed was recorded, to assess whether the patient may have an indirect contact with organochlorine pesticides. However, due to the small sample size of this study, the particulars of the partner's occupation have not been included in the results. Approximately 30% of the women involved did not have a partner at the time of interview, and due to this no account was made of the women who had partners in the past and their respective employment details.

The patients were asked if they used herbicides or pesticides around the home, if so, which brands of chemicals were used, how often the chemicals were used, and if handled personally, whether any protective equipment was used to minimize their exposure. Approximately 62% of the women involved in the study did not report using herbicide around their household and due to this and the relatively small sample size, the results only indicate whether herbicide was used and whether the patient handled the chemical themselves or not. Approximately 80% of the women involved in the study had reported using pesticide around the home. Of these women 74% had handled the pesticides themselves. The information documented for the frequency of use of herbicides and pesticides, as well as the use of protective equipment was not included in the results section due to disparity of patient's answers as well as frequent missing information. The brands of chemicals used as herbicides and pesticides were documented and coded into four categories; (1) pyrethroids, (2) carbamates, (3) organophosphates, and (4) organochlorines. However this information has not been included in the results. Also the recall of types of herbicide and or pesticide and particular brands is complicated for some women, especially for those women who did not handle the chemicals themselves. Therefore the results section only documents the patient's use of pesticides around the home (if applicable) and whether the chemicals were handled by the patients or by someone else.

The patients were asked if they had ever lived or worked on a farm where chemicals might have been used for treating animals or crops, and if so, how long they had lived or worked on these farms, whether they handled any of the chemicals, the types of chemicals that were used, and if they washed the potentially contaminated clothing. Approximately 87% and 82% of women reported that they had never lived or worked on a farm with animals or crops respectively. Due to the low number of women that had lived or worked on farms with animals or crops, the results only document the time spent (on average) on these farms, whether the patient had handled the chemicals themselves and if not whether they may have had potential exposure to the chemicals used by washing contaminated clothing.

The participants were asked to recall if they had ever lived in a house that had been treated with pesticides, for what reason the house was treated, and if known the brands of

chemicals used to treat the house. The reasons for having the house treated with pesticide varied, and the brands of chemicals used to treat the homes was largely unknown owing to the fact that many people employed the use of licensed pest controllers to treat their homes. For these reasons the results section only documents whether the patients had lived in a house that had been treated or not. The participants were also asked if they were aware of their local councils or shires spraying in the area. Approximately 80% of the women involved in the study reported never seeing anyone in the area spraying chemicals or that they didn't know how often the council or shire sprayed. For these reasons this assessment was not included in the results section.

2.2.11 Statistical analyses

All clinical pathology, questionnaire and breast biopsy pesticide concentration data were statistically analysed using SPSS computer software (Chicago, IL). Organochlorine pesticide concentrations in the breast biopsy tissue produced a skewed distribution when analysed for normality and were subsequently transformed logarithmically to mitigate the skewed effect. The data generated from the questionnaire, the clinical pathology and the analyses of breast tissue were analysed, firstly, by descriptive statistics, to determine frequencies and the associated variance of each variable. This was also performed to determine which factors could act as potential cofounders in the final regression analyses.

The logarithmically transformed pesticide data and other continuous variables were cross tabulated against the breast disease continuum, using one-way analysis of variance, to test for variance of means between groups and for any linear relationships throughout the disease continuum. The data derived from the questionnaire, requiring coding for yes or no answers or for multiple response answers before analysis, was cross-tabulated against the breast disease continuum using chi-square contingency tables. Significance was determined using Pearson's statistic.

Between 1995 and 1999, 157 women presenting for breast abnormalities and who underwent routine biopsy at St Vincent's Hospital, Peter MacCallum Cancer Institute and the Mercy Hospital were recruited and met criteria for inclusion in the study.

The chemical exposure and risk factor data from the questionnaire and transformed pesticide concentrations were analysed by a multiple logistic regression model fitted through a forward stepwise variable selection procedure with the use of SPSS software. Logistic regression models were appropriate for the present study because the outcomes were categorical rather than continuous. Variables were entered into the regression equation in a stepwise fashion, in the order of strongest significance to weakest significance (with p values less than 0.05) to observe what happened to each variable once entered into the model, and if the change was insignificant it was removed from the model. The variables were continuously entered into and out of the regression and the effects of each variable on the others already in the model were monitored for change until there were no variables remaining with a significance value less than $p = 0.05$. The variables entering into the regression models were also screened for chance or random variables by considering previous one-way comparative analyses and the frequency data. If random variables enter into the regressions, the model ceases (George Rennie, personal communication). The dependent variable for the regression model was the breast disease continuum. The model was not adjusted for any confounding variables because of the small numbers of subjects in some of the disease categories.

2.3 RESULTS

The subjects were aged between 26 and 85 years of age. They were predominantly postmenopausal women and had borne children (77% and 87% respectively). The women of the study resided in metropolitan and rural Victoria, and some country New South Wales (77% metropolitan dwellers and 23% rural dwellers), and they were mostly Australian born women (72%). Fifty two percent of the women meeting the inclusion criteria had smoked at some time or were currently smoking.

Breast adipose tissue was analysed for nine organochlorine pesticides: hexachlorobenzene (HCB), β isomer of hexachlorocyclohexane (β BHC), heptachlor epoxide, *trans* and *cis* isomers of chlordane, p,p'-DDE, dieldrin, p,p'-DDD and p,p'-DDT. Residues of all the organochlorine compounds listed, except p,p'-DDT were found in the breast adipose tissue samples (Table 14). Residues of p,p'-DDE were found the most frequently in the samples of breast adipose tissue (92%), followed by hexachlorobenzene (64%). The two

isomers of chlordane, *trans* and *cis*, were detected least frequently of all the pesticides analysed (9% and 14% respectively). The average concentration of organochlorine pesticides in the breast adipose samples was also highest for p,p'-DDE followed by the beta isomer of benzene hexachloride (2.86µg/g and 1.65µg/g respectively). Again, the lowest concentrations of organochlorines detected in the breast adipose were for the *trans* and *cis* isomers of chlordane (17.9ng/g and 54.7ng/g respectively).

Table 14, The average concentrations and the frequency of detection of each organochlorine pesticide being evaluated in the breast tissue of all study participants.

Organochlorine compound	No. of samples with pesticide detected	Mean concentration (ng/g) (SD)	Concentration range (ng/g) ^a
HCB	100	802 (1604.9)	0 - 13073.41
βBHC	29	1654.5 (8410.6)	0 - 54432.71
heptachlor epoxide	30	105.2 (382.6)	0 - 1582.56
<i>t</i> chlordane	14	17.9 (77.9)	0 - 572.28
<i>c</i> chlordane	22	54.7 (256.9)	0 - 1958.02
p,p'-DDE	145	2855.5 (3465.1)	0 - 19226.02
dieldrin	51	186.3 (561.8)	0 - 4234.15
p,p'-DDD	32	571.1 (2486.1)	0 - 17064.79
total pesticides	155	6247.3 (11768.3)	0 - 97513.46

^a Residues of p,p'-DDT were not detected in any breast adipose tissue samples.

2.3.1 Clinical Pathology

A summary of the pathology of cancerous tumours, as classified by the three pathology teams at the collaborating hospitals, is presented in Table 15. Eighty-one of the 114 (71%) breast cancer cases were positive for oestrogen receptors, 24% (28 of the 114 cases) of the breast cancer cases were oestrogen receptor negative and 4% (5 of the 114) of tumours had an unknown oestrogen receptor component. Oestrogen receptor status was classified as unknown if there was insufficient biological material to be used for the oestrogen receptor determinations in the pathology laboratory. In situ disease was included in the malignant classification and accounts for the subjects for whom oestrogen receptor tests were deemed unknown.

Table 15, Clinical pathology data of samples classified as malignant, from the study population, from Victoria Australia between 1995 and 1999.

Variable	Crude Frequency (n = 114)
Oestrogen receptors	
positive	81
negative	28
unknown	5
Progesterone receptors	
positive	76
negative	31
unknown	7
Tumour Size (mm)	
< 5	7
> 5 -10	20
> 10 - 20	50
> 20 - 50	30
> 50	4
unknown	3
Percentage nodes positive for cancer	
0	96
1 - 50%	22
50 - 100%	5

Sixty-seven percent (76 of the 114) of the breast cancers were positive for progesterone receptors, twenty-seven percent (31 of 114) of the cancer cases were progesterone receptor negative and seven of the 114 (6%) were unclassified in terms of progesterone receptor status. Like the oestrogen receptors, progesterone receptors were classified as unknown if there was insufficient material for the receptor determinations.

Both tumour size and percentage of axillary nodes positive for cancer are important indicators of prognosis. In this study, the majority of tumours (44%) were 10 to 20 mm in diameter, 26 percent were 20 to 50 mm and 4 percent were greater than 50 mm in diameter. The study results indicate a good prognosis for most patients (44%) and as the size of the tumour increases in diameter, the prognosis of disease worsens. The involvement of greater than 50% of axillary nodes in the disease process was only evident in a small percentage of women (less than 5%).

2.3.2 Comparison of demographic, reproductive, clinical, lifestyle and chemical exposure data between groups, with special reference to the breast disease continuum.

According to Dupont and Page (1985) there is a continuum of disease states from a relatively normal state, through to invasive cancer. These disease states, as listed below, form the basis in which the questionnaire and clinical data are compared. The disease states include women who had normal tissue (n = 6) as obtained from breast reduction procedures; women diagnosed with non-proliferative benign disease (n = 19); women diagnosed with proliferative benign lesions such as sclerosing adenosis (n = 13); women with atypical hyperplasia (n = 5); women diagnosed with in situ carcinoma of ductal and lobular types (n = 12); and women diagnosed with graded cancers (1, 2 and 3; n = 32, n = 42 and n = 27 respectively).

2.3.2.1 Reproductive factors

The study subjects were classified into the continuum of disease states and breast cancer risk factors concerning reproductive capacity and medical history are presented in Table 16. Women with normal breast conditions, as derived from breast reduction, or non-proliferative benign disease were on average younger at entry into the study, compared with the pre-cancer groups (proliferative benign, atypical hyperplasia and in situ carcinoma) and with the graded cancers but these differences were not significant ($p = 0.060$). The age at menarche for all groups did not vary greatly (12 to 13 years on average; $p = 0.403$) but the women with atypical hyperplasia tended to be slightly older at menarche (14 years on average). Overall, most of the study population were postmenopausal, with slightly more of the study population being diagnosed with in situ carcinoma, grade 1 or grade 2 cancer (83%, 88% and 86% respectively) compared to 60-68% for the other disease states.

The duration of time being a mother did not vary significantly along the disease continuum; 20.7 years on average for non-proliferative benign disease to 34.7 years on average for grade 1 carcinoma ($p=0.071$) however is nearly reaching significance. A larger proportion of women diagnosed with in situ or invasive cancers were nulliparous compared with the normal, benign and atypical hyperplasia groups but this was not

statistically significant ($p=0.556$). However, among the women who had a full term pregnancy a greater percentage (55 to 94% according to disease condition) had breastfed their offspring, except for the women who were diagnosed with atypical hyperplasia, where a larger percent did not breastfeed (60%; $p = 0.206$). The total duration of breastfeeding was slightly longer for in situ (12.8 months), grade 1 (10.5 months), and grade 3 (15.5 months) cancers compared with the benign groups and atypical hyperplasia group (less than 10 months on average), but the difference was not significant ($p = 0.160$).

Oophorectomy and hysterectomy procedures were not reported by a large portion of the study groups, whether benign or cancer ($p = 0.969$ and $p = 0.435$ respectively), and there was no difference between groups who reported having previous liver disease and those who did not ($p = 0.259$).

Table 16, Reproductive and general medical history variables of women in respect to the breast disease continuum.

Variable	Normal	Non-Prolif. Benign	Proliferative Benign	Atypical Hyperplasia	In situ Cancer	Grade 1 Cancer	Grade 2 Cancer	Grade 3 Cancer	p value
Age (mean) (SD) ^a	51.5 (9.89)	51.5 (14.5)	54.3 (13.0)	57 (9.46)	61 (9.29)	62.1 (9.77)	57.8 (12.1)	54 (14.6)	0.060†
Age at Menarche (mean) (SD)	12.3 (1.5)	12.4 (2.04)	12.5 (1.66)	14 (1.58)	13.5 (2.07)	12.9 (1.29)	13.1 (2.08)	13.4 (1.76)	0.403†
Menopausal status (%)									
Premenopausal ^d	33	32	38	40	17	12	14	33	0.226‡
Postmenopausal	67	68	62	60	83	88	86	67	
Total no. years a mother (mean) (SD) ^c	29.7 (10.7)	20.7 (16.8)	28.4 (14.0)	32.4 (9.1)	32.2 (14.8)	34.7 (14.9)	23.8 (17.9)	26.1 (18.3)	0.071†
No. Full Term Births (%)									
Nulliparous ^b	0	16	0	0	8	9	24	15	0.556‡
1 or more	100	84	100	100	92	91	76	85	
Breast fed (%)									
Yes	83	68	62	40	92	72	55	74	0.206‡
No	17	32	38	60	8	28	45	26	
Total months lactation (mean) (SD)	6.5 (5.8)	9.7 (19.2)	7.4 (10.8)	2.6 (3.7)	12.8 (9.2)	10.5 (9.8)	7.4 (10.5)	15.5 (15.1)	0.160†
Oophorectomy(%)									
Yes	17	21	15	20	17	12	10	15	0.969‡
No	83	79	85	80	83	88	90	85	
Hysterectomy (%)									
Yes	17	32	15	20	42	22	12	26	0.435‡
No	83	68	85	80	58	78	88	74	

Variable	Normal	Non-Prolif. Benign	Proliferative Benign	Atypical Hyperplasia	In situ Cancer	Grade 1 Cancer	Grade 2 Cancer	Grade 3 Cancer	p value
Previous liver disease (%)									
Yes	0	5	8	0	17	0	10	0	0.259†
No	100	95	92	100	83	100	90	100	
Oral Contraceptive Use (past or present) (%)									
Yes	83	68	46	60	58	66	52	70	0.594‡
No	17	32	54	40	42	34	48	30	
Time oral contraceptives taken (mean no. months)(SD)	117 (114)	54 (61.7)	97.8 (135)	18 (19.9)	64.2 (81.9)	49 (85.9)	58 (84.3)	72 (85.4)	0.425†
HRT use (past or present) (%)									
Yes	33	32	38	40	25	53	29	37	0.550‡
No	67	68	62	60	75	47	71	63	
Time HRT taken (mean no. of years) (SD)	2.3 (3.67)	1.4 (2.73)	3.6 (7.59)	2.2 (4.38)	3.2 (6.25)	4.1 (6.56)	1.8 (3.90)	2.0 (4.27)	0.573†
Family history (%)									
No History	67	53	85	60	67	66	74	70	0.659‡
Some degree	33	47	15	40	33	34	26	30	
Previous breast disease (%)									
Yes	33	21	18	0	33	9	31	11	0.118‡
No	67	79	92	100	67	91	69	89	

Key: † The analysis of variance test was used

‡ The chi-square test was used

a age at entry into the study

^b includes women who had miscarriages or stillbirths

^c variable derived from age at entry into the study minus the age at first full term birth

^d women were considered as premenopausal if they were still menstruating

SD Standard Deviation

Oral contraceptive use and hormone replacement therapy use revealed very little difference between most groups ($p = 0.594$ and $p = 0.550$ respectively). However, for the normal group, 83%, and for the grade 3 cancers, 70%, had used oral contraceptives at some stage. The in situ and grade 2 cancer patients had not used hormone replacement therapy as often as the other groups (75 and 71% respectively). The duration of time that these hormone therapies were taken did not vary statistically for each of the groups ($p = 0.425$ for oral contraceptive use and $p = 0.573$ for HRT use). The normal group and the proliferative benigns had taken oral contraceptives for 117 months and 97.8 months on average, respectively, and the atypical hyperplasia group only used oral contraceptives for 18 months on average. The grade 1 cancer patients tended to use HRT for the longest

period of time on average, compared to the non-proliferative benign patients who used HRT for the least amount of time (4.1 and 1.4 years respectively).

A majority of the study group did not have a family history of breast cancer, nor did they report any previous breast disease ($p = 0.659$ and $p = 0.118$ respectively). A family history of breast cancer was more likely to be reported by women diagnosed with non-proliferative or atypical hyperplasia (47% and 40% respectively) when compared to the other disease states (15% to 34%). However these were not statistically different. The women with a history of previous breast disease were women who had undergone reduction mammoplasties, or surgery for in situ carcinoma or grade 2 cancer (approximately 30% respectively). Interestingly, the women with atypical hyperplasia reported no previous breast disease.

2.3.2.2 Lifestyle Factors

The lifestyle and geographical factors that may increase a woman's risk of breast cancer, as classified by the disease continuum, are presented in Table 17. A greater percentage of the whole study group were city dwellers, except for the women diagnosed with atypical hyperplasia, where more women lived in regional areas. Of those women who did live in regional areas, though, more were diagnosed with invasive cancers than with the benign conditions. These observations were strongly significant ($p = 0.005$). Overall most of the study population were born in Australia, except for women diagnosed with in situ carcinoma, where more were born elsewhere in the world. Interestingly though, of the women born outside of Australia, more were diagnosed with benign diseases than with graded cancers ($p = 0.017$). This observation is also related to the number of years resident in Australia, whereby women with graded cancers had a longer residency (> 49.4 years) in Australia compared with the benign groups (< 44.7 years; $p = 0.0009$).

Very little variation was found for the disease continuum, in terms of height and body mass index ($p = 0.518$ and $p = 0.711$ respectively). However, the weight of patients was slightly higher for the benign groups (74 and 90.8kg) compared to the graded cancers (68.4, 68 and 67.5kg) but this was not significant ($p = 0.927$). Out of a possible score of 67 for the short fat questionnaire, all study groups had a moderate number of fat

consumption events in their diet, but these events did not vary greatly between the groups ($p = 0.952$).

The use of prescribed medication was higher overall for the pre-cancer groups and for the women with graded cancers, possibly owing to the treatment regimes that they were undertaking for their breast conditions. The women, who were classified as normal, were generally non-users of prescribed medication (83 %; $p = 0.021$). The use of non-prescribed medications did not vary greatly along the disease continuum ($p = 0.376$), even though the women in the normal group (83%) used the medications more so than the other groups (< 78%).

Table 17, Geographical and lifestyle factors of women classified by the disease continuum, accrued from Victoria, Australia between 1995 and 1999.

Variable	Normal	Non-Prolif. Benign	Proliferative Benign	Atypical Hyperplasia	In situ cancer	Grade 1 cancer	Grade 2 cancer	Grade 3 cancer	p value
Area of residence (%) ^a									
metropolitan	100	84	92	40	100	84	71	56	0.005†
regional	0	16	8	60	0	16	29	44	
Birth country (%)									
Australia	67	58	62	100	33	81	79	81	0.017†
Elsewhere	33	42	38	0	67	19	21	19	
Years resident in Australia (mean) (SD)	44.7 (8.45)	43.6 (18.7)	37.9 (18.5)	56.4 (10.1)	45.9 (9.04)	59.5 (11.7)	51.4 (16.7)	49.4 (16.9)	0.0009 ‡
Height (cm) (mean) (SD)	162.5 (6.1)	163.4 (7.3)	160.2 (5.6)	156.6 (5.3)	161 (8.6)	161 (6.6)	162.3 (7.1)	160.3 (6.1)	0.518‡
Body mass index (mean) (SD) ^b	27.8 (4.4)	26.4 (5.2)	28.1 (9.2)	29.3 (5.9)	25.7 (4.5)	26.3 (5.0)	25.6 (3.9)	26.4 (5.6)	0.711‡
Weight (kg) (mean) (SD)	74 (15.5)	70.8 (17.8)	73.1 (27.1)	72.4 (18.4)	67.3 (17.2)	68.4 (14.4)	68 (12.4)	67.5 (13.0)	0.927‡
Short fat ques. score (mean) (SD) ^c	25.2 (5.1)	26.8 (6.2)	27.7 (8.0)	28 (4.4)	27.1 (6.1)	26.7 (5.7)	25.6 (6.6)	26.6 (6.0)	0.952‡
Use of prescribed medication (%)									
Yes	17	68	85	80	75	75	88	70	0.021†
No	83	32	15	20	25	25	12	30	
Use of non-prescribed medication (%)									
Yes	83	63	46	40	58	53	62	78	0.376†
No	17	37	54	60	42	47	38	22	
Alcohol consumption (%)									
Yes ^d	50	84	77	40	67	75	64	59	0.388†
No	50	16	23	60	33	25	36	41	

Smoke consumption (past or present) (%)									
Yes	50	42	54	40	50	44	50	56	0.983†
No	50	58	46	60	50	56	50	44	
No. of years Smoking (past or present) (mean) (SD)									
	13.3	5.7	5.9	14	8.5	15.2	12.6	6	0.134‡
	(15.6)	(8.8)	(8.8)	(18.5)	(13.4)	(18.1)	(16.3)	(8.6)	
No. of smokes per day (mean) (SD)									
	8.3	9.8	5.9	6.6	6.4	7.3	6.4	6.3	0.948‡
	(10.3)	(14.3)	(9.6)	(8.5)	(8.7)	(9.4)	(8.1)	(10.0)	

Key: † The chi-square test was used

‡ The analysis of variance test was used

^a Defined by the protocol of assigning postcodes by Australia Post

^b Body mass index is calculated as the weight in kilograms divided by the square of the height in metres

^c Short fat questionnaire score is equal to the number of fat related events in the diet, adapted from Dobson *et al.*, 1993

^d also includes social drinkers

SD Standard Deviation

The consumption of alcohol and cigarettes in the past or presently revealed very little variation throughout the disease continuum ($p = 0.388$ and $p = 0.983$ respectively). However, the non-proliferative benign, proliferative benign and grade 1 cancer groups were greater consumers of alcohol than the other groups (84, 77 and 75% respectively). No statistical differences were found for the number of years the patients had or currently were smoking, or the number of cigarettes smoked per day ($p = 0.134$ and $p = 0.948$ respectively). Interestingly though, more cigarettes were consumed by the benign groups (8.3 and 9.8) compared with the graded cancer patients (< 7.3).

2.3.2.3 Organochlorine pesticide exposure data

The potential exposure to chemicals, both privately and occupationally, and how they relate to the disease continuum are presented in Table 18. Most of the study group, reside in areas that are defined as residential only. A small portion of women from throughout the disease continuum did live in areas where the potential exposure to chemicals from industry was low. However, of the women who lived in areas where the potential exposure to chemicals from industry was high, a greater proportion were diagnosed with atypical hyperplasia (60%; $p = 0.718$). The patient's occupation and the industry in which the patient worked revealed very little difference between the groups of the disease continuum ($p = 0.433$ and $p = 0.587$ respectively).

Table 18, Chemical exposure data of women diagnosed by the breast disease continuum between 1995 and 1999.

	Normal	Non-Prolif. Benign	Proliferative Benign	Atypical Hyperplasia	In situ cancer	Grade 1 cancer	Grade 2 cancer	Grade 3 cancer	p value
Industries Near Residence (%)									
Residential Only	83	47	61	40	75	63	71	56	0.718†
Potential Low ^a	0	5	8	0	8	6	0	7	
Potential High ^b	17	47	31	60	17	31	29	37	
Patient's Occupation (%)									
Professional	17	11	8	20	25	9	26	15	0.433†
Retired/ unemployed/ home duties	33	63	85	60	58	69	64	67	
Administrative/ clerical/ sales	33	26	8	20	17	22	7	15	
Trades/ labour	17	0	0	0	0	0	2	4	
Industry the Patient Works (%)									
Potential Low ^a	83	68	85	60	92	81	83	70	0.587†
Potential High ^b	17	32	15	40	8	19	17	30	
Reported Herbicide Use Around the Home (%)									
Yes	50	53	23	60	50	41	33	30	0.502†
No	50	47	77	40	50	59	67	70	
Patient Handled the Herbicide Around the Home (%)									
Yes	17	32	0	40	25	28	17	15	0.723†
No	33	21	23	20	25	13	17	15	
Not Applicable	50	47	77	40	50	59	67	70	
Reported Pesticide Use Around the Home (%)									
Yes	83	89	85	100	67	84	76	78	0.685†
No	17	11	15	0	33	16	24	22	
Patient Handled the Pesticide Around the Home (%)									
Yes	50	89	77	100	67	72	71	74	0.255†
No	33	0	8	0	0	12	5	4	
Not Applicable	17	11	15	0	33	16	24	22	
Lived or Worked on Farm with Animals Where Chemicals May Be Used (%)									
Yes	0	26	0	40	17	16	7	11	0.145†
No	100	74	100	60	83	84	93	89	
Time on Farm With Animals (mean no. years) (SD)									
	0	8 (17.2)	0	6.6 (13.7)	2.5 (5.8)	3.4 (9.4)	1.2 (5.3)	2.8 (11.6)	0.252‡

	Normal	Non-Prolif. Benign	Proliferative Benign	Atypical Hyperplasia	In situ cancer	Grade 1 cancer	Grade 2 cancer	Grade 3 cancer	p value
Patient Handled									
Chemicals on a Farm with Animals (%)									
Yes	0	5	0	0	8	0	2	4	0.096†
No	0	21	0	40	0	16	5	7	
Unknown	0	0	0	0	8	0	0	0	
Not Applicable	100	74	100	60	83	84	93	89	
Patient Washed									
Clothes that were worn to Apply Chemicals on Animal Farm (%)									
Yes	0	10	0	20	0	9	2	7	0.239†
No	0	16	0	20	8	6	5	4	
Unknown	0	0	0	0	8	0	0	0	
Not Applicable	100	74	100	60	83	84	93	89	
Lived of Worked on Farm with Crops Where Chemicals May Be Used (%)									
Yes	33	32	0	20	0	22	10	26	0.080†
No	67	68	100	80	100	78	90	74	
Time on Farm with Crops (mean no. years) (SD)	3.3 (6.8)	7.8 (16.8)	0	0.8 (1.8)	0	2.8 (6.3)	1.6 (5.5)	4.8 (12.8)	0.172‡
Patient Handled									
Chemicals on a Farm with Crops (%)									
Yes	17	11	0	0	0	0	2	4	0.155†
No	17	21	0	20	0	22	7	22	
Not Applicable	67	68	100	80	100	78	90	74	
Patient Washed									
Clothes that were worn to Apply Chemicals on Crop Farm (%)									
Yes	17	16	0	20	0	3	2	15	0.171†
No	17	16	0	0	0	19	7	11	
Not Applicable	67	68	100	80	100	78	90	74	
House Sprayed (%)									
Yes	17	58	23	40	42	38	33	52	0.446†
No	0	0	8	20	0	12	7	11	
Unknown	83	42	69	40	58	50	60	37	

Key: † The chi-square test was used

‡ The analysis of variance test was used

^a Potential low exposure to the chemicals being studied. This includes industries consisting of business, commercial, transport, clothing manufacture, metalwork, health and welfare, education and public service ventures.

^b Potential High exposure to the chemicals being studied. This includes industries consisting of agriculture, chemical manufacture, cleaning, dry cleaning, wood production, food processing, electrical components and textile production.

SD Standard Deviation

There was little difference between all groups for their reported use of herbicides and pesticides around the home ($p = 0.502$ and $p = 0.685$). The women diagnosed with atypical hyperplasia used more herbicides and pesticides than subjects from the other disease groups (60% for herbicides and 100% for pesticide use). There was no statistical variation between the disease groups if they had themselves handled these herbicides or pesticides around the home ($p = 0.723$ and $p = 0.255$ respectively). Interestingly though, of the women who had reported using these chemicals around the home, a greater percentage had handled the pesticides, in comparison to the herbicides, which were handled by the patient as well as someone else in the household.

A greater percentage of women diagnosed with atypical hyperplasia had resided or worked on a farm where chemicals may have been used on animals, but not significantly so ($p = 0.145$). In terms of women living or working on a farm where chemicals may have been used on crops, there was very little difference between the disease groups. Women diagnosed with proliferative benign and the in situ cancer conditions had not to resided or worked on crop farms (100%; $p = 0.080$). The time spent on animal or crop farms revealed no statistical variation between the disease groups ($p = 0.252$ and $p = 0.172$ respectively), however the women in the non-proliferative benign group had spent the longest period of time on these farms (8 years on animal farms and 7.8 years on crop farms). There was little variation between disease states if the patients themselves had handled the chemicals used on animals or crops ($p = 0.096$ and $p = 0.155$ respectively), nor was there any statistical difference if the patients had washed exposed clothing ($p = 0.239$ clothes worn on animal farms; $p = 0.171$ clothes worn on crop farms). Interestingly though there was a slightly larger proportion of women in the benign groups (17 and 11%) who had handled chemicals used on crops, compared to the graded cancers (0, 2 and 4%).

The concentration of organochlorine pesticides in biopsy specimens was compared between the different diseases along the breast disease continuum. Figure 7 represents the mean concentrations of each pesticide tested for each breast disease state within the continuum. Hexachlorobenzene and DDE were the only pesticides detected at all stages of disease along the continuum. Overall, the amount of pesticide detected was highest for

DDE, regardless of disease state. However the difference in mean concentration of DDE between each disease state was not statistically significant ($p = 0.159$). The difference in mean concentration for all pesticides tested between disease states was not statistically significant ($p = 0.316$ for HCB, $p = 0.224$ for beta isomer of benzene hexachloride, $p = 0.078$ for heptachlor epoxide, $p = 0.236$ for trans chlordane, $p = 0.357$ for cis chlordane, $p = 0.727$ for dieldrin and $p = 0.397$ for DDD). Large error bars were produced for some pesticides owing to the small number of study participants in the particular disease groups, for example, women with normal breast conditions totalled 6.

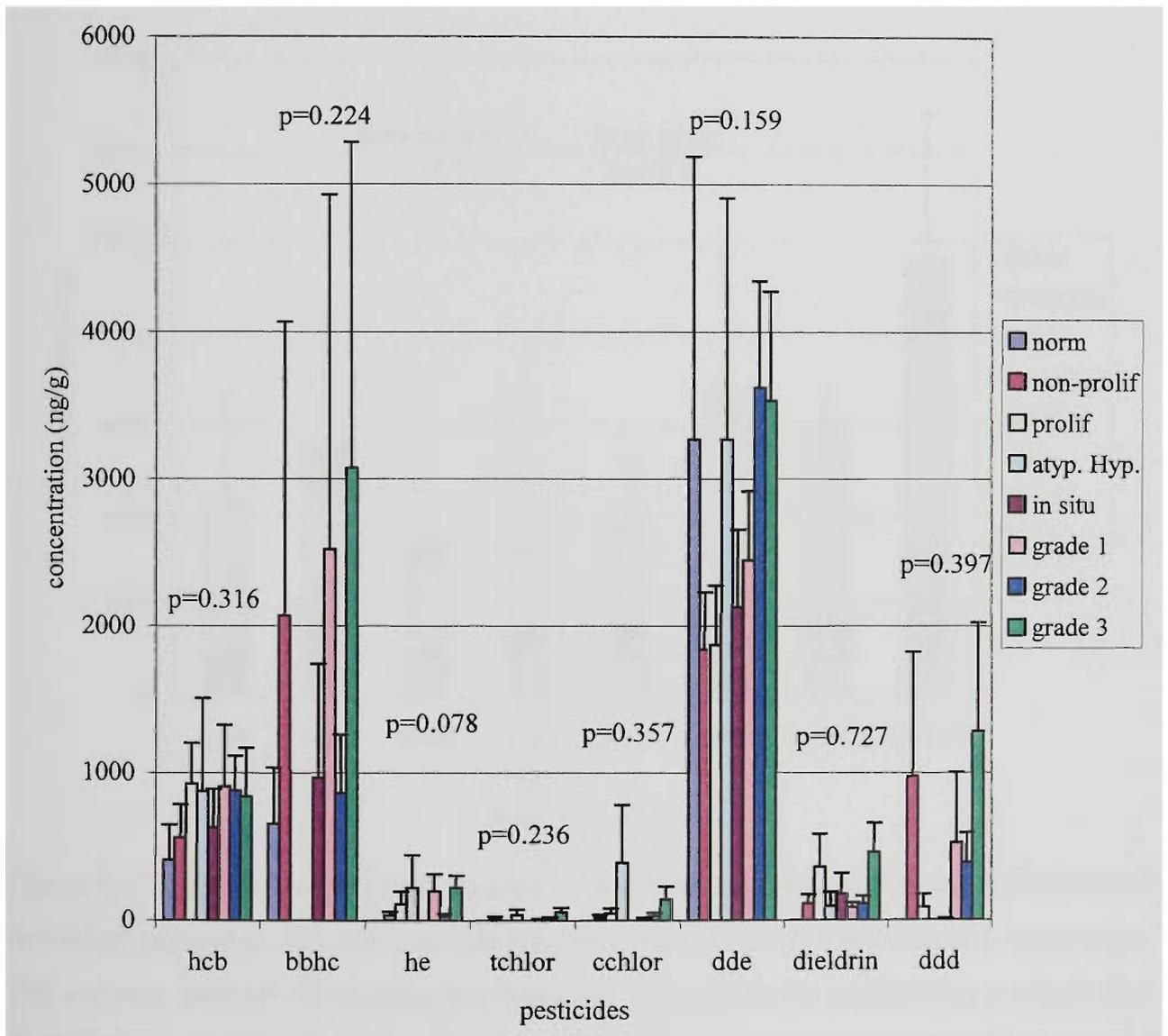


Figure 7, The comparison of pesticide types and concentrations with respect to the breast disease continuum. The one-way analysis of variance test was used to compute the significance p values that appear on the graph for each pesticide. Key: hcb = hexachlorobenzene; bbhc = beta isomer of benzene hexachloride; he = heptachlor epoxide; tchlor = trans isomer of chlordane; cchlor = cis isomer of chlordane; dde = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene; ddd = 1,1-dichloro-2,2-bis(chlorophenyl)ethane; norm = normal tissue; non-prolif = non-proliferative

benign lesions; prolif = proliferative benign lesions; atyp hyp = atypical hyperplasia of the breast; in situ = in situ carcinomas of ductal and lobular types; grade 1, 2 and 3 = graded cancers from 1 to 3.

The individual pesticide concentrations were also assessed as the total amount of pesticide for each disease state along the breast disease continuum (Figure 8). Statistically there was no difference in total organochlorines between disease groups ($p = 0.332$), however there is a linear relationship with total organochlorine pesticide levels and disease along the continuum ($p = 0.037$).

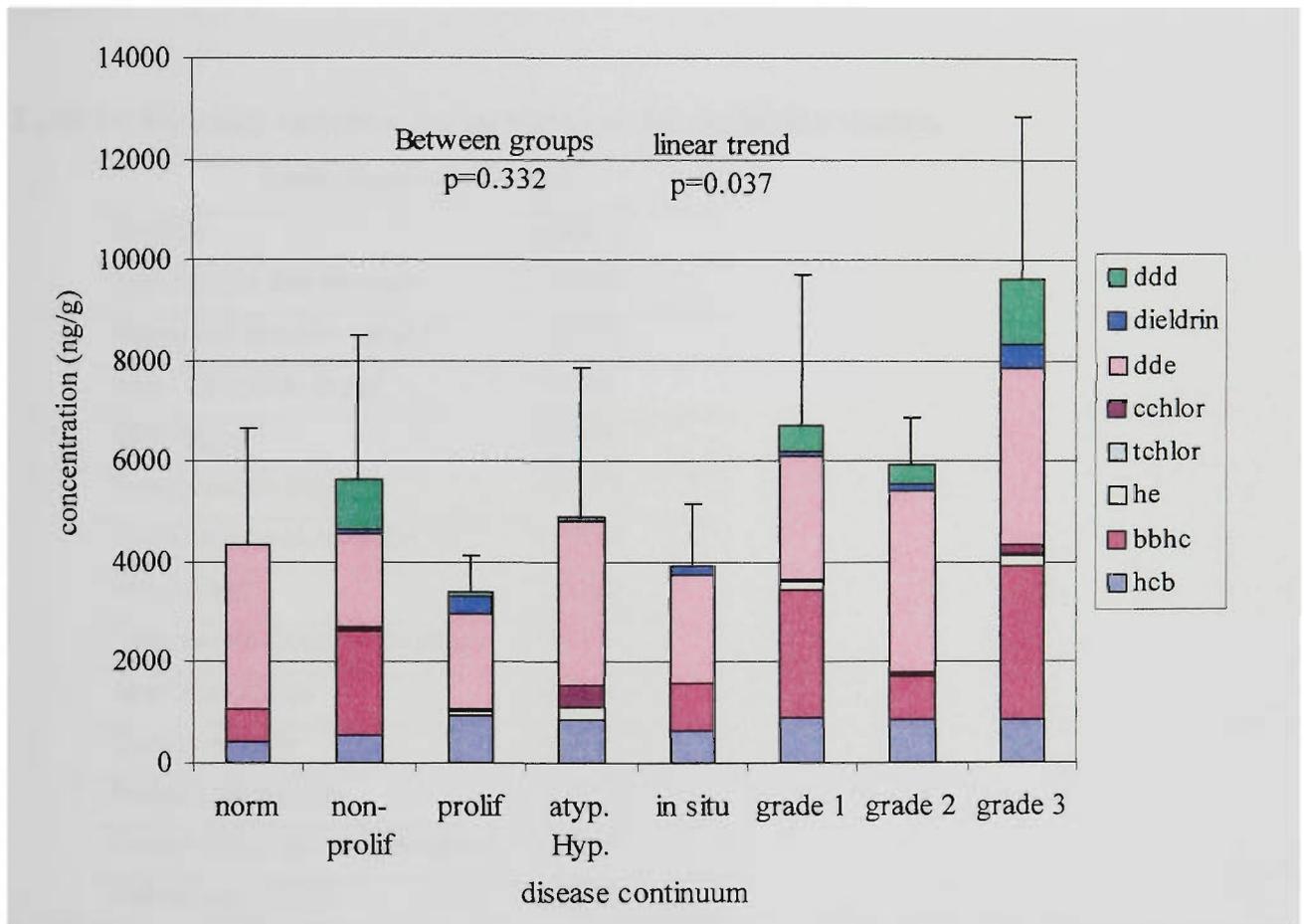


Figure 8, Comparison of the total amount of pesticide and the specific concentrations of individual pesticides that make up this total with respect to the breast disease continuum. The one-way analysis of variance test was used to compute the significance p values that appear on the graph for each pesticide. Key: hcb = hexachlorobenzene; bbhc = beta isomer of benzene hexachloride; he = heptachlor epoxide; tchlor = trans isomer of chlordane; cchlor = cis isomer of chlordane; dde = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene; ddd = 1,1-dichloro-2,2-bis(chlorophenyl)ethane; norm = normal tissue; non-prolif = non-proliferative benign lesions; prolif = proliferative benign lesions; atyp hyp = atypical hyperplasia of the breast; in situ = in situ carcinomas of ductal and lobular types; grade 1, 2 and 3 = graded cancers from 1 to 3.

2.3.3 Comparison of questionnaire and breast tissue organochlorine pesticide concentration data along the breast disease continuum

As stated earlier, the analysis of all facets of data collected in the present study required multiple logistic regression models fitted through forward stepwise variable selection procedures. The dependent variable of the regression model was the breast disease continuum coded as a continuous variable. The independent variables that may be considered in the regression model, on the basis of significant values, are presented in table 19.

Table 19. Potential variables for inclusion in the regression models.

Breast disease continuum	
Variable	p value
Year of entry into the study	0.0009
Heptachlor epoxide (ng/g) *	0.0709
<i>trans</i> - Chlordane (ng/g) *	0.0881
DDE (ng/g) *	0.0308
Total pesticide (ng/g) *	0.1015
Years resident in Australia	0.0081
Weight (kg)	0.1098
Total months lactation (months)	0.0961
Area of residence	0.0050
Country of birth	0.0169
Patient's occupation	0.1097
Current use of oral contraceptives	0.0969
Current use of HRT	0.0390
Use of prescribed medication	0.1098
Patient handled herbicides used around the home	0.1073

Key: * All pesticide levels are the logarithmically transformed values
HRT Hormone replacement therapy

According to the significance values for the breast disease continuum data, the most significant value was that of the year of entry into the study ($p = 0.0009$). However this variable was known, prior to the regression analyses, to be a bias of the study, so it was not included in the final regression model. A previous regression model (data not shown)

using the total amount of pesticide (logarithmically transformed totals) as the dependent variable suggests that there may be a weak association with the year of entry into the study ($p = 0.044$). The regression model of the breast disease continuum as the dependent variable against all variables tested, excluding the year of entry into the study is presented in Table 20.

Table 20. The sequential order of variables entered into the regression model with respect to the breast disease continuum.

Step number of the regression	Variable	p value
1st	Area of residence	0.0002‡
2nd	Reported herbicide brands used around the home ¥	0.063‡
3rd	Time on a farm with animals (years) ¥	0.009‡
4th	Years resident in Australia	0.009‡
5th	Knowledge of the local council spraying in the area	0.014‡
6th	Age at menarche (years)	0.028‡

Key; * All pesticide values are the logarithmically transformed values

† A previous model found that total pesticides were related to year of entry into the study, which was removed from the variable list. Therefore this model proceeded with the removal of this variable.

‡ The significance value at the end of the regression model.

¥ According to the one-way comparative tests, this variable is likely to be a random event in the model, therefore any variables entering the equation after this one are not included in the final model.

The area of residence was the first variable to enter the regression equation (after the removal of the total pesticides), suggesting that women living in regional areas tend to be diagnosed with graded cancers compared with women living in metropolitan areas. The reported types of herbicides used around the home entered the model in the next step. This variable, when cross checked with the one-way comparative analyses, does not make clinical sense, as the brands of herbicides recorded were largely not applicable to most women in the study as they did not use herbicides, or the brand used was a class of herbicide not under investigation in this study. The types of herbicides used may only

have been recalled by a small number of women, so there may be some recall bias. Therefore the reported herbicide brands were treated as a random variable in the equation and hence any variables entered into the regression after it, were not included in the final model.

The final regression model, therefore only consists of the area of residence (whether it be metropolitan Melbourne or regional areas of Victoria and neighbouring border towns of New South Wales) with respect to the breast disease continuum ($p = 0.002$). This model should also take into account the removed variables, such as year of entry into the study and total pesticide concentration, as these may be potential confounders linked to the area of residence with respect to the breast disease continuum.

2.3.4 Determination of possible synergy between the number of pesticides detected, disease state and the data obtained from the questionnaire

The number of different pesticides present in each biopsy specimen was calculated and each specimen classified according to the disease state. The average number of pesticides detected was compared for the breast disease continuum, to determine if more than one pesticide (possibly working in synergy with each other) affects breast cancer progression (Figure 9).

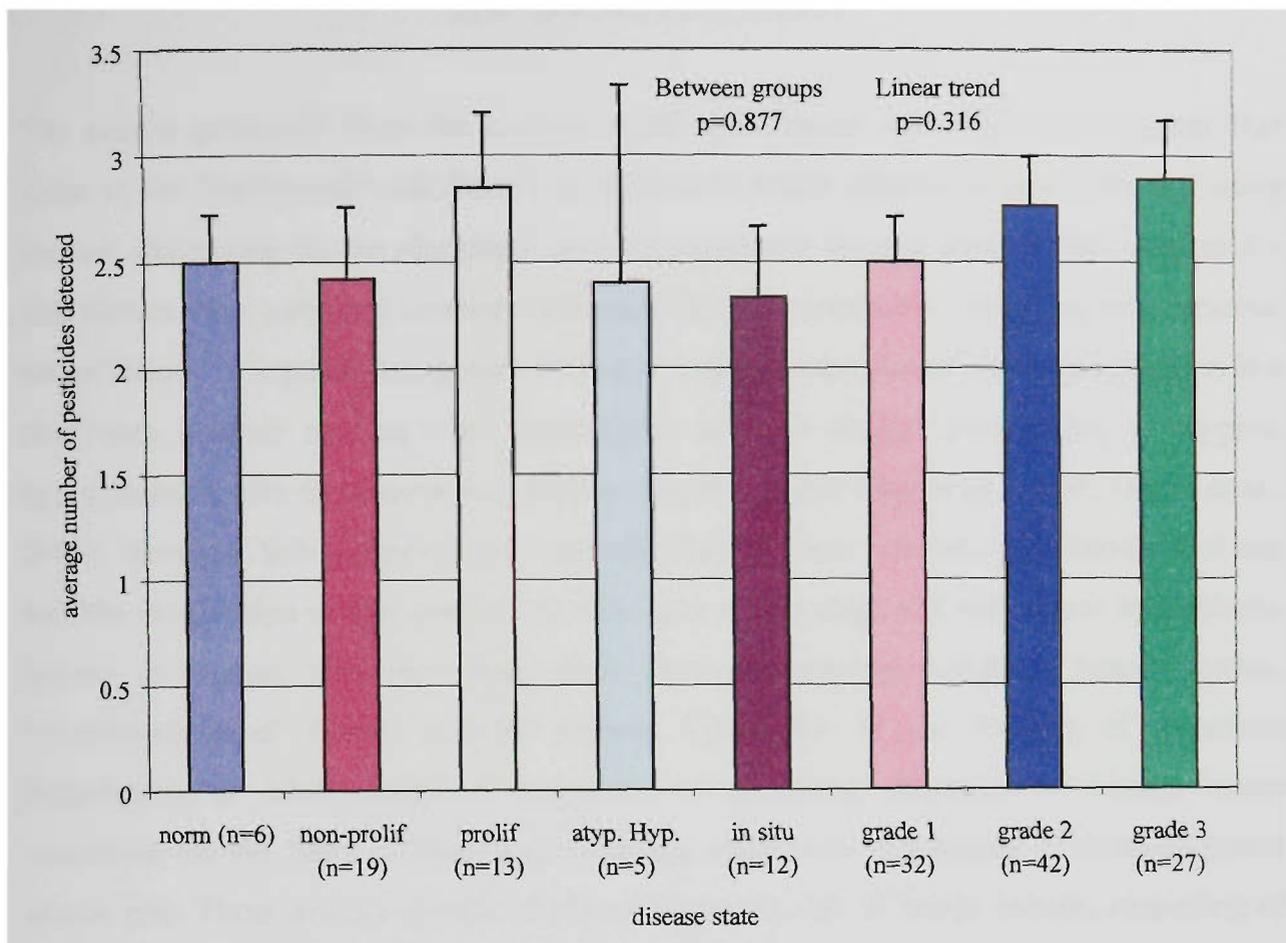


Figure 9. The average number of pesticides according to the diseases along the breast disease continuum. Key; norm = normal breast tissue; non-prolif. = non-proliferative benign breast lesions; prolif. = proliferative benign breast lesions; atyp. Hyp. = atypical hyperplasia; in situ = in situ carcinoma of ductal and lobular types; grade 1, 2 and 3 = graded cancers according to the Scarff Bloom Richardson scale

The mean number of organochlorines detected for each disease along the breast disease continuum is graphed in Figure 9. A mean number of 2 to 3 pesticides were detected in all disease states. Overall, there was a tendency for an increase in number of pesticides with progression through the breast disease continuum although this observation was not statistically significant (linear trend $p = 0.316$) and there were not any obvious differences in the number of pesticides between groups ($p = 0.877$).

2.4 DISCUSSION

The results generated from the questionnaire and clinical pathology data suggest that some of the "traditional" risk factors are related to breast disease in this study and some are not, depending on the classification of the patient's disease state. In this chapter the risk factors were analysed against the breast disease continuum, including precancerous breast lesions in separate categories. Research suggests that breast cancer progression is a multi-step process starting with hyperplasia without atypia, progressing to atypical hyperplasia, in situ carcinoma and finally invasive cancer (Bai *et al.*, 2001; Gayat *et al.*, 2003). However this progression is unpredictable as there are unknown factors that can halt the progression of this continuum of events at any stage and may cause hyperplastic lesions to regress, thus preventing them from undergoing malignant transformation. Fitzgibbons *et al.* (1998) and the Cancer Committee of the College of American Pathologists of 1985 described categories for grouping diagnoses of benign breast conditions on the basis of pathologic findings relative to the degree of invasive breast cancer risk. These groups consist of (1) no increased risk of breast cancer, consisting of conditions such as adenosis other than sclerosing adenosis, ductal ectasia, fibroadenoma without complex features, fibrosis, mastitis, mild hyperplasia without atypia, ordinary cysts, simple apocrine metaplasia without hyperplasia or adenosis and squamous metaplasia; (2) slightly increased risk of breast cancer (1.5-2.0 times increased risk), consisting of fibroadenoma with complex features, moderate or florid hyperplasia without atypia, sclerosing adenosis, and solitary papilloma without co-existent atypical hyperplasia; (3) moderately increased risk of breast cancer (4-5 times increased risk) consisting of atypical ductal and lobular hyperplasia; (4) markedly increased risk of breast cancer (8-10 times increased risk) consisting of in situ ductal and lobular carcinoma (Fitzgibbons *et al.*, 1998; Wang *et al.*, 2004). The present research study used these categories for identifying groups of women who may have an increased risk of breast cancer, however the numbers of women in some of these groups was small. The present study consisted of 6 women who were at no increased risk of breast cancer due to having breast reduction surgery and 19 women being diagnosed with non-proliferative benign breast conditions. Thirteen women were diagnosed with proliferative benign conditions, giving them a slightly increased risk of breast cancer, whilst 5 women were diagnosed with atypical hyperplasia and 12 women diagnosed with in situ carcinomas giving them

moderately to markedly increased risks. A great majority of the present study's participants were diagnosed with graded cancers; 32, 42 and 27 diagnoses for grade 1, grade 2 and grade 3 breast carcinomas respectively. Due to the uneven spread of participants between these groups, it is difficult to draw any conclusive findings of the known risk factors for breast cancer, such as age at menarche and menopausal status, as well as the potential of organochlorine pesticide exposure in relation to breast cancer risk along the disease continuum. Ideally for this comparison to occur a larger study group would be required, therefore the following discussion of the presented results is purely observational. In saying this however, some interesting observations were made in particular reference to the factors associated with atypical hyperplasia, as the results indicate and other researchers suggest that this condition may be an important factor for breast cancer risk determinations (Gayat et al., 2003; Reis-Filho and Lakhani, 2003).

The aetiology of breast cancer has led to the suggestion that there is a strong hormonal influence in the development of the disease and that the development and differentiation of the breast is under the tight regulation by hormones at different periods of a woman's reproductive life. Previous research suggests that factors such as age at menarche, menopausal status, parity-related events, family history and the use of exogenous hormones, are significant factors in the development of breast cancer (Stavraky and Emmons, 1971; Helmrich *et al.*, 1983; Colditz *et al.*, 1996). It is not surprising that these factors have been implicated considering that they are influenced or directly influence themselves, the hormonal milieu of the female human body. The severity of disease, from relatively normal to invasive breast cancer increased with age, although this was not significant ($p = 0.060$). This non-significant finding was the result of the number of disease categories that were used in the analysis and the correspondingly small number of patients classified in each group. An age adjustment of the data pertaining to the continuum of breast disease events was not performed because of these small numbers of participants. The present study also found that women diagnosed with atypical hyperplasia had a later age at menarche compared with the other groups (14 years compared to 12-13 for the other groups collectively), suggesting that in this study group that a later commencement of menarche is not a protective factor against the risk of breast cancer.

Traditionally if a woman is diagnosed with breast cancer at a young age they tend to have a more aggressive cancer compared to older women, possibly owing to the strong links with family inheritance (Clark *et al.*, 1984; Hall *et al.*, 1990). These findings in the literature however cannot be supported with certainty as the classification of disease states used in these studies investigating breast cancer in younger women also includes the lesions that predispose to breast cancer development. Previous research efforts have been undertaken to elucidate whether there are shared epidemiologic profiles between women with breast cancer and women with various benign breast conditions, including pre-cancerous lesions (Ernster, 1981; Fleming *et al.*, 1982; Wang and Fentiman, 1985). The present study found that women diagnosed with atypical hyperplasia in general had different breast cancer risk factor profiles in relation to the reproductive factors compared to the other groups; (1) a greater proportion were pre-menopausal (40% compared to 12-38% for the other groups collectively, $p=0.226$); (2) of those who had full-term births, a smaller percentage had breast-fed (40% compared to 55-92% for the other groups collectively, $p=0.206$); (3) lower time for lactation (2.6months on average compared to 6.5-15.5months on average for the other groups collectively, $p=0.160$); and (4) of those using oral contraceptives, a shorter time of use (18months on average compared to 49-117months for other groups collectively, $p=0.425$). These observations above suggest that given a larger study group, atypical hyperplasia is an important benign breast condition that should be isolated from other benign conditions for the association with breast cancer risk. In contrast to this however, longer durations of time for lactation were observed for women diagnosed with graded cancers (7.4 to 15.5months on average) which are amongst the highest durations reported for all the present study groups, indicating that a long duration is not protective for breast cancer risk, and further indicating that different disease categories potentially have different associated risk factors. In a review, Ernster (1981) suggests that a late age at natural menopause tended to be associated with women diagnosed with benign breast conditions, but this group of women is likely to include those with proliferative disease. Research by Carter and colleagues (1988) however suggests that risk of breast cancer among women with benign breast conditions is associated with the degree of epithelial atypia, in their study of 16,692 women with benign breast disease classified into 5 disease categories. Their notion of breast cancer risk dependent on benign classification is also supported by the later work of Gayat *et al.* (2003) and Wang and colleagues (2004). Gayat and colleagues (2003) found that women

diagnosed with atypical hyperplasia and younger than 50 years had a 4.5fold increased risk of breast cancer compared with women of the same age from the general population. They also found that among 40-69 year old women, atypical hyperplasia incidence increased over time at a rate of 13.6% per year from 1994 to 1999, and at a rate of 15.4% per year amongst postmenopausal women, which is significantly greater than the incidence of malignant disease among the same age strata (Gayat et al., 2003). Wang et al. (2004) suggest that approximately seventy percent of women presenting for breast biopsies would be classified as non-proliferative benign lesions, but their association with breast cancer risk is less well known than associations with atypical hyperplasia and in situ carcinomas. Their findings suggest that the risk of breast cancer associated with non-proliferative benign breast disease is independent of that associated with the key breast cancer risk factors. Future studies, however should endeavour to explore the risk factors for breast cancer, associated with different categories of benign breast disease.

The role of oestrogen as a causal factor for breast cancer does not explain the interracial and international variations in breast cancer incidence apparent in many studies (McMichael and Giles, 1988; Mayberry and Stoddard-Wright, 1992; Chen *et al.*, 1994; Weiss *et al.*, 1995). The results presented in table 17 of the present study suggest that women diagnosed with invasive breast cancer in general are resident in Australia for longer periods of time. This observation is interesting considering that it is an association between age at entry into the study and the assessment of breast disease in migrant women to Australia. The highly significant ($p < 0.005$) nature of these findings suggests that older Australian-born women are more likely to develop breast cancer than migrant women of the same age. Conversely, migrant women to Australia may be protected from breast cancer to an extent, depending on which country they were born, or by factors derived from their country of birth such as environment, lifestyle and diet. The data of this study however suggest that of the women born outside of Australia, a larger proportion tended to be diagnosed with benign breast conditions ($p = 0.017$). This finding however must be taken into consideration with caution as some of the categories of the breast disease continuum contain less than 10 participants. The report of McMichael and Giles (1988) suggests that, as an example, breast cancer mortality among Italian-born migrants to Australia, tends to vary as a function of duration of residence, such that a residence of 17 years or more confers a mortality rate similar to Australian-born women.

The report of Australia's Health in 2000 also suggests that illness among migrant groups increases with length of residence in Australia (AIHW, 2000). If there is some degree of protection afforded to immigrants compared to Australian born it is likely to be related to diet. As dietary fat consumption was the only dietary variable addressed in this study (and was not significantly associated with breast cancer, $p=0.952$), it is not clear where the potential protection may lie for the migrant women with benign breast disease. Descriptive epidemiological data presented by McMichael and Giles (1988) suggests that some Southern European migrant women have diets high in all sources of dietary fibre (much higher than the traditional Australian intake) and the consumption of saturated fats was much lower, and may offer protection against breast cancer. Research by Baghurst and Rohan (1994) suggests that a diet high in fibre reduces the risk of breast cancer by reducing the deconjugation and reabsorption of oestrogen excreted via the biliary system. Diet may be an important determinant in risk among migrants, as research has shown that first generation migrants tend to maintain the dietary practices of their country of birth (Haenszel and Kurihara, 1968; McMichael and Giles, 1988). On the other hand, second generation migrants have been shown to adopt some of the dietary behaviours of the country of migration, and thus potentially increase their risk (Haenszel and Kurihara, 1968; McMichael and Giles, 1988).

The role of oestrogens as a causal factor for breast cancer does not explain the differences in rates reported for women living in rural areas compared to metropolitan areas. This study reports that, although most of the study population reside in metropolitan Victoria, slightly more women residing in regional areas of Victoria tend to be diagnosed with the graded breast cancers ($p = 0.005$). Also of interest is the higher number of women diagnosed with atypical hyperplasia that reside in regional/ rural areas (60%, compared to 0-16% for other benign groups collectively and 0-44% for in situ and graded cancers collectively). In a report of Australia's Health, the health of populations living in rural and remote areas of Australia tends to be worse than that of populations in metropolitan areas. The Australian Institute of Health and Welfare (2000) suggest that this is likely to be attributed to poorer access to health services and professionals, lower socioeconomic status and employment levels, and exposure to comparatively harsher environmental and occupational hazards. A study of the distribution of medical practitioners throughout Australia, has found that rural and remote areas are under-represented by health

practitioners compared to metropolitan areas (144 per 100 000 for rural and remote compared to 306 per 100 000 for metropolitan areas) (AIHW, 2000). Thus, rural women may need to travel to metropolitan areas to access specialist services and if this is the case, these women are also more likely to delay seeking medical advice.

The trends between pesticide exposure and the association with breast cancer have been presented. As stated, the level of pesticides detected in the breast adipose tissue of all participants was dependent on the type of pesticide. DDT was not detected in any of the breast adipose specimens. DDT was the active compound used in the past for the control of vector-borne disease, to combat head lice and for agricultural and household purposes, however, this compound is unstable and is degraded to the stable metabolite, DDE. DDE was detected the most frequently of the pesticides tested and at the highest concentrations ($2.86\mu\text{g/g} \pm 3.46$ of breast adipose tested). The levels of DDE were higher in the biopsy tissue taken from women with invasive cancer compared with the benign breast biopsies but this was not statistically significant ($p=0.159$). Of particular interest was the relatively high level of DDE in tissue of women diagnosed with atypical hyperplasia (levels similar to women that underwent reduction mammoplasties). This observation raises the possibility that organochlorine pesticides, in particular DDE and other associated metabolites of DDT, may not be important risk factors for breast cancer development in relation to a continuum of breast disease events. In the literature however the data is scarce in terms of any trends in chemical exposure data in relation to a continuum of disease events. And even though the average levels of DDE were similar in the breast biopsies from women who underwent reduction mammoplasties, atypical hyperplasia and invasive cancers, there exists a linear trend ($p=0.037$) of increasing concentrations of organochlorine pesticides, which is mainly attributed to the DDE levels, through the breast disease continuum, thus indicating that there is the potential of organochlorine pesticides to influence breast cancer risk.

The levels of organochlorine pesticides detected in this study are fairly consistent with the findings depicted in previous studies suggesting that there is a reduction in concentrations of pesticide residues over time, considering that the active chemicals are no longer used. Research compiled by Loganathan and colleagues (1993) investigating the temporal trends of organochlorine residues in human adipose tissue supports the notion of reduced

concentrations of pesticide residues over time, in line with the phasing out of DDT use. For example, research of organochlorine levels in women from Poland was 27 $\mu\text{g/g}$ on average in 1979 for DDE whilst in 1990 the DDE levels were reduced to 13 $\mu\text{g/g}$ on average (Loganathan *et al.*, 1993; Tanabe *et al.*, 1993). Research by Wasserman and colleagues in 1968 detected 6.3 $\mu\text{g/g}$ of DDE and 2.5 $\mu\text{g/g}$ of DDT in adipose tissue of the abdominal wall of people with no known exposure to pesticides in Western Australia. The levels reported in adipose tissue in the studies mentioned are from people of the general population and not related to specific disease states. The focus of the present study however was to assess levels of organochlorine pesticides in breast adipose tissue and relate these levels, along with reported chemical exposure data, to the relative risk of breast cancer (chapter 3). The study of a continuum of breast disease events in this chapter, in relation to chemical exposure and the possible risk of breast cancer is quite unique. In terms of organochlorine exposure, other research has focused on these links between controls (either age-matched populations or women with benign breast disease) and breast cancer patients, or in women with significant occupational exposure. The results of the present study indicate that most of the chemical exposure variables measured were not statistically associated with breast cancer risk along the breast disease continuum. However, one important point to note was that there were some interesting observations that may highlight particular subsets of people with higher susceptibilities to breast cancer development than others. Women diagnosed with atypical hyperplasia reported of industries near their place of residence, where the potential exposure to chemicals was quite high, to a greater extent than women of the other groups collectively (60% compared to 17-47% of the other groups). Industries that would fall into this category include agriculture, for example market gardens or semi-rural properties, chemical manufacture companies, such as oil refineries, cleaning companies where a range of potentially toxic cleaning agents would be used, dry cleaning, wood production which includes saw mills and pulping factories, food processing which includes the washing/ sterilisation of raw food ingredients and any modifications of foods for incorporation into processed foods, electrical components where in the past the manufacture of electrical components utilised chlorine based materials such as polychlorinated biphenyls (PCBs), and textile production where the potential for exposure to chemicals is high through dyeing processes and the production of synthetic materials. These industries have been isolated from others, as in the past, chlorine-based chemicals

were used in these types of industries, and the potential for air-drift of these chemicals or their breakdown products to reach residents in the immediate area, even though there is no direct contact with the chemicals, is quite high (McDuffie, 1994). Research by Zahm and colleagues (1993) suggests that the relative risk of Non-Hodgkin's Lymphoma (NHL) varied depending on how and for what purpose the chlorinated pesticides were used. Hence, given a larger sample size for the present study, comparisons of breast cancer risk would have been made on the basis of industries near the place of residence, industries of occupation and other potential sources of pesticide or herbicide exposure. In the present study, women diagnosed with atypical hyperplasia reported living or working on a farm where chemicals may have been used on animals to a greater extent than the other categories of the disease continuum (40% compared to 0-26%, $p=0.145$), however similar observations were not seen for women living or working on farms where crops may have been treated with chemicals. The findings of Zahm and colleagues (1993) support these observations, such that the relative risk of NHL was 3.0 (95% CI: 0.9-9.3) for pesticides used on dairy cattle compared with an odds ratio of 1.6 (95% CI: 0.5-4.3) for the use of pesticides on farm buildings or lots. Another interesting observation of the present study was among women diagnosed with atypical hyperplasia, a greater percentage had used herbicides or pesticides around the home, compared to the other groups (60% compared to 23-50%, and 100% compared to 67-89% for the other groups collectively in relation to herbicide ($p= 0.502$) and pesticide ($p=0.685$) use respectively). In studies of agriculturally exposed women researchers have found elevated risks of multiple myeloma, ovarian cancer and Non-Hodgkin's lymphoma attributable to herbicide and pesticide use (Donna *et al.*, 1983; Eriksson and Karlsson, 1992; Zahm *et al.*, 1992; Zahm *et al.*, 1993).

2.5 CONCLUSION

As indicated in this chapter, the accuracy of breast disease classification plays an important role in determining trends in the aetiological factors that may be involved in breast cancer development. Research suggests that the risk of breast cancer varies depending on the classification of benign lesions, and this is further complicated by the different histologic criteria used to classify benign breast disease. The problem with classifying the participants into the eight categories of the disease continuum is that the

number of patients in some subgroups was not sufficiently large enough to permit a detailed statistical analysis of subgroups. Therefore as a consequence, most of the in depth statistical analyses to follow in chapter 3 focus on the true benign and true cancer categories. Some researchers suggest that the risk of breast cancer is not uniform in women with benign breast disease, such that the risk is concentrated in women with benign proliferative lesions. Hence, the women of this study with diagnoses classified as proliferative benign, atypical hyperplasia or in situ carcinoma were not included in the final statistical analyses (to follow in chapter 3). In previous studies the term “benign breast disease” has been used loosely to describe women who have undergone breast biopsies but do not have invasive or in situ carcinoma, and may account for discrepancies in estimating breast cancer risk. Of particular interest in this study however, were the noticeable observations between a diagnosis of atypical hyperplasia and many of the chemical exposure variables. Several variables tested by the questionnaire, to ascertain chemical exposure, would place women diagnosed with atypical hyperplasia in a high risk exposure category and thus may increase their risk of breast cancer. This study however found that most of the accepted risk factors for breast cancer were not associated with any elevation in risk along the breast disease continuum, owing to the small sample sizes in each category. It would be ideal to confirm these observations in a study with a larger number of subjects in each category, particularly with more true benigns as controls, such as those recruited from breast reduction surgery.

Noteworthy observations that were significant however, were those associated with lifestyle choices, such as area of residence and number of years residency in Australia. And one particular trend of importance was the total linear increase of organochlorine pesticides with increasing severity of breast disease such that women with grade III breast cancer tended to have a higher total body burden of organochlorine pesticides compared with women undergoing reduction mammoplasty (normal group).

CHAPTER 3
ORGANOCHLORINE PESTICIDES AS A RISK
FACTOR FOR BREAST CANCER

3.1 INTRODUCTION

Organochlorine pesticides are persistent lipophilic substances used for the control of vector-borne diseases, to control pests in and around the home, and in industry. Studies of these pesticides in animal models in the laboratory and in the field have yielded results indicating that they are potentially toxic, and as a result, have led to their banning in most parts of the world. However, due to their persistence, they are still being detected in the environment and in wildlife. Studies in America have found that these pesticides are potential endocrine disruptors and thus may be involved in many hormone and endocrine-mediated diseases, such as breast cancer.

Many studies have evaluated the effects of organochlorine pesticides and their potential to increase the risk of breast cancer in women. However, even though there have been numerous studies, there is still some uncertainty regarding the potential role of organochlorines as causative agents for breast cancer development. The conflicting results in the literature regarding the issue of organochlorine pesticides as a risk factor for breast cancer may be the result of differences in the types of study (and hence the study groups) used, as well as the biological medium used to evaluate breast cancer risk attributable to pesticide exposure.

The presence of organochlorine pesticides in human serum and adipose tissue has been documented in many studies. Owing to their chemical structure, organochlorine pesticides are primarily stored in adipose tissue, but have also been detected at lower concentrations bound to serum proteins and in other tissues (Eyster *et al.*, 1983). Research by Archibeque-Engle and colleagues (1997) evaluated the comparative concentrations of organochlorine pesticides in serum and adipose tissue. Their results suggest that whilst most of the pesticides they tested were found in the adipose tissue, much lower levels were detected in the serum (Archibeque-Engle *et al.*, 1997). Archibeque-Engle and colleagues (1997) suggest that serum is a useful biological medium for evaluating the mobilisation of the organochlorine pesticides and would indicate recent exposure or acute exposure if detected at substantially high concentrations. On the other hand the levels of organochlorine pesticides detected in adipose tissue are thought to represent body burden

and so provides a better indicator of long-term pesticide exposure. Significant associations between DDE and PCBs and breast cancer risk have been reported from studies that used serum, adipose tissue or both as a measurement medium. The investigation by Wolff and colleagues (1993), which involved the measurement of pesticides in a cohort of women accrued from 1985 to 1991 in a New York University Women's Health Study found that breast cancer was strongly associated with DDE levels detected in serum. However Krieger and colleagues (1994) found no association between serum DDE or PCB exposure and increased breast cancer risk within their cohort. The work of Falck and colleagues (1992) also found that the levels of DDE were higher in the women diagnosed with cancer compared with controls, while the levels in adipose tissue were substantially higher than in serum ($1877\text{ng/g} \pm 1283\text{ng/g}$ from women with cancer compared to $1174\text{ng/g} \pm 630\text{ng/g}$ in controls). This study by Falck and colleagues (1992) and the case control study of Bagga and colleagues (2000) found that total DDT levels were elevated in women with breast cancer but there was no increase in breast cancer risk when consideration was given to the established risk factors or other confounding factors.

Previous research by Petrek and colleagues (1994) suggested that the levels of fatty acids measured in adipose tissue may vary depending on the source of the tissue, and could be a good indicator for choosing the most appropriate medium for the measurement of lipophilic substances. Petrek and colleagues (1994) measured the fractions of different fatty acids in breast and abdominal adipose tissue and their research suggests that the levels of saturated and monounsaturated fats were higher in the breast adipose tissue than in the abdominal tissue. Oka and Yoshimura (1986) suggested that the levels of these fats may be important for the normal differentiation, proliferation and development of breast epithelial cells. These studies have been conducted after the severe restriction of use of these pesticides had taken place, thus making it likely that the levels of organochlorines being stored in the body would be reduced over time. The restriction in use of these organochlorine pesticides occurred at an earlier time point in the United States than in Australia, which would account for the findings of high levels of pesticide being detected in the current Australian population. However there have only been a limited number of Australian studies to date, undertaken to investigate the appropriate choice of biological medium so it is likely that the findings of pesticide levels in Australia under-represents the true body burden of the organochlorines and therefore the possible risk of breast

cancer (Bick, 1967; Brady and Siyali, 1972; Siyali, 1972; Siyali, 1973; Stacey and Tatum, 1985; Sim and McNeill, 1992).

3.1.1 Aims

To identify the factors other than organochlorine pesticides that may contribute to the risk of breast cancer in order to adjust for any confounding or effect modification that may occur in relation to pesticide exposure.

To determine if there is an increased risk of breast cancer associated with organochlorine pesticide exposure from measurements of pesticides in adipose tissue when adjusted for other potential factors as assessed from a questionnaire on lifestyle of women admitted to hospital for breast surgery.

To determine if any potential interactions or synergy exist between organochlorine pesticide exposure, breast cancer risk and the established risk factors for breast cancer in women who had been admitted to hospital for breast surgery.

3.2 MATERIALS AND METHODS

The organochlorine pesticide concentrations in breast biopsy tissue, questionnaire data assessing the risk factors and any potential confounders for breast cancer, and pathology data (all as presented in chapter 2) were combined and statistically analysed. The statistical analyses involved logistic regression equations to determine if breast cancer risk was increased with organochlorine pesticide exposure and an analysis of the types of potential interactions between variables.

3.2.1 Statistical Analyses

Firstly, all variables from the questionnaire and the logarithmically transformed pesticide data were cross tabulated against sample type (crude benign or cancer) to determine any difference. The logarithmically transformed pesticide concentration data and other continuous variables, such as age, weight and time spent on a crop or animal farm, were analysed, using student's t-tests for independent samples, against benign and cancer categories. The data requiring coding for yes or no answers, or in the case of multiple response answers, were analysed against benign and cancer categories by chi-square contingency tables, using Pearson's statistic to assess significance.

Secondly, all questionnaire, pathology and logarithmically transformed data were analysed against "true benign" and "true cancer" groups, excluding conditions that were deemed precursors to breast cancer development. The logarithmically transformed pesticide data and other continuous data were statistically analysed against the conditions categorised as "true" benign and those categorised as "true" cancer by one-way analysis of variance. The variables comprising categorical data obtained from the questionnaire were analysed against "true" benign and "true" cancer categories by chi square contingency tables, using Pearson's statistic to assess significance.

The chemical exposure and risk factor data from the questionnaire and transformed pesticide concentrations were analysed by a multiple logistic regression model fitted through a forward stepwise variable selection procedure with the use of SPSS software. Logistic regression models were appropriate for the present study because the outcomes were categorical rather than continuous. Variables were entered into the regression equation in a stepwise fashion, in the order of strongest significance to weakest significance (with p values less than 0.05) to observe what happened to each variable once entered into the model, and if the change was insignificant if it was removed from the model. The variables were continuously entered into the regression and the effects of each variable on the others already in the model were monitored for change until there were no variables remaining with a significance value less than $p = 0.05$. The variables entering into the regression models were also screened for chance or random variables by considering previous one-way comparative analyses and the frequency data. If random

variables enter into the regressions, the model ceases (George Rennie, personal communication). The dependent variable for the regression model was true benign/ true cancer. The breast conditions classified as crude benign/ cancer are both likely to include conditions considered as precursors to breast cancer development, so these statistics will not be presented as regression models in the results. The results will only reflect the regression models for the true benign/ true cancer. Regression models were also calculated with an adjustment for age, as it is a strong risk factor for breast cancer. The age data collected in this study however produced a skewed distribution and because it is a variable that is being forced into the regression equation, the age data was subsequently transformed (squared) to produce a normal distribution.

In the final instance, the number of pesticides detected in the breast tissue specimens was compared to the disease status and the variables collated from the questionnaire. The number of pesticides was determined to assess if there was any synergy with more than one pesticide and its correlation with disease state. Again these were cross analysed with the data obtained from the questionnaire by one-way analysis of variance with coded data and by two-tailed Pearson's correlations with continuous numerical data. Logistic regression analysis was not performed using the total number of pesticides detected as the dependent variables.

3.3 RESULTS

3.3.1 Comparison of demographic, reproductive, clinical, lifestyle and chemical exposure data between groups, with special reference to benign and cancer categories.

In the first instance the data generated from the questionnaire was compared between benign and cancer groups. The benign group consists of women who underwent breast reduction procedures, women diagnosed with non-proliferative lesions such as fibroadenoma, cysts, women diagnosed with proliferative lesions such as sclerosing adenosis, and atypical hyperplasia. The women classified in the cancer group consisted of

those diagnosed with cancer that could be graded using the Scarff, Bloom and Richardson scale, and women diagnosed with in situ disease.

3.3.1.1 Reproductive factors

The breast cancer risk factors concerning reproductive capacity and general medical history are summarised in Table 21. Women presenting with breast cancer were older than women with benign breast conditions. The mean age at entry into the study of women with benign and malignant breast conditions was statistically different ($p=0.011$) at 53 years and 58.6 years respectively. Breast cancer patients had a slightly older age at menarche than benign patients, 13.1 years and 12.6 years respectively, but this difference was not statistically significant ($p=0.122$). The women enrolled in the study were predominantly postmenopausal, however amongst the premenopausal women, a larger percentage had benign breast conditions (35% compared to 18%) ($p=0.027$).

Table 21, Reproductive and general medical history variables collated on the basis of benign and malignant classification between 1995 to 1999.

Variable	Cancer (n = 114)	Benign (n = 43)	p value
Age (mean)(SD) ^a	58.6 (12.08)	53 (12.74)	0.011†
Age at Menarche (mean)(SD)	13.1 (1.80)	12.6 (1.83)	0.122†
Menopausal status (%)			
Premenopausal ^d	18	35	0.027‡
Postmenopausal	82	65	
No. Full Term Births (%)			
nulliparous ^b	16	7	0.446‡
1 or more	84	93	
Total no. years a mother (mean)(SD) ^c	28.6 (17.29)	25.6 (14.83)	0.326†
Breast fed (%)			
Yes	68	65	0.747‡
No	32	35	
Total months lactation (mean)(SD)	10.9 (11.91)	7.7 (14.20)	0.163†
Oophorectomy(%)			
Yes	13	19	0.378‡
No	87	81	
Hysterectomy (%)			
Yes	23	23	0.931‡
No	77	77	
Previous liver disease (%)			
Yes	5	5	0.885‡
No	95	95	
Oral Contraceptive Use (past or present) (%)			
Yes	61	63	0.825‡
No	39	37	

Variable	Cancer (n = 114)	Benign (n = 43)	p value
Time oral contraceptives taken (mean no. months) (SD)	60.5 (85.24)	71.9 (97.06)	0.475†
Hormone replacement therapy use (past or present) (%)			
Yes	37	35	0.771‡
No	63	65	
Time hormone replacement therapy taken (mean no. of years) (SD)	2.6 (5.13)	2.3 (4.90)	0.653†
Family history (%)			
No History	70	65	0.699‡
Some degree of history	30	35	
Previous breast disease (%)			
Yes	20	16	0.596‡
No	80	84	

Key: † The student's t-test was used

‡ The chi-square test was used

^a age at entry into the study

^b includes women who had miscarriages or stillbirths

^c variable derived from age at entry into the study minus the age at first full term birth

^d women were considered as premenopausal if they were still menstruating

SD Standard Deviation

The number of full term pregnancies and the total number of years being a mother did not differ significantly between women with malignant or benign conditions ($p = 0.446$ and $p = 0.326$ respectively). Among the women who had a full term birth, a greater percentage had breast-fed their offspring, but there was no statistical difference between cancer and benign patients. The total duration of breastfeeding however, was slightly longer for the case patients (10.9 months) than the benign patients (7.7 months) but the difference was not significant ($p = 0.163$).

The removal of one or both ovaries, or the removal of the uterus was not associated with any difference in the likelihood of being diagnosed with benign disease or breast cancer ($p = 0.378$ and $p = 0.931$ respectively), nor was having had previous liver disease ($p = 0.885$).

Oral contraceptive use and hormone replacement therapy use was not significantly different between cases and benign patients, when considered as ever (past or present) or never used. However, it is interesting to note that the current use of hormone replacement therapy was statistically higher ($p = 0.012$) amongst women with benign conditions (16%) than for women with breast cancer (4%) (data not shown). The duration of time for which

these hormone therapies were taken was not significantly different between cancer cases and patients with benign disease ($p = 0.475$ for oral contraceptive use and $p = 0.653$ for HRT use). A family history of breast cancer of any kinship and previous diagnosis of breast disease were not correlated ($p = 0.699$ and $p = 0.596$ respectively) with any difference in likelihood of breast cancer or benign disease.

3.3.1.2 Lifestyle Factors

Lifestyle factors include factors related to dietary consumption and other consumptions of alcohol, cigarette smokes, medications and their relation to body mass indicators. Lifestyle also encompasses geographical locations in which the women were born and currently live (Table 22).

Table 22, Geographical and lifestyle factors of women classified as benign or cancer, accrued from Victoria, Australia between 1995 and 1999.

Variable	Cancer (n = 114)	Benign (n = 43)	p value
Area of Residence (%) ^a			
Metropolitan	74	84	0.195†
Regional	26	16	
Birth Country (%)			
Australia	75	65	0.228†
Elsewhere	25	35	
Years Resident in Australia (mean) (SD)	52.8 (15.33)	43.5 (17.18)	0.001‡
Weight (kg)(mean) (SD)	68 (13.5)	72 (20.2)	0.237‡
Height (cm)(mean) (SD)	161.4 (6.85)	161.5 (6.62)	0.933‡
Body mass index (mean) (SD) ^b	26.03 (4.65)	27.47 (6.53)	0.127‡
Short fat ques. score (mean) (SD) ^c	26 (6.15)	27 (6.35)	0.486‡
Use of prescribed medication (%)			
Yes	79	67	0.126†
No	21	33	
Use of non-prescribed medication (%)			
Yes	62	58	0.680†
No	38	42	
Alcohol consumption (%)			
Yes ^d	66	72	0.472†
No	34	28	
Smoke consumption (past or present) (%)			
Yes	50	47	0.732†
No	50	53	
No. of years Smoking (past or present) (mean) (SD)	11.5 (15.48)	7.8 (11.33)	0.107‡
No. of smokes per day (mean) (SD)	6.6 (8.88)	8.1 (11.71)	0.416‡

Key: † The chi-square test was used

‡ The student's t-test was used

^a Defined by the protocol of assigning postcodes by Australia Post

^b Body mass index is calculated as the weight in kilograms divided by the square of the height in metres

^c Short fat questionnaire score is equal to the number of fat related events in the diet, adapted from Dobson *et al.*, 1993

^d also includes social drinkers

SD Standard Deviation

A greater percentage of women with benign breast conditions were metropolitan dwellers compared with breast cancer patients, whilst those women living in regional areas, more had breast cancer. These observations were not statistically significant. Similarly women who were born in Australia were more likely to fall into the breast cancer group, but of the women born elsewhere in the world, there was a greater proportion with benign disease (35% compared with 25% for breast cancer cases, $p = 0.228$). Although the birth country was not of any statistical significance for breast cancer and benign patients, the number of years resident in Australia tended to be proportionately higher amongst cancer cases (52.8 years) compared to benign cases (43.5 years, $p = 0.001$).

In terms of body mass indicators, weight (in kilograms), height (in centimetres) and body mass index (kg/m^2) showed very little variation between cancer and benign groups ($p = 0.237$, $p = 0.933$ and $p = 0.127$ respectively). A short fat questionnaire was administered to participants of the study to gauge their behaviour in respect to fat intake. Out of a possible score of 67, breast cancer and benign patients both had a moderate number of fat consumption related behaviours in their diet of 26 and 27 respectively ($p = 0.486$). The use of prescribed and non-prescribed medication both were higher amongst breast cancer patients, possibly owing to the various treatments that they were undertaking for their conditions but this result was not significant ($p = 0.126$ and $p = 0.680$ respectively).

There was no statistical difference between benign and cancer groups with respect to alcohol and cigarette consumption ($p = 0.472$ and $p = 0.732$ respectively). There was also no statistical variation detected between groups for the number of years the patients had been smoking or for the number of cigarettes smoked per day ($p = 0.107$ and $p = 0.416$ respectively), although more cigarettes on average were consumed by the benign group per day and the mean number of years smoking was higher for the cancer patients.

3.3.1.3 Pesticide exposure factors

In the first instance the pesticide exposure data generated from the questionnaire was compared between benign and cancer groups (Table 23).

Most of the study group, regardless of benign or cancer categories live in areas that are residential only. But of those women that do live in areas where the potential risk of exposure to chemicals is classified as high, a greater proportion were benign subjects (40% compared to 30% for cancers, $p=0.474$). The patient's occupation and the industry in which the patient works revealed very little variation between the cancer and benign patients ($p = 0.599$ and $p =0.526$ respectively). The benign patients reported the use of herbicides and pesticides around the home more so compared with cancer patients, however there was no association statistically. There was also no statistical difference between cancer and benign patients if they had themselves, handled these pesticides or herbicides around the home ($p=0.296$ and $p=0.670$ respectively).

The patients were asked if they had ever lived or worked on a farm where chemicals were used on animals or crops, how long they have been in this living or working situation, whether they had handled the chemicals used and whether they washed clothing that was exposed to chemicals. A slightly greater percentage of benign patients had resided or worked on farms where chemicals may have been used on animals or crops ($p = 0.402$ and $p = 0.518$ respectively), however the time spent on these farms revealed no statistical difference between the two groups. There was no statistical difference between study groups if the patients had themselves, handled the chemicals used on animals or crops ($p = 0.635$ and $p = 0.439$ respectively), nor was there any statistical difference if the patients had washed exposed clothing ($p = 0.692$ for clothes used on a farm with animals; $p = 0.502$ for clothes worn on a crop farm) even though there was a larger percentage in the benign group.

Table 23, Chemical exposure data of the total study population, accrued from Victoria, Australia between 1995 and 1999.

Variable	Benign (n = 43)	Cancer (n = 114)	p value
Industries Near Residence (%)			
Residential Only	56	66	
Potential Low Exposure ^a	5	4	0.474†
Potential High Exposure ^b	40	30	

Variable	Benign (n = 43)	Cancer (n = 114)	p value
Patient's Occupation (%)			
Professional	12	18	0.599†
Retired/ unemployed/ home duties	65	66	
Administrative/ clerical/ sales	21	14	
Trades/ labour	2	2	
Industry the Patient Works (%)			
Potential Low Exposure ^a	74	79	0.526†
Potential High Exposure ^b	26	21	
Reported Herbicide Use Around the Home (%)			
Yes	44	37	0.378†
No	56	63	
Patient Handled the Herbicide Around the Home (%)			
Yes	23	20	0.670†
No	21	17	
Not Applicable	56	63	
Reported Pesticide Use Around the Home (%)			
Yes	88	77	0.122†
No	12	23	
Patient Handled the Pesticide Around the Home (%)			
Yes	81	70	0.296†
No	7	7	
Not Applicable	12	23	
Lived or Worked on Farm with Animals Where Chemicals May Be Used (%)			
Yes	16	11	0.402†
No	84	89	
Time on Farm With Animals (mean no. years) (SD)	4.3 (12.8)	2.3 (8.3)	0.341‡
Patient Handled Chemicals on a Farm with Animals (%)			
Yes	2	3	0.635†
No	14	8	
Unknown	0	2	
Not Applicable	84	87	
Patient Washed Clothes That Were Worn to Apply Chemicals on Animal Farm (%)			
Yes	7	5	0.692†
No	9	5	
Unknown	0	3	
Not Applicable	84	87	
Lived or Worked on Farm with Crops Where Chemicals May Be Used (%)			
Yes	21	17	0.518†
No	79	83	
Time on Farm with Crops (mean no. years) (SD)	4 (11.8)	2.5 (7.8)	0.359‡

Variable	Benign (n = 43)	Cancer (n = 114)	p value
Patient Handled Chemicals on a Farm with Crops (%)			
Yes	7	3	0.439†
No	14	14	
Not Applicable	79	83	
Patient Washed Clothes that Were Worn to Apply Chemicals on Crop Farm (%)			
Yes	12	6	0.502†
No	9	10	
Not Applicable	79	83	
House Sprayed (%)			
Yes	39	39	0.686†
No	56	52	
Unknown	5	9	

Key: † The chi-square test was used

‡ The student's t-test was used

^a Potential low exposure to the chemicals being studied. This includes industries consisting of business, commercial, transport, clothing manufacture, metalwork, health and welfare, education and public service ventures.

^b Potential High exposure to the chemicals being studied. This includes industries consisting of agriculture, chemical manufacture, cleaning, dry cleaning, wood production, food processing, electrical components and textile production.

SD Standard deviation

The levels of individual organochlorine pesticides in breast adipose tissue were compared between benign and cancer groups. The graph presented as Figure 10 shows the average concentration of pesticide (in ng/g) and associated error versus the types of pesticides analysed for the benign and cancer groups. Overall, most of the pesticides analysed were slightly higher in concentration in the breast tissue of women diagnosed with cancer compared to levels found in women with benign breast conditions, but these differences were not significantly different ($p > 0.05$ for all pesticides).

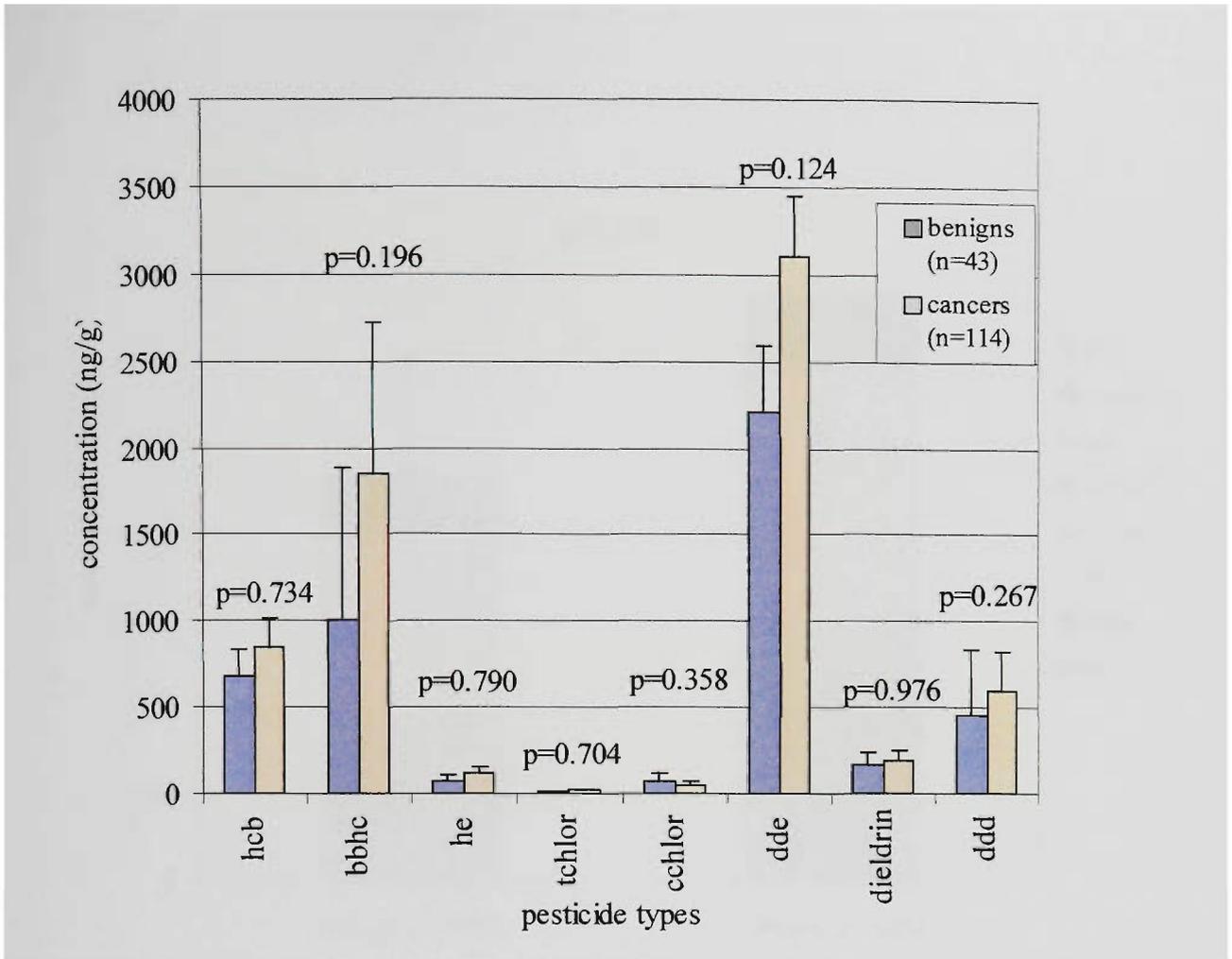


Figure 10, The types of pesticides and respective concentrations detected in breast adipose from women with benign and cancer conditions. The two-tailed student's t-test was used to compute the significance p values that appear on the graph for each pesticide.

Key: hcb = hexachlorobenzene; bbhc = beta isomer of benzene hexachloride; he = heptachlor epoxide; tchlor = trans isomer of chlordane; cchlor = cis isomer of chlordane; dde = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene; ddd = 1,1-dichloro-2,2-bis(chlorophenyl)ethane.

The total amount of pesticide per participant and the mean concentrations calculated per group (benign or cancer) are shown in Figure 11. This figure represents the mean concentration of total pesticides for the benign and cancer groups and the amount of individual pesticides that make up the total. Overall, the total amount of pesticide was higher in the women diagnosed with cancer than the women in the benign group but not to a statistically significant level ($p = 0.120$).

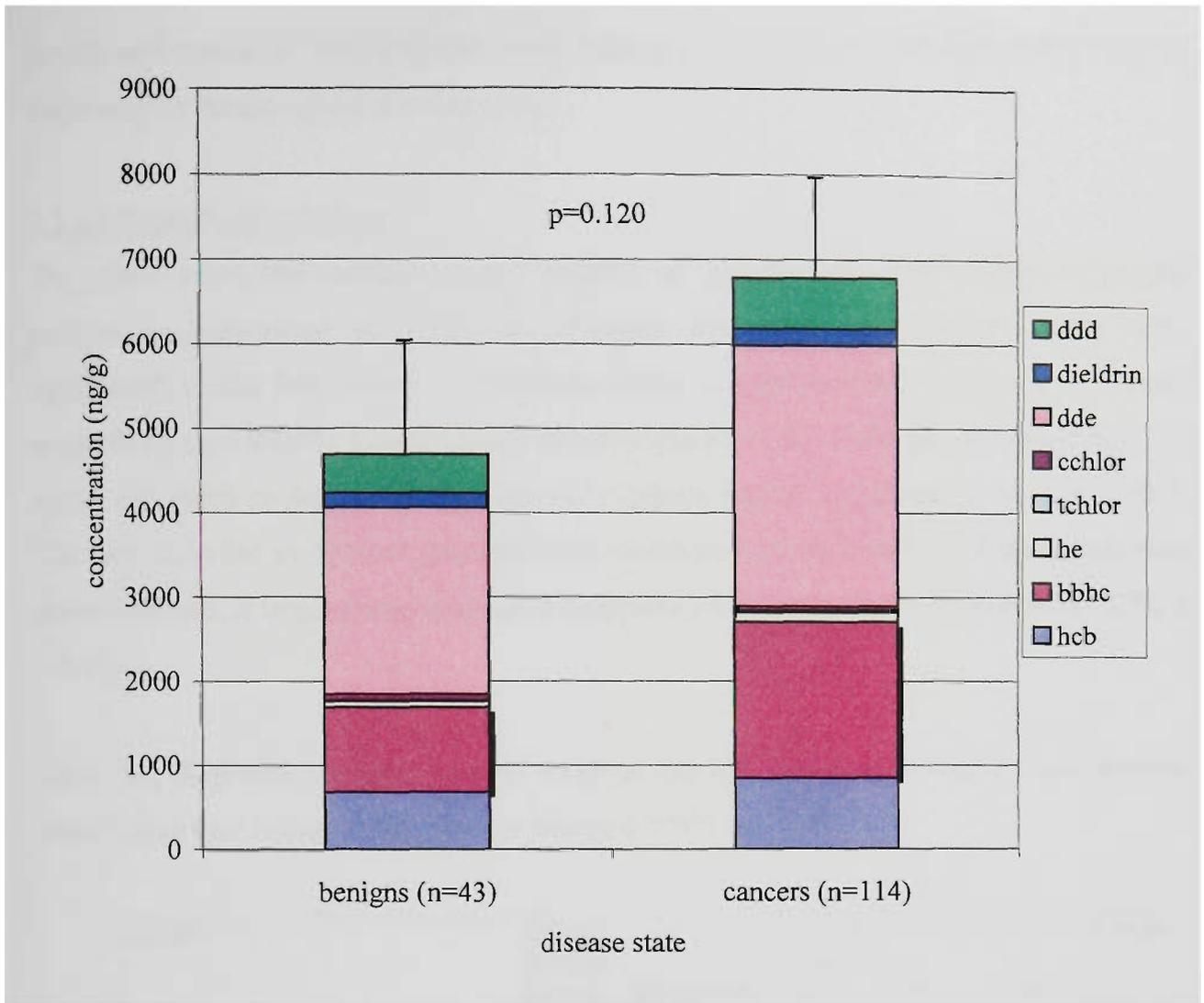


Figure 11, The comparison of the total amount of pesticide and the amount of individual pesticides that contribute to this total, with respect to benign and cancer categories. The two-tailed student's t-test was used to compute the significance p values that appear on the graph for each pesticide. Key: hcb = hexachlorobenzene; bbhc = beta isomer of benzene hexachloride; he = heptachlor epoxide; tchlor = trans isomer of chlordane; cchlor = cis isomer of chlordane; dde = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene; ddd = 1,1-dichloro-2,2-bis(chlorophenyl)ethane.

3.3.2 Comparison of demographic, reproductive, clinical, lifestyle and chemical exposure data between groups, with special reference to true benign and true cancer categories.

Some of the disease states included in both the crude benign and cancer categories of the previous section (3.3.1) contain precursor events to breast cancer development. Given this information and that there were no distinct observations with statistical significance this section differentiates the disease states into true benign (non-proliferative conditions)

(n=25) and cancer (n=101) and uses these categories to re-analyse the factors that may be important for breast cancer development..

3.3.2.1 Reproductive factors

The reproductive and medical history variables as classified for true benign and graded cancers are presented in Table 24. Women diagnosed with breast cancer were significantly older than women with benign breast conditions (58.3 years and 51.5 years respectively; $p = 0.025$). Breast cancer patients had a slightly older age at menarche (13.1 years) compared to the benign patients (12.4 years) but not significantly so ($p = 0.407$). The women in the study were predominantly postmenopausal, however of those that were premenopausal, a larger proportion were diagnosed with benign breast conditions (32%; $p = 0.151$).

Table 24, Reproductive and general medical history variables collated from women classified as true benign or true cancer between 1995 and 1999.

Variable	Benign (n =25)		Cancer (n =101)			p value
	Normal	Non-Prolif.	G1	G2	G3	
Age (mean) (SD) ^a	51.5 (9.9)	51.5 (14.5)	62.1 (9.8)	57.8 (12.1)	54.5 (14.6)	0.025†
Age at Menarche (mean) (SD)	12.3 (1.5)	12.4 (2.0)	12.9 (1.3)	13.1 (2.1)	13.4 (1.8)	0.407†
Menopausal status (%)						
Premenopausal ^d	8	24	4	6	9	0.151‡
Postmenopausal	16	52	28	36	18	
No. Full Term Births (%)						
nulliparous ^b	0	12	3	10	4	0.364‡
1 or more	24	64	29	32	23	
Total no. years a mother (mean) (SD) ^c	29.7 (10.7)	20.7 (16.8)	34.7 (14.9)	23.8 (17.8)	26.1 (18.3)	0.031†
Breast fed (%)						
Yes	20	52	23	23	20	0.341‡
No	4	24	9	19	7	
Total months lactation (mean) (SD)	6.5 (5.8)	9.7 (19.2)	10.5 (9.8)	7.4 (10.5)	15.5 (15)	0.140†
Oophorectomy(%)						
Yes	4	16	4	4	4	0.806‡
No	20	60	28	38	23	
Hysterectomy (%)						
Yes	4	24	7	5	7	0.421‡
No	20	52	25	37	20	
Previous liver disease (%)						
Yes	0	4	0	4	0	0.187‡
No	24	72	32	38	27	

Variable	Benign (n =25)		Cancer (n =101)			p value
	Normal	Non-Prolif.	G1	G2	G3	
Oral Contraceptive Use (past or present) (%)						
Yes	20	52	21	22	19	0.397‡
No	4	24	11	20	8	
Time oral contraceptives taken (mean no. months) (SD)	117 (113.8)	54 (61.7)	49 (85.9)	58 (84.3)	72 (85.4)	0.408†
Hormone replacement therapy use (past or present) (%)						
Yes	8	24	17	12	10	0.277‡
No	16	52	15	30	17	
Time hormone replacement therapy taken (mean no. of years) (SD)	2.3 (3.7)	1.4 (2.7)	4.1 (6.6)	1.8 (3.9)	2.0 (4.3)	0.228†
Family history (%)						
No History	16	40	21	31	19	0.899‡
Some degree of history	8	36	11	11	8	
Previous breast disease (%)						
Yes	8	16	3	13	3	0.113‡
No	16	60	29	29	24	

Key: † The analysis of variance test was used

‡ The chi-square test was used

^a age at entry into the study

^b includes women who had miscarriages or stillbirths

^c variable derived from age at entry into the study minus the age at first full term birth

^d women were considered as premenopausal if they were still menstruating

SD Standard Deviation

The number of full term births did not vary greatly between benign and cancer groups ($p = 0.364$). However the total number of years the patients were mothers was significantly higher for the women with cancer (27.9 years on average) compared to the benign patients (22.8 years; $p = 0.031$) owing to the older age at entry into the study for women in the cancer group. Among the women who had a full term pregnancy, a slightly larger percentage of the benign patients had breastfed their young compared to the cancer patients ($p = 0.341$). The total duration of breastfeeding however, was slightly longer for the cancer group (10.5 months on average) than the benign group (9 months on average), but the difference was not significant ($p = 0.140$).

Oophorectomy and hysterectomy procedures were not to be reported by a large portion of the study group. However of those that did report having these procedures, a slightly larger portion were diagnosed with benign breast conditions ($p = 0.806$ for oophorectomy

and $p = 0.421$ for hysterectomy). There was no significant difference for those who reported having previous liver disease ($p = 0.187$).

Oral contraceptive use and hormone replacement therapy use, in the past or currently, was not significantly different between cancer and benign patients ($p = 0.397$ and $p = 0.277$ respectively). The duration of time that each of these hormone therapies were taken also revealed no significance between cases and benigns ($p = 0.408$ oral contraceptive use and $p = 0.288$ HRT use), even though oral contraceptives were taken for a slightly longer period by the benign patients (69.1 months compared to 58.9 months for cancers). A family history of breast cancer, of any kinship, was not related to any difference between breast cancer and benign subjects ($p = 0.899$). Although there was no statistical difference between cancers and benigns for family history, a greater proportion of patients with benign conditions had some degree of breast cancer history (44%). Previous diagnosis of breast disease was not associated with any variation between breast cancer and benign patients ($p = 0.113$).

3.3.2.2 Lifestyle Factors

The lifestyle and geographical factors pertaining to breast cancer risk as classified by "true" benign and graded cancer states are summarised in Table 25. A greater proportion of women with benign conditions resided in metropolitan areas (88%), whilst of the women who resided in regional areas a greater percentage were diagnosed with breast cancer (29%). This observation was statistically significant ($p = 0.041$). Overall most of the study population were born in Australia (80% of cancers and 60% of benigns). Interestingly though, of women born elsewhere in the world, 40% were diagnosed with benign breast disease ($p = 0.308$). Although this observation was not statistically significant, it can be related to the number of years resident in Australia, whereby women with breast cancer had a longer residency (53.5 years) in Australia compared with benign patients (43.9 years; $p = 0.007$).

Table 25, Geographical and lifestyle factors of women classified as benign or cancer, accrued from Victoria, Australia between 1995 and 1999.

Variable	Benign (n =25)		Cancer (n =101)			p value
	Norm.	Non-Prolif.	G1	G2	G3	
Area of Residence (%) ^a						
Metropolitan	24	64	27	30	15	0.041†
Regional	0	12	5	12	12	
Birth Country (%)						
Australia	16	44	26	33	22	0.308†
Elsewhere	8	32	6	9	5	
Years Resident in Australia (mean) (SD)	44.7 (8.4)	43.6 (18.7)	59.5 (11.7)	51.4 (16.6)	49.5 (16.9)	0.007‡
Weight (kg)(mean) (SD)	74 (15.5)	70.8 (17.8)	68.4 (14.4)	68 (12.4)	67.5 (13.0)	0.814‡
Height (cm)(mean) (SD)	162.5 (6.1)	163.4 (7.3)	161.0 (6.6)	162.3 (7.1)	160.3 (6.1)	0.552‡
Body mass index (mean) (SD) ^b	27.8 (4.4)	26.4 (5.2)	26.3 (4.9)	25.6 (3.9)	26.4 (5.6)	0.824‡
Short fat ques. score (mean) (SD) ^c	25.2 (5.1)	26.8 (6.2)	26.7 (5.7)	25.6 (6.6)	26.6 (6.0)	0.893‡
Use of prescribed medication (%)						
Yes	4	52	24	37	19	0.004†
No	20	24	8	5	8	
Use of non-prescribed medication (%)						
Yes	20	48	17	26	21	0.294†
No	4	28	15	16	6	
Alcohol consumption (%)						
Yes ^d	12	64	24	27	16	0.273†
No	12	12	8	15	11	
Smoke consumption (past or present) (%)						
Yes	12	32	14	21	15	0.881†
No	12	44	18	21	12	
No. of years Smoking (past or present) (mean) (SD)	13.3 (15.6)	5.7 (8.8)	15.2 (18.1)	12.6 (16.3)	6.0 (8.6)	0.069‡
No. of smokes per day (mean) (SD)	8.3 (10.3)	9.8 (14.3)	7.3 (9.4)	6.4 (8.1)	6.3 (10.0)	0.763‡

Key: † The chi-square test was used

‡ The analysis of variance test was used

^a Defined by the protocol of assigning postcodes by Australia Post^b Body mass index is calculated as the weight in kilograms divided by the square of the height in metres^c Short fat questionnaire score is equal to the number of fat related events in the diet, adapted from Dobson *et al.*, 1993^d also includes social drinkers

SD Standard Deviation

Very little variation was revealed between cancer and benign patients in relation to weight, height and body mass index ($p = 0.814$, $p = 0.552$ and $p = 0.824$ respectively). The short fat questionnaire administered as part of the main questionnaire also revealed

very little variation ($p = 0.893$) between cases and benigns, with moderate fat related behaviours in the diets of both groups. The use of prescribed medications was significantly more so by the breast cancer patients (79%) than the benign patients (56%; $p = 0.004$). The use of non-prescribed medications did not to vary greatly between case and benign subjects ($p = 0.294$).

The consumption of alcohol and cigarettes in the past or presently was not different between cancer and benign groups ($p = 0.273$ and $p = 0.881$ respectively). There was no statistical variation detected for the number of years the patients had or currently were smoking, or for the number of cigarettes smoked per day ($p = 0.067$ and $p = 0.763$ respectively). Interestingly though, the cancer patients smoked for a longer duration of time (11.66 years on average) than the benign patients (7.56 years; $p = 0.067$) although this was not significant.

3.3.2.3 Pesticide exposure factors

The potential exposure to chemicals, personally and occupationally, and their relationship in terms of true benign and graded cancer classification are presented in Table 26. Regardless of benign or cancer groups, most of the study group resided in areas that were residential only. Of those women who resided in areas where the potential exposure to chemicals from industry was high, a slightly larger percentage were diagnosed with benign breast diseases, but this was not significant ($p = 0.524$). The patient's occupation and the industry in which they worked revealed very little difference between cases and benign subjects ($p = 0.178$ and $p = 0.575$ respectively).

The women with benign breast disease reported using herbicides and pesticides around the home more so than the cancer patients, but this was not significant ($p = 0.497$ and $p = 0.744$ respectively). There was also no association between cancer and benign patients if they had themselves handled the herbicides or pesticides around the home ($p = 0.788$ and $p = 0.155$ respectively).

A greater percentage of women diagnosed with benign breast disease had resided or worked on farms where chemicals may have been used on animals or crops ($p = 0.237$ and $p = 0.228$ respectively). The time spent on these farms was not statistically different

($p = 0.180$ for animal farms and $p = 0.233$ for crop farms) even though the benign patients had spent a longer time period on both animal and crop farms (6.08 years and 6.72 years respectively). There was no variation ($p = 0.477$ chemicals on animal farms; $p = 0.218$ chemicals on crop farms) between the cases and benigns if they had themselves handled the chemicals used on animals or crops. Although there were no statistical differences, the patients with benign conditions (12%) had handled the chemicals used on crops more so than the cancer patients (2%). This observation also applied to patients that may have washed exposed clothing, whereby the benign patients were exposed to potentially contaminated clothing more than the cancer patients, but this result was not significant ($p = 0.573$ clothes worn on animal farms; $p = 0.294$ clothes worn on crop farms).

Table 26, Chemical exposure data of women classified in the true benign and true cancer categories, accrued from Victoria, Australia between 1995 and 1999.

Variable	Benign (n =25)		Cancer (n =101)			p value
	Norm	Non-Prolif	G1	G2	G3	
Industries Near Residence (%)						
Residential Only	20	36	20	30	15	0.524†
Potential Low Exposure ^a	0	4	2	0	2	
Potential High Exposure ^b	4	36	10	12	10	
Patient's Occupation (%)						
Professional	4	8	3	11	4	0.178†
Retired/ unemployed/ home duties	8	48	22	27	18	
Administrative/ clerical/ sales	8	20	7	3	4	
Trades/ labour	4	0	0	1	1	
Industry the Patient Works (%)						
Potential Low Exposure ^a	20	52	26	35	19	0.575†
Potential High Exposure ^b	4	24	6	7	8	
Reported Herbicide Use Around the Home (%)						
Yes	12	40	13	14	8	0.497†
No	12	36	19	28	19	
Patient Handled the Herbicide Around the Home (%)						
Yes	8	24	9	7	4	0.788†
No	4	16	4	7	4	
Not Applicable	12	36	19	28	19	
Reported Pesticide Use Around the Home (%)						
Yes	20	68	27	32	21	0.744†
No	4	8	5	10	6	
Patient Handled the Herbicide Around the Home (%)						
Yes	12	68	23	30	20	0.155†
No	8	0	4	2	1	
Not Applicable	4	8	5	10	6	

Variable	Benign (n =25)		Cancer (n =101)			p value
	Norm	Non-Prolif	G1	G2	G3	
Lived or Worked on Farm with Animals Where Chemicals May Be Used (%)						0.237†
Yes	0	20	5	3	3	
No	24	56	27	39	24	
Time on Farm With Animals (mean no. years) (SD)	0	8 (17.2)	3.4 (9.4)	1.2 (5.3)	2.8 (11.6)	0.180‡
Patient Handled Chemicals on a Farm with Animals (%)						0.477†
Yes	0	4	0	1	1	
No	0	16	5	2	2	
Not Applicable	24	56	27	39	24	
Patient Washed Clothes that were worn to Apply Chemicals on Animal Farm (%)						0.573†
Yes	0	8	3	1	2	
No	0	12	2	2	1	
Not Applicable	24	56	27	39	24	
Lived or Worked on Farm with Crops Where Chemicals May Be Used (%)						0.228†
Yes	8	24	7	4	7	
No	16	52	25	38	20	
Time on Farm with Crops (mean no. years) (SD)	3.3 (6.8)	7.8 (16.8)	2.8 (6.3)	1.6 (5.5)	4.8 (12.8)	0.233‡
Patient Handled Chemicals on a Farm with Crops (%)						0.218†
Yes	4	8	0	1	1	
No	4	16	7	3	6	
Not Applicable	16	52	25	38	20	
Patient Washed Clothes that were worn to Apply Chemicals on Crop Farm (%)						0.294†
Yes	4	12	1	1	4	
No	4	12	6	3	3	
Not Applicable	16	52	25	38	20	
House Sprayed (%)						0.264†
Yes	4	44	12	14	14	
No	20	32	16	25	10	
Unknown	0	0	4	3	3	

Key: † The chi-square test was used

‡ The analysis of variance test was used

^a Potential low exposure to the chemicals being studied. This includes industries consisting of business, commercial, transport, clothing manufacture, metalwork, health and welfare, education and public service ventures.

^b Potential High exposure to the chemicals being studied. This includes industries consisting of agriculture, chemical manufacture, cleaning, dry cleaning, wood production, food processing, electrical components and textile production.

SD Standard Deviation

The organochlorine pesticide concentrations in breast tissue was compared between true benign and true cancer (Figure 12). The mean concentration of pesticides overall, was higher in the tissue of women diagnosed with breast cancer compared to the benign group, with the exception of DDD, but the difference seen for DDD was not statistically significant ($p = 0.808$). There was statistically no difference between the mean concentrations of pesticides between benign and cancer groups, except DDE. The mean amount of DDE was significantly elevated in the tissue of women diagnosed with cancer compared to women with benign breast conditions ($p = 0.013$).

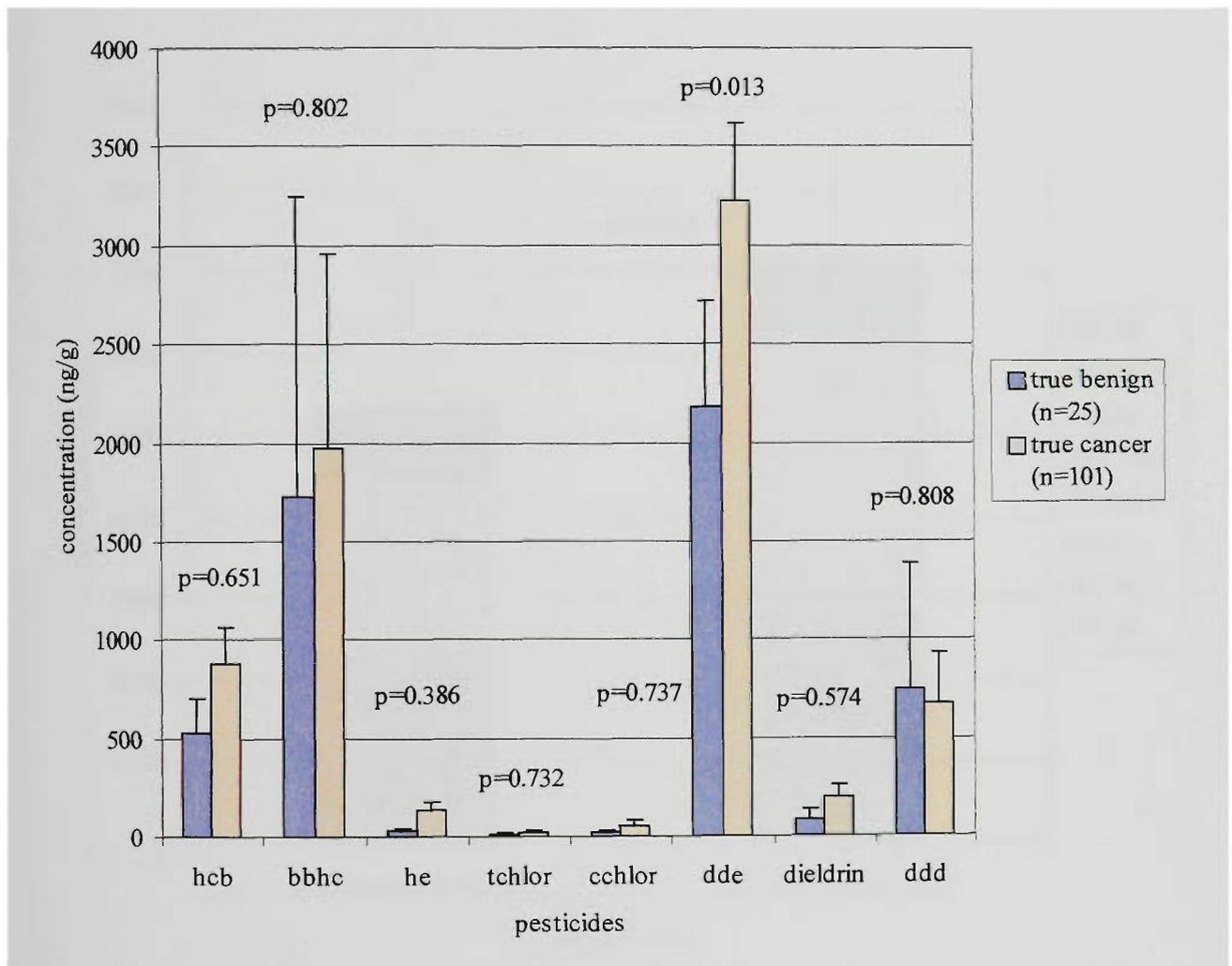


Figure 12, Comparison of organochlorine pesticide concentrations between benign and cancer groups. The one-way analysis of variance test was used to compute the significance p values that appear on the graph for each pesticide. Key: hcb = hexachlorobenzene; bbhc = beta isomer of benzene hexachloride; he = heptachlor epoxide; tchlор = trans isomer of chlordane; cchlор = cis isomer of chlordane; dde = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene; ddd = 1,1-dichloro-2,2-bis(chlorophenyl)ethane.

The individual organochlorine pesticide concentrations (as presented in Figure 12) was combined to determine the total amount of organochlorines and these totals were then compared between groups. Figure 13 represents this comparison of mean total concentration of organochlorine between true benign and true cancer groups. The figure indicates that the mean total concentration of organochlorines is higher for the women diagnosed with breast cancer compared to the women with benign breast conditions but the association was statistically weak ($p = 0.041$) compared to the association for DDE alone.

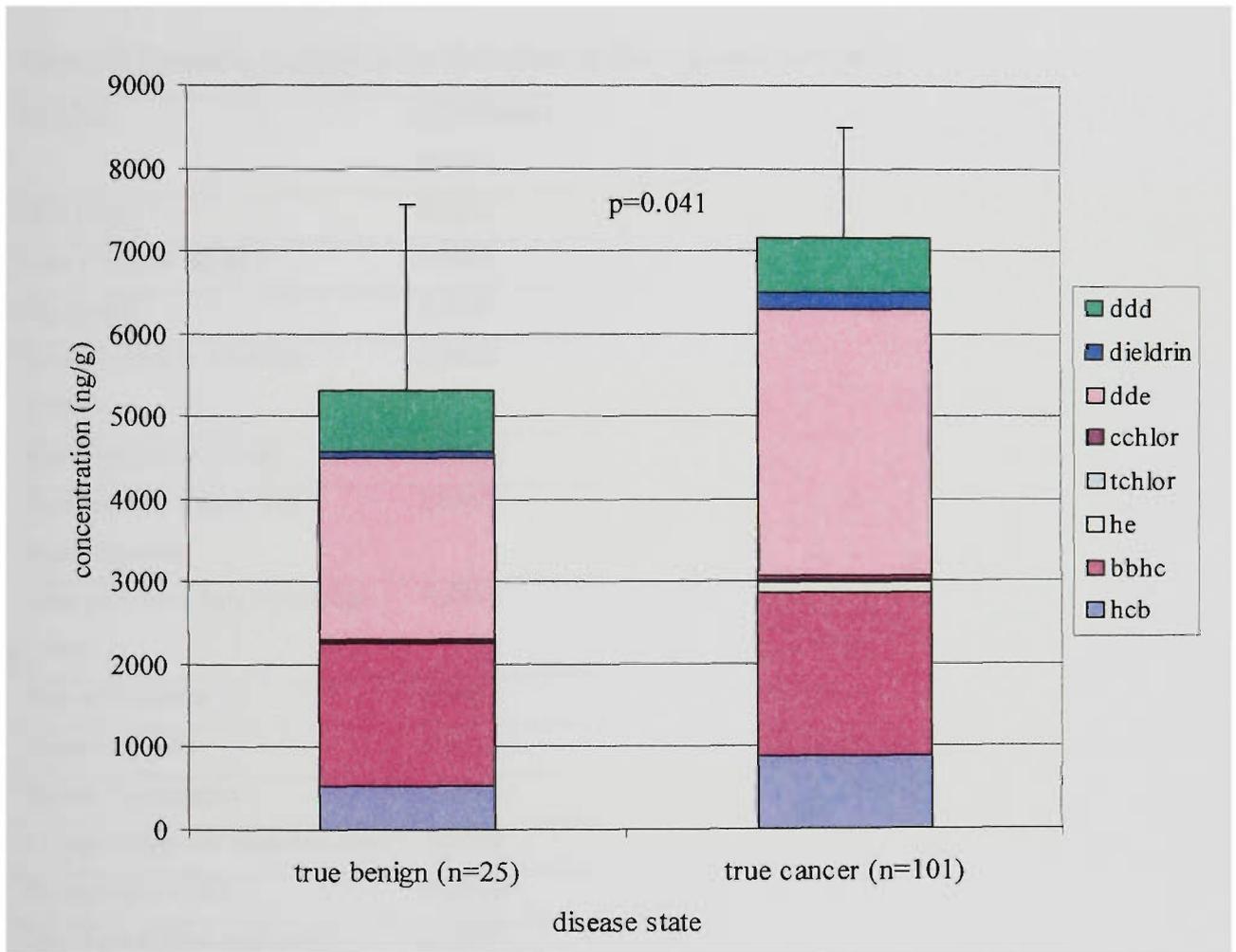


Figure 13, Comparison of the total amount of pesticide and the specific concentrations of individual pesticides that make up this total with respect to true benign and true cancer groups. The one-way analysis of variance test was used to compute the significance p values that appear on the graph for each pesticide. Key: hcb = hexachlorobenzene; bbhc = beta isomer of benzene hexachloride; he = heptachlor epoxide; tchlor = trans isomer of chlordane; cchlor = cis isomer of chlordane; dde = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene; ddd = 1,1-dichloro-2,2-bis(chlorophenyl)ethane.

3.3.3 Regression modelling with special reference to the “true” benign and “true” cancer groups.

As stated earlier, the analysis of all facets of data collected in this study required multiple logistic regression models fitted through forward stepwise variable selection procedures. The dependent variable of the regression model was true benign/ true cancer. The independent variables that may be considered in the regression model, on the basis of significant values, are presented in table 27.

Table 27. Potential variables for inclusion in the regression models.

Variable	Significance Value‡
DDE (ng/g) *	0.0132
Total pesticide (ng/g) *	0.0405
Age (years)	0.0181
Years resident in Australia (years)	0.0088
Age at menarche (years)	0.0889
Time spent on a farm with animals (years)	0.0997
Time spent on a farm with crops (years)	0.0803
Area of residence	0.0898
Country of birth	0.0429
Patient's occupation	0.0983
Current use of oral contraceptives	0.0382
Current use of HRT	0.0525
Use of prescribed medication	0.0157
Use of herbicides around the home	0.1021
Herbicide brands used around the home	0.0259
Patient handled herbicides used around the home	0.1025
Patient handled chemicals used on a crop farm	0.0606

Variable	Significance Value‡
Patient washed clothes worn on a crop farm	0.1031

Key: * All pesticide levels are the logarithmically transformed values

‡ Significant p values

HRT Hormone replacement therapy

Prior to the analyses, and as stated in the previous section, the year of entry into the study was known to be a biased variable, due to the focus towards recruitment of patients diagnosed with cancer in the early years (1995 and 1996) of the study, and was thus excluded from the regression analyses. At the first step of the regression model, (Table 28) the number of years resident in Australia was entered. The second step consisted of the logarithmically transformed DDE concentrations expressed as nanograms per gram of fat extracted. At the third step the time spent on a farm with crops entered the regression equation. This variable produced a negative coefficient B and the estimated odds ratio was below 0.999 (variables with an Odds ratio below the assigned 0.999 cutoff value produce a negative coefficient B) when entered into the regression, indicating that this variable should not be considered an important contributor to the regression model. The one-way comparative analysis (used as a screening tool for chance events) of time spent on a farm with crops and true benign/ cancer revealed that only about 20% of the study group had actually spent any time on a crop farm. In terms of the regression, the entering of this variable into the regression model is likely to be a random event due to the small number of subjects assessed by this variable. Therefore the regression model ceased at this step.

Table 28. The sequential order of variables entered into the regression model with respect to true benign/ cancer.

Variable	Model 1		Model 2		Model 3		Model 4		Model 5	
	Est.	95%	Est.	95%	Est.	95%	Est.	95%	Est.	95%
	OR	CI	OR	CI	OR	CI	OR	CI	OR	CI
Years resident in Australia	1.04	1.01-1.06	1.04	1.01-1.06	1.04	1.01-1.07	1.04	1.01-1.07	1.04	1.01-1.07
DDE (ng/g)†			1.62	1.19-2.04	1.67	1.23-2.11	1.73	1.27-2.15	1.80	1.35-2.26
Time spent on a farm with crops*					0.96	0.92-1.00	0.92	0.87-0.98	0.91	0.86-0.97
Area of residence							10.26	8.12-12.39	15.11	12.9-17.32
Patient handled herbicide around the home*									0.70	0.40-1.01

Key; * These variables produced negative coefficient values when entered into the regression. Also according to the one-way comparative tests, these variables do not make clinical sense and are likely to be random variables entered into the regression equation. Therefore any variables entered into the model after these, are not considered as part of the final model.

† The concentration of DDE entered into the regression model pertains to the logarithmically transformed values.

Two variables, area of residence and whether the patient handled herbicide around the home, entered the regression equation at steps 4 and 5 respectively, owing to their significance values being below the cutoff score of 0.05. However due to the random variable entered at step 3 these variables are not included in the final regression model.

Two variables, years of residence in Australia and levels of DDE (ng/g) in breast biopsy tissue, remain in the final regression model, fitted according to true benign and true cancer categories. The model suggests that there was a 4% increase in breast cancer risk for every year resident in Australia. There was also a 67% increase in risk for every ten-fold (as the pesticide levels were logarithmically transformed) increase in DDE concentration.

Age is a strong determinant for breast cancer risk and therefore regression models need to adjust for age to determine the true risk attributable to the factors being tested, such as organochlorine pesticide levels. Table 29 contains the regression model that has been adjusted for age. Both the untransformed and transformed age data were entered into the regression, to demonstrate the effects of age on the regression model if transformation of the data did not occur. The untransformed age data had an estimated odds ratio below 0.999 (variables with an odds ratio below the assigned 0.999 cutoff value produce a negative coefficient B) when entered into the regression, indicating that this variable should not be considered an important contributor to the regression model. The transformation of the age data mitigated this effect, thereby forcing the regression model to accept age as an important contributor to the model. . If the participant had ever lived or worked on a farm with animals, was entered in at the first step of the regression model (Table 29). The second step consisted of the number of cigarettes that may have been smoked in the past. At the third step the number of full term pregnancies entered the regression equation. The fourth and final steps of the regression with age adjustment consisted of whether the house in which the participant resides had been treated with chemicals and the tissue levels of hexachlorobenzene (in nanograms per gram of tissue) respectively. The following variables, untransformed age data, the past number of cigarettes smoked, number of full term pregnancies, and having the house of residence treated with chemicals, all had estimated odds ratios below the 0.999 cutoff value, which meant that the coefficient was negative and that these variables should not be considered as important contributors to the model. Therefore the regression model with age adjustment should only contain the variable that reports whether the participant has ever lived or worked on a farm with animals.

Table 29. Logistic regression model with respect to true benign and true cancer categories with age adjustment.

Variable	Model 1		Model 2		Model 3		Model 4		Model 5	
	Est. OR	95% CI	Est. OR	95% CI	Est. OR	95% CI	Est. OR	95% CI	Est. OR	95% CI
Age*	0.93	0.61- 1.25	0.91	0.57- 1.26	0.93	0.56- 1.30	0.85	0.47- 1.23	0.78	0.37- 1.19
T Age	1.07	0.79- 1.34	1.08	0.77- 1.38	1.07	0.74- 1.40	1.16	0.82- 1.49	1.24	0.88- 1.60
Lived or worked on a farm with animals	6.17	5.0- 7.34	6.9	5.67- 8.12	5.11	3.82- 6.40	4.78	3.44- 6.13	3.97	2.58- 5.36
Past # of cigarettes *			0.92	0.86- 0.98	0.91	0.85- 0.97	0.90	0.84- 0.96	0.90	0.83- 0.96
# FTP*					0.66	0.26- 1.05	0.64	0.23- 1.04	0.60	0.17- 1.04
House treated*							0.27	-0.97- 1.51	0.22	-1.08- 1.52
HCB (ng/g) †									1.50	1.09- 1.90

Key; * These variables produced negative coefficient values when entered into the regression. Therefore any variables entered into the model after these, are not considered as part of the final model.

† The concentration of HCB entered into the regression model pertains to the logarithmically transformed values.

T Age = Square transformed age data

FTP = Full term pregnancies

3.3.2 Determination of possible synergy between the number of pesticides detected, disease state and the data obtained from the questionnaire

The number of different pesticides present in each biopsy specimen was calculated and each specimen classified according to the disease state. The average number of pesticides

detected was compared for the true benign and true cancer categories, to determine if more than one pesticide (possibly working in synergy with each other) affects breast cancer development (Figures 14). The mean number of pesticides detected in the benign group was 2.44 compared to the mean for the cancer group, 2.76, but statistically there was no difference ($p = 0.319$).

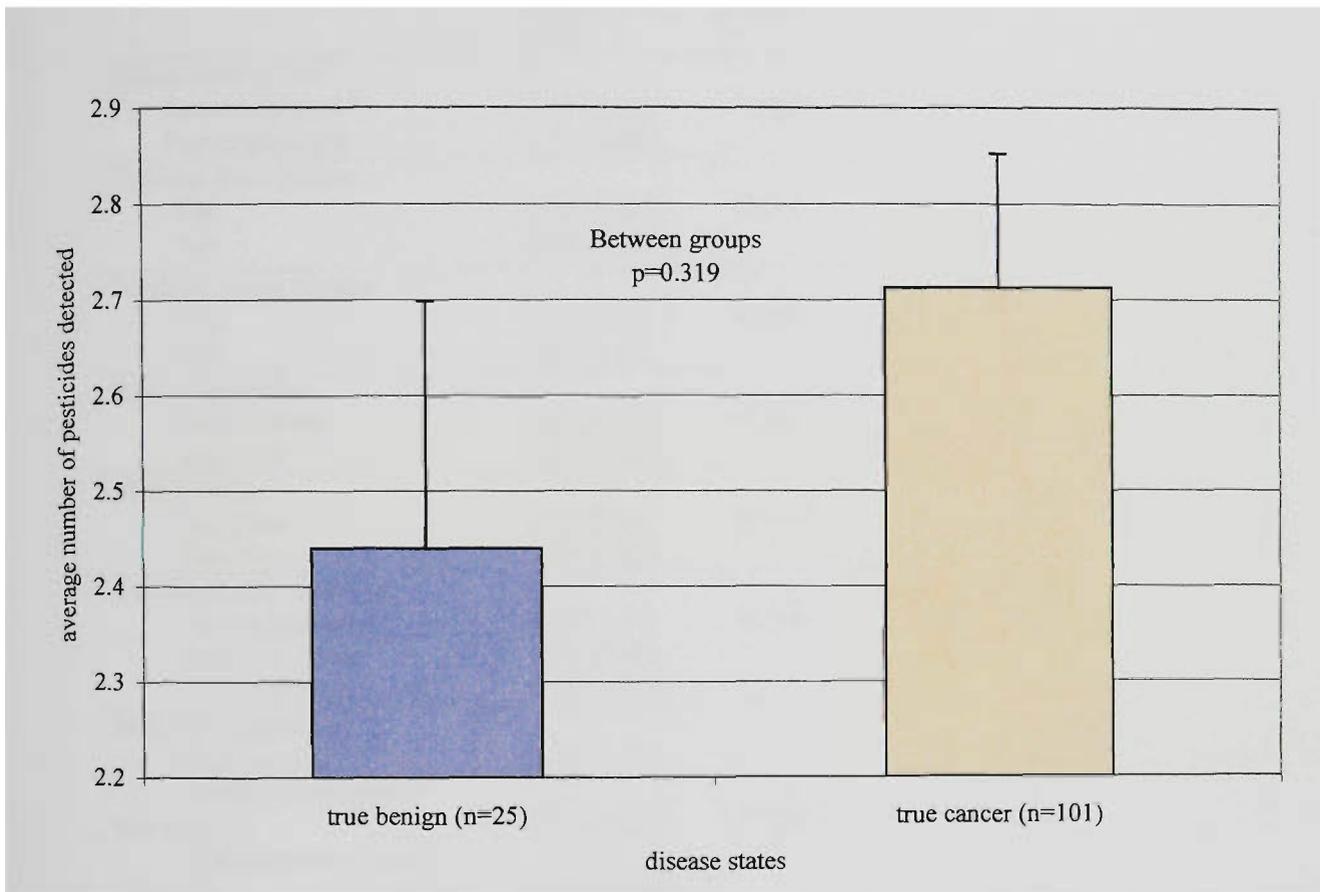


Figure 14. The average number of pesticides in breast tissue according to the true benign and true cancer classification.

The average number of pesticides detected was also compared with the data collated from the questionnaire. Tables 30 and 31 present some of the variables assessed from the questionnaire. Statistically there was no difference in the number of pesticides present between premenopausal and postmenopausal women ($p = 0.665$). There were slightly more pesticides detected for the postmenopausal women (2.73) than premenopausal women (2.59). Women who reported having previous liver disease had more pesticides than women without previous liver but the number of pesticides detected was not statistically different between groups ($p = 0.436$). Women reporting previous breast disease had fewer numbers of pesticides than those who had no previous breast conditions ($p = 0.054$).

Table 30. Comparison of medical and pesticide data with true benign/ true cancer groups, with respect to the number of pesticides detected in the breast tissue.

Variable	True benign/ true cancer Mean no. pesticides (SD)	p value [†]
Menopausal status		
Premenopausal ^a	2.59 (1.31)	0.665
Postmenopausal	2.73 (1.50)	
Previous liver disease		
Yes	3.20 (2.39)	0.436
No	2.68 (1.42)	
Previous breast disease		
Yes	2.20 (1.35)	0.054
No	2.82 (1.46)	
Area of residence ^b		
Metropolitan	2.65 (1.41)	0.522
Regional	2.84 (1.59)	
Birth Country		
Australia	2.73 (1.48)	0.700
Elsewhere	2.61 (1.38)	
Industries near residence		
Residential only	2.58 (1.37)	0.316
Potential Low ^c	2.40 (0.89)	
Potential High ^d	2.98 (1.64)	
Patients occupation		
Professional	2.52 (1.33)	0.477
Retired/ unemployed/ home duties	2.82 (1.57)	
Administrative/ sales/ clerical	2.57 (1.08)	
Trades/ labour	1.67 (1.15)	
Reported herbicide use around the home		
Yes	2.46 (1.20)	0.144
No	2.85 (1.58)	
Reported pesticide use Around the home		
Yes	2.74 (1.47)	0.490
No	2.52 (1.42)	
Lived or worked on farm with animals where chemicals may be used		
Yes	2.62 (1.71)	0.825
No	2.71 (1.42)	
Lived or worked on farm with crops where chemicals may be used		
Yes	3.07 (1.64)	0.133
No	2.60 (1.39)	
House sprayed		
Yes	2.50 (1.29)	0.372
No	2.88 (1.49)	
Unknown	2.60 (1.96)	

Key: [†] The one way analysis of variance test was used.

^a Women were considered as premenopausal if they were still menstruating

^b Defined by the protocol of assigning postcodes by Australia Post

^c Potential low exposure to the chemicals being studied. This includes industries consisting of business, commercial, transport, clothing manufacture, metalwork, health and welfare, education and public service ventures.

^d Potential High exposure to the chemicals being studied. This includes industries consisting of agriculture, chemical manufacture, cleaning, dry cleaning, wood production, food processing, electrical components and textile production.

Interestingly women residing in regional areas had a higher number of pesticides present in their breast tissue compared to women in metropolitan areas but this observation was not significant ($p = 0.522$). There was no difference in mean number of pesticides according to country of birth with respect to disease states ($p = 0.700$). There was no statistical difference in number of pesticides detected according to industries near the area of residence ($p = 0.316$). However there were a slightly higher number of pesticides present in the breast tissue of women living in residential areas only or in areas of potential low chemical exposure.

The patients occupations showed very little variation in terms of the number of pesticides detected ($p = 0.477$). Interestingly women classified as working in the trades and labour areas had a lower number of pesticides (1.67) than other groups (2.40 to 2.82).

The number of organochlorines found in the breast tissue of women who reported using herbicide around their homes was lower than those who did not use herbicide but this result was insignificant ($p = 0.144$). On the other hand, women who reported using pesticides around their homes had a slightly higher number of pesticides detected in their breast biopsies ($p = 0.490$).

There was no significant difference in the number of pesticides detected for women if they had ever lived or worked on a farm with animals or crops ($p = 0.825$ for animal farm, and $p = 0.133$ for crop farm). However the women who reported living or working on a crop farm had a slightly higher number of pesticides in their breast tissue compared to the women who did not live or work on these farms although this was not statistically significant.

The women who had reported having their homes sprayed tended to have a fewer number of organochlorines in their tissues compared to those who did not have their homes sprayed ($p = 0.372$).

The numerical data obtained from the questionnaire was also compared to the number of pesticides detected for the true benign and true cancer categories (Table 31). These values were generated using the Pearson Correlation test that measures the linear association between variables. The Pearson's Correlation Coefficient is a value that indicates the strength as well as the direction of the relationship.

The number of pesticides detected was positively correlated with age for true benign/cancer data but the strength of the association was not significant ($p = 0.444$). A weak negative association between age at menarche and number of pesticides was produced for the true benign/ cancer data ($p = 0.388$).

Table 31. Comparison of numerical questionnaire data with the true benign/ true cancer groups with respect to the number of pesticides detected in the breast tissue.

Variable	True benign/ true cancer Pearson's correlation coefficient	p value‡
Age ^a	0.069	0.444
Age at menarche	-0.077	0.388
Total no. years a mum ^b	0.129	0.140
Time oral contraceptives Taken	0.190	0.032
Years resident in Australia	0.109	0.223
Weight (kg)	0.178	0.046
Body Mass Index (BMI) ^c	0.213	0.016

Key: ‡ The two tailed Pearson's Correlation test was used.

^a The age at entry into the study

^b Variable derived from age at entry into the study minus the age at first full term birth.

^c Body Mass Index is calculated as the weight in kilograms divided by the square of the height in metres.

The number of organochlorines present in the breast tissue of women with a longer duration of time as a mother was greater than those who were mothers for shorter periods of time, but not significantly so ($p = 0.140$). Women who had taken oral contraceptives

for a long period of time had a significantly higher number of pesticides in their breast tissue ($p = 0.032$).

The number of years resident in Australia was not significantly associated with the number of pesticides ($p = 0.223$).

The number of pesticides present in breast tissue was positively associated with the patient's weight ($p = 0.046$). A similar observation was evident for body mass index, where a significant positive relationship was determined with the number of pesticides detected in the breast tissue ($p = 0.016$).

3.4 DISCUSSION

The following discussion of the results will proceed by firstly highlighting the significant variables that should be considered as confounders for the relative risk of breast cancer, followed by the assessment of variables that have emerged in the regression models with and without age adjustment.

3.4.1 Identification of possible confounding variables

Previous studies have shown that age is a strong determinant of breast disease (Kelsey, 1979; Colditz, 1993), and using the crude benign and cancer categories the present study also indicates that age was a significant factor ($p = 0.011$). However it deserves mention that in the first analyses conducted in the current study there may have been the inclusion of pre-cancerous breast lesions in both groups, which predispose to breast cancer development at differing rates. Other investigators who have studied the risk factors associated with breast cancer have not defined the conditions that are included in the benign classification, which may lead to spurious results. The present study mitigated the effect of the pre-cancerous breast lesions on breast cancer risk attributable to age by re-analysing the data against true benign (subjects diagnosed with non-proliferative benign conditions and those who underwent reduction mammoplasties) and true cancer (grade 1

through to grade 3) conditions. The finding of 'age at entry into the present study' as a strong determinant of breast cancer risk was also evident for the conditions just mentioned ($p=0.025$). Therefore, regression analyses to follow should be age adjusted for determining the breast cancer risk attributable to pesticide exposure.

The analyses of crude benign and cancer categories also suggest that a greater proportion of women with breast cancer were postmenopausal and women with benign conditions were premenopausal ($p = 0.027$). This observation was also evident in the analyses among women in the true benign and cancer categories but was not statistically significant ($p=0.151$). The research of Wang and Fentiman (1985) also suggests that premenopausal women are more commonly diagnosed with benign breast disease than controls.

The total number of years being a mother was correlated with breast cancer. Women diagnosed with true breast cancer were mothers for a significantly longer period of time (27.9 years) compared to women with benign breast conditions (22.8 years; $p = 0.031$). This variable is strongly correlated with age and age at first full term pregnancy, and thus taking this into account, women diagnosed with breast cancer tended to start their families at slightly older ages than women with benign conditions (data not shown). The fact that these women didn't have their first child until a later age, even though they were mothers for a longer period of time, probably accounts for their contribution to risk. Research by MacMahon and colleagues (1970) suggests that women whose first birth occurs at 30 to 34 years of age had approximately the same breast cancer risk as nulliparous women. The results of this study suggest that there were slightly more women who were nulliparous in the breast cancer group, than with benign breast conditions, but this was not significant ($p > 0.05$). Research also suggests that an early age at first birth confers a protective effect against breast cancer risk and subsequent births offer some additional protection depending on the subject's age at the time of the births (MacMahon *et al.*, 1982; Lambe *et al.*, 1994).

This study found that the number of years resident in Australia was consistently related to with breast disease ($p < 0.01$ for both analyses presented)). The presented results suggest that women who are diagnosed with cancer tend to be residents in Australia for a longer period of time (52.8 years) than women diagnosed with benign conditions (43.5 years).

Women diagnosed with breast cancer resided in regional areas more so than women diagnosed with benign conditions (29% compared to 12%, $p=0.041$). A residence in regional or rural surroundings would increase the likelihood of exposure to chemicals that may be used for treating crops or cattle.

3.4.2 Relative risk of breast cancer.

The relative risk of breast cancer without adjustment for confounding variables was influenced by two factors within the population of women studied here, the number of years resident in Australia and levels of DDE in breast adipose tissue. A discussion of these factors, although inappropriate in terms of true relative risk estimates which have to account for age, highlights the potential of environmental factors influencing breast cancer risk, given a larger study population. The number of years resident in Australia increased the risk of breast cancer by 4% with every year of residence and secondly the breast cancer risk was increased by 67% for every ten-fold increase in DDE concentration detected in the breast adipose tissue. Each of these factors will be discussed in detail.

3.4.2.1 Residence in Australia

Overall, the women diagnosed with benign breast conditions were residents in Australia for lesser periods of time than women diagnosed with breast cancer. Earlier research suggests that there is a changing pattern of cancer incidence and mortality with length of residence in the host country (McCredie *et al.*, 1994; Tyczynski *et al.*, 1994; Kliewer and Smith, 1995; McCredie *et al.*, 1999). Research by McCredie and colleagues (1999) suggests that this changing pattern of incidence and mortality may be due to the influence of birthplace, such that it is already known that the breast cancer incidence rates vary between countries and are largely higher in Westernised countries. They also suggest that the effect of duration of residence on breast cancer incidence is mainly due to environmental factors in the host country but may also be influenced by socio-economic factors related to the reasons for migration, living circumstances, inheritance, exposure to chemicals early in life before migration and retained cultural and lifestyle factors (McCredie *et al.*, 1999). The work by Kliewer and Smith (1995) indicates that the mortality rates of breast cancer for migrants from low and high-risk countries displayed

patterns of convergence to the host country rate (Australia). The trend was particularly evident for women migrating from countries where the mortality rates were greater or lower than 25% of that for Australian born women (Kliwer and Smith, 1995). When the duration of stay in the host country was considered, however, there was an increasing trend of risk with longer durations of residence. Therefore the risk of breast cancer is largely dependent on the country of origin and the associated breast cancer risk of that country, which is quite possibly attributable to different environmental and lifestyle factors.

One of the classic studies on the migration effect and its potential to influence disease, was data published on Japanese women migrating to the United States. Research by Haenszel and Kurihara (1968) investigated the comparison of Japanese migrant women to Hawaii and continental United States, compared to white and non-white American women and the mortality rates from cancer and other diseases. Haenszel and Kurihara (1968) suggested that the breast cancer risks of Japanese migrants to the United States and their United States born offspring failed to approach the rate of the host population at any point in the lifespan. The persistence of low breast cancer rates among second generation Japanese migrants suggests that there is a strong role for genetic factors in influencing breast cancer risk. Studies by Hemminki and Li (2002) however, suggest that incidence rates of breast cancer can vary between different generations of migrants, such that the first two decades of life set the pattern and subsequent risk of breast cancer later in life. They suggest that birth in the new host country results in acquiring the cancer risk of that country, irrespective of the nationality of descent, whilst if the woman migrated in her twenties, her risk would assume that of the country of origin (Hemminki and Li, 2002). The discrepancies in breast cancer risk for second-generation migrants may lie with the country of origin, but more importantly, lifestyle and environmental factors of the migrant's country of origin, and the magnitude of change in these variables in the host country.

Many studies suggest that lifestyle and local environmental conditions may be major determinants of risk associated with women migrating from a country with low breast cancer risk to a country with high breast cancer risk (Kliwer and Smith, 1995; Ziegler *et al.*, 1993). However, there have not been many studies that have documented if there is a

lowering of risk if a woman migrates from a high-risk country to a country with a low breast cancer risk. Research by Kliewer and Smith (1995) investigated the risk of breast cancer mortality in migrants to Australia and Canada from countries with both high and low cancer risks. Their research suggests that women migrating from a country with lower breast cancer risk than the Australian rate tend to assume the Australian breast cancer rate after residing in the country for more than 30 years (Kliewer and Smith, 1995). Conversely, if the woman migrates from a country with a high breast cancer rate and resides in Australia for greater than 29 years, their mortality rate is lower than that of their birth country (Kliewer and Smith, 1995). In summary, if migrants become long term residents in Australia their breast cancer risk converges towards the rate of the Australian born women. Other studies suggest that the risk may also be influenced by the fact that there is familial clustering, such that there is a shared exposure to similar lifestyle, cultural and environmental behaviours (Ottman *et al.*, 1986; Andrieu *et al.*, 1991; Colditz *et al.*, 1993).

3.4.2.2 The influence of DDE in breast adipose tissue

The second factor found in the present study to be linked with increasing the risk of breast cancer were the concentrations of DDE in breast adipose tissue. Breast cancer risk was increased by 67% for every 10-fold increase in DDE concentration detected in the breast adipose tissue. Women diagnosed with benign breast disease had lower DDE concentrations in their tissue on average than women diagnosed with breast cancer (2182 ± 536 ng/g detected in benign tissue compared to 3223 ± 392 ng/g detected in breast cancer tissue). Historical research that investigated the links between various organochlorine pesticides and breast cancer has yielded conflicting data regarding such a connection. A pilot study undertaken by Falck and colleagues (1992) found 50-60% higher levels of PCBs, and DDT and its metabolite DDE in the breast adipose tissue of women with breast cancer ($n = 20$) compared with women with benign breast disease ($n = 20$). The study by Wolff and colleagues (1993) also found that levels of DDE and PCBs were higher for breast cancer patients ($n = 58$) than for control subjects ($n = 171$). After adjustment for some of the known risk factors for breast cancer, the statistical analyses found a fourfold increase in relative risk of breast cancer for elevations of DDE concentrations from 2ng/ml to 19ng/ml (Wolff *et al.*, 1993). A prospective study by Krieger and colleagues (1994) however found no association between organochlorine

pesticides and relative risk of breast cancer. The work of Krieger and colleagues (1994) was prospective which allowed for the collection of unbiased samples of serum, however adequate data was not collected for age at first pregnancy and lactation, which have both been found to be inversely related to breast cancer risk, and lactation offers a chief route for the excretion of organochlorine pesticides. These limitations of Krieger and colleagues (1994) study cannot rule out the possibility of relative risks in the same order of magnitude as those presented above and previously in chapter one. A recent study by Bagga and colleagues (2000) also found no correlation between organochlorine pesticides and breast cancer. Bagga and colleagues (2000) did however find a significant difference in DDE levels between breast cancer patients ($n = 73$) and control subjects ($n = 73$) (on a lipid basis 800ng/g of DDE in breast cancer patients compared to 709ng/g of DDE in control subjects, $p = 0.006$). When these differences in DDE levels were adjusted for other factors, such as age, there was no significant relationship found with breast cancer risk (Bagga *et al.*, 2000). Consequently this may be interpreted as the increased propensity to accumulate organochlorines with age, which may also be related to increased body mass with age, but there is also the possibility of decreasing the body burden of these pesticides through lactation, which doesn't appear to have been included in their regression model. The present study, like Bagga and colleagues (2000) study was made up of two groups of women, women with pathologically confirmed breast cancer and a control group mainly consisting of women who underwent reduction mammoplasty. It is believed that these women are not at any increased risk of breast cancer and there appears to be no reports suggesting an association between reduction mammoplasty and the incidence of breast cancer, and assumes that these women represent relatively healthy women.

The disparity observed between studies in relation to calculating relative risk of breast cancer attributable to organochlorine pesticide exposure may be partly due to the type of biological tissue or fluid used for the study. Organochlorine pesticides are sequestered in lipid-rich tissues where their long half-life results in their accumulation and allows them to persist for an individual's lifetime (Morgan and Roan, 1971). Early research by Morgan and Roan (1971) suggests that organochlorine pesticide stability within the human body is largely dependent on the efficiency of excretory mechanisms and transport of the different isomers of organochlorine pesticides in and out of fat depots. Their

research suggests that levels of DDT isomers are comparable between adipose and serum but in terms of DDE, DDD and their isomers there is a less accurate relationship (Morgan and Roan, 1971). These findings are largely owing to the fact that DDT is very unstable and degrades rapidly to DDE or DDD. DDD readily degrades through a series of reactions to a form that is easily excreted and rarely found stored in adipose tissue (Morgan and Roan, 1971). DDE by contrast does not degrade further to an excretable form and according to Morgan and Roan (1971) has a very slow removal from adipose tissue. Some of the previously mentioned studies have measured organochlorine pesticide levels in serum or plasma whilst others have used adipose tissue collected from the breast or other sites of the body. Significant relationships between breast cancer and organochlorine pesticides have been found in studies using both breast adipose tissue and serum (Falck *et al.*, 1992; Wolff *et al.*, 1993).

Research by Archibeque-Engle and colleagues (1997) suggest that blood used as the medium to determine the levels of organochlorines may not be a reliable indicator of body burden and thus the relative risk of breast cancer. They also suggest that as blood contains only a small portion of fatty material, it acts as a transport medium for the organochlorine pesticides, until the blood circulates to an area in the body with a high fat content (Archibeque-Engle *et al.*, 1997). Levels of organochlorines detected in the blood or its components would therefore be an underestimation of total body burden, unless the exposure to pesticide was recent. However some studies, especially prospective designed studies, have used blood as a medium, as it is easy to acquire and can be stored (potentially tainted with organochlorine pesticides) with minimal degradation of the pesticides for many years (Krieger *et al.*, 1994; Archibeque-Engle *et al.*, 1997).

Other studies have used adipose tissue from various sites of the body to estimate exposure to organochlorine pesticides and subsequently determine breast cancer risk. A study by van't Veer and colleagues (1997) measured DDE exposure in adipose tissue aspirated from the buttocks and found that DDE concentrations were lower for women with breast cancer compared to control women, and there was no association with breast cancer risk. Past Australian studies have measured organochlorine pesticides in subcutaneous body fat from laparotomy, perirenal fat taken at autopsy, adipose from caesarean section, breast milk and blood components but have not related their findings to risk of breast cancer or

other diseases (Bick, 1967; Brady and Siyali, 1972; Siyali, 1972; Siyali *et al.*, 1974; Stacey and Tatum, 1985; Sim and McNeil, 1992; Stevens *et al.*, 1993). Studies by Petrek and colleagues (1994) suggests that there may be differences in fatty acid profiles for particular tissues of the body, which may mean that some tissues are more suitable than others for estimating body burden of chemicals and thus can be used to estimate breast cancer risk. Studies performed by Petrek and colleagues (1994) found that total saturated fat was higher in breast adipose than abdominal adipose tissue of the same individual. Another study also found similar effects for other fatty sites of the body (Malcom *et al.*, 1989). Victor and colleagues (1993) found that the fatty acid profiles of the breast may be altered with neoplastic change and may be used to determine the extent to which organochlorine pesticides can accumulate in the body. Therefore pesticide accumulation may be a marker of altered metabolic activity rather than contribute to the development of breast cancer. If this is the case and adipose stored in different areas of the body is proportional to dietary fat concentrations, then breast adipose tissue would be more suitable for estimating breast cancer risk than other tissues. The present study however did utilise breast adipose tissue as a medium for measuring organochlorine pesticides in order to relate the findings to breast cancer risk, thus the most appropriate medium had been used. Also an assessment of dietary fat, as it explains adipose deposition, would need to be included in the analyses. The present study included as part of a comprehensive questionnaire, a section to assess the number of dietary related events in an individual's routine (Dobson *et al.*, 1993). The studies that present conflicting results may not have accounted for the potential of organochlorines to be stored differentially depending on the medium used, as well as an account of dietary fat intake, which would also depict type of adipose tissue obtained. However, although the present study included dietary data, it did not prove to be significant in the relationship between breast cancer and pesticides. In future studies it may be important to investigate the types of different lipids in the diet and thus, their propensity to determine the deposition of adipose in the body and how that might relate to potential chemical accumulation.

The levels of DDE reported in studies vary between studies and depends on the medium used. Presented in Table 32 are the average levels of DDE detected in various studies over time, using blood and adipose. Part A of Table 32 presents the mean levels of DDE in studies that used blood or its components, whilst part B of Table 32 presents those

studies that have used adipose tissue. Overall the studies reporting levels of DDE in blood components were substantially lower than the levels reported by studies that used adipose tissue. This is as expected because the lipid levels in blood are much lower than in fats and there is a partition ratio operating between blood and adipose tissue. Furthermore, of the studies that used blood and were conducted in the United States there was a slight reduction in levels over time. This holds true for the studies conducted in the United States that used adipose tissue. The present study however reported substantially higher levels of DDE than the previous studies mentioned in Table 32. One possible reason for this discrepancy is that the use of DDT in Australia was totally banned at a much later date (the ban began in 1981, but a total ban wasn't enforced until 1987) than that in the United States. This later banning of DDT in Australia means that there is potentially more time for DDT to accumulate and may result in the detection of higher levels of its more stable metabolite DDE. However when considering the past Australian studies investigating the levels of DDE in the healthy population in comparison, the levels found in the present study indicate that over time there is the gradual elimination of these persistent chemicals from the body.

Table 32, A, Mean concentrations of DDE in blood components from patients with breast cancer and from control subjects of various studies.

Investigators	Country of Study	DDE levels in control subjects (ng/ml)	DDE levels in breast cancer patients (ng/ml)
Wolff <i>et al.</i> , 1993	USA	7.7	11
Krieger <i>et al.</i> , 1994	USA	43.1	43.3
Hunter <i>et al.</i> , 1997	USA	6.97	6.01
Schechter <i>et al.</i> , 1997	Vietnam	16.67	12.17

Table 32, B, Mean concentrations of DDE in adipose tissue from patients with breast cancer and from control subjects of various studies.

Investigators	Country of Study	DDE levels in control subjects (ng/g)	DDE levels in breast cancer patients (ng/g)
Falck <i>et al.</i> , 1992	USA	1487	2200
Van'tVeer <i>et al.</i> , 1997	Europe	1030-3130	1020-2560
Liljegren <i>et al.</i> , 1998	Sweden	1026	767
Bagga <i>et al.</i> , 2000	USA	709	800
Present study	Australia	2182	3223

With an adjustment for age the relative risk of breast cancer attributable to the factors discussed above was removed. The number of years resident in Australia is a factor of age at entry into the study combined with the number of years lived in Australia. Therefore age is a stronger risk factor for breast cancer alone rather than considered as part of the number of years resident in Australia variable. Age adjustment in this circumstance also removes the association of breast cancer risk to the factors associated with migration, for example shared lifestyle, environmental and cultural behaviours. The association between risk of breast cancer and concentrations of DDE in the breast tissue was also removed when adjusted for age. Exposure to DDT in the past would account for the presence of DDE levels in the present study, however the presence of these levels is likely to be influenced by age, such that an older woman has a longer lifetime exposure and accumulation of such materials than a younger woman. The data presented in table 31 also suggests that age is positively correlated with an increase in number of pesticides detected in the breast tissue ($p=0.444$) and a strong positive correlation was also observed between the number of pesticides and body mass index (BMI). This finding suggests that with an increase in BMI, there is the propensity to accumulate more organochlorine pesticides. But this finding also relates to the ability to accumulate more pesticides with increasing age, as several investigations suggest that with an increase in age there is an increase in body size, and an increase in breast cancer risk (LeMarchand *et al.*, 1988; Smith *et al.*, 1994; Kumar *et al.*, 1995). The other potential confounding variables highlighted in section 3.4.1 were not adjusted for because of the small numbers of controls compared to the breast cancer cases, as the degrees of freedom would not allow for such adjustments to be made. Given a larger study group however, all confounders

would have been adjusted for. Therefore, even though the levels of DDE were higher in the breast tissue of women with breast cancer than women with benign conditions, DDE exposure is not considered an important risk factor for breast cancer development in this study. With age adjustment the only factor that emerged in the regression equation was whether the participant had lived or worked on a farm where animals may have been treated with chemicals (estimated O.R=3.97 at the end of the regression model, 95% CI 2.58-5.36, p=0.047). This variable will be discussed in detail.

3.4.2.3 Living or working on a farm where chemicals may have been used on animals

Women diagnosed with truly benign breast conditions lived or worked on farms with animals more often than women diagnosed with cancer, which does not explain how breast cancer risk is increased. However when the area of residence is considered, more women diagnosed with breast cancer reported living in regional areas than women diagnosed with benign conditions. The present study's findings on this matter are difficult to interpret owing to the small number of participants who have spent extended periods of time living or working in regional areas. Given a larger sample size for this study, it would have been important to test whether the area of residence confounds the effects of living or working on a farm with animals.

It is a commonly held perception that women living in rural areas are not engaged in farming or agricultural ventures as an occupation (McDuffie, 1994). The theory is that these women may be at increased risk of breast cancer (compared to women living in metropolitan regions) from exposure to potentially carcinogenic chemicals that are used to treat crops or animals for pests. Although rural women may not directly handle the chemicals used on their farms, their exposure may be quite considerable if they are bystanders whilst someone else is applying the chemicals, or if they come into contact with contaminated clothing (McDuffie, 1994).

Organochlorine pesticides have traditionally been used for a variety of agricultural purposes to control pest insects, weeds or crop diseases. In India DDT and BHC were used to ensure increased quality and quantity of agricultural products (Kashyap *et al.*, 1994). In Australia organochlorine pesticides were used for treating all sorts of crop pests and treating animal stock (Kannan *et al.*, 1994). Studies by Kannan and colleagues (1994)

suggest that the prolonged use of DDT on vegetable, fruit and cotton growing areas left considerable pesticide residues in the soil, which contaminated crop produce. This type of contamination may be a significant route of exposure for most of the population to organochlorine pesticides and potential health problems. Further, studies by Kannan and colleagues (1994) found that of a series of foodstuffs tested for organochlorine pesticides, that fish, meat and fat, and dairy products, overall were the most contaminated food products with total DDT. Kannan and colleagues (1994) suggest that meat and meat produce contributed the most of the foodstuffs investigated to the intake of PCBs and DDTs, fish was the main contributor to chlordane intake, and fish and beverages were the main contributors of Aldrin and dieldrin in the diet. They estimate that plant products however only contribute less than ten percent of the dietary intake of organochlorine pesticides (Kannan *et al.*, 1994). Previous studies by Wan and colleagues (1989) also suggest that plant products contribute to low-level environmental contamination. In their study Wan and colleagues (1989) suggest that DDT levels in the land used for tropical fruit production were higher than those found in the soils used for vegetable production. The differences reported are most likely due to the level of use of DDT in these regions as well as the localised climatic variations (Iwata *et al.*, 1993). A study by Corrigan and Seneviratna (1990) found that some meat consignments of Australian meat destined for export contained DDT residues above the maximum residue limit (MRL). Chemicals at or above the MRL may cause significant public health concerns because of the potential carcinogenicity, mutagenicity and teratogenicity, but also may pose problems to international trade (Corrigan and Seneviratna, 1990). There are a variety of ways that cattle could be exposed to organochlorines; traditionally cattle have been dipped for a variety of insect pests, the cattle may be housed in holding facilities where structural supports may have been treated, animals feeding on contaminated grain, or from spray drift from neighbouring farms (Corrigan and Seneviratna, 1990; Pratchett, 1990).

This is not to say that women in metropolitan areas are not potentially at risk of being exposed to carcinogenic chemicals. Some occupations that women are engaged in, regardless of geographical areas of residence, may increase their potential exposure to carcinogenic chemicals, and thus their breast cancer risk. The present study assessed the types of industries and the types of occupations that may potentially expose women to high levels of organochlorine chemicals. These industries include agriculture, chemical

manufacturing plants, cleaning and dry cleaning, industries utilising electrical components, food processing, textile production, metalwork, some forms of transport and the production of wood products. However this research found no correlation ($p > 0.05$) between the type of industry in which the patient works and breast cancer risk. It is interesting to note though, that women working in potentially high-risk industries were more frequently diagnosed with benign conditions. It may be the case that although these women may be employed in a high risk industry, that their direct contact with potentially harmful chemicals is minimal, especially if they are employed in the clerical, sales, professional or office duties part of the business. This sort of trend was also witnessed for reported pesticide and herbicide use around the home, personal handling of these chemicals, living or working on animal or crop farms, washing exposed clothing and time spent on these farms. As the present study was of a retrospective design, it may have been subject to recall bias. The recall of events many years ago, especially if there is the perception of no connection with disease or other life altering events, may be difficult among some women, especially those with benign breast disease. Women with breast cancer however may be searching for answers as to why they have contracted the disease and may tend to recall events with more accuracy. In Melbourne many houses are treated for termites, so this would put metropolitan women at an increased risk of exposure, and these women may be unaware that their houses have been treated. An analysis of the types of chemicals that were used by these women (data not shown) reveals that women with benign breast conditions tend to favour using pyrethroid-based chemicals, as opposed to the use of organophosphate and organochlorine-based chemicals by women diagnosed with breast cancer. For many it is a formidable task to determine the levels of contamination in plant produce owing to the various chemicals that are available to control pests but also because the actual chemicals used are seldom known. This was a problem with the present study as many of the participants were unaware of the chemicals used (if they were used) because they did not handle them themselves, but as previously mentioned, may have been inadvertently exposed by washing contaminated clothing or by inhaling spray drift from neighbouring properties. A study by Beard and colleagues (1995) investigated the amount of pesticides in ambient air in the Coffs Harbour region of New South Wales, Australia and the subsequent exposure to humans. The research indicates that although three organochlorine and three organophosphate pesticides were detected in samples of ambient air, that when the acceptable daily intakes (ADI) were

considered, that exposure was very low, even in areas of high agricultural pesticide use (Beard *et al.*, 1995). The detection of the pesticides was not related to their use in agricultural settings but rather to the application as termiticides in the area. This finding is of particular importance as it highlights the potential for these pesticides to drift and expose non-target organisms.

3.4.3 Possible additive effects of multiple pesticides detected in the breast adipose tissue.

The characterisation of a single environmental factor as a risk or potentially preventable factor for breast cancer is confounded by the complexity of environmental factors, and the complexity of the interaction and exposure to many different chemicals. As already highlighted, there are many ways in which a person can be exposed to chemicals which may potentially put them at risk of developing breast cancer; through the food that is consumed; the use of chemicals to control pests or indirectly by inhaling spray drift or washing contaminated clothing. To investigate all these chemicals in the one study and their potential to increase breast cancer risk would be cumbersome, let alone require a substantial study population. The present study, even though it investigated a series of organochlorine pesticides and the total effects of these pesticides on breast cancer development, only one pesticide was found at high levels in the tissue from women with breast cancer but was not related to increasing breast cancer risk. When the effects of total pesticides detected in the breast tissue was considered, most of the effect observed was due to the detection of DDE, but again this factor is not a causative agent for breast cancer development. Research by Payne and colleagues (2001) suggest that even though there are exposures to a multitude of agents with potentially oestrogenic or oestrogen-like activity in a person's lifetime, there is no conclusive evidence that these agents produce combination effects when present as mixtures. Such that organochlorine pesticides are lipophilic in nature and will accumulate in adipose tissue, but are likely to act with each other as well as other endogenous oestrogens. The research of Payne and colleagues (2001) suggests that if a mixture of the different isomers of DDT and its metabolites are formed and added to cells in culture, that the proliferative effect observed in the cells is due to the most potent mixture component. It is interesting to point out however, that the

levels at which Payne and colleagues (2001) observed these effects were considerably lower than levels found in human serum. Payne and colleagues (2001) also compared the concentrations of single agents that are needed to elicit a cell proliferation response and how they compared with levels found in human tissue. This work found that concentrations of individual agents were substantially higher than levels found in human serum and are unlikely to induce cell proliferation individually. This will be discussed further in chapter 4, where the present study attempted to elucidate the whether DDE or DDT elicits cell proliferation, and if so what impact these chemicals have on specific genetic pathways.

The findings presented suggest that the number of pesticides detected in tissues is not related to the risk factors for breast cancer. It would be expected however that the pesticides under investigation in the present study and their respective concentrations in the breast adipose tissue would be higher in women living or working in areas where these chemicals would have been used. The finding of the present study indicate this, such that women who have lived or worked on a farm where chemicals may have been used to treat crops, recorded a non-significantly higher number of pesticides compared to women who hadn't lived or worked in such areas. Surprisingly though, women involved with manual labour occupations, where the expected exposure to chemicals would be high, recorded lower numbers of pesticides than women employed in professional, administrative/ clerical areas or whom were retired or engaged in home duties. This observation suggests that (I) women who were classified as retired may have engaged in potentially high risk occupations which were unknown to the present study's investigators, or (II) there may be different exposure routes to chemicals on the basis of occupation (which may be an indicator of socioeconomic class differences) and also on the basis of area of residence. Research by Gridley and colleagues (1999) investigated the potential healthy worker effect for cancer incidence among women in Sweden. Their work suggests that there is an increased breast cancer risk among full time employed women, younger working women, women who worked in urban areas and for higher socioeconomic levels (based on the women's job title, not education and income). The work of Gridley and colleagues (1999) thus found no evidence of a healthy worker effect for cancer incidence, rather their work suggests the opposite. They also investigated cancer incidence among women who were employed on the basis of residence and their

work suggests that the breast cancer risk was higher among women who lived in urban areas (Gridley *et al.*, 1999).

3.5 CONCLUSIONS

These observations tend to suggest that each woman may present with her unique set of factors that may or may not put her at increased risk of breast cancer. Risk factors are an important component in research determining relative risk of breast cancer attributable to chemical exposure, however there is often difficulty in obtaining accurate data via recall and the assessment of exposure therefore has to be made by biological tests, such as measuring pesticide levels in adipose tissue. The present study, although a relatively small one in comparison to some of the benchmark studies in this area of research, found a negative association between organochlorine pesticides and breast cancer risk. The levels of DDE were increased in the breast tissue of women diagnosed with breast cancer compared to those in women with benign breast conditions, however with consideration of age DDE levels were not causative factors for breast cancer development. The present study included a comprehensive questionnaire to assess breast cancer risk factor information, dietary behaviours in relation to fat intake and chemical exposure assessments. A large amount of information from this questionnaire was gathered, however all the data collected could not be controlled for in the statistical analyses because of the small number of participants in this study. The lack of Australian data in the literature pertaining to chemical exposures and the risk of breast cancer, meant that it was not possible to predict at the onset how many participants would be required for the study. The power to find an association between DDE and breast cancer risk is therefore limited by the small number of participants in the study. The high levels of DDE in women with breast cancer ($p=0.013$) still raises the question as to whether DDE has the potential to initiate proliferation and be involved in breast cancer development, given that some of the larger studies conducted on this matter have found increased risks attributable to DDE exposure. Chapter 4 will present data, which investigates how DDE interacts with breast cancer cells, to initiate proliferation, as well as the effects of DDE on the genes and pathways involved in breast development.

There was also an association found between risk of breast cancer and increased duration of residence in Australia when age was not adjusted. However as this is a factor of age combined with the number of years lived in Australia, an adjustment for age alone removes this factor as a risk for breast cancer development.

CHAPTER 4
ORGANOCHLORINE PESTICIDES AND GENE
EXPRESSION

4.1 INTRODUCTION

The previous chapters have shown that organochlorine pesticides are present in human tissue and that the relative risk of breast cancer was increased with increasing levels of DDE detected when age was not adjusted in the regression model. The presence of these pesticides, alarming as it may be, does not prove causation of breast cancer. Neither does the presence of pesticides in human tissue through a continuum of breast disease events explain the disease process. Researchers have suggested that these environmental pollutants are still of considerable concern because of their ability to mimic steroid hormones such as oestrogen. It has been suggested that if chemicals like DDT are able to mimic endogenous oestrogen then they have the potential to interfere with processes that are normally regulated by oestrogen and result in alterations in cellular reproduction and increase the susceptibility to cancer. The mechanisms by which oestrogen and oestrogenic substances effect proliferation of certain cells are not well understood, although some researchers have begun to investigate this. Shekhar and colleagues (1997) have suggested that oestrogen may induce the expression of regulatory genes of the cell cycle, which implicates the oestrogen receptor in regulating cell cycle progression. Research by Dees and colleagues (1997a) supports the theory that DDT is able to mimic oestradiol and stimulate cells to enter the cell cycle. Dees and colleagues (1996 and 1997a) have conducted a number of experiments to show that DDT (a mixture containing 85% p,p'-isomer and 15% o,p'-isomer) was able to increase the synthesis of cyclin D1. Cyclin D1 regulates the normal development and proliferation of breast epithelium and regulates entry of MCF-7 cells into the cell cycle (Weinstat-Saslow *et al.*, 1995; Dees *et al.*, 1997a). The over expression of cyclin D1 on the other hand may be a useful prognostic indicator with respect to breast cancer invasiveness (Weinstat-Saslow *et al.*, 1995).

Research by Shekhar and colleagues (1997) have suggested that pesticides, such as DDT and DDE, can stimulate growth and oestrogen receptor function in human breast cells. They found that p,p'-DDT was able to stimulate the proliferation of MCF-7 cells in culture to a greater capacity than the o,p'-DDT isomer (Shekhar *et al.*, 1997). Other researchers have suggested that the p,p'-isomers of DDT as well as DDE are non-oestrogenic compared to the o,p'-isomers of DDE and DDT (Kupfer and Bulger, 1980; Bulger and Kupfer, 1983; Shekhar *et al.*, 1997). Research by Soto and colleagues (1995),

testing environmental chemicals for oestrogenic activity using the E-Screen assay found that *o,p'*-DDT displayed oestrogenic activity at 1 μ M and 10 μ M in MCF-7 cells, whilst *p,p'*-DDT only exhibited oestrogenic qualities at 10 μ M. The work by Shen and Novack (1997) investigated the effects of DDT on growth factor gene expression, and found that at lower concentration (0.1 and 0.01 μ M) *p,p'*-DDT was able to increase epidermal growth factor receptor tyrosine phosphorylation, which contributes to cell proliferation and differentiation. There is continuing debate as to which isomers of DDT and its metabolites exert effects on breast epithelial cells.

The work presented in this chapter will investigate the effects that *o,p'*- and *p,p'*-isomers of DDE and DDT may have on breast cells in culture. The present study utilized four cell lines, each derived from pleural effusions of different breast conditions, two cell lines were oestrogen receptor positive, MCF-7 and T47D, and two cell lines were oestrogen receptor negative, MDA-MB-231 and MDA-MB-435. These cell lines were selected to determine whether DDT exerts an effect via the oestrogen receptor pathway or by some other route. Previous research, suggests that DDT and its metabolites are involved in the cell cycle and thus may play a role in promoting growth rather than causing mutational aberrations. Hence, the present study utilized cell lines that express a transformed phenotype as opposed to normal untransformed cells. In the first experiment, the pesticides were introduced into a cell culture system containing human breast cells, to investigate cell proliferation or inhibition in response to exposure to different isomers of DDT and DDE. A mixture of breast cell lines, oestrogen receptor positive and oestrogen receptor negative, were used for this experiment to determine the differential effects on growth of these cells in response to exposure to the isomers of DDE and DDT in two different media, as described subsequently. In the second experiment, the cell lines were exposed to the agents and the capacity of the cells to survive and produce colonies in response to exposure was measured. This experiment utilized two cell lines, one oestrogen receptor positive and one oestrogen receptor negative, to determine the differential effects of the pesticides (two isomers of DDE and DDT) on the colony forming capacity. In the final experiment a microarray system was used to investigate the effects of DDE on up-regulation or down-regulation of genes expressed in treated and untreated breast cells.

4.1.1 Aims

To determine the response of breast cells to DDT and DDE isomers, by proliferation studies and cell clonal assays (or cell survivability assays).

To determine the concentrations and length of exposure at which this response occurs.

To determine the precise nature of this response by investigating the genes that may be regulated by exposure and whether they are linked to breast cancer in some way.

To examine the potential pathways by which DDT may be involved in producing breast cancer.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals and reagents

The DDT isomers and metabolites were purchased from Chemservice (West Chester, PA). The DDT isomers and metabolites used include the two isomers of DDT, o,p'-DDT and p,p'-DDT, and the major metabolites of these compounds, o,p'-DDE and p,p'-DDE. DDE was prepared as a concentrated stock solution in absolute ethanol, dimethylsulfoxide (DMSO) and dioxane. Sulforhodamine B (SRB) was initially purchased as a powder from Sigma Chemical Co. (St Louis, MO), but for the purposes of this study was supplied by M. Waltham at St Vincent's Institute of Medical Research, Victoria Australia.

4.2.2 Cell lines and maintenance

These studies utilised four cell lines, MCF-7 (St Vincent's), T-47D, MDA-MB-231 (LP'95) and MDA-MB-435 (PMCI). The MCF7 cells are isolated from a pleural effusion

obtained from a patient with adenocarcinoma of the breast. This line expresses cytoplasmic oestrogen receptors (Soule *et al.*, 1973). The T-47D line was isolated from a pleural effusion obtained from a patient with infiltrating ductal carcinoma of the breast. This line contains receptors for oestrogen, 17β -oestradiol, other steroid hormones and calcitonin. The MDA-MB-231 cells are isolated from pleural effusion obtained from a patient with adenocarcinoma of the breast. MDA-MB-435 cells were an evolved line from a pleural effusion obtained from a patient with metastatic, ductal adenocarcinoma of the breast. The MDA-MB231 and MDA-MB-435 lines do not express the oestrogen receptor, however the MDA-MB-231 cells express receptors for epidermal growth factor and transforming growth factor. Both, oestrogen receptor positive and negative cell lines were used to determine if the carcinogenic agent exerts an effect through the oestrogen receptor pathway or by some other route.

All cells were grown and passaged as monolayer cultures in T75 flasks in RPMI-1640 growth media (herein after referred to as "growth media") supplemented with 10% foetal calf serum (FCS) and 50 μ g/ml gentamycin, at 37°C in 5% carbon dioxide atmosphere. Experimental media consisted of phenol red free RPMI-1640 (Gibco BRL) supplemented with 10% charcoal stripped foetal bovine serum (csFBS; Hyclone) and 5 μ g/ml gentamycin. The growth patterns of each cell line in the growth and experimental media was determined over a 5 day period as described by the method of Skehan *et al.*, (1990) detailed in section 4.2.3). This method utilizes sulforhodamine B, which binds to macromolecular counterions in cells fixed with trichloroacetic acid (TCA) and is a commonly used method to determine cytotoxicity to particular drugs or other agents. The growth patterns of each cell line in response to the two different media used, was important to establish because the growth media contains growth factors and dyes that may mask the effects of growth promotion by DDT. These growth studies were performed in parallel with the cell proliferation studies and consisted of measurements of optical density in quadruplicate at each end of a 96 well plate (therefore 8 measurements in total).

4.2.3 Cell proliferation studies

In these studies, the sulforhodamine B assay, as described in Skehan *et al.*, (1990) was used to determine the degree of cellular proliferation or cytotoxicity of breast cells exposed to DDT and DDE isomers, by measurement of optical density.

For the growth studies of cells exposed to DDE and DDT, the cells were removed from the flasks with trypsin/ EDTA after a five day growth period, counted on a haemocytometer, and seeded with the experimental medium into 96 well plates. The cells were seeded at densities depending on their proliferative capacity (MCF7, T-47D and MDA-MB-435 seeded at 2000 cells per well, and MDA-MB-231 seeded at 1000 cells per well). All plates were incubated for approximately 3 days at 37°C in 5% carbon dioxide atmosphere, to allow the cells to adhere to the base of the wells. After the cells adhered to the base of the wells, the treatments with pesticide commenced. Two assays were performed. In the first assay, different concentrations of the *o,p'*-DDE isomer, ranging from 0.1µM to 50µM were trialled to determine the range in which identifiable changes in cellular proliferation occurred for all four cell lines. Subsequently, a second assay utilizing somewhat higher concentrations was performed for both *o,p'*- and *p,p'*-isomers of DDE and DDT to compare their effects.

In the first assay working solutions of different concentrations of DDE were diluted in growth and experimental media and added to the 100µl of cells media already in the 96 well plates. The final concentrations used for the first cell proliferation study are listed below (Table 33) and a sample layout is included in Figure 15.

In the second assay, solutions of the main isomers of DDE and DDT were used, not only to repeat the first experiment, but also to investigate whether there were differences in proliferation according to the isomers used as well as to determine if the parent compound (DDT) produced a different effect. Working solutions of the *p,p'*- and *o,p'*- isomers of DDE and DDT were made up at differing concentrations in experimental media and growth media. These working solutions of pesticide were added to the 100µl of cells and media in the 96 well plates and would produce the concentrations of interest listed below (Table 33). Each concentration of pesticide was studied in at least triplicate. The results

presented will consist of those pertaining to the second cell proliferation study, as the parameters tested included o,p'- and p,p'- isomers of both DDE and DDT, tested in four cell lines and in growth and experimental media.

Table 33, Final concentrations of pesticides used for the first and second SRB cell proliferation assays.

SRB assay #1 o,p'-DDE	SRB assay #2 o,p'-DDE and DDT p,p'-DDE and DDT
Cells only control	Cells only control
50µM	100µM
10µM	50µM
5µM	10µM
1µM	5µM
0.1µM	1µM
0.01µM	0.5µM
0.001µM	0.1µM
Diluent control (@1%)	Diluent control (@ 1%)

Key: SRB = sulforhodamine B; diluent control for SRB #1 = mix of dimethylsulfoxide and ethanol; diluent control for SRB #2 = dioxane

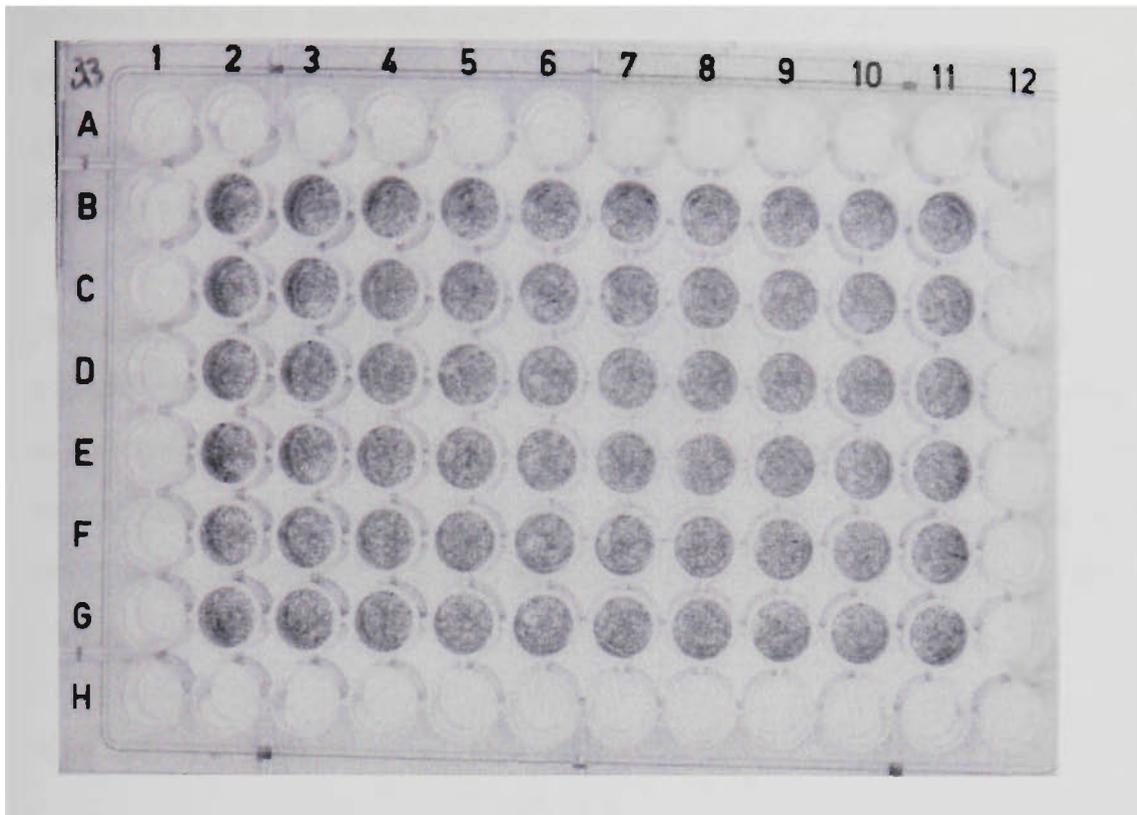


Figure 15, Sample layout for the SRB assays on a 96 well plate, as per assay 1.

- Key;
- Columns 1 and 12 and rows A and H were left blank (no cells and no agents)
 - Columns 2 and 3, were cells only controls (0 μ M agent controls)
 - Column 4 contained 50 μ M agent
 - Column 5 contained 10 μ M agent
 - Column 6 contained 5 μ M agent
 - Column 7 contained 1 μ M agent
 - Column 8 contained 0.1 μ M agent
 - Column 9 contained 0.01 μ M agent
 - Column 10 contained 0.001 μ M agent
 - Column 11 contained diluent control at 1%

Each day after addition of DDE, DDT, solvent or cells only, a plate was removed from the incubator for each cell line. The detailed methodology for the sulforhodamine B (SRB) assay has been described by Skehan *et al.* (1990). Briefly, to fix the cells in situ, 50 μ l of cold 50% (w/v) trichloroacetic acid (TCA) was added to each well and the plates were incubated for 60 minutes at 4°C. After such time, the supernatant was removed, the cells were washed five times with deionised water, and the plates were allowed to dry at room temperature. One hundred microlitres of SRB solution (0.4% w/v in 1% acetic acid) was added to each well, and the plates incubated at room temperature for 10 minutes. The

unbound SRB was removed and the cells were washed five times with 1% acetic acid. The plates were allowed to air dry overnight. The stain was solubilised by adding 150 μ l of 10 mM unbuffered Tris solution (pH 10.5) to each well, and slightly agitating the plates on a gyratory shaker for 10-15 minutes, to make the stain intensity uniform.

The optical density (OD) of each well was read on an automated microtitre plate reader at a single wavelength of 595nm. Optical density is proportional to the concentration of cells in the media and was measured for each treatment (all concentrations of DDE, DDT, solvent and controls). The OD of the “cells only” controls were used as a reference for determining cell proliferation or inhibition in the cells treated with DDE, DDT or solvent.

4.2.4 Soft agar studies

Soft agar assays were conducted to investigate the survivability of cells and capacity to form colonies in the presence of toxicants, such as DDE and DDT. These assays are different from the cell proliferation assays in the fact that the latter possess unlimited proliferation potential in liquid media, whereas the former are restricted in their proliferation capacity by the solid medium. This assay can identify cells with the selective advantage of growth given the media and agents provided, to “survive” the conditions and produce colonies. This experiment was conducted using two approaches: (I) cells were grown in the presence of different concentrations of DDE and then introduced into the agar, and (II) growth of the cells and introduction into the agar with the addition and replenishment of pesticides after the cells have established.

4.2.4.1 Soft agar assay approach # 1

MCF-7 cells were grown in flasks exposed to seven different concentrations of o,p'-DDE ranging from 0 μ M to 50 μ M DDE in experimental media, but only three concentrations are presented in the results as there were concerns that the incubation of cells with agent prior to being added to agar would not allow for efficient growth of colonies. The control and the two highest concentrations of agent used were selected for measurement, because if there were any inhibition of colony forming capacity, it would be expected with the high concentrations tested. After three days of exposure to o,p'-DDE, the media was

poured out of the flask and the cells were washed twice with phosphate buffered saline (PBS). A small amount (enough to cover the cell layer) of trypsin/EDTA was added to cells to remove any dead material and the excess was poured off. The flasks were returned to the CO₂ incubator for ten minutes for treatment with trypsin. Five millilitres of RPMI-1640 media containing 10% FCS were added to suspend the cells. This cell solution was transferred to a Falcon tube and spun at 400xg for five minutes. The supernatant was discarded and the pellet resuspended in fresh RPMI-1640 media containing 10% FCS. A cell count of each treatment was performed by pipetting 9 μ l to a haemocytometer and counting under a microscope. Due to the low number of cells for each treatment, the amount of cells required for the assay was taken from the stock solutions rather than being diluted to 1ml in media before addition to agar. Cells in solution (amount depending on the cell counts) were added to 3ml of 2 x DME (Dulbecco's enriched modification media). An equal volume (3ml) of 0.7% agar was added to the medium containing the cells. In six well plates, 0.25ml of agar solution (0.5% agar, 1x RPMI-1640 and 10% foetal bovine serum) was pipetted per well to provide a base layer. In a simplified procedure, 1.5ml of this agar/cell mix was pipetted into each well, so that each concentration was assayed in triplicate. These plates were incubated for 14 days at 37°C in 5% carbon dioxide atmosphere. After 14 days, the cells were stained with crystal violet. Briefly, 0.5ml of a 0.05% crystal violet solution (containing methanol and water) was added to each well and left for one hour prior to determining cell survivability. The base of each well was scored to produce a grid, to facilitate counting of different areas of the well. Four quadrants per well were counted under 100 times magnification and the objective was focused to incorporate counting at different planes in the agar.

4.2.4.2 Soft agar assay approach # 2

The low number of cells encountered with the experiment may have been a limiting factor when trying to look at survivability of cells or the ability of cells to form colonies from exposure to different agents. The fact that these cells were exposed to agents before the addition of agar, may have hindered the production of colonies, as the cells were adjusting their growth in response to the agents as well as trying to survive in agar, which can be a hostile environment for particular cells. To try and negate this effect a second experiment was undertaken to determine the survivability of these cells in response to

exposure to different agents. This second experiment allowed the cells to independently establish in the agar with concurrent exposure (and replenishment) to pesticides (unlike experiment 1 where the cells were grown in media in the presence of agents). In order to determine if the survivability response was mediated by oestrogen, some oestrogen receptor negative cells (MDA-MB-231) were included in the assay, so that both ER positive and ER negative cells were exposed to DDE or DDT isomers and to the biologically potent oestrogen, 17β -oestradiol.

MCF-7 and MDA-MB-231 cells were grown and harvested from experimental media. The cells were seeded into 3 x 6 well plates (per cell line) to achieve 20 000 MCF-7 and 5000 MDA-MB-231 cells per well. A base layer of agar solution (0.5% agar, 1 x RPMI-1640 media, 10% FBS; 0.25ml per well) was pipetted into each well and allowed to set. Meanwhile working stock solutions of each agent of interest were prepared such that the concentration was twice that required in the assay, to allow for diffusion of the agent through the top and base layers of agar. Both isomers of DDE and DDT were used to determine the effect of the isomers on cellular growth. DDT was also included in the assay, as the initial exposure to pesticide would be to DDT and secondary effects would be from the metabolised form, DDE. Each isomer of DDE and DDT was assayed in duplicate at $10\mu\text{M}$ and 17β -oestradiol was assayed in triplicate at 10nM . Wells containing cells only were used as controls.

Twenty microlitres of pesticide (from a 10nM stock), 5ml of media plus cells and 5ml of 0.7% agar solution were combined and 1.5ml added to each duplicate well, and the plates incubated in a 5% CO_2 atmosphere. After around 14 days incubation the pesticides and oestradiol were replenished at the same concentrations already in the wells. Five hundred microlitres of $10\mu\text{M}$ pesticide and 10nM oestradiol were added to each well and the plates incubated for a further 7 days in the CO_2 incubator. After a total of 3 weeks incubation, the cells were stained with crystal violet as described previously. The base of each well was scored to produce a grid, to facilitate counting of different areas of the well. Four quadrants per well were counted under 100 times magnification and the objective was focused to incorporate counting at different planes in the agar.

The first attempt at this second approach to the soft agar assay was not successful. The MCF-7 and the MDA-MB-231 cell lines, both failed to produce colonies and remained suspended in the agar as singular cells. The second approach to the soft agar assay was repeated using similar conditions to the first attempt except as outlined next; (1) the media used in the base and top agar was 2XDME, rather than 2XRPMI-1640; (2) the agents being tested were assayed in growth media and in experimental media (charcoal stripped serum and phenol red free media) for the cell lines listed above; (3) the control plates for both cell lines consisted of tests for 10nM 17 β -oestradiol and 2xDME, both in triplicate; and (4) each isomer of DDE and DDT was assayed at 0, 0.1, 1 and 10 μ M concentrations for both cell lines in duplicate.

4.2.5 Microarray

Oestrogen receptor positive and negative cells were grown in the presence of 0, 0.1 and 10 μ M DDE for comparative gene expression analyses using microarray technology. This technology enables the user to identify altered gene expression by comparing two different RNA samples such as normal versus cancer, or as the case here, with different concentrations of treatment with DDE.

4.2.5.1 RNA preparation

MCF-7 and MDA-MB-231 cells were grown in RPMI-1640 media containing 10% FCS. After reaching approximately 80% confluency (whilst cells were still in the logarithmic phase of growth), the cells were washed in PBS and the media replaced with phenol red free RPMI-1640 and charcoal stripped FCS, containing o,p'-DDE at 0, 0.1 and 10 μ M. The cells were exposed for 3 days (as determined from the cell proliferation assays), after which time the media was removed and the cells were washed with warmed PBS. The procedure for preparing RNA from cell cultures was that accompanying the Qiagen RNeasy kit. Briefly, RLT (RNeasy lysis buffer) buffer containing 10 μ l β -mercaptoethanol per 1ml of RLT buffer was added to the flasks and the cells were scraped using a rubber policeman. The cell lysate was collected and sheared through a 19-gauge needle and stored in a -70°C freezer. The cells were thawed on ice and 4ml of cold 70% ethanol was added and the tube shaken vigorously. The cell solution (maximum of 4ml at a time)

was then applied to RNeasy midi-spin columns (which were provided in 15ml centrifuge tubes) and centrifuged at 3000-5000xg for 5 minutes. Four millilitres of RWI (second lysis buffer) buffer was added to the columns and centrifuged at 3000-5000xg for 5 minutes. After such time, 2.5ml of RPE (wash buffer) buffer was applied to the columns and centrifuged at 3000-5000xg for 2 minutes. RPE buffer was applied to the columns for a second time and again spun at 3000-5000xg but for 5 minutes. The column was removed from the centrifuge tube and inserted into a fresh tube. Approximately 200-250 μ l of RNase free water was applied to the column, let stand for a couple of minutes and then spun at 3000-5000xg for 5 minutes. The eluent was collected and subjected to UV analysis and gel electrophoresis to verify RNA quantity and quality.

4.2.5.2 GeneFilter preparation

The array membranes used for this experiment were supplied by Research Genetics and were Human Breast Specific GeneFilters. These filters include approximately 5000 genes that are known to be expressed in normal and malignant breast tissue from human and other animal origins. The genes are represented as DNA from cDNA clones containing the 3' end of a gene. The membranes also contain housekeeping genes and total genomic DNA, which were used as control points for hybridisation, homogeneity of hybridisation and used as a basis for normalisation when investigating differences in expression.

Three filters containing human breast specific genes were used in this experiment, and because RNA was extracted from MCF-7 cells under three different treatments (0, 0.1 and 10 μ M DDE), three cross hybridizations were conducted to test filter reliability between each hybridization. The DDE concentrations that were used on each filter for each hybridization are listed in Table 34.

Table 34, Concentration of DDE treated MCF-7 cells on Human Breast Specific GeneFilters #19, #20 and #21 for each hybridization.

	Hybridisation #1	Hybridisation #2	Hybridisation #3
GF225 #19	10 μ M DDE	10 μ M DDE	0 μ M DDE
GF225 #20	0.1 μ M DDE	0 μ M DDE	0.1 μ M DDE
GF225 #21	0 μ M DDE	0.1 μ M DDE	10 μ M DDE

Key: GF225 = refers to the type of GeneFilter, Human Breast Specific.

The protocol for hybridisation of test RNA (as prepared in the previous section, 4.2.5.1) to the GeneFilter microarrays, was provided by Research Genetics. Before the use of the GeneFilter membranes, they were washed and agitated in boiling 0.5% sodium dodecyl sulphate (SDS) for five minutes. Washing the membranes before use rids them of any residual material that may interfere with hybridisation.

Prehybridisation takes approximately 2 hours. The GeneFilters were placed into hybridisation roller tubes with the DNA side facing the interior of the tube. The MicroHyb hybridisation solution was preheated at 42°C, to prevent precipitation of the solution, and 5mL added to the roller tubes. The hybridisation tubes were then rolled to saturate the membranes and the following agents were added;

Cot-1 DNA is a blocking agent for repeat sequences and non-specific sites on the membrane, and is used to reduce background signals of the hybridisations. Cot-1 DNA was boiled for 5 minutes and 5µg (20µl) was added to the roller tubes.

PolydA (5µg) was also added to the roller tubes containing the GeneFilters. The tubes were vortexed to ensure uniform mixing of the solutions and the GeneFilters were positioned in the tubes so as to remove any bubbles that may have occurred between the filter and the inside of the tube. The roller tubes were then placed into a hybridisation roller oven set at 42°C and allowed to prehybridise for 2 hours.

The labelling of RNA with a radioactive probe, prior to the application to the GeneFilters, takes approximately 2 hours. In a microcentrifuge tube total RNA, OligodT (1µg/µl) and diethylpyrocarbonate (DEPC) treated water were combined. This RNA mixture was incubated at 70°C for 10 minutes, after which time the tubes were placed on ice. To the microcentrifuge tubes containing the RNA, the following were added;

6µl of 5x first strand buffer

1µl of DTT (0.1M)

1.5µl of dNTP mix (containing 20mM dATP, dGTP and dTTP)

1.5µl of reverse transcriptase (superscript II)

10µl of ³³P dCTP (concentration 10mCi/ml with a specific activity of 3000 Ci/mmol)

The contents of the microcentrifuge tubes were mixed thoroughly by pipette and pulse centrifuged to collect any drops from the sides of the tubes. The microcentrifuge tubes were then incubated at 37°C for 90 minutes.

The probe (the contents of the microcentrifuge tube) was purified by passage through a Biospin 6 Chromatography Column (Bio-Rad). The manufacturer's protocol was followed for this procedure, which removes any unincorporated nucleotides. Briefly, the columns were prepared by resuspending the gel, snapping off the tips and centrifuging for 4 minutes at 1000xg. Fifty microlitres of water was added to each microcentrifuge tube (prepared above) to give a total volume of approximately 80µl. A small amount (approximately 2µl) of each treated mix was set aside for scintillation counting (labelled as BEFORE purification of probe). The remainder of the 80µl was applied to the Biospin columns and centrifuged for 5 minutes at 1000xg. A small amount (approximately 2µl) of eluent from centrifugation was set aside for scintillation counting (labelled as AFTER purification of probe) to assess the efficiency of incorporation of the radiolabel. The counts per minute were measured for the BEFORE and AFTER samples and the amount of incorporated radiolabel recovered was calculated.

Hybridisation of the probe to the GeneFilters takes approximately 12 to 18 hours. Briefly, the purified probe was placed into a boiling water bath for 3 minutes to allow for denaturation. The probe was pipetted into the roller tube, taking care not to pipette directly onto the membrane. The solution was mixed by vortexing and the filters incubated at 42°C for at least 12 to 18 hours in a hybridisation roller oven. After such time, the hybridisation solution was discarded and the membranes were washed with differing stringency buffers. The first and second washes consisted of 30 to 40ml of 2x SSC, 0.5-1% SDS and an incubation of 42-50°C for 20-30 minutes. After each wash the wash solution was discarded. The third and fourth washes consisted of 30 to 40ml of 0.5x SSC, 0.5-1% SDS and an incubation of 42-60°C for 15 to 30 minutes. A final rinse of the GeneFilter arrays was performed with 2x SSC to remove any residual SDS that may interfere with the image plate. After washing, the GeneFilters must be prevented from drying, by placing them onto moistened filter papers (cut to the size of the membranes and moistened with deionised water) and wrapping these in plastic wrap. Creases and bubbles between the GeneFilters and the plastic wrap must be avoided in this process as

they may interfere with imaging. The wrapped GeneFilters were placed into a cassette and carefully aligned with the phosphor-imaging screen, to facilitate the use of imaging software. The exposure time of the filters to the image plate was 72-128 hours. The exposure in the phosphor imager then produces an image of the treated filters, which were then imported into Pathways software (version 3, Research Genetics) for gene expression analyses.

The images for the GeneFilters were overlaid to compare the intensities for each hybridization, such that #19 was compared to #20, #19 compared with #21, and #20 compared with #21. A Chen test at the 95% confidence interval was calculated within the Pathways software for each comparison. The Chen test is a statistical test that determines whether or not the intensities of two samples are different based upon a desired confidence interval. This test essentially eliminates genes that are expressed at similar intensities between the two filters (and hence fall under the bell curve for normal distribution) and retains the 2.5% of genes differentially expressed at each end of the normally distributed bell curve.

4.2.6 Statistical analyses

The data collected for the proliferation (section 4.2.3), the colony forming studies (section 4.2.4) and gene array (section 4.2.5) were analysed using SPSS computer software (Chicago, IL). The optical densities obtained from the cell proliferation studies and the numbers of colonies formed from the soft agar studies produced skewed distributions when analysed for normality and were subsequently transformed (by square root) to mitigate the skewed effect. The data collected from the cell proliferation studies was subjected to univariate analyses of the transformed OD data against cell line (MCF-7, T47D, MDA-MB-231 and MDA-MB-435), types of agents used (DDE, DDT and isomers of both), concentrations of agents used, media type (growth or experimental) and exposure time (1-5 days), and significance of any differences between cell proliferation and the other variables tested was assessed using p values of less than 0.05 from between subject and within subject analysis of variance. The types of cells used, exposure time to agents, the types of agents used and the concentrations of agents were subsequently

subjected to post hoc analyses using the Tukeys HSD test to determine which means are different.

The data collected for the soft agar assay was analysed by univariate analyses of the transformed number of colonies against size of colonies (for assay approach number 1 only), cell type (MCF-7 and MDA-MB-231), media type (growth or experimental), type of agent used (DDE or DDT of o,p'- or p,p'-isomers) and concentration of agent. The differences between the above variables and the number of colonies were assessed using p values of less than 0.05. The agent used and concentration of agent were subjected to post hoc analyses using the Tukeys HSD test, to determine where the difference lies between the variables.

The data collected for the microarray experiments was also analysed by statistics. The differences in intensity between one array and another were statistically analysed by students t-tests.

4.3 RESULTS

As part of the cell proliferation studies, using the sulforhodamine B assay, the growth features of each cell line were studied. The growth curves for each cell line under investigation were used to determine appropriate times for seeding out of the cells for the various assays described in the methods and also to determine the response of each cell line to the growth and experimental media (Figures 16 to 19).

In comparison to the other cell lines studied, the MCF-7 cells (Figure 16) tended to produce lower intensities of cell density, as determined by absorbance over the five day period. There was also a slight inhibition of cell density with growth in the experimental media (phenol red free RPMI media and charcoal stripped foetal bovine serum) compared with the growth media. This inhibition of cell growth in the experimental media was also evident for the T47D cells (Figure 17). The conditions provided by the growth media tended to favour the growth of the T47D cells to a greater extent than the MCF-7 cells.

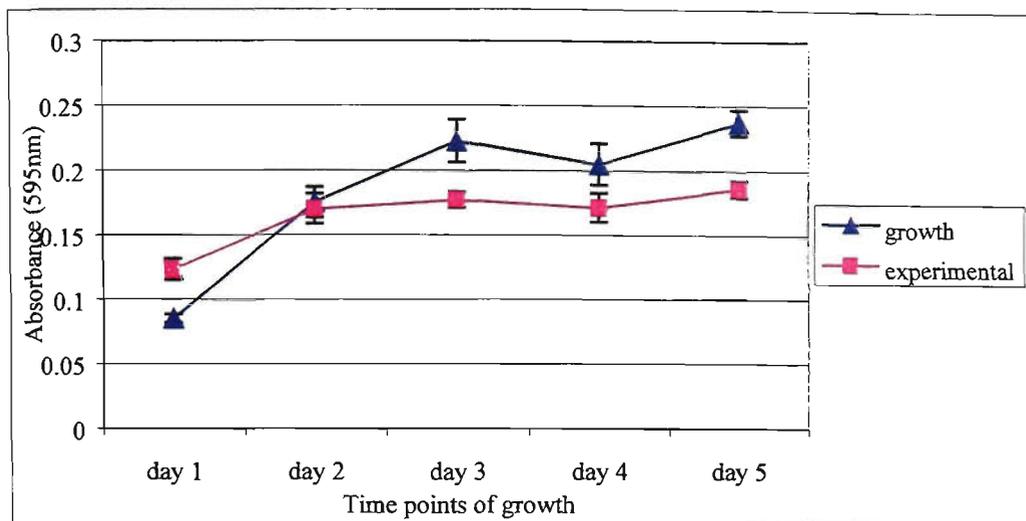


Figure 16, The cell density of MCF-7 cells over time in growth and experimental media, as recorded using the sulforhodamine B assay.

Key: growth media = RPMI media containing 10% foetal bovine serum

Experimental media = phenol red free RPMI media containing 10% charcoal stripped foetal bovine serum.

Each point on the graph represents the mean absorbance of 16 replicates, 8 replicates taken from each end of the 96 well plate. Error bars represent the standard error of the mean.

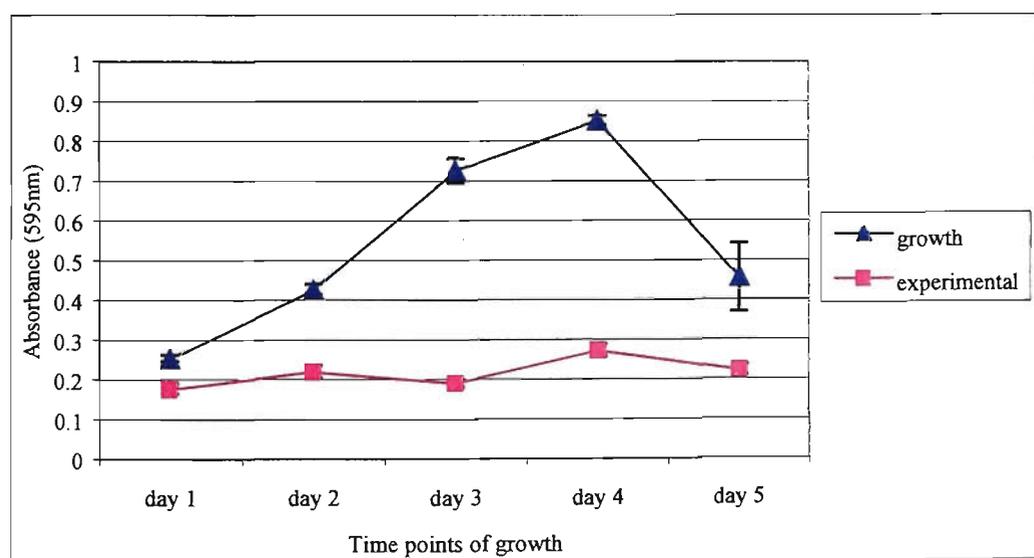


Figure 17, The cell density of T47D cells over time in growth and experimental media, as recorded using the sulforhodamine B assay.

Key: growth media = RPMI media containing 10% foetal bovine serum

Experimental media = phenol red free RPMI media containing 10% charcoal stripped foetal bovine serum.

Each point on the graph represents the mean absorbance of 16 replicates, 8 replicates taken from each end of the 96 well plate. Error bars represent the standard error of the mean.

The growth curves of both oestrogen receptor negative cell lines, MDA-MB-231 and MDA-MD-435 (Figures 18 and 19 respectively), were not affected by the type of media. Both cell lines tended to grow to a greater extent (as determined by the higher recorded absorbances, and thus higher cell densities) than the oestrogen receptor positive cell lines (MCF-7 and T47D).

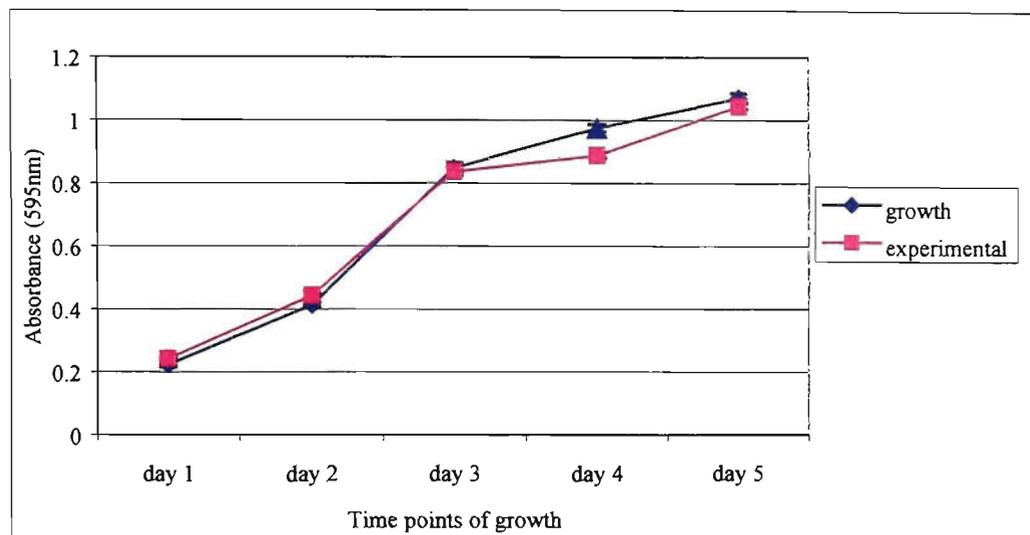


Figure 18, The cell density of MDA-MB-231 cells over time in growth and experimental media, as recorded using the sulforhodamine B assay.

Key: growth media = RPMI media containing 10% foetal bovine serum

Experimental media = phenol red free RPMI media containing 10% charcoal stripped foetal bovine serum.

Each point on the graph represents the mean absorbance of 16 replicates, 8 replicates taken from each end of the 96 well plate. Error bars represent the standard error of the mean.

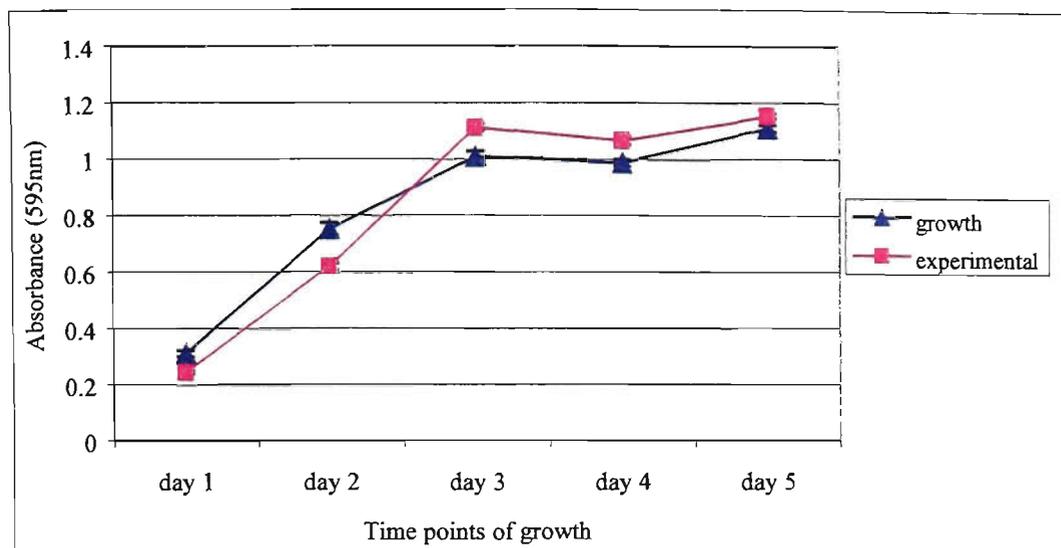


Figure 19, The cell density of MDA-MB-435 cells over time in growth and experimental media, as recorded using the sulforhodamine B assay.

Key: growth media = RPMI media containing 10% foetal bovine serum

Experimental media = phenol red free RPMI media containing 10% charcoal stripped foetal bovine serum.

Each point on the graph represents the mean absorbance of 16 replicates, 8 replicates taken from each end of the 96 well plate. Error bars represent the standard error of the mean.

4.3.1 Cell proliferation studies

4.3.1.1 Individual cell line effects

To determine the effects of DDT and DDE on the growth of cells, MCF-7, T47D, MDA-MB-231 and MDA-MB-435 cells were exposed to different concentrations of the *o,p'*- and *p,p'*-isomers of DDE and DDT over a five-day period and cell density recorded. Representative graphs of results for each cell line are presented in figures 20 to 23 (other graphs generated for each isomer of agent and the different cell lines can be located in Appendix E). Each graph records the cell response to the agents at different concentrations relative to the response of cells with no agent, such that the growth absorbance for untreated cells were subtracted from the absorbance for treated cells. Any absorbance reading above zero was described as proliferation resulting from exposure to agent, whereas any figures below zero were described as inhibition because of exposure.

Figure 20 represents the growth of MCF-7 cells exposed to p,p'-DDE in growth media. The graph indicates that most concentrations tested tended to produce maximum proliferation at day 2, and thereafter the growth of the cells was inhibited. The lowest concentration tested tended to inhibit the growth of MCF-7 cells over time until day 5 where it recorded the maximum proliferation of all concentrations tested. Interestingly, the cell density at the highest concentrations tested declined over time, possibly due to a toxic effect on the cells. The effects on cell growth in experimental media, exposed to the same agent varied from this result (Appendix E). At all concentrations tested, the cells achieved maximum proliferation at day 3, and thereafter the cell density declined with time. The response of MCF-7 cells to o,p'-DDE did not vary to the same extent as the p,p'-DDE isomer when grown in growth media compared to the experimental media. There were no obvious trends between the different concentrations tested, but at day 3 the cells in experimental media recorded the most proliferation of the time period of these experiments, whereas the cells in the growth media tended to be inhibited until about day 4 where the cell response varied depending on concentration of agent. The MCF-7 cells treated with both isomers of DDT at varying concentrations, tended not to vary as much as the DDE, with concentration over time (Appendix E).

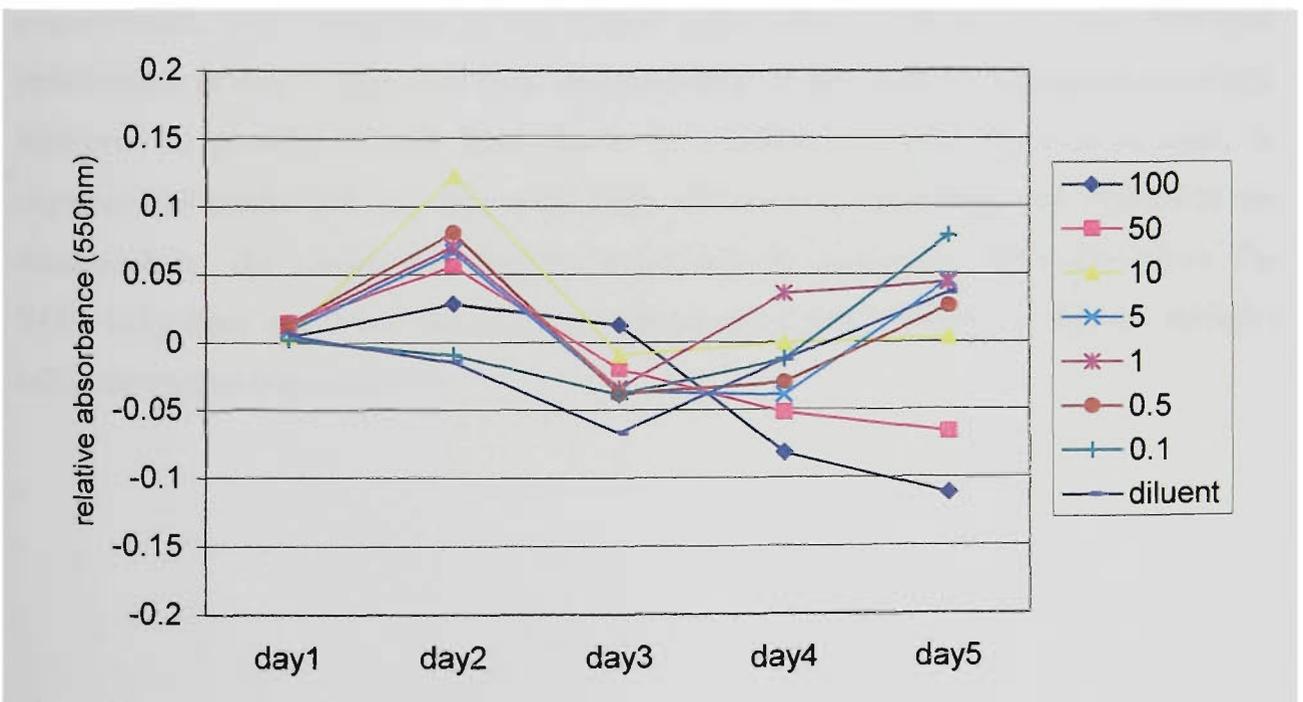


Figure 20. The cell density of MCF-7 cells in growth media over time, exposed to different concentrations of p,p'-DDE.

- Key: Each point on the graph represents the average absorbance of four replicates
 Dil. = diluent control, does not exceed 1% in the final cell suspension
 0.1 to 100 = represent μM concentrations of agent in cells

Figure 21 presents the cell density of T47D cells in growth media exposed to p,p'-DDE over time. The graph indicates that with most concentrations tested, that there is an increase in proliferation with time up to day 4, with the exception of 50 and 100 μ M concentrations where the cell density is inhibited from day 1. In comparison, the cell density of T47D cells in experimental media did not differ with concentration over time (Appendix E). The o,p'-DDE isomer had a varied effect on cell density in growth media (Appendix E). The lowest concentrations tested (0.1 and 0.5 μ M) inhibited growth from about day 2, with the most profound inhibition recorded at day 3, but by day 5 there was a reduction in inhibition. Again, in contrast, the cells exposed to agent in experimental media, did not vary greatly with concentration over time (Appendix E). The T47D cells exposed to p,p'-DDT in growth media tended towards inhibition by day 4 but there were no obvious differences with concentration over time, with the exception of the cells exposed to 50 and 100 μ M concentrations, where the growth was inhibited consistently from day 1. Like previously, the cells exposed to the same agent but in experimental media did not vary with concentration over time and were slightly proliferative. The T47D cells exposed to o,p'-DDT in growth media tended not to vary over time with concentration, with exception to the lowest concentrations where the cells increased proliferation at day 4, and also with the exception of the highest concentrations which inhibited the growth of cells from day 1. In contrast, the cells exposed to agent in experimental media did not vary over time, however the response was dependent on concentration. The lowest and highest concentrations tended to inhibit growth of the T47D cells from day 2 but the middle concentrations tested tended to slightly increase cell proliferation (Appendix E).

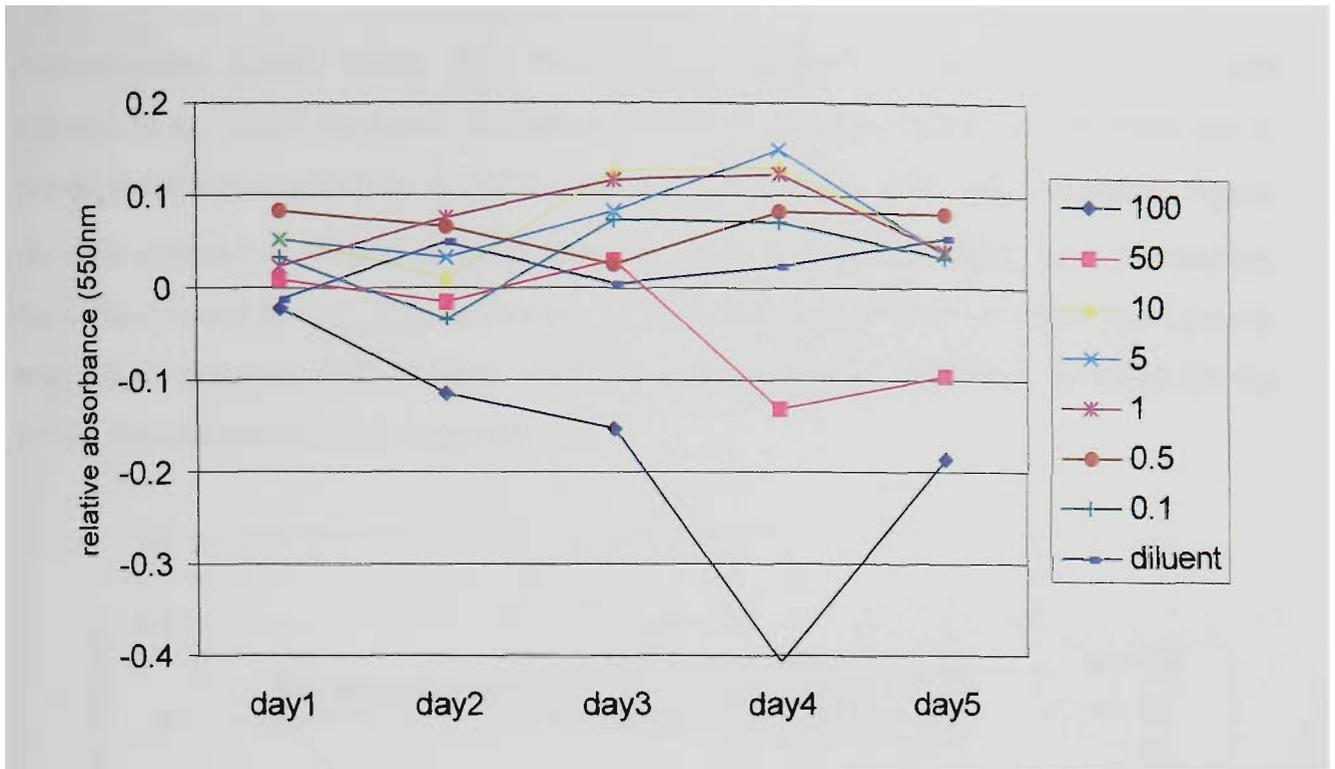


Figure 21. The cell density of T47D cells in growth media over time, exposed to different concentrations of p,p'-DDE.

Key: Each point on the graph represents the average absorbance of four replicates

Dil. = diluent control, does not exceed 1% in the final cell suspension

0.1 to 100 = represent μM concentrations of agent in cells

Figures 22 and 23 represent the cell densities of MDA-MB-231 and MDA-MB-435 cells respectively, in growth media over time. For both graphs there does not appear to be any noticeable inhibition of growth dependent on concentration of p,p'-DDE over time, however with exposure to $100\mu\text{M}$ of agent inhibition of cell density was evident from day 1. There was no obvious difference in cell densities (for both MDA-MB-231 and MDA-MB-435 cells) recorded in comparison with the cells exposed to p,p'-DDE in experimental media (Appendix E). The MDA-MB-231 cells exposed to o,p'-DDE in growth media did not vary greatly in response to concentration over time, with the exception of the lower concentrations tested, whereby there was a slight inhibition of growth at day 3. In contrast however, the cells in experimental media tended not to vary in cell density with concentration over time, except for the higher concentrations where the cells were inhibited from day 2 (Appendix E). The MDA-MB-231 cells exposed to p,p'-DDT in both growth and experimental media did not vary greatly with concentration over time, except for exposure to $100\mu\text{M}$ concentration of agent in the growth media, where the cells were inhibited from growth from day 1. The cells in experimental media, for most concentrations over time tended to be proliferative, except for the higher

concentrations tested, where they were slightly inhibitive. The MDA-MB-231 cells exposed to o,p'-DDT for most concentrations tested tended to inhibit growth from day 2, but the 0.1 μ M concentration tended to increase proliferation of the cells with time. Again, the cells exposed to 100 μ M of agent were inhibited from growth from day 1. In contrast, the cells exposed to o,p'-DDT in experimental media, tended to be inhibited from growth with all concentrations over time, with the most profound inhibition recorded for the lowest concentration tested (Appendix E).

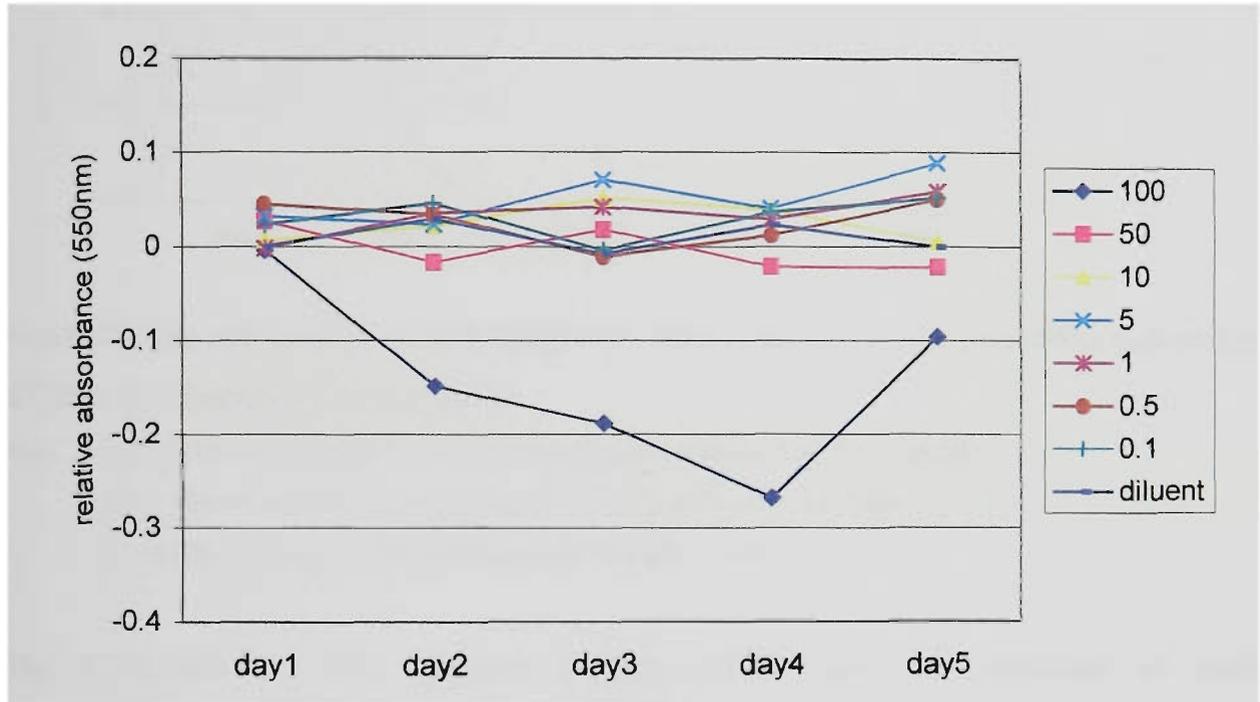


Figure 22 The cell density of MDA-MB-231 cells in growth media over time, exposed to different concentrations of p,p'-DDE.

Key: Each point on the graph represents the average absorbance of four replicates
 Dil. = diluent control, does not exceed 1% in the final cell suspension
 0.1 to 100 = represent μ M concentrations of agent in cells

The MDA-MB-435 cells exposed to o,p'-DDE in growth media tended to proliferate with all concentrations tested over time, except for the lower concentrations where there was inhibition of growth at day 2 (Appendix E). In contrast, the cells exposed in experimental media proliferated with all concentrations except for 50 and 100 μ M concentrations, where cell growth was inhibited from day 2, with a maximum inhibition at day 3.

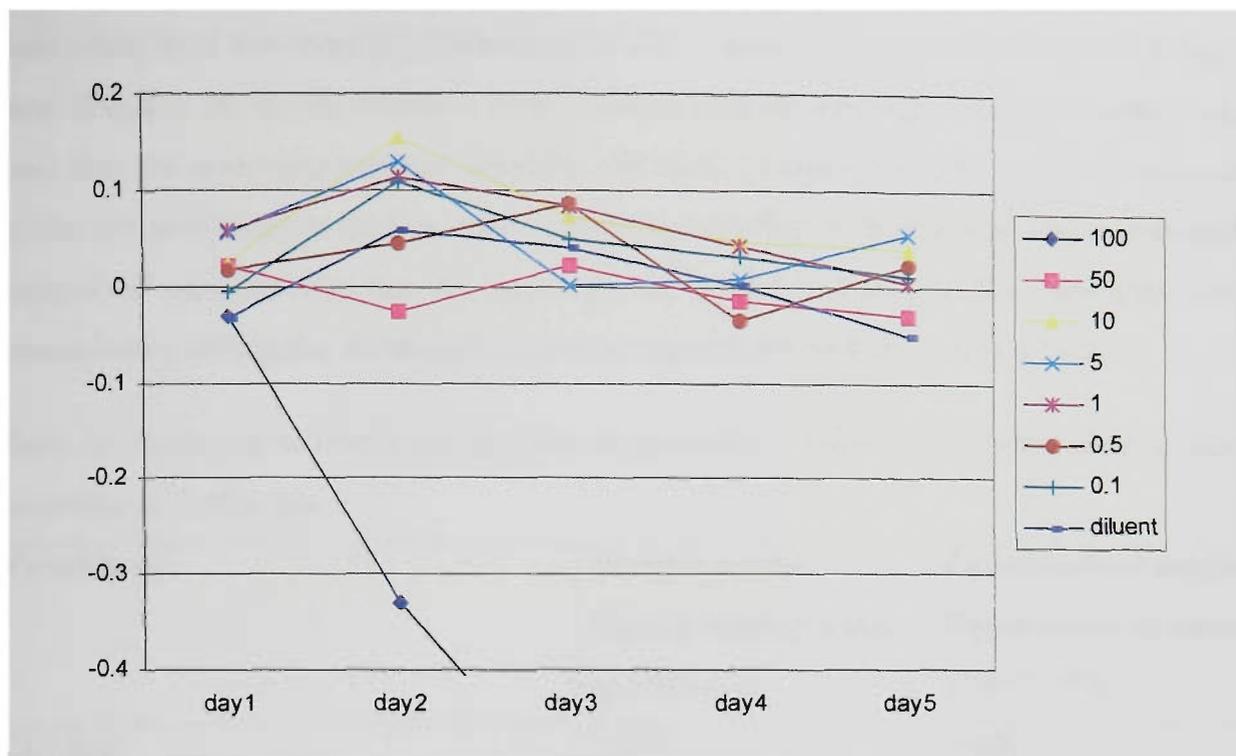


Figure 23. The cell density of MDA-MB-435 cells in growth media over time, exposed to different concentrations of p,p'-DDE.

Key: Each point on the graph represents the average absorbance of four replicates

Dil. = diluent control, does not exceed 1% in the final cell suspension

0.1 to 100 = represent μM concentrations of agent in cells

The MDA-MB-435 cells exposed to p,p'-DDT tended to proliferate at most concentrations over time, however the cells exposed to 100 μM agent at day 3 were markedly inhibited from growth. In contrast the cells exposed to p,p'-DDT in experimental media did not vary greatly in response to exposure at the concentrations over time (Appendix E). The MDA-MB-435 cells exposed to o,p'-DDT tended to proliferate with time at most concentrations tested (especially at day 3 and onwards), except for the highest concentration, whereby the cells were inhibited over time. In contrast the cells exposed to agent in the experimental media proliferated over time, with exception of the lowest concentrations tested, which had an inhibitory effect on the cells.

4.3.1.2 Differences between cell lines

The previous section highlighted that within individual cell lines there were subtle differences in cell response depending on treatment, concentration of agent, and time of exposure to agent. Even though the results were subtle, they still prove useful for the following studies and form the basis of conditions that should be tested. It has already

been established that there are differences in cell response depending on the type of media used (Figures 16 to 19), which is more evident with the oestrogen receptor positive cell lines than the oestrogen receptor negative cell lines. Consequently, the statistical analyses of the cell proliferation studies, were grouped according to media type and a univariate analysis of variance was run on each type of media (Table 35). The univariate tests proceeded by testing the differences between variables in relation to cell density.

Table 35. Summary of results for between subject effects for the cell proliferation studies according to media type.

Variable (s)	Growth media Significance (p value at 95% CI)	Experimental media Significance (p value at 95% CI)
Cell line	0.000	0.000
Exposure time (days)	0.000	0.000
Agents (pesticide, diluent, isomers)	0.007	0.090
Concentration of agents	0.000	0.014
Cells vs exposure	0.000	0.000
Cells vs agents	0.000	0.165
Cells vs concentration	0.004	0.954
Cells vs exposure vs agents	0.000	0.996
Cells vs exposure vs concentration	1.000	1.000
Cells vs concentration vs agent	0.081	0.993
Cells vs exposure vs agents vs concentration	1.000	1.000
Exposure vs agents	0.000	0.930
Exposure vs concentration	0.030	1.000
Exposure vs agents vs concentration	0.968	1.000
Agents vs concentration	0.000	0.148

Key: The dependent variable within each statistical set of data was the average absorbance of four replicates.

According to the results in table 35, there was a statistically significant difference in cell density dependent on the type of cells. In the growth media all cells were statistically different from each other in cell response ($p < 0.05$), but in the experimental media the

MCF-7 cells and the T47D cells were not statistically different from each other ($p=0.123$, 95% CI= -0.0718-0.0057) but were different from the other cell lines tested ($p<0.05$). There was also a statistical difference in cell density over time of exposure to the agents in both growth and experimental media ($p<0.05$). In growth media all time points of exposure produced different cell densities in comparison to each other, except for day 4 and 5, where there was no statistical difference ($p=0.999$, 95% CI= -0.0296-0.0242). In contrast the cell density measured at the various time points in experimental media, were not statistically different for day 3 and day 4 ($p=0.968$, 95% CI= -0.0538-0.0335).

There was a statistically significant difference in cell density with the different agents used in the growth media ($p=0.007$) but not in the experimental media ($p=0.090$). In the growth media statistically significant differences in cell density were noted between *p,p'*-DDE and both isomers of DDT ($p= 0.000$) and *o,p'*-DDE and both isomers of DDT ($p=0.000$) but not between the two isomers of DDE ($p=0.797$). Statistically significant differences were also noted between the diluent and the *p,p'*- and *o,p'*-DDE ($p=0.006$ and $p=0.000$ respectively) but not DDT($p>0.05$). In the experimental media only statistically significant differences were found between the diluent and *p,p'*-DDE, *o,p'*-DDE and *p,p'*-DDT ($p=0.001$, $p=0.001$ and $p=0.007$ respectively). Very weak differences in cell density were found between *o,p'*-DDE and *o,p'*-DDT ($p=0.063$, 95%CI=-0.0014-0.0860).

There was a statistically significant difference in cell density dependent on the concentration of pesticide used in the growth media (0.000) and in the experimental media ($p=0.014$). In the growth media statistically significant differences were noted between cells treated at all concentrations of pesticide and cells treated at 100 μ M ($p=0.000$), possibly due to the toxic effect of 100 μ M on the cells. Significant differences were also recorded between cells treated at 0.1 μ M and 1 μ M, 5 μ M and 10 μ M ($p=0.018$, $p=0.061$ and $p=0.063$ respectively). Interestingly, in the experimental media the only concentrations, which produced a significant difference in cell density, were between 0.1 μ M and 1, 5, 10, 50 μ M ($p=0.022$, $p=0.003$, $p=0.003$ and $p=0.022$ respectively).

In terms of interaction between the variables tested, in experimental media most interactions were not statistically significant ($p>0.05$), with the exception of cells versus exposure time. In the growth media most of the interactions tested (Table 35) were

statistically significant ($p < 0.05$), except cell type versus exposure time versus concentration of agent ($p = 1.000$), exposure versus agent versus concentration ($p = 0.968$) and cells versus exposure versus agents versus concentration ($p = 1.000$).

4.3.2 Soft agar studies

To determine the effects of DDE on colony forming capacity and thus cell survivability in soft agar, MCF-7 cells were used. In the first instance, colony forming capacity was measured with MCF-7 cells grown in experimental media exposed to *o,p'*-DDE and then added to the agar. Seven concentrations of DDE exposed cells were added to agar, but only three concentrations were analysed due to the concerns mentioned in the methods (Figure 24). Using the first assay to determine colony forming capacity, two sizes of colonies were formed in the agar, small colonies of less than 400 μm in diameter and large colonies of greater than 400 μm in diameter. Statistically there was no difference in size of colonies formed between the different concentrations analysed in response to *o,p'*-DDE exposure ($p = 0.072$). Hence, small and large colonies were combined for one count of colony forming units in the second assay to colony formation studies.

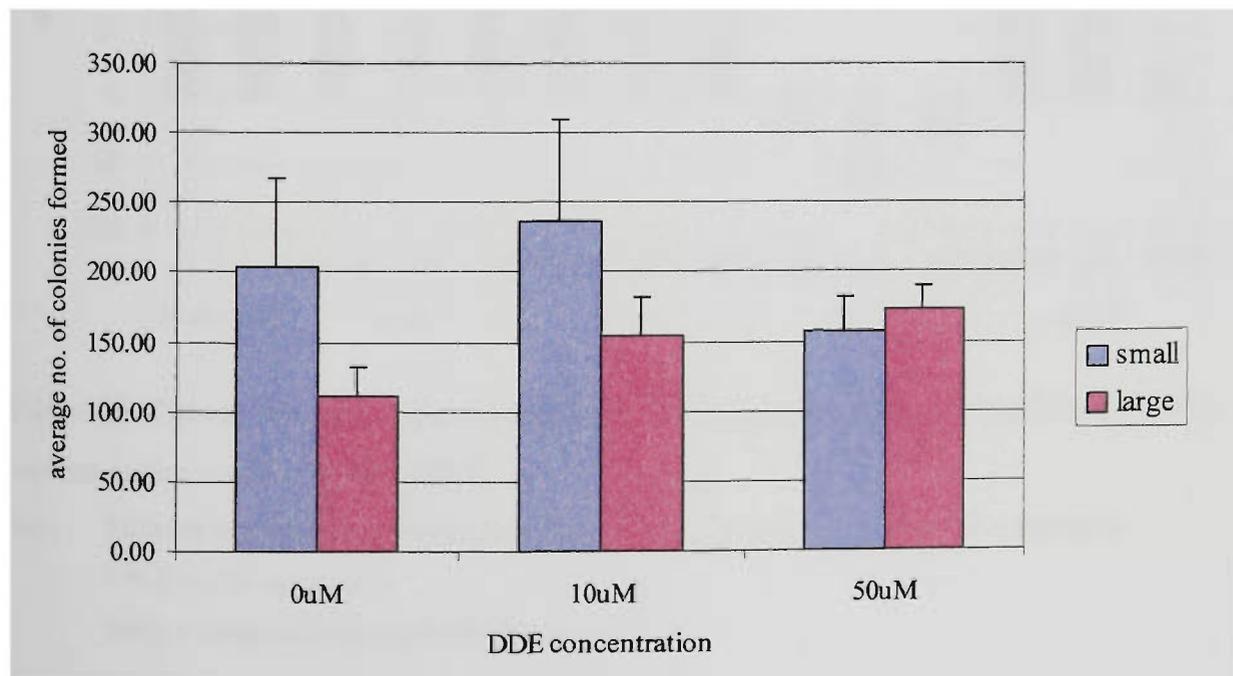


Figure 24, Number of colonies formed by MCF-7 cells in response to three concentrations of *o,p'*-DDE.

Key: small colonies refer to those less than 400 μm in diameter

Large colonies refer to those greater than 400µm in diameter

The bars in the graph represent the average number of colonies counted from four fields of view per well and from three replicates.

The second approach to colony formation studies utilised MCF-7 cells and compared the colony forming capacity with that of MDA-MB-231 cells. The second approach also included the analysis of colony formation in growth and experimental media, and with exposure to o,p'- and p,p'- isomers of DDE and DDT. The number of colonies formed by MCF-7 cells in growth media as a result of exposure to the isomers of DDE and DDT is depicted in Figure 25.

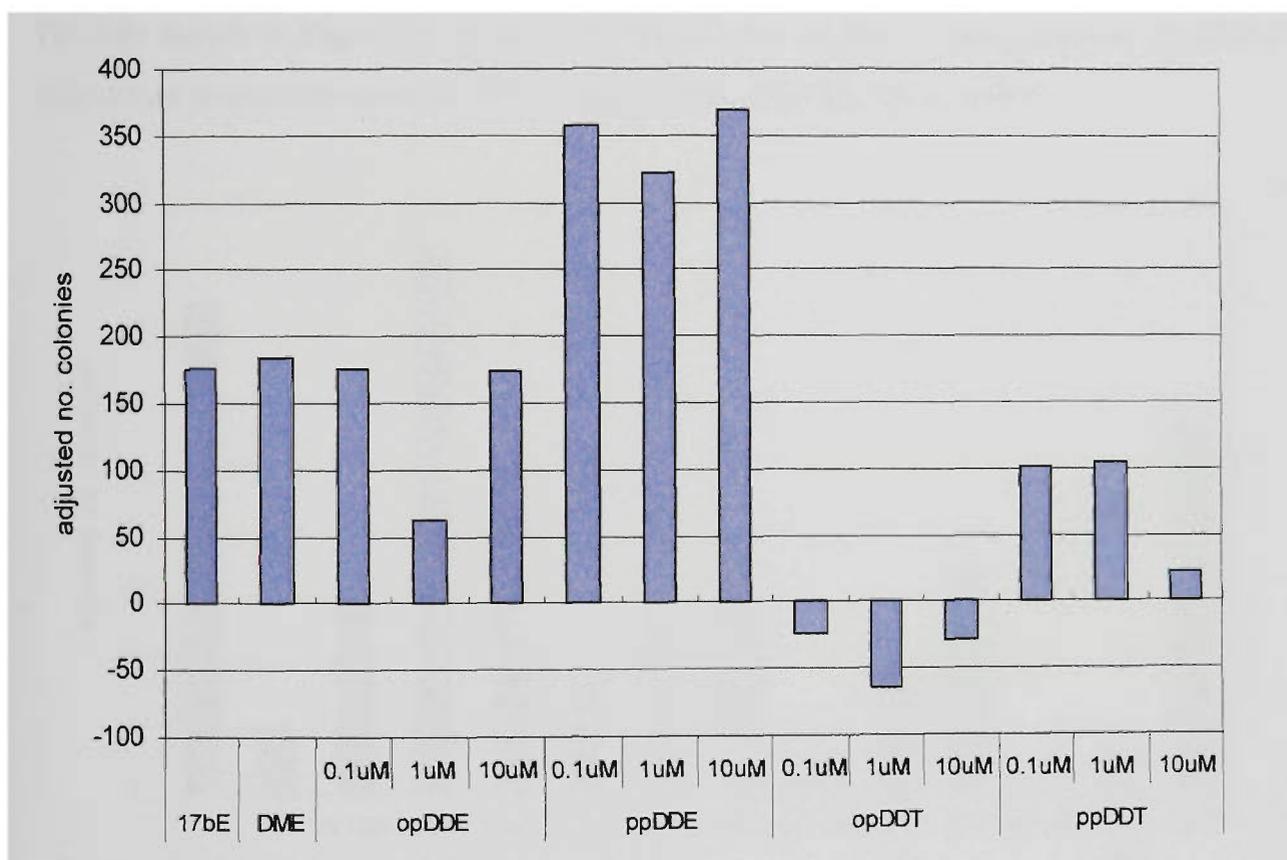


Figure 25. Colony forming capacity of MCF-7 cells grown in growth media exposed to various isomers of DDE and DDT.

Key: Each bar represents the average number of colonies counted per well for two replicates.

17b-E = 17β-oestradiol

DME = Dulbeccos enriched modification media

As can be seen in Figure 25, more colonies were formed by cells exposed to DDE than those exposed to DDT, however there was only a statistical difference between cells exposed to p,p'-DDE and o,p'-DDT (p=0.003). This is not surprising considering that in

the human body exposures to DDT will result in the detection of its more stable metabolite DDE, however it is uncertain as to whether the same response would be observed in vitro. Also there was a tendency for more colonies to be formed for the p,p'-isomers of both DDE and DDT than for the o,p'-isomers. In terms of concentration effects of the agents used, the statistical analyses indicated no dose response relationships with colony formation by MCF-7 cells and type of agents used. It is also interesting to note that the number of colonies formed by exposure to p,p'-DDE was higher than the number produced with exposure to 17 β -oestradiol, but this result was not statistically significant (p=0.848).

The data shown in Figure 26, in contrast, depicts the colony forming capacity of MCF-7 cells grown in experimental media, exposed to the same agents as above.

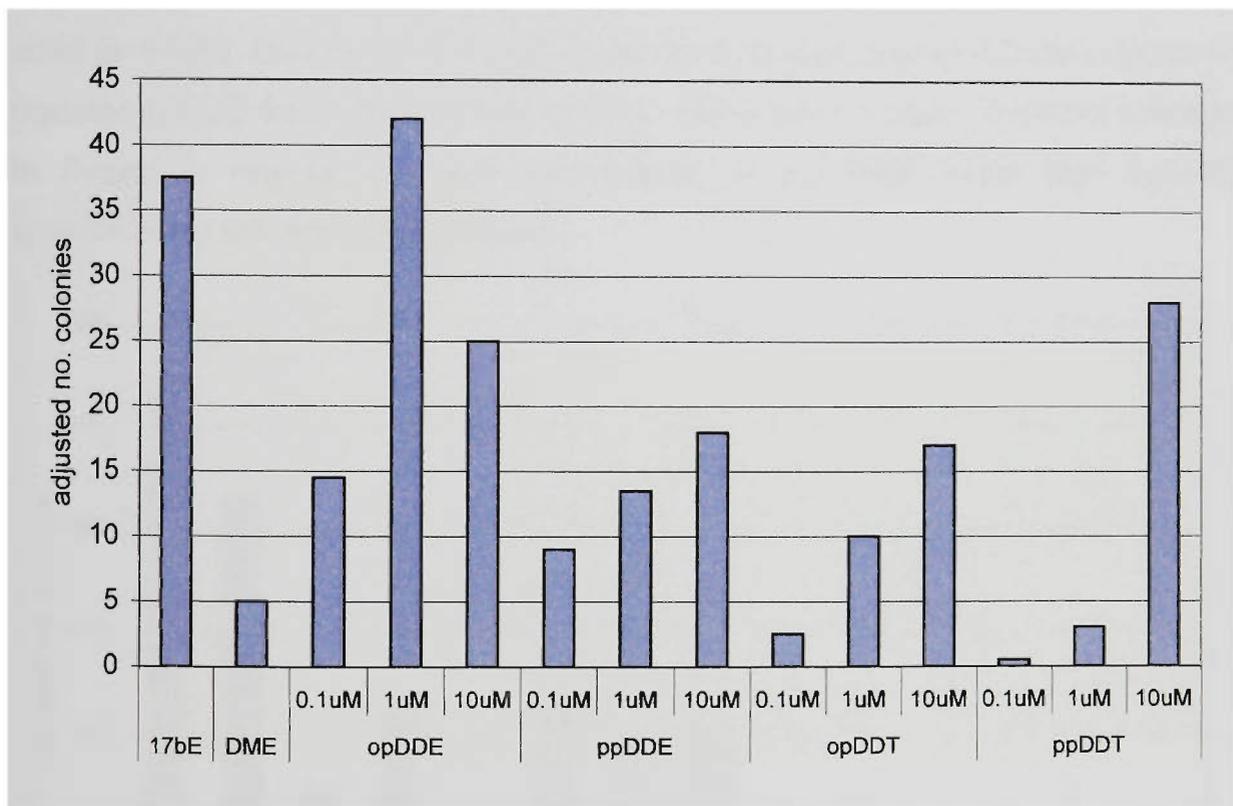


Figure 26. Colony forming capacity of MCF-7 cells grown in experimental media exposed to various isomers of DDE and DDT.

Key: Each bar represents the average number of colonies counted per well for two replicates.

17b-E = 17 β -oestradiol

DME = Dulbeccos enriched modification media

Again, the data indicated that more colonies formed by the cells exposed to the DDE than the DDT, however differences between the isomers used are not so apparent. For the

different isomers of agents tested there appears to be a dose response, where an increase in concentration of agent is associated with an increase in colony formation, although *o,p'*-DDE is an exception. The concentration of agent however, did not show a significant effect ($p=0.951$). Only *o,p'*-DDE at $1\mu\text{M}$ produced a cell response exceeding that of 17β -oestradiol, but this result was not statistically different ($p>0.05$). In terms of the media used however, the MCF-7 cells produced fewer colonies when grown in the experimental media, than in the growth media which is a strongly significant result ($p=0.000$).

The data shown in Figure 27 represents the colony forming capacity of MDA-MB-231 cells grown in growth media, exposed to different isomers of DDE and DDT. In comparison to the MCF-7 cells, the MDA-MB-231 cells did not form colonies as efficiently, thus there is a significant difference in cell response of exposure to the agents tested ($p=0.006$). Like the MCF-7 cells however, these cells produced more colonies with exposure to DDE than with exposure to DDT. There was a tendency for more colonies to be formed in response to each concentration of *p,p'*-DDE rather than *o,p'*-DDE, stimulation but this was not significant.

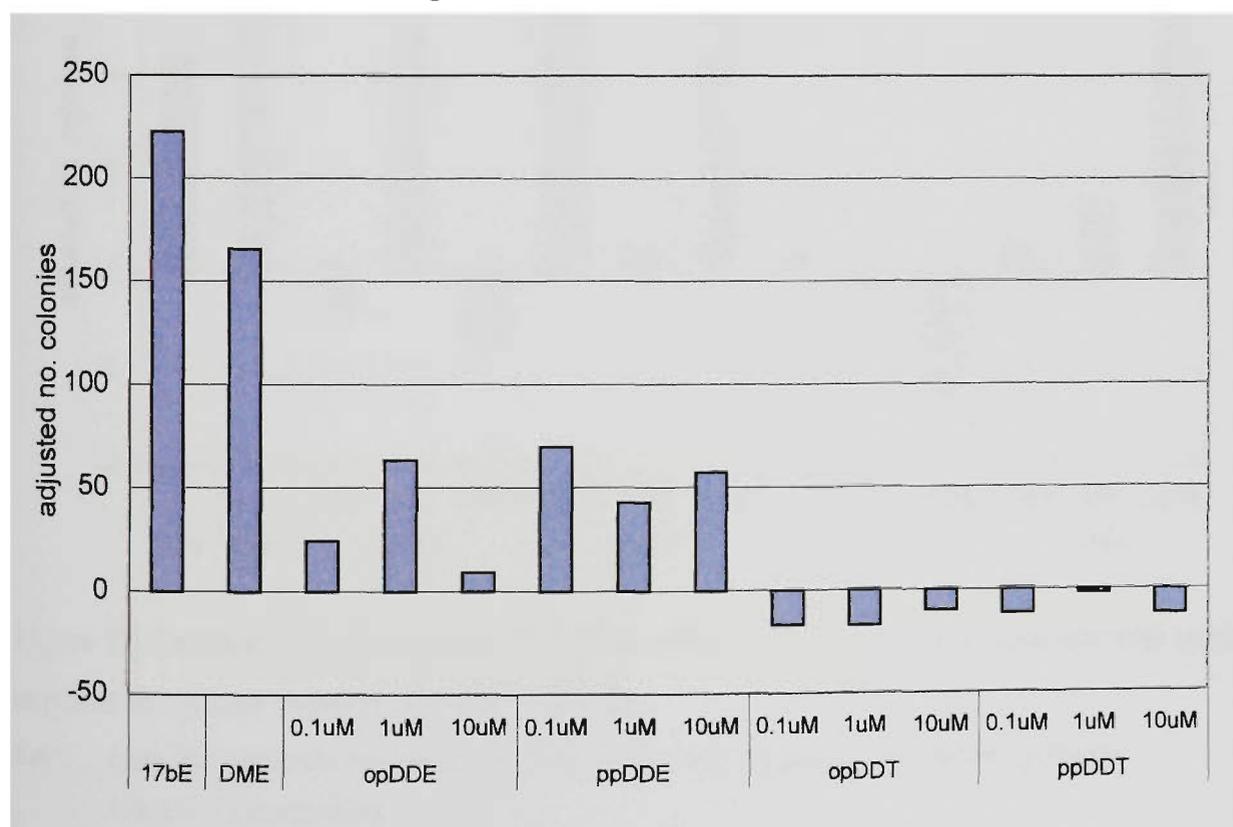


Figure 27. Colony forming capacity of MDA-MB-231 cells grown in growth media exposed to various isomers of DDE and DDT.

Key: Each bar represents the average number of colonies counted per well for two replicates.

17b-E = 17β -oestradiol

DME = Dulbeccos enriched modification media

Data for colony forming capacity of MDA-MB-231 cells grown in experimental media is shown in Figure 28. Again, compared to the MCF-7 cells grown in the same media, there was a significant reduction in numbers of colonies recorded for both isomers of DDE and DDT ($p < 0.05$). Slightly more colonies were formed for the *p,p'*- isomers of both DDE and DDT, but there were no obvious differences in numbers of colonies (as a whole) between DDE and DDT, except between *p,p'*-DDE and *o,p'*-DDT ($p = 0.003$). An indication of a dose response relationship was apparent for DDT, however for *o,p'*-DDT there was a decline in colonies formed with increasing concentration but this was not statistically proven ($p > 0.05$). A strong statistically significant difference was noticed in colony forming capacity for MDA-MB-231 cells dependent on the type of media used ($p = 0.000$).

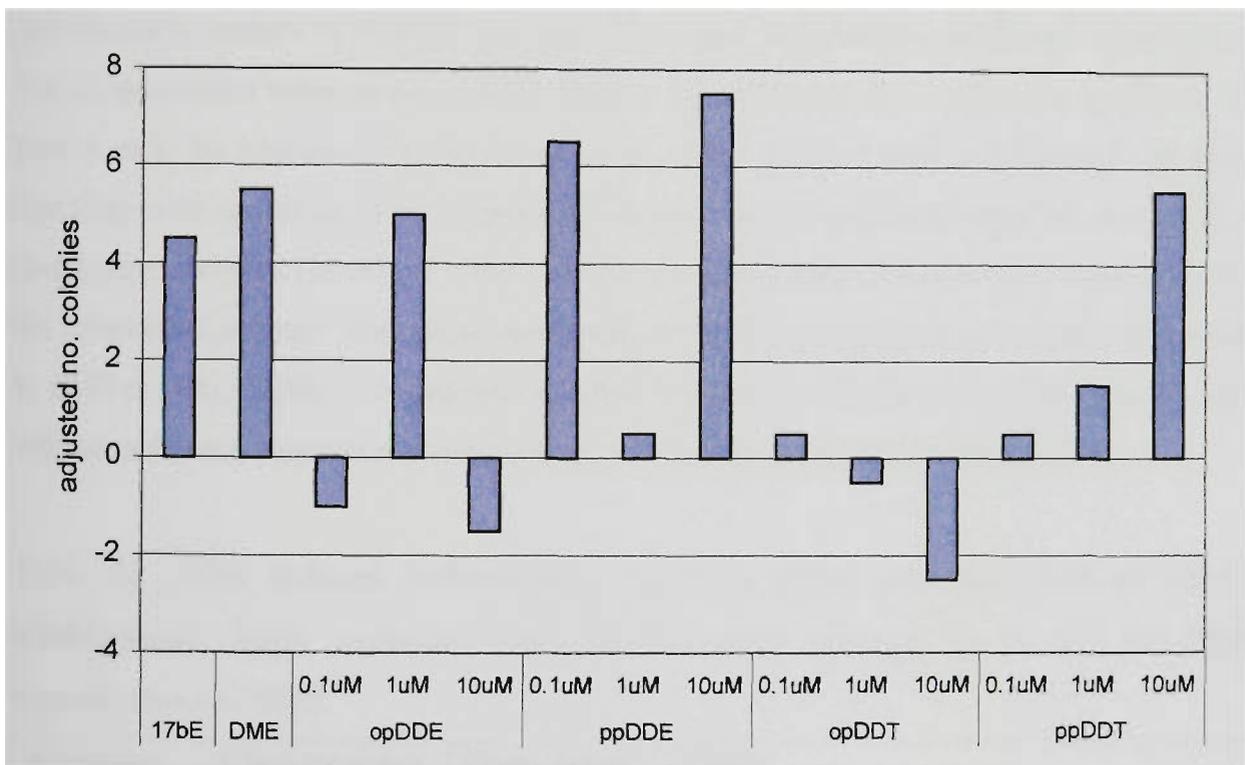


Figure 28. Colony forming capacity of MDA-MB-231 cells grown in experimental media exposed to various isomers of DDE and DDT.

Key: Each bar represents the average number of colonies counted per well for two replicates.

17b-E = 17 β -oestradiol

DME = Dulbeccos enriched modification media

4.3.3 Microarray

Messenger RNA was extracted from MCF-7 and MDA-MB-231 cells that had undergone exposure to 0.1 μ M and 10 μ M DDE, as well as cells that had not been exposed to DDE. Only the mRNA from the oestrogen receptor positive cells were used for the microarray experiments because one of the objectives for using this technology was to determine if DDT and its metabolites act through the oestrogen receptor pathway. If this were not the case then it would seem appropriate to analyse the genes that may be affected in the oestrogen receptor negative cell line and thus the other potential pathways in which DDT may act, which may be the objective of future studies. The filters used within each hybridisation (refer to Table 34) were compared and generated a list of genes that were differentially expressed at the 95% confidence interval. The lists of genes for each comparison within any one hybridization round were then compared against other hybridization rounds to identify the genes that were consistently expressed differentially, that is, genes that were deemed differentially expressed on more than one occasion. The reason why the analyses was approached in such a manner was to eliminate any genes that may have appeared to be expressed due to some experimental error or as a result of faulty gene spots on the arrays. Table 36 lists the genes that were common for at least 2 of the hybridizations, and were either up-regulated or down-regulated as a result of exposure to DDE at either of the concentrations tested. Included with the list below were 18 genes with an unknown identity and function, and 15 expressed sequence tags (ESTs).

Table 36. DDE induced differentially expressed genes resulting from at least 2 hybridisations. RNA extracted from MCF-7 cells exposed to 0, 0.1 or 10 μ M concentrations of DDE.

Accession	Chromosome: Location	Gene Name	Title
R58991	X:1,e,23,1	SAT	Spermidine/spermine N1-acetyltransferase
AA424743	14:1,g,5,4	BRF1	Butyrate response factor 1 (EGF-response factor 1)
W94868	1:1,g,9,6	RNF11	Ring finger protein 11
AA130584	19:1,g,29,8	CEACAM5	Carcinoembryonic antigen related cell adhesion molecule 5

Accession	Chromosome: Location	Gene Name	Title
AA680300	2:1,a,18,9	EPAS1	Endothelial PAS domain protein 1
AA400973	9:1,h,21,2	LCN2	Lipocalin 2 (oncogene 24p3)
R40920	7:2,a,1,11	CALN1	Calneuron 1
AA449301	13:2,d,8,2	FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
N54470	17:1,b,20,3	KIAA0130	KIAA0130 gene product
AA633901	5:1,b,14,7	TGFBI	Transforming growth factor, beta induced 68kD
AA487192	12:2,h,11,6	CPM	Carboxypeptidase M
W47485	9:1,d,5,3	SR-BP1	Sigma receptor (SR31747 binding protein 1)
AA428778	X:1,d,22,5	EFNB1	Ephrin-B1
AA479102	16:1,f,24,2	PRKCB1	Protein kinase C, beta 1
AA488432	7:1,f,13,9	PSPH	Phosphoserine phosphatase
N53512	3:1,g,8,7	CACNA2D2	Calcium channel, voltage dependent, alpha 2/delta subunit 2
H23081	19:1,g,16,8	ZNF264	Zinc finger protein 264
AA191488	9:1,a,14,9	SLC31A1	Solute carrier family 31 (copper transporters), member 1
AA167273	1:1,a,4,10	KIAA0468	KIAA0468 gene product
AA436163	9:1,h,5,10	PTGES	Prostaglandin E synthase
AA664135	10:2,a,5,3	NMT2	N-myristoyltransferase 2
AA196287	14:2,a,4,7	ALDH6A1	Methylmalonate-semialdehyde dehydrogenase
AA918818	6:2,e,15,3	TNFRSF21	Tumour necrosis factor receptor superfamily, member 21
AA485088	18:2,e,29,9	ATP5A1	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, alpha subunit, isoform 1 cardiac muscle
AI291437	1:2,h,20,4	TTC4	Tetratricopeptide repeat domain 4
AA937895	YX:1,b,19,10	MIC2	Antigen identified by monoclonal antibodies 12E7, F21 and O13
AA598601	7:1,c,13,5	IGFBP3	Insulin-like growth factor binding protein 3
H07926	18:1,c,6,8	ACAA2	Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)
N71982	6:1,f,29,10	H2BFA	H2B histone family, member A
R61295	3:2,b,22,1	SLC25A6	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6
AA428607	6:2,h,28,5	TSPYL	TSPY-like
AA464743	13:1,e,21,2	RPL21	Ribosomal protein L21
AI018613	3:1,e,26,11	RPL29	Ribosomal protein L29
H46425	5:1,g,29,3	PURA	Purine-rich element binding protein A

Accession	Chromosome: Location	Gene Name	Title
T69926	22:1,g,29,4	MYH9	Myosin heavy polypeptide 9, non-muscle
AA485226	12:1,h,8,4	VDR	Vitamin D (1,25-dihydroxyvitamin D3) receptor
N51018	X:1,h,13,6	BGN	biglycan
AA862954	9:2,a,28,2	SAS	N-acetylneuraminic phosphate synthase; sialic acid synthase
H84657	19:2,c,20,8	GRWD	Glutamate rich WD repeat protein GRWD
AI291772	14:2,h,21,4	PSMA3	Proteasome (prosome, macropain) subunit, alpha type 3
W81191	17:1,a,30,3	SC65	Nucleolar autoantigen (55kD) similar to rat synaptonemal complex protein

Indicated in Figure 29 are the genes that were common for at least two hybridizations and were up regulated as a result of exposure to 0.1 μ M DDE. The bars that are in the direction above zero represent those genes that are up-regulated with exposure to 0.1 μ M DDE. With exposure to 0.1 μ M DDE, only two genes were commonly expressed in more than one hybridization, nucleolar autoantigen and N-myristoyltransferase 2. The levels of expression of nucleolar autoantigen in cells that were untreated compared to treated cells were significantly different ($p=0.003$). The intensity levels of N-myristoyltransferase 2 between untreated and treated cells were also significantly different ($p=0.026$). There were no down-regulated genes for this exposure level of DDE.

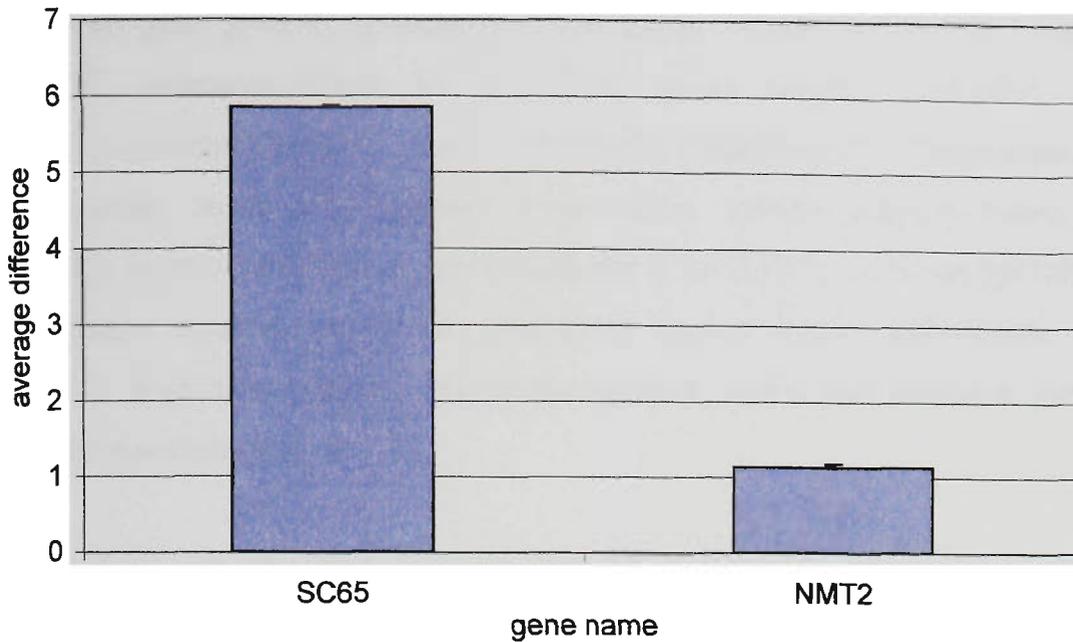


Figure 29. Genes up-regulated with exposure to 0.1 μ M DDE.

Key: the bars represent the average difference in expression between the untreated cells and the cells exposed to 0.1 μ M DDE, for each of the hybridisations. SC65 is a nucleolar autoantigen (55kD) similar to rat synaptonemal complex protein. NMT2 is N-myristoyltransferase 2. Error bars represent the standard error of the mean.

The following graph (Figure 30) represents the genes up and down-regulated with exposure to 10 μ M DDE relative to no DDE exposure. The bars in the positive direction from zero represent the genes that are up-regulated with the exposure, whilst the bars in the negative direction from zero represent the genes that are suppressed with exposure. Statistical analyses of the results showed that only five genes were up-regulated with exposure to 10 μ M DDE, in comparison to untreated cells. These include antigen identified by particular monoclonal antibodies, H2B histone family member A, solute carrier family 25, ribosomal protein L21 and ribosomal protein L29. Of these five genes only one (H2B histone family member A) was expressed at levels in the cells exposed to 10 μ M DDE that were significantly different to that in untreated cells ($p=0.001$).

Of the genes that were suppressed in expression with exposure to DDE at 10 μ M the following were expressed at levels that were significantly different between the treated and untreated cells: spermidine/ spermine N1 acetyltransferase ($p=0.027$), butyrate response factor 1 ($p= 0.009$), ring finger protein 11 ($p=0.001$), carcinoembryonic antigen related cell adhesion molecule 5 ($p=0.006$), endothelial PAS domain protein 1 ($p=0.013$),

lipocalin 2 (p=0.004), calneuron 1 (p=0.001), fms related tyrosine kinase 1 (p=0.006), KIAA0130 gene product (p=0.001), transforming growth factor, beta induced 68kD (p=0.014), carboxypeptidase M (p=0.011), sigma receptor (p=0.009), ephrin B1 (p=0.031), protein kinase C, beta 1 (p=0.042), phosphoserine phosphatase (p=0.049), solute carrier family 31, member 1 (p=0.021), tetratricopeptide repeat domain 4 (p=0.030), acetyl coenzyme A acyltransferase 2 (p=0.022), tspy-like (p=0.013), purine rich element binding protein A (p=0.039), myosin heavy polypeptide non-muscle (p=0.013), N-acetylneuraminic phosphate synthase, sialic acid synthase (p=0.001) and nucleolar autoantigen (p=0.029).

A comparison was also made between the cells treated with a low concentration of DDE (0.1 μ M) and a high concentration of DDE (10 μ M) (Figure 31). The genes that were down-regulated with exposure to 10 μ M DDE in this comparison were essentially the same as those in the comparison between untreated cells and cells exposed to 10 μ M DDE. Only two genes were still up-regulated with the exposure, antigens identified by specific monoclonal antibodies (p=0.071) and ribosomal protein L29 (p=0.138). Of the genes that were suppressed in expression with exposure to DDE at 10 μ M the following were expressed at levels that were significantly different between the low concentration of exposure and the high concentration of exposure: spermidine/ spermine N1 acetyltransferase (p=0.005), butyrate response factor 1 (p= 0.012), ring finger protein 11 (p=0.022), carcinoembryonic antigen related cell adhesion molecule 5 (p=0.003), endothelial PAS domain protein 1 (p=0.011), lipocalin 2 (p=0.003), calneuron 1 (p=0.022), fms related tyrosine kinase 1 (p=0.005), KIAA0130 gene product (p=0.003), transforming growth factor, beta induced 68kD (p=0.003), carboxypeptidase M (p=0.005), sigma receptor (p=0.007), ephrin B1 (p=0.007), protein kinase C, beta 1 (p=0.002), phosphoserine phosphatase (p=0.017), calcium channel, voltage dependent, alpha 2/ delta subunit 2 (p=0.010), zinc finger protein 264 (p=0.002), solute carrier family 31, member 1 (p=0.003), KIAA0468 gene product (p= 0.022), prostaglandin E synthase (p= 0.018), methylmalonate-semialdehyde dehydrogenase (p=0.005), tumour necrosis factor receptor superfamily, member 21 (p= 0.001), ATP synthase (p=0.010), tetratricopeptide repeat domain 4 (p=0.047), vitamin D receptor (p=0.030), biglycan (p=0.030), N-acetylneuraminic phosphate synthase, sialic acid synthase (p=0.037), proteasome (p=0.002) and nucleolar autoantigen (p=0.042).

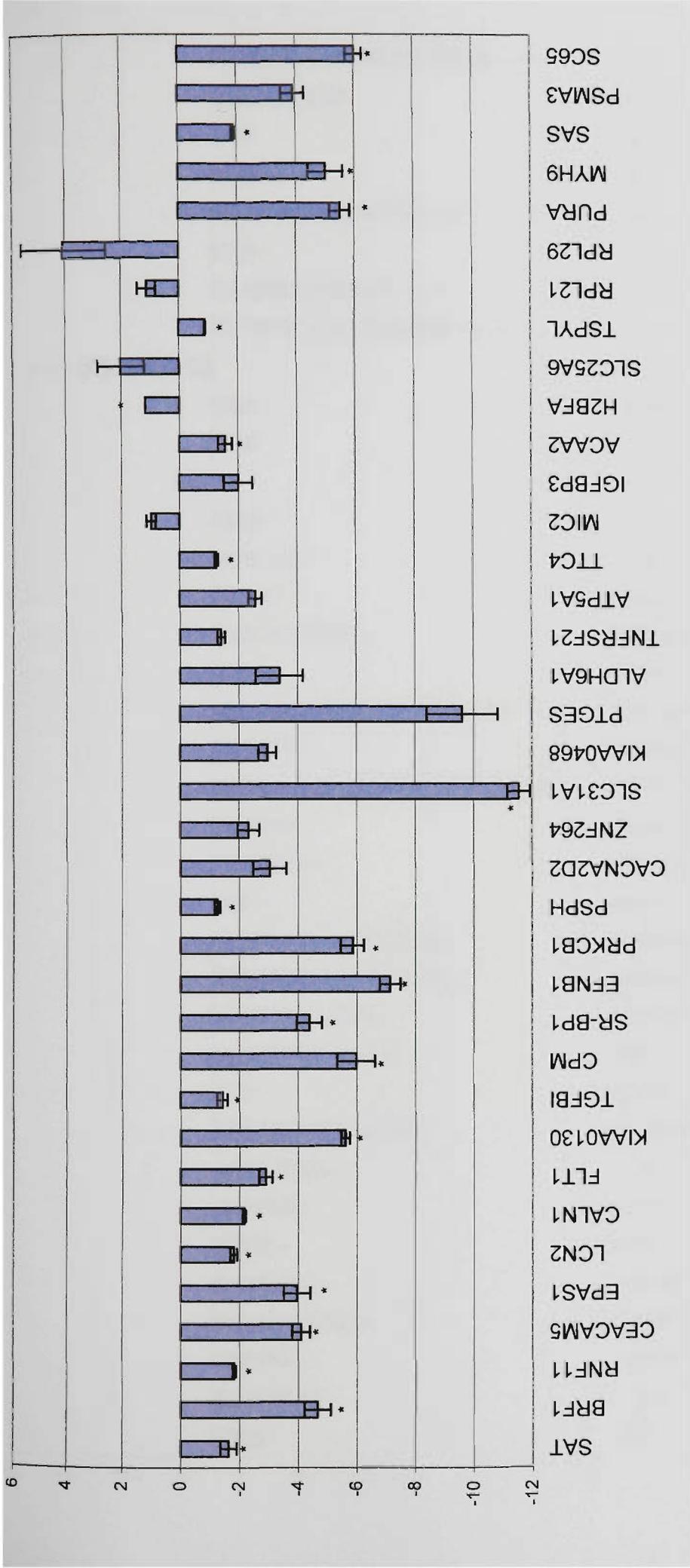


Figure 30. Genes up and down-regulated with exposure to 10µM DDE.

Key; The bars represent the average difference in expression between the untreated cells and the cells exposed to 10µM DDE, for more than one hybridisation. The bars in the negative part of the graph represent genes that had a higher expression in the untreated cells compared to DDE. For elaboration on the gene names used on the above figure please refer to Table 35.

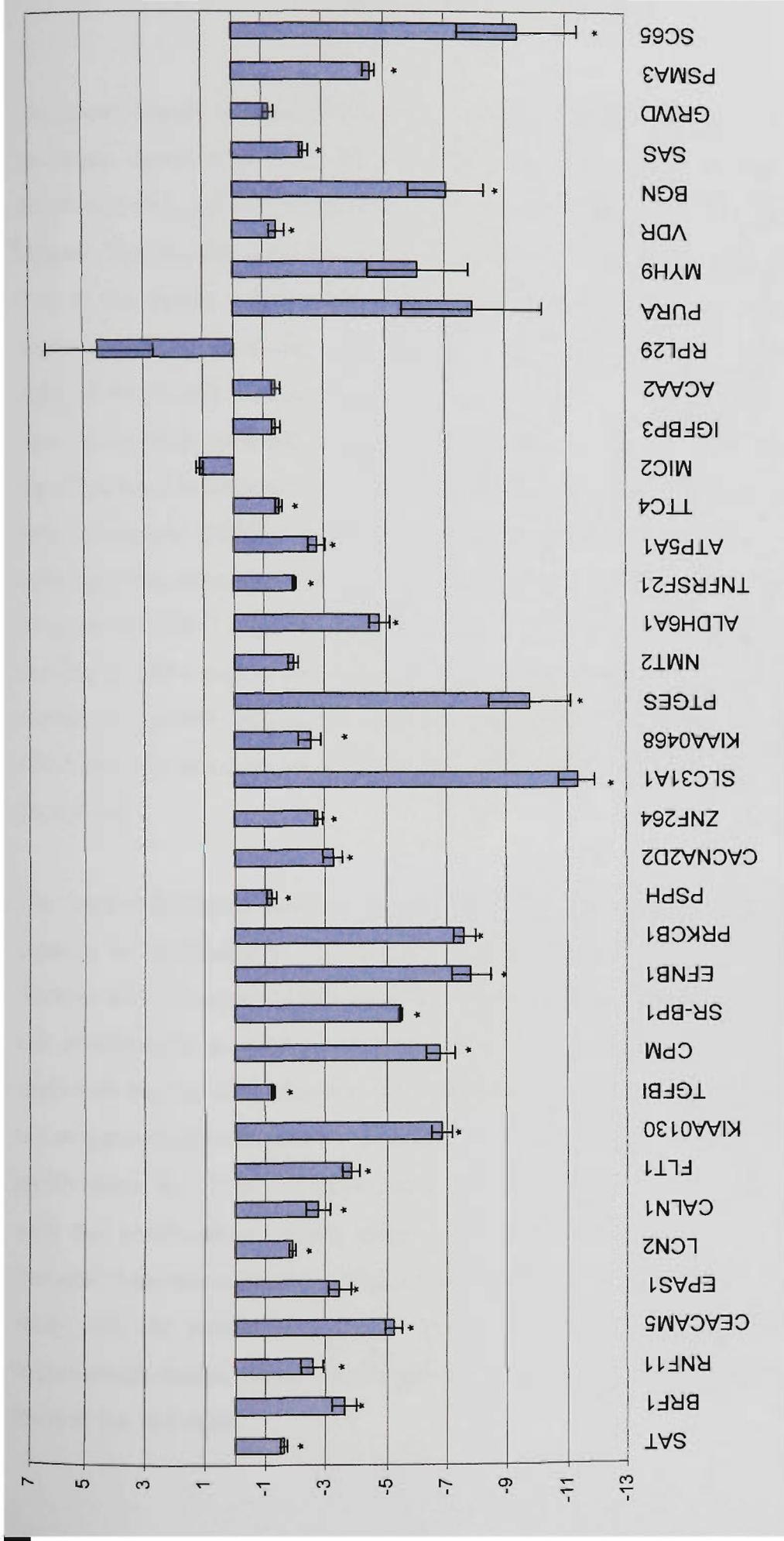


Figure 31. Genes up and down regulated with exposure to 10 μ M DDE in comparison to cells exposed to 0.1 μ M DDE.

Key; The bars represent the average difference in expression between the untreated cells and the cells exposed to 10 μ M DDE, for more than one hybridisation. The bars in the negative part of the graph represent genes that had a higher expression in the cells exposed to 0.1 μ M DDE compared to the cells exposed to 10 μ M DDE. For elaboration on the gene names used on the above figure please refer to Table 35.

4.4 DISCUSSION

The present study monitored the effects of DDE and DDT on the cellular proliferation of oestrogen receptor positive MCF-7 and T47D cells and oestrogen receptor negative MDA-MB-231 and MDA-MB-435 cells over time. This is in line with the first aim of this chapter. The present study found that there were differences in response of the above cell lines to the agents tested which was dependent on the type of media used. The growth media contains phenol red, a pH indicator dye, and foetal calf serum that have not been stripped of growth factors, which may be required for the viability of some cells and account for their proliferative capacity (Shekhar *et al.*, 1997). The experimental media on the other hand is stripped of these growth factors and does not contain phenol red. Dees and colleagues (1997b) suggest that phenosulfothiazine (or phenol red) is a weak oestrogen that stimulates the growth of human breast cells and can bind to the oestrogen receptor in MCF-7 cells. So the present study determined the cell densities of oestrogen receptor positive and negative cells in both types of media to (I) allow for the cells that require the growth factors for viability and (II) to determine the potential proliferative effect of cells in response to DDE and DDT without the added oestrogenic impact of phenol red.

The results indicated that, in general that all cells were slightly suppressed in their capacity to proliferate in the experimental media, however not to the extent by which Shekhar and colleagues (1997) suggest. Almost all previous studies that have investigated cell proliferation as a result of exposure to environmental chemicals have based their studies on the use of media that was free of phenol red dye but contained serum that was not stripped of growth factors. Also the present study did not include assessments of cell proliferation as a result of exposure to 17β -oestradiol, which could be used to compare with the proliferation results from exposure to DDE and DDT. Other studies have included these measurements. This makes it difficult to compare the results of the present study with the results of previous studies, so any comparisons will be based on the experimental media, which would produce similar outcomes in terms of proliferation to those in the literature.

Overall, the results of the present study failed to produce reliable trends in terms of cell proliferation or inhibition with exposure to DDE and DDT over time. The MCF-7 cells in growth media tended to produce mixed results depending on concentration of agent and for all isomers of pesticides tested, whereas the T47D cells and the oestrogen receptor negative cells produced similar trends in growth/ or suppression of growth for each isomer of pesticide with each concentration tested. This may suggest that MCF-7 cells are very sensitive to stimulus provided by the pesticides tested at the various concentrations, which would indicate that they may be the most appropriate cell line to use for sensitivity measures. The trends of proliferation/ or inhibition in experimental media however, were not apparently different between the oestrogen receptor positive and negative cells. Previous studies found an increase in cell number (or proliferation) in a dose dependent manner with exposure to DDE and DDT, but this was not the case in the present study (Steinmetz *et al.*, 1996; Shekhar *et al.*, 1997; Payne *et al.*, 2001). Steinmetz and colleagues (1996) found no increased cell numbers observed with oestrogen receptor negative cells in response to β -HCH. This result would be expected considering that oestrogen receptor negative cells are not reliant on oestrogen or an oestrogen derivative for the stimulation of growth. Previous studies have shown that β -HCH has a low or no affinity for binding to the oestrogen receptor (Steinmetz *et al.*, 1996; Soto *et al.*, 1994). In contrast DDT and its isomers have been shown to bind to the oestrogen receptor, although the affinity of binding is different for each isomer therefore it is possible that DDT may interact with proteins of another pathway to induce cancer development (Soto *et al.*, 1994; Soto *et al.*, 1995; Bradlow *et al.*, 1997). These differences in the binding of DDT or DDE to the oestrogen receptor may help to explain the inconclusive results of the cell proliferation experiments in the present study.

Dees and colleagues (1997a) have suggested that DDT can stimulate the growth of MCF-7 cells under serum-free conditions without insulin, but the number of cells produced by this exposure was not different to that found when the cells were exposed to 17β -oestradiol. In experiments in which insulin was present in the media, exposure to DDT inhibited the growth and number of cells compared to that of 17β -oestradiol. Insulin is a key factor for regulating cell growth and research suggests that there is crosstalk between growth factor signal transduction pathways (insulin-like growth factor) and oestrogen receptor pathways (Dees *et al.*, 1997a; Shen and Novak, 1997; Rajah *et al.*, 2002). Based

on the results of Dees and colleague's study (1997a), insulin decreased the response of cells to DDT exposure, indicating that growth factors may interfere with the efficacy of weak oestrogenic compounds and their involvement in processes that lead to uncontrolled cell growth. The lack of cellular growth in response to DDE in experimental media in this study may explain why there were no obvious differences observed between oestrogen receptor negative and positive cells since this media contains lower amounts of these growth factors.

The present study also monitored the ability of MCF-7 and MDA-MB-231 cells to form colonies following exposure to DDE and DDT. As before, both types of cells struggled to form colonies when exposed to the agents in experimental media, due to the absence of the growth factors. Soft agar assays have been used in previous studies to determine the expression of particular genes that have been transfected into cells (Liu *et al.*, 1997; Coppola *et al.*, 1999) or to study the sensitivity of cells to chemical agents (Benard *et al.*, 1983; Lemieux *et al.*, 1993; Stausbol-Gron *et al.*, 1995). The present study used the soft agar method to complement the cell proliferation assay for assessing the ability of oestrogen receptor positive and oestrogen receptor negative cells to survive following exposure to DDE and DDT. The results of the present study suggest that DDE interacts with the oestrogen receptor positive cells to a greater extent than the oestrogen receptor negative cells, to produce colonies on agar. This also suggests that DDE is able to interact in the oestrogen receptor pathway to "promote" growth to a greater capacity than DDT. This result differs from the research by Zava and colleagues (1997) where they showed that a lower concentration of DDT than DDE is required to half saturate the oestrogen receptor. These results indicate that DDT is capable of reducing 17β -oestradiol binding to the oestrogen receptor to a greater extent than DDE. Zava and colleagues (1997) also suggested that DDT is unable to induce pS2 or progesterone receptor expression, both of which are indicators of oestrogenic activity, however DDT was able to down-regulate oestrogen receptor expression. Research by Soto and colleagues (1995; 1997) also suggests that DDT has a stronger oestrogenic potential than DDE, but have a much lower affinity for the oestrogen receptor compared with 17β -oestradiol. In support of these findings, Tiwari and colleagues (1994) suggested that that indole-3-carbinol, a micronutrient present in cruciferous vegetables, reduced the number of colonies formed on agar by MCF-7 cells with increasing concentration of exposure, compared with MDA-

MB-231 cells. This finding is significant, as other studies have shown that indole-3-carbinol, curcumin and genistein, derived from cruciferous vegetables, turmeric and soybeans respectively, are capable of inhibiting the growth of oestrogen receptor positive cells induced by DDT or other pesticides (Verma *et al.*, 1997; Zava and Duwe, 1997; Verma and Goldin, 1998; Verma *et al.*, 1998). Because two cell lines with differing numbers of oestrogen receptors were used it is most likely that the oestrogen responsiveness of these cell lines is the determining factor in relation to chemically induced cell growth (Tiwari *et al.*, 1994; Bradlow *et al.*, 1997). These results also suggest that oestrogen receptor negative cells are still responsive to oestrogen stimulus in terms of colony formation. Tiwari and colleagues (1994) also found that oestrogen receptor negative cells are able to metabolise oestradiol, which was also supported by the observation that MDA-MB-231 cells have high constitutive levels of cytochrome P4501A1, which is responsible for oxidative metabolism of oestrogen. The current study also found that oestrogen receptor negative cells were able to respond to stimulus by 17 β -oestradiol to form colonies in agar.

In terms of the two isomers of DDE or DDT, the present study failed to produce conclusive results as to which isomers are able to promote cell growth. The MCF-7 cells produced more colonies with exposure to the *para para* isomers of DDT or DDE in growth media. This trend was also observed for the oestrogen receptor negative cells in experimental media. However when cells were grown in the experimental media, the MCF-7 cells produced more colonies following exposure to the *ortho para* isomers. This tends to suggest that the result achieved in the growth media for MCF-7 cells may be the result of interaction between factors in the growth media and the pesticides to induce colony growth. In agreement with the present studies findings, Soto and colleagues (1997) also found that the *ortho para* isomers of DDT have a greater proliferative effect than the *para para* isomers of DDT.

4.4.1 Gene expression patterns by array experiments

Previous research utilizing gene arrays as a tool for determining candidate genes or markers of disease especially in relation to cancer have been reported by a number of

investigators. Welsh and colleagues (2001) investigated the gene expression profiles in normal and neoplastic ovarian tissue, with the view to identify the genes that can be used as molecular markers for potential disease screening. Gruvberger and colleagues (2001) utilized gene arrays to determine gene expression profiles in oestrogen receptor positive and negative breast cancers and subsequently found that these tumours display different genetic phenotypes, which are not solely explained by oestrogen responsiveness. Mellick and colleagues (2002) investigated gene expression patterns in tumours and the mesenchyme of late stage oestrogen receptor negative breast tumour biopsies.

The uses of gene array technology to determine gene expression profiles from chemical exposure are few. Larkin and colleagues (2002) determined the expression of genes in largemouth bass following exposure to oestradiol, nonylphenol and p,p'-DDE. They found that male largemouth bass exposed to DDE expressed oestrogen responsive genes but in female bass some of these genes were down-regulated, which suggests that DDE may affect gene regulation differently in males and females. In female largemouth bass, it has been suggested that DDE acts on a pathway other than through the oestrogen receptor, such as the aryl hydrocarbon receptor pathway. Zeyton and colleagues (2002) analysed the effects of dioxin (TCDD), another toxicant known to bind to the aryl hydrocarbon receptor, on apoptosis, cytokine production and angiogenesis using pathway specific cDNA arrays. They found that the levels of chemically induced gene expression differed depending on the tissue used. They also showed that dioxin was involved in cytokine production and angiogenesis in the pathways screened by microarray.

The present study was undertaken to determine which genes (if any) in human breast specific arrays were differentially expressed following exposure to DDE. A second aim of the present study was to determine if DDE was involved in regulation of key elements that are known to promote breast cancer development. The results of this study indicate that a low level of exposure to DDE (0.1 μ M) affected two genes, both of which were up-regulated with the exposure. These included Nucleolar autoantigen which has similar homology to the rat synaptonemal complex protein and is a protein that is specifically expressed in meiotic prophase, where chromosomes are arranged in an orderly manner along axial elements. As prophase proceeds these axial elements are incorporated into zipper-like structures known as synaptonemal complexes (Offenberg *et al.*, 1998). The

role of nucleolar autoantigen in cancer development is unknown, however it is speculated that this gene is involved in the assembly of the synaptonemal complexes and as a consequence may contribute to chromosomal abnormalities (Tureci *et al.*, 1998).

This study also showed that N-myristoyltransferase 2 (NMT2) was up-regulated by low level exposure to DDE. NMT2 is an enzyme which catalyses the covalent transfer of myristic acid to the amino-terminal glycine residues of eukaryotic cellular proteins (Rajala *et al.*, 2000; Kolluri *et al.*, 2001). Rajala and colleagues (2000) suggest that myristoylation of proteins may be involved in the induction of apoptosis, and thus may be implicated in the pathogenesis of cancer. Kolluri and colleagues (2001) investigated the effects of dioxin on gene expression of the aryl hydrocarbon receptor by hepatoma cells and found that NMT2 was a gene induced by exposure to dioxin. Rajala *et al.* (2000) and Kolluri *et al.* (2001) also suggested that the inappropriate myristoylation of proteins, caused by the dioxin exposure, may be associated with several forms of cancer and are likely to play key roles in cancer pathogenesis. These earlier studies raise the possibility that DDE may interact with many receptor molecules in multiple pathways, including the aryl hydrocarbon receptor and the oestrogen receptor, to elicit cell proliferation or cancer development. Research by Wormke and colleagues (2000) suggests that chemicals similar to dioxin, which activate the aryl hydrocarbon receptor, have the capacity to disrupt multiple endocrine signaling pathways. T47D human breast cancer cells express oestrogen receptors as well as aryl hydrocarbon receptors, and treatment of these cells with dioxin resulted in down regulation of the aryl hydrocarbon receptor (AhR) and degradation of the oestrogen receptor protein (Wormke *et al.*, 2000). This inhibition of oestrogen receptor function following the degradation of oestrogen receptor protein was thought to be involved in the inhibition of growth of oestrogen dependent mammary tumours in rodent models (Wormke *et al.*, 2000). Thus there are contradictory results with respect to the action of dioxin. Conversely, it was suggested that DDE may bind to and activate the oestrogen receptor, leading to the induction of oestrogen dependent tumour growth, which would result in a mechanism of action which is opposite to that proposed for dioxin (Chen *et al.*, 1997).

The exposure of MCF-7 cells to high levels of DDE (10 μ M) altered the expression of different genes compared to that found at the low levels of exposure (0.1 μ M). A

significant proportion of genes on the arrays were down-regulated at the high level of exposure which may have been due to a toxic effect at this concentration. Only one of the up-regulated genes was expressed at statistically different levels between the exposed cells and the unexposed cells. This gene was identified as H2B histone family member A. Previous research has shown that histones are essential components of eukaryote chromosomes and play roles in chromosome maintenance, replication and function (Zheng *et al.*, 2003). Research by Cheung and colleagues (2003) suggests that post-translational modification to histones can influence chromatin folding thus indicating that histones may be associated with apoptosis. In other studies, Cheung and colleagues (2003) detected H2B histone phosphorylation in HL-60 leukaemic cells undergoing apoptosis. This view was also supported by the work of Zheng and colleagues (2003) who suggested that H2B histones are involved in cell cycle progression and are predominantly expressed in a S-phase and DNA replication dependent manner.

This study also showed that many genes that expressed at statistically different levels between untreated cells and cells exposed to 10 μ M DDE. These include spermidine/spermine N1 acetyltransferase, butyrate response factor 1, ring finger protein 11, carcinoembryonic antigen related cell adhesion molecule 5, endothelial PAS domain protein 1, lipocalin 2, calneuron 1, fms related tyrosine kinase 1, KIAA0130 gene product, transforming growth factor, beta induced 68kD, carboxypeptidase M, sigma receptor, ephrin B1, protein kinase C, beta 1, phosphoserine phosphatase, solute carrier family 31, member 1, tetratricopeptide repeat domain 4, acetyl coenzyme A acyltransferase 2, tspy-like, purine rich element binding protein A, myosin heavy polypeptide non-muscle, sialic acid synthase and nucleolar autoantigen. These genes are discussed below.

Spermidine/spermine N1-acetyltransferase (SAT) is a polyamine enzyme that catabolises the effects of spermine in cells and is found in nearly all vertebrate cells (Seiler *et al.*, 2000; Tomitori *et al.*, 2002). Research by Tomitori and colleagues (2002) suggests that SAT is involved in the regulation of cell growth and in eliciting stress responses in cells, possibly by the negative regulation of polyamines. Faaland and colleagues (2000) also suggest that this may be the case, by showing that an increase in SAT activity did result in a depletion of natural polyamines and inhibits cell growth. Faaland and colleagues (2000)

also suggest that polyamines are elevated in breast cancer cells compared to neighbouring normal tissues. In the present study SAT was down-regulated by DDE, which may have been due to cellular stress. Conversely, Faaland and colleagues (2000) and Seiler and colleagues (2000) suggested that in cells where SAT activity is increased, the production of hydrogen peroxide may induce apoptosis through an increase in polyamine oxidation. The present study does not support this proposal since the results showed that with a reduction in SAT activity, there would be a decline in the production of hydrogen peroxide and therefore the degree of apoptosis induction would be lower. Our study therefore raises the possibility that DDE is able to regulate part of the cell cycle by interacting with genes that are involved in apoptosis.

Butyrate response factor 1 (BRF1) is a zinc finger protein that regulates mRNA turnover and is involved in the mitochondrial apoptosis pathway (Stoecklin *et al.*, 2002). Sodium butyrate, a fermentation product of dietary fibre, can repress transcription of BRF1 in human colorectal cancer cell lines, which in turn can effect colorectal cancer cell proliferation by inducing growth arrest, differentiation and apoptosis (Maclean *et al.*, 1998). In the present study BRF1 was down-regulated by DDE, suggesting that DDE may be involved in reducing the likelihood of apoptosis by interacting with key regulatory genes and tending to promote cancer development.

Ring finger protein 11 (RNF11) is a variant zinc finger motif mapped to chromosome 1p31-p32 region (Seki *et al.*, 1999). The human RNF11 protein was mapped to a region where frequent alterations have been identified in T-cell acute lymphoblastic leukaemia (Seki *et al.*, 1999). Research by Watanabe and colleagues (2002) suggests that the function of RNF11 is relatively unknown, but similarly related genes were reported to be involved in cell growth, cell motility and cancer invasion.

Carcinoembryonic antigen related cell adhesion molecule (CEACAM) is expressed on epithelial and endothelial cells as well as on some lymphoid and myeloid cells (deJonge *et al.*, 2002). These CEACAM molecules bind to opacity-associated proteins, which in turn play a critical role in adhesion of bacteria (especially *Neisseria spp.*) to epithelial and endothelial cells, where the bacteria can invade the body (Swanson *et al.*, 2001; deJonge *et al.*, 2002). CEACAM is related to the carcinoembryonic antigen (CEA) which is a

glycoprotein that has been proven to be a suitable target antigen for the detection of primary and metastatic colorectal cancers and some other carcinomas (Hammarstrom, 1999).

Endothelial PAS domain protein 1 (EPAS1) is a member of the transcription factor family expressed in vascular endothelial cells (Maemura *et al.*, 1999). EPAS1 plays a key role in angiogenesis, which is a critical step in tumour growth because in the absence of angiogenesis there is a lack of oxygen at the centre of the tumour, which would result in apoptosis and necrosis (Blancher *et al.*, 2000). Up-regulation of EPAS1 would therefore result in an increase in angiogenesis and thus would be implicated in the development of aggressive metaplasia (Liang *et al.*, 2002). In the present study though, DDE down-regulated EPAS1 expression, thereby reducing angiogenesis.

Lipocalin 2 (LCN2) is an extracellular carrier for lipophilic molecules such as retinoids, steroids and fatty acids (and potentially fat-soluble xenobiotics) all of which are involved in the regulation of mammary cell growth (Seth *et al.*, 2002). Research by Seth and colleagues (2002) suggested that lipocalin expression was controlled by oestrogen, since the levels of expressed lipocalin vary during the oestrus cycle in mice, with the highest expression correlating with the oestradiol surge at proestrus. In the present study lipocalin 2 was down-regulated with exposure to DDE, which suggests that in this case DDE is not interacting with the oestrogen receptor to elicit an oestrogenic response.

Calneuron 1 (CALN1) is a member of the calmodulin superfamily (linked to calcium binding proteins) and is expressed at high levels in the brain (Wu *et al.*, 2001). It has a likely functional role in neuron development and its expression is consistent with a role in normal cognitive development, learning and memory (Wu *et al.*, 2001). As it binds calcium, CALN1 would also play an important role in intracellular physiological processes, triggering cardiac contraction and controlling hormone secretion from endocrine cells.

Fms related tyrosine kinase 1 (FLT1) is an angiogenic receptor, which interacts with the vascular endothelial cell growth factor (VEGF) to bring about angiogenesis (Wang *et al.*, 2000; Aguayo *et al.*, 2001). VEGF is expressed during embryonic development, during

wound healing and in pathologies that are dependent on neovascularisation, such as cancer (Wang *et al.*, 2000). Research by Aguayo and colleagues (2001) suggests that there is a strong correlation between FLT1 and white blood cell counts and absolute lymphocyte counts. They and others also suggest that VEGF has an increased expression in leukaemic cells (Aguayo *et al.*, 2001; Dias *et al.*, 2001). Research by Ferrera and colleagues (1998) investigated the molecules that are involved in or that act as mediators of corpus luteum angiogenesis. Their research unexpectedly found that treatment of cells with soluble FLT1 receptors, which inhibited VEGF bioactivity, also resulted in suppression of corpus luteum angiogenesis. Like EPAS1 described earlier, a down regulation of FLT1 would result in increased VEGF bioactivity and an increase in angiogenesis. Ferrera and colleagues (1998) also suggest that a suppression of corpus luteum angiogenesis was associated with inhibition of progesterone release and maturation of the endometrium. As progesterone release is related to the secretion of oestrogen at different stages of the menstrual cycle, DDE a potential oestrogenic compound, is likely to interfere with this oestrogenic signal by down-regulating FLT1 expression.

The KIAA0130 gene product is alternatively known as the thyroid hormone receptor associated protein (TRAP) and enhances thyroid hormone receptor expression (Wang *et al.*, 2002). TRAP is associated with the vitamin D receptor, the glucocorticoid receptor and the oestrogen receptor, and is thus involved in steroid hormone signalling pathways (Wang *et al.*, 2002). Studies reported by Wang and colleagues (2002) suggest that over-expression of TRAP enhanced androgen receptor-mediated transcription *in vivo*. They and others also suggest that TRAP complexes with other molecules to form ligands with nuclear hormone receptors and thus facilitate expression of target genes that regulate cell growth, differentiation and neoplastic conversion (Zhang and Fondell, 1999; Zhu *et al.*, 1999; Wang *et al.*, 2002). The involvement of TRAP in steroid hormone signalling suggests that it may be a likely candidate for involvement in steroid hormone-dependent cancer such as breast cancer. Zhu and colleagues (1999) suggest that molecules like TRAP are amplified in breast tumours and breast cancer cell lines however the results obtained in the present study are not consistent with this suggestion.

Transforming growth factor beta induced (TGFBI) is a growth factor that is produced and controlled by androgens, which mediate proliferation through growth factor signalling pathways (Bruckheimer and Kyprianou, 2001). Earlier studies by Oh and colleagues (1995) suggest that TGFBI is produced and hormonally regulated in some oestrogen receptor positive cell lines, which is interesting considering that oestrogen receptor negative cell lines possess the receptors specific for TGFB. Other studies by Oh and colleagues (1995) suggest that antioestrogens such as tamoxifen, can induce biologically active TGFB in MCF-7 cells, and also induces IGFBP3 expression. Furthermore, TGFB stimulates the mRNA and protein expression of IGFBP3 and thus inhibits monolayer cell growth in human breast cancer cells. The present study found that TGFB was down-regulated with DDE exposure, in line with some of the work of Bruckheimer and Kyprianou (2001), where a loss of TGFB function or down-regulation of TGFB contributes to the absence of TGFB mediated growth inhibition and apoptosis during tumourigenesis.

Carboxypeptidase M (CPM) is a widely distributed membrane-bound carboxypeptidase thought to be responsible for removal of COOH-terminal basic amino acids. It is also involved in protein digestion and activation, and can regulate peptide hormone activity (Tan *et al.*, 1989). Because it is a membrane-bound molecule, CPM has a neutral pH and can therefore act on peptide hormones and proteins in many tissue sites, thereby controlling their activity before and after interaction with specific membrane receptors (Tan *et al.*, 1989).

Sigma receptors (SRBP1) are ligands which are highly expressed in the liver and kidney, and in tumour cell lines derived from various tissues (Bowen, 2000). Research by Bowen (2000) and others suggests that a possible role for SRBP1 is in sterol metabolism as progesterone has a high affinity for sigma receptors (Schaefer *et al.*, 2000). Work by Crawford and Bowen (2002) suggests that SRBP1 is absent in normal breast tissue but are strongly expressed in breast tumour biopsy tissue and are speculated to be involved in apoptosis. As SRBP1 modulates intracellular calcium, which in itself plays a role in cytotoxicity, alterations in this modulation of calcium is thought to induce apoptosis. Bowen (2000) also suggested that the number of sigma receptors increases when cells enter a state of rapid proliferation (which may explain why they are absent in normal

breast tissue) and thus are likely to be involved in the control mechanisms of cell proliferation or the cell cycle.

Ephrin B1 (EFNB1) belongs to the largest known subfamily of receptor protein tyrosine kinases (Varelias *et al.*, 2002). Varelias and colleagues (2002) and others suggest that a likely function of ephrin is to coordinate processes of cell migration and membrane motility in axon guidance of neurons (Moreno-Flores *et al.*, 2002). Nakamoto and Bergemann (2002) suggested that because ephrin is involved in cell motility, adhesion and cytoskeleton arrangement, that these molecules are likely to be an important component in carcinogenesis. Nakamoto and Bergemann (2002) suggested that this may be the case because ephrin molecules have been found over-expressed in a wide range of cancers, and possibly are involved by mediating angiogenesis or promoting metastasis. The findings of the present study however found that ephrin was down-regulated with DDE exposure.

Protein kinase C (PRKCB1) has been involved in apoptotic pathways and has been found to be responsible for the resistance of colon cancer cells to apoptotic drugs such as paclitaxel (Cesaro *et al.*, 2001). Clinically, paclitaxel is the active agent in the treatment of many cancers including breast cancer. Research by Cesaro and colleagues (2001) suggests that low level expression of protein kinase C, beta form is linked with loss of differentiated phenotype in colorectal cancer, whereas over-expression is associated with recovery of cell proliferation control and the acquisition of the differentiated phenotype. The present study found a reduction in PRKCB1 expression with DDE exposure.

Phosphoserine phosphatase (PSPH) is speculated to be activated by amyloid beta in neuroblastoma cells, which subsequently is suggested to be involved in the pathogenesis of Alzheimers disease (Heese *et al.*, 2000). Research by Strunck and colleagues (2001) suggests that molecules with identical cDNA sequences to PSPH are speculated to be involved in endometrial carcinogenesis. PSPH is regulated by contact of endometrial cells to the basement membrane, components of which are crucial for endometrial cell function. Loss of PSPH regulation through the non-contact of endometrial cells to the basement membrane would result in dysregulated synthesis of peptides or molecules

representing precursors of signal transducers and the dysregulated growth of abnormal cells (Strunck *et al.*, 2001).

Copper is required for growth and development, and when in excess, mediates free radical production and oxidation of lipids, proteins and DNA (Kuo *et al.*, 2001). Solute carrier family 31 (copper transporters) (SLC31A1) acts as a chaperone or binds copper to facilitate copper uptake in mammalian cells and has a specialized role in epithelial cells and in connective tissue (Kuo *et al.*, 2001; Garrick *et al.*, 2003). Excess copper not only produces oxidative damage but also contributes to cell death. In the present study, solute carrier was down-regulated with DDE exposure, suggesting that there may have been inadequate means for facilitating copper uptake in the cells. This unstable regulation of copper homeostasis would also affect the trafficking of ATPases that are involved in copper metabolism (Garrick *et al.*, 2003).

Tetratricopeptide repeat domain 4 (TTC4) is a member of the tetratricopeptide repeat motif involved in cellular stress responses, interferon responses, transport of proteins across membranes and transcription suppression (Su *et al.*, 1999). Because these molecules share homology to stress-inducible proteins and map within the chromosomal region associated with the loss of heterozygosity in breast cancer, this may be a potential molecule that is implicated in breast tumourigenesis (Su *et al.*, 1999; Poetsch *et al.*, 2000; Su *et al.*, 2000). Su and colleagues (1999) and Irwin and colleagues (2002) suggest that TTC4 is involved in cell cycle control as some of the cell division cycle genes contain tetratricopeptide repeats. In the present study TTC4 was down-regulated with DDE exposure and would potentially result in loss of cell cycle control and the possibility of inducing carcinogenesis.

Acetyl coenzyme A acyltransferase 2 (ACAA2) is involved in fatty acid metabolism. Research by McLean and colleagues (1996) suggests that with enhanced expression of ACAA, steroid levels are reduced in diabetic animals, which is a result of sterol carrier protein mediated cholesterol transport loss.

TSPY-like (TSPYL) shares homology to DENTT, which is detected in a variety of normal tissue, with the highest level being detected in the brain and testis (Ozbun *et al.*,

2001). As this protein is found at high levels in the testes, it likely has a role in regulating meiotic differentiation and may also be involved in cell proliferation.

Myosin heavy polypeptide 9 (MYH9) encodes a non-muscle myosin that is found prevalent in the liver and kidney. D'Apolito and colleagues (2002) suggest that MYH9 plays an important role in the structure and function of platelets and may be important in the pathogenesis of MYH9 related disorders such as cataracts and nephritis.

N-acetylneuraminic phosphate synthase; sialic acid synthase (SAS) is located on chromosome 12q13-14, in a region that includes a number of growth related genes including GLI proto-oncogene and the p53 associated protein MDM (murine double minute) (Jankowski *et al.*, 1994; Shadan *et al.*, 2000). Abnormalities in this region of chromosome 12q have been reported to be associated with a range of benign soft tissue tumours and sarcomas (Shadan *et al.*, 2000; Nikitakis *et al.*, 2001). In the present study, SAS was down-regulated with DDE exposure which possibly indicates that there may be interruption to the growth related cellular processes.

Many of the genes that were differentially expressed, whether it be up- or down-regulated, between untreated cells and cells exposed to 10 μ M DDE were also expressed differentially between low level and high level exposure to DDE. However there were a handful of genes that were only expressed differently between the two levels of DDE exposure, all of which were expressed at higher levels in the low DDE treated cells. The genes that were expressed at statistically different levels between high and low levels of exposure to DDE include a calcium channel subunit, zinc finger protein 264, KIAA0468 gene product, prostaglandin E synthase, methylmalonate semialdehyde dehydrogenase, tumour necrosis factor receptor superfamily, ATP synthase, vitamin D receptor, biglycan and proteasome subunit.

Voltage dependent calcium channels (CACNA2D2) are found in plasma membranes and mediate calcium influx into and out of cells (Barclay and Rees, 2000; Gao *et al.*, 2000). The gene encoding voltage dependent calcium channel has been identified as a potentially new tumour suppressor gene as it is located on chromosome 3p21.3, in an area where frequent allele loss and deletions are found in several cancers (Gao *et al.*, 2000). Gao and

colleagues (2000) also suggest that as calcium channels control calcium influx, there may be a plausible role for these channels in apoptosis.

Zinc finger protein 264 (ZNF264) is located on chromosome 19q13.4 and is detected at high levels in the adult testes. Not much is known about this zinc finger protein and why it may have differentially expressed between low and high level exposure to DDE, except that it is an imprinted gene from paternal alleles (Kim *et al.*, 2001).

KIAA0468 gene product, also known as syndecan, is a member of the heparan sulfate proteoglycans that may function as extracellular matrix receptors and coreceptors for growth factors and signaling molecules (Kosher, 1998). Kosher (1998) suggests that syndecan may mediate the interaction of cells with extracellular components and molecules that control cell shape, adhesion, proliferation and differentiation. Research by Russo and colleagues (2001) suggests that administration of oestradiol to ovariectomised rat models induced a rapid increase in uterine syndecan mRNA. This rapid rise in syndecan may therefore result in syndecan being classified as an early growth response gene in the uterus. However in the present study this gene product was down regulated with exposure to a high concentration of DDE, which suggests that at a level that is close to the physiological dose DDE (ie, the low level of DDE used in the present study) may have interacted with the oestrogen receptor in place of oestrogen to increase syndecan expression. Expression of syndecan may also alter the expression of genes encoding transcription factors and oncogenes that may be implicated in cancer development.

Prostaglandin E synthase (PTGES) is located on chromosome 9q34.3 and has been implicated in the inflammation process and also as a key mediator of ovulation (Filion *et al.*, 2001). Filion and colleagues (2001) suggest that levels of prostaglandins are increased in the hours preceding ovulation and that they are involved in the activation of proteolytic and collagenolytic events leading to follicle rupture. Yoshimatsu and colleagues (2001) suggest that elevated levels of prostaglandins have been found in a variety of tumours, as they possess the properties that promote malignant growth (such as stimulation of angiogenesis and inhibition of immune surveillance).

Methylmalonate semialdehyde dehydrogenase (ALDH6A1) belongs to the superfamily of NAD (P)⁺ dependent enzymes that catalyse the oxidation of endogenous and exogenous aldehydes (Vasiliou and Pappa, 2000). Research by Vasiliou and Pappa (2000) suggests that polymorphisms in ALDH6A1 are associated with metabolic diseases characterized by neurologic complications.

Tumour necrosis factor receptor superfamily (TNFRSF21) or otherwise known as death receptor 6, is capable of initiating signaling pathways once activated by a specific ligand, leading to activation of the caspase cascade and cell death (Pan *et al.*, 1998; Bridgham *et al.*, 2001). Daniel and colleagues (2001) suggest that this receptor and its associated ligand are capable of programmed cell death by regulating tissue homeostasis and their ability to kill malignant cells. However earlier research by Pan and colleagues (1998) suggests that death receptor 6 is unable to induce apoptosis in the oestrogen receptor positive cell line, MCF-7. This is most likely due to the fact that MCF-7 cells lack caspase 3 protein, which cannot be activated to trigger programmed cell death (Weitsman *et al.*, 2003). The results of the present study indicate that exposure to DDE at high levels down regulates the expression of death receptor 6 in comparison to low level exposure. These results suggest that at a high concentration DDE may have been toxic to the cells, and thus induced a stress response. Death receptor 6 has also been implicated in activating pathways linked to an immune or stress response (Pan *et al.*, 1998; Bridgham *et al.*, 2001).

Research by Chen and colleagues (2001) suggests that ATP synthase (ATP5A1) was down regulated by a factor of two after treatment with oestradiol plus dioxin, in comparison to expression levels after treatment with oestradiol alone. ATP5A1 contains motifs for binding transcription factors such as Sp1, which in itself is involved in activating housekeeping genes, cell cycle regulated genes and is required to prevent methylation of CpG islands (Suske, 1999).

Research strongly suggests that vitamin D receptor (VDR or 1,25-dihydroxyvitamin D3), with its ligand bound, may be involved in reducing tumour incidence and inhibiting tumour progression (Wang *et al.*, 2000; Weitsman *et al.*, 2003). Weitsman and colleagues (2003) suggest that it does so by causing direct cytostatic and cytotoxic effects on cancer

cells, by causing cell cycle arrest followed by induction of apoptosis in tumour cells and by potentiating the cytotoxic action of different mediators of the immune system. The work of Thomson and colleagues (2002) is in agreement with these statements and also suggests that liganded VDR is able to cause cell cycle arrest via the induction of cell cycle regulatory proteins, p21 and p27 and by the repression of anti-apoptotic factors, Bcl-2. Thomson and colleagues (2002) also suggest that VDR is capable of binding xenobiotics that may be implicated in cancer initiation. By binding xenobiotics, VDR induces the cytochrome P450 enzymes to catalyse the transformation of hydrophobic chemicals into more hydrophilic forms, which can be more readily excreted (Thomson *et al.*, 2002).

Biglycan is a member of the small leucine rich proteoglycans family, that have been found expressed in the stroma of normal breast tissues (Leygue *et al.*, 2000). Leygue and colleagues (2000) however suggest that decorin, a proteoglycan in the same subgroup as biglycan, was inversely regulated in tumour tissue relative to adjacent normal tissue. It is believed that biglycan and the other members of the proteoglycan family are involved in matrix assembly and structure through the interaction with fibronectin and type V collagen, and control cell growth as they bind to transforming growth factor beta with high affinity (Ungefroren and Krull, 1996; Leygue *et al.*, 2000).

In research on dendritic cells, proteasome (PSMA3) has been implicated in the non-lysosomal degradation of proteins and the generation of peptides that bind to molecules for antigen presentation (Matsunaga *et al.*, 2002).

4.4.2 Pathways of DDE action

As discussed in the previous section, there are many different genes whose expression in oestrogen receptor positive cells is altered by exposure to DDE. According to Vogelstein and Kinzler (2004) there are nine known pathways to date, through which alterations in gene expression lead to the neoplastic process, and the apoptosis pathway is one of them. The pathways involved in the neoplastic process are listed below and are intrinsically linked by common genes.

1. Receptor Tyrosine Kinase pathway (RTK)
2. Retinoblastoma pathway (Rb)
3. p53 pathway
4. Apoptosis pathway
5. Hypoxia Inducible Transcription Factor pathway (HIF1)
6. Adenomatous Polyposis Coil pathway (APC)
7. Glioma Associated Oncogene pathway (GLI)
8. Phosphoinositide-3-Kinase pathway (PI3K)
9. SMAD pathway

(Vogelstein and Kinzler, 2004).

The Rb pathway, p53 and Apoptosis pathway are linked by their interactions in cell cycle control. The Rb pathway involves genes, such as cyclin DI and colk4, which directly control the transition from resting stage (Go or G1) to the replicating phase (S phase) of the cell cycle (Volgelstein and Kinzler, 2004). The p53 pathway involves the p53 protein, a transcription factor, which regulates cell death and inhibits cell growth when induced by cellular stress. The p53 pathway can be disrupted through point mutation, infection with DNA tumor viruses whose products bind to p53 and inactivate it, or by amplification of the MDM2 gene, which can lead to apoptosis (Volgelstein and Kinzler, 2004). The apoptosis pathway then proceeds, or is interrupted and involves genes such as the caspases.

On the basis of the results found in the present study it is likely that the apoptosis pathway appears to be involved in the DDE induced changes in the oestrogen receptor positive cell line. The apoptosis pathway is closely related to the Rb pathway of cell cycle control and the p53 pathway that regulates cell death induced by cellular stress. In the present study a number of genes that were differentially expressed following exposure to DDE can potentially be included in these pathways. The genes that have been found in this study include BRF1 which is involved in mRNA turnover in the mitochondrial apoptosis pathway (Stoecklin *et al.*, 2002), SRBP1 that modulates calcium and indirectly induces apoptosis (Crawford and Bowen, 2000), SAS since it is located in the same chromosomal region where MDM and GLI proto-oncogene are found (Jankowski *et al.*, 1994; Shadan *et al.*, 2000), and TNFRSF21 which initiates signaling pathways that lead to the activation

of caspases and cell death (Pan *et al.*, 1998; Bridgham *et al.*, 2001). Other genes of interest in this study include nucleolar autoantigen and TSPY-L, which are both related to regulation of meiotic cell cycle, and ATP5A1, which contains motifs for binding transcription factors that are involved in the activation of cell cycle regulatory genes (Suske, 1999). Additional genes to the ones described above include SAT, which is involved in the regulation of cell growth and elicit stress response (Tomitori *et al.*, 2002) and TTC4 which plays a role in the stress response and shares homology with proteins that map to the chromosomal region associated with the loss of heterozygosity in breast cancer (Su *et al.*, 1999; Poetsch *et al.*, 2000; Su *et al.*, 2000).

The signal transduction pathways, RTK, HIF1, APC, GLI, PI3K and SMAD, have been shown to be of considerable clinical value in the development of targeted therapies against tumours, which arise from mutations involving particular genes and gene products. The RTK pathway, involves genes such as Ras, ErbB2 and FLT, which have been reported to be involved in the activation of transcription factors. The HIF1 pathway involves genes such as VEGF and ARNT and culminates in angiogenesis. Oncogenes and tumour suppressor genes have been implicated in angiogenesis, where blood flow and oxygenation are essential to the understanding of tumour growth and metastatic potential (Liang *et al.*, 2002; Vogelstein and Kinzler, 2004). The APC and GLI pathways are linked to the Rb pathway by activation of cyclin D1, but also contains cadherin and catenin genes, wnt and N-myc genes (APC and GLI pathways respectively) which are potentially involved in adhesion and cell motility (Vogelstein and Kinzler, 2004). The PI3K pathway includes bcl2, a gene, which is also involved in the apoptosis pathway, whilst other genes in this pathway lead to transcription and translation, making this common to the RTK pathway. The SMAD pathway includes TGF β , which is also involved in transcription.

The HIF1 pathway is important as it can help determine the metastatic potential of particular tumours. Under normal circumstances and in the absence of oxygen, the pathway culminates in angiogenesis through the expression of cytokines such as VEGF. In the present study, three genes that were differentially expressed following exposure to DDE including, EPAS1, FLT1 and PTGES, raise the possibility that the HIF1 pathway may be involved in the mechanism of action of DDE on oestrogen receptor positive cells.

Previous studies have shown that EPAS1 is a member of the transcription family expressed in vascular endothelial cells (Maemura *et al.*, 1999), while FLT1 is a known angiogenic receptor that interacts with the vascular endothelial cell growth factor (VEGF) to bring about angiogenesis (Wang *et al.*, 2000; Aguayo *et al.*, 2001). PTGES has been implicated in the inflammation process and prostaglandins have been found to possess properties that promote malignant growth through the stimulation of angiogenesis (Filion *et al.*, 2001; Yoshimatsu *et al.*, 2001).

It is possible that many other pathways are involved in the mechanism of action of DDE, such as calcium modulation pathways, which also indirectly induce apoptosis (and include calneuron 1 and CACNA2D2), and possibly adhesion, carrier or matrix assembly pathways (including CEACAM, PSPH, syndecan, biglycan, lipocalin 2 and SLC31A1).

It needs to be emphasized that the differentially expressed genes that were found in this study, following DDE exposure were detected using microarray and were not confirmed using other techniques such as Northern blotting or real time polymerase chain reaction (RT-PCR). However, as described in the methods section for the microarray experiments, positive results were only reported if the microarray experiments did indicate genes that were common from at least 2 of 3 hybridisation rounds, which suggests that they are real gene products as a result of the hybridization and not artifacts. However, as indicated above further experiments involving Northern blots or real time polymerase chain reaction (RT-PCR) experiments would be needed to confirm the genes that were found to be differentially expressed in the DDE treated cells. This work would have been undertaken but was not done because of time constraints. However a preliminary RT-PCR experiment was undertaken for one gene, IGFBP3 in the MCF-7 cell cultures, which showed that this gene was down-regulated following exposure to DDE. The preliminary data supported the findings of the present work, that is, the difference between untreated and treated cells was not statistically significant. Further work involving RT-PCR and Northern blots would be needed to support the changes that were found using microarray.

4.5 CONCLUSIONS

The observations in this study suggest that DDE, an organochlorine pesticide, does induce changes in gene expression in oestrogen receptor positive breast cancer cells. The genes that were identified using microarray have been shown to be part of a number of pathways that alter growth and differentiation. The data presented here, however are only those of the microarray experiments from oestrogen receptor positive cells, so there is the possibility of pathways in oestrogen receptor negative cells other than those presented here, of being regulated by DDE exposure. The mechanisms by which DDE may increase a woman's risk of breast cancer can involve multiple genes that may be regulated by a multitude of pathways. DDE in itself may interact with different signalling pathways to bring about altered gene expression and thereby the potential of carcinogenesis.

GENERAL DISCUSSION

Study Population

The present study was undertaken to determine whether organochlorine pesticide levels increase the risk of breast cancer. Women who underwent surgery for breast abnormalities or for reduction mammoplasty were recruited for the study. Over a four-year period, in which this study was undertaken, 157 women met the criteria for inclusion as part of this research. For this particular type of research however, where a questionnaire containing 116 questions about breast cancer risk factors, chemical exposure data and lifestyle behaviours was used to assess cancer risk, the study population was small. An analysis of expected numbers of participants was not conducted at the onset of this study merely because the investigators did not know what to expect, and with the lack of Australian data in this respect, it was not possible to predict the numbers of women that would need to be recruited for the study. If time permitted, a preliminary study would have been conducted to test and validate the recruitment process in order to achieve a greater compliance. The pathology staff at each of the hospitals largely dictated the recruitment process and thus only a small sample size was achieved. The recruitment process in the present study had its share of problems and future work would need a greater commitment and involvement of clinical staff at the collaborating hospitals in order to obtain a greater number of participants. The number of participants was limited, as a result of these problems with recruitment and also because this research is the basis of a doctoral degree and had time constraints placed upon it.

Women who underwent surgery for breast abnormalities or for reduction mammoplasty were recruited for the study. At the onset of the present study, the recruited women were classified, on the basis of their pathological diagnoses, into two crude categories being benign ($n = 43$) and cancer ($n = 114$). The problem with these two categories is that there is no distinction made for women who may be diagnosed with pre-cancerous conditions which can be classified as neither benign or cancer. It only became apparent during the recruitment phase of this study that all participating women should be further classified into groups that make the distinction between true benign, pre-cancerous conditions and graded cancers. The current research presented the findings of the analysis of breast cancer risk factors, chemical exposure data and lifestyle behaviours in relation to a continuum of breast disease events (chapter 2). The breast disease continuum contained eight categories ranging from relatively

normal breast conditions (such as those obtained from breast reduction surgeries) through to invasive breast cancer (grade 3). The study of the disease continuum in relation to pesticide exposure was quite unique and highlighted specific diagnoses that should be considered separately from others, in assessing breast cancer risk. In particular, atypical hyperplasia and its associated factors from the questionnaire were highlighted as being an important consideration for risk of breast cancer development. However, like the present study as a whole, the analyses of pesticide exposure and breast cancer risk factors in relation to this continuum of breast disease events was limited by the small study size, hence the findings reported in chapter 2 were merely observational. Research by Bai *et al.* (2001) and Gayat and colleagues (2003) suggests that atypical hyperplasia is a high risk factor for breast cancer as it shares some of the proliferative and apoptotic features of in situ carcinomas, which are recognised to elevate breast cancer risk. However, much less is known about breast cancer risk associated with non-proliferative breast lesions. Wang and colleagues (2004) evaluated the risk of breast cancer associated with non-proliferative benign breast disease and found that the risk was significantly elevated, independent of that associated with the key breast cancer risk factors. Therefore the classification of benign breast disease into categories according to the degree of epithelial proliferation, and the assessment of breast cancer risk on the basis of these categories is important. The work of Page and colleagues (1978), Dupont and Page (1985), Yu and colleagues (1992) and Goehring and Morabia (1997) suggests that there is insufficient evidence to link some benign breast diseases with an increased breast cancer risk, higher than that of the general population. Some studies that have used women with benign breast disease as controls have not found any association between pesticides and breast cancer risk, whilst others have found a modest increased risk (Falck *et al.*, 1992; Schechter *et al.*, 1997; Guttes *et al.*, 1998; Liljegren *et al.*, 1998). These inconsistencies are possibly owing to each study's definition of benign breast disease, which may cover a wide spectrum of pathologically defined lesions that predispose to breast cancer development at differing rates. Hence, future work should endeavour to group women according to their pathological diagnoses into a continuum of breast disease events, for the assessment of risk factors for breast cancer.

Given that some of the categories in the breast disease continuum contained less than 10 subjects, further analyses were conducted on groups that consisted of truly benign conditions (those obtained from breast reduction surgeries and non-proliferative benign conditions) compared to graded cancers, with the elimination of any precancerous diagnoses. In this study, women who were classified as having a benign breast condition acted as controls (n=25), whilst those diagnosed with graded cancers were classed as the cases (n=102). In case-control studies it is generally expected that the number of controls should, at least, be equal to or greater than the number of cases. In the present study this was not the case, even though it was the intention and every attempt was made to recruit more control subjects, but for the reasons highlighted above, the study proceeded with the numbers recruited. Control patients and cases were recruited in the same manner, as they presented to the follow-up clinics at the collaborating hospitals, so attempts were made to alleviate potential bias. In the first and second years of the study however, women diagnosed with various grades of breast cancer were mainly recruited from one hospital. In the third and fourth years of the study, two other hospitals joined the collaboration and more women diagnosed with benign breast conditions were recruited. As the control subjects were mainly derived from one hospital and the cases derived from the other two hospitals, there is the potential for inter-hospital differences (and thus bias) in recruitment. Given the small sample size of the present study it was not possible to stratify the analyses on the basis of hospital from where the participants were recruited. There is also the potential for bias in the interviewing process between cases and controls from the different hospitals. Women diagnosed with breast cancer are more likely to attend follow-up clinics for the treatment of their condition and would have been interviewed at that time, in person. Women diagnosed with benign breast conditions classified as the control group, especially those obtained from the Mercy Hospital For Women, would have been interviewed over the telephone. Attempts were made to ensure that the interviews, regardless of whether they were in person or over the telephone, proceeded in the same manner, where the questions were asked in sequential order as they appear in the questionnaire. There is also the potential for bias given that at the commencement of the study mainly women with breast cancer were recruited, and the control group was recruited at a later date. Attempts were made to eliminate these hospital differences in recruitment and the staging of recruitment over time by drafting a common set of guidelines for hospital

staff to adhere to for the collection of tissue specimens. Adherence to these guidelines was therefore beyond the control of the principal investigator of this study (the PhD candidate).

Some studies, including the present study, have used women with benign breast conditions as controls. There is evidence however that women, with a previous history of benign breast disease, have a higher predisposition to breast cancer than women from the general population, which may indicate that they are not a suitable control group (Black *et al.*, 1972; Ernster, 1981; Hunter *et al.*, 1997). It is quite possible that if the control group was selected on the basis of age matching from the general population, that a greater number of controls could have been included in the study.

Risk Factors

There is still uncertainty as to the causes of breast cancer however there are factors that are thought to increase a woman's risk of developing the disease. These factors include age, gender, family history of the disease especially if first degree relatives are affected, history of benign breast disease, age at menopause, age at menarche, number of full term pregnancies and the use of hormone therapies.

The present study investigated the potential for organochlorine pesticides to be risk factors for breast cancer. This study was conducted by measuring the levels of a series of organochlorine pesticides in breast adipose tissue, as well as by administering a questionnaire that contained questions to assess breast cancer risk factors, chemical exposure data and lifestyle behaviours. The amount of data collected for the small study group imposes limitations on the comparisons that can be made between the cases and controls and the risk factors investigated. However in saying this, the present study found high levels of DDE in the breast adipose tissue of women diagnosed with breast cancer compared to women without cancer. The present study also found that with a longer time of residence in Australia there was also an increased risk of breast cancer (not adjusted for age), which suggests the potential link with lifestyle and environmental factors. Age adjustment of the data removes any previous association of the levels of DDE and the years of residence in Australia with increasing breast cancer risk. As already discussed in chapter 3, both of these variables are likely to be age dependent, such that with increasing age there is

potentially a longer lifetime exposure and accumulation of DDE, and age is a factor in combination with the number of years resident in Australia. Age is known to be a strong risk factor for breast cancer and therefore any analyses of other potential factors for increasing the risk of breast cancer, should account for age. Therefore breast adipose tissue levels of DDE and other organochlorine pesticides are not risk factors for breast cancer development. The results of other studies that have used alternative control populations, for example hospital controls, women undergoing reduction mammoplasties or community based controls, also have failed to reach a consensus about any connection between breast cancer risk and tissue levels of DDE (Mussalo-Rauhamaa *et al.*, 1990; Van't veer *et al.*, 1997; Bagga *et al.*, 2000). Therefore there doesn't appear to be any significant differences in the relationship between tissue levels of DDE and breast cancer risk based on the type of control population used. The present study also investigated the tissue DDE levels through a continuum of breast disease events even though it proved not to be associated with breast cancer risk from the regression model, as a means of identifying those conditions with differing predispositions to breast cancer development. A relationship between organochlorine chemical exposure and breast cancer is possible based on the fact that all relative risks of breast cancer cannot be explained by the known risk factors. However, differences in study design including the medium used to determine organochlorine levels, and the choice of control populations and hence research findings and the adjustments for confounding variables for breast cancer have created uncertainty about this issue. The present study found an increased risk of breast cancer associated with living or working on a farm where animals may be treated with chemicals, when age was adjusted. However it is not known how this association exists with increasing the risk of breast cancer as more women diagnosed with benign breast conditions had lived or worked on farms with animals. This variable is likely to be related with area of residence, as more women diagnosed with cancer reported living in regional areas than women with benign conditions. The data however was not adjusted for this potentially confounding variable. Given a larger study group to assess the differences in risk factors between rural and metropolitan dwellers there is the possibility of organochlorine pesticide exposure increasing the risk of breast cancer.

The determination of current levels of pesticides in the breast adipose tissue may be a poor surrogate of lifetime exposure and therefore a poor estimate of associated breast cancer risk, because lipid metabolism can change over time. Another factor which needs to be closely investigated in relation to organochlorine contamination in the body is diet, as it is one of the major routes of exposure to these chemicals that may help to explain the deposition of adipose in the body and hence the propensity of organochlorine accumulation. Previous studies on this matter have not investigated dietary factors in detail. The present study involved an analysis of dietary fat, in particular saturated fats, as part of the questionnaire, but due to the small number of participants in this study, could not be analysed in detail. Future work undertaken to elucidate a role for organochlorine pesticides in increasing the risk of breast cancer would need to focus on the different types of dietary fat and how these may influence the rate of accumulation of exogenous lipophilic chemicals. This being the case, and that breast cancer has a long latency, means that there are limitations on the findings of the present study. This study also does not take into account early life exposure to organochlorine pesticides, which could prove to be important indicators for breast cancer risk in the future.

Genetic effects of DDE exposure

The relationship between breast cancer and organochlorine pesticides is also possible given that the organochlorine class of chemicals have structures similar to that of the female hormone, oestrogen, which is involved in the promotion of breast cell growth. The present study found higher levels of DDE and other organochlorine pesticides in the breast adipose tissue of women with breast cancer compared to women with benign breast conditions. However the high levels of these pesticides was not associated with increasing the risk of breast cancer. But this finding does not explain the effects that the presence of these pesticides may have on the breast and does not explain why women with breast cancer have higher levels of these pesticides than women with benign conditions. The final part of the current study found that proliferation of oestrogen receptor positive breast cancer cells and the expression of a number of genes were altered with exposure to DDE. Some of these genes were found to be involved in the apoptosis pathway, others involved in signal transduction pathways and some found to alter calcium influx, which also can be involved in the modulation of the apoptotic process. Some genes that were down regulated with

exposure to DDE could not be easily linked to known pathways. The genes that were expressed in response to DDE exposure and that are involved in the apoptotic pathway could help to explain the differences between pesticide levels in women diagnosed with breast cancer and women with benign conditions. This suggests that women with high levels of DDE have breast cancer because the existence of DDE in their breast tissue has altered the normal cascade of genetic events that induces apoptosis. This theory however is only on the basis of results from microarray experiments using one cell line, MCF-7 breast cancer cells. The present study utilised four breast cancer cell lines for the proliferation studies, however due to limited resources only one of these cell lines could be applied to the microarray to test for differential gene expression. Future studies would need to investigate the differentially expressed genes following DDE exposure in oestrogen receptor negative breast cancer cells as a comparison to those studied here. Future work could also include similar studies as those described in chapter 4 utilising a non-transformed cell line to investigate the potential for DDE to interact with non-hormone and non-cancerous pathways. Also, further work needs to be done to verify the expression patterns of the differentially expressed genes found in the present study, which would likely involve real time polymerase chain reaction experiments or Northern blotting.

Other Recommendations

Although this was a relatively small study, the research found that the levels of DDE were higher in the breast adipose tissue of women with breast cancer than women with benign breast conditions. These DDE levels, although they did not increase the risk of breast cancer, affected gene expression when breast cancer cells were exposed. DDT and most other organochlorine chemicals are banned from use around the world, it is studies like this that can provide useful information before the registration of chemicals in the future. Studies investigating the effects of chemicals on human health should be on display for members of the general public, so that they can make an informed decision about their personal use of chemicals. Future work also needs to include an analysis of other chemicals that have been suspected of mimicking oestrogen or interacting with the oestrogen receptor and how they affect gene expression. The pesticides in use today should be investigated to determine their effects on gene expression and thus their potential to affect human health.

APPENDIX A

VICTORIA UNIVERSITY OF TECHNOLOGY

IN CONJUNCTION WITH

ST VINCENT'S HOSPITAL AND MERCY HOSPITAL FOR WOMEN

ENVIRONMENTAL PESTICIDES AND THE RISK OF BREAST CANCER

INTRODUCTION

This study is investigating the relationship of pesticides and related chemicals in body tissues, to breast disease. We recognise that many of the questions are about very personal issues, however to investigate the area targeted we have been careful to ask only questions absolutely necessary for the research. The answers are confidential.

A presumption of your completion of this questionnaire is that you have been referred to the project by a medical practitioner and signed the 'Patient Declaration Form.'

One of the research team will be present to assist you with the completion of the form and answer any queries.

INSTRUCTIONS

Please enter information in the space provided (either boxes or lines). Where alternatives are provided (Yes/No), please tick the appropriate box.

PART 1. BASIC INFORMATION

1. Code number:									
2. Date entered study (DD/MM/YY):									
3. Family Name:				Given name(s):					
4. Date of birth (DD/MM/YY):									
5. Postcode of address:									
6. Country of birth:									
7. Years of residence in Australia:									
8. Weight (kg):					Height (cm)				

9. Type of industry (if any) close by your place of residence (tick box):

- | | |
|---|--|
| <input type="checkbox"/> a. Agriculture | <input type="checkbox"/> h. Metalwork & engineer. |
| <input type="checkbox"/> b. Business and commercial | <input type="checkbox"/> i. Residential only |
| <input type="checkbox"/> c. Chemical manufacture | <input type="checkbox"/> j. Textile production |
| <input type="checkbox"/> d. Cleaning/dry cleaning | <input type="checkbox"/> k. Transport |
| <input type="checkbox"/> e. Clothing manufacture | <input type="checkbox"/> l. Wood products/building |
| <input type="checkbox"/> f. Electrical components | <input type="checkbox"/> m. Other (Please state) |
| <input type="checkbox"/> g. Food processing and preparation | |

10. Your occupation currently (tick box):

- | | |
|--|--|
| <input type="checkbox"/> a. Professional (eg, teacher) | <input type="checkbox"/> d. Administrative |
| <input type="checkbox"/> b. Clerical/sales | <input type="checkbox"/> e. Trades/labour |
| <input type="checkbox"/> c. Home & family duties | <input type="checkbox"/> f. Unemployed |

11. Type of industry you work or worked in (tick box):

- | | |
|---|---|
| <input type="checkbox"/> a. Agriculture | <input type="checkbox"/> i. Health & welfare |
| <input type="checkbox"/> b. Business and commercial | <input type="checkbox"/> j. Metalwork/engineering |
| <input type="checkbox"/> c. Chemical manufacture | <input type="checkbox"/> k. Public service |
| <input type="checkbox"/> d. Cleaning/dry cleaning | <input type="checkbox"/> l. Textile production |
| <input type="checkbox"/> e. Clothing manufacture | <input type="checkbox"/> m. Transport |
| <input type="checkbox"/> f. Education | <input type="checkbox"/> n. Wood products |
| <input type="checkbox"/> g. Electrical components | <input type="checkbox"/> o. Other (Please state) |
| <input type="checkbox"/> h. Food processing and preparation | |

12. Your partner's occupation currently (tick box):

- | | | | | | |
|--------------------------|----|----------------------------|--------------------------|----|----------------|
| <input type="checkbox"/> | a. | Professional (eg, teacher) | <input type="checkbox"/> | d. | Administrative |
| <input type="checkbox"/> | b. | Clerical/sales | <input type="checkbox"/> | e. | Trades/labour |
| <input type="checkbox"/> | c. | Home & family duties | <input type="checkbox"/> | f. | Unemployed |

13. Type of industry your partner works or worked in (tick box):

- | | | | | | |
|--------------------------|----|---------------------------------|--------------------------|----|-----------------------|
| <input type="checkbox"/> | a. | Agriculture | <input type="checkbox"/> | i. | Health & welfare |
| <input type="checkbox"/> | b. | Business and commercial | <input type="checkbox"/> | j. | Metalwork/engineering |
| <input type="checkbox"/> | c. | Chemical manufacture | <input type="checkbox"/> | k. | Public service |
| <input type="checkbox"/> | d. | Cleaning/dry cleaning | <input type="checkbox"/> | l. | Textile production |
| <input type="checkbox"/> | e. | Clothing manufacture | <input type="checkbox"/> | m. | Transport |
| <input type="checkbox"/> | f. | Education | <input type="checkbox"/> | n. | Wood products |
| <input type="checkbox"/> | g. | Electrical components | <input type="checkbox"/> | o. | Other (Please state) |
| <input type="checkbox"/> | h. | Food processing and preparation | | | |

PART 2: FAMILY HISTORY OF BREAST CANCER

14. State any history of breast cancer in a first or second degree relative (tick box):

- | | | | | | |
|--------------------------|----|----------|--------------------------|----|------------------------|
| <input type="checkbox"/> | a. | none | <input type="checkbox"/> | e. | aunt (maternal) |
| <input type="checkbox"/> | b. | mother | <input type="checkbox"/> | f. | aunt (paternal) |
| <input type="checkbox"/> | c. | daughter | <input type="checkbox"/> | g. | grandmother (maternal) |
| <input type="checkbox"/> | d. | sister | <input type="checkbox"/> | h. | grandmother (paternal) |

PART 3: PERSONAL HEALTH HISTORY

GENERAL HISTORY

15. Have you ever had any liver disease? Yes No
go to Q21
16. If yes, state name of the condition:
17. When did this occur? Year Month
18. How long did you have this condition for? Weeks
19. What treatment did you have?
- a. None
 - b. Surgery
 - c. Medication Give name
 - d. Other Give name
20. State date of any treatment. Year Month
21. Have you ever had other breast disease? Yes No
go to Q27
22. If yes, state name of the condition:
23. In which breast? a. Right
 b. Left
 c. Both
24. How long did you have the condition for? Weeks
25. What treatment did you have for the breast disease?
- a. Surgery

- b. Medication Give name:
- c. Vitamin therapy
- d. Natural therapies Give name:
- e. Other Give name:

26. State date of treatment (year and month) Year Month

GYNAECOLOGICAL HISTORY

27. Please state your age at first menstrual period. Years Months

28. Are you post-menopausal (past the "change of life")? Yes No
go to Q30/31

29. If yes, state the time since LMP. Years Months

30. If still menstruating, when was your last menstrual period (LMP)? Weeks

31. Have you had regular menstrual periods in the past year? Yes No

32. Are you currently taking oral contraceptives (the pill) or injected contraceptives?
 Yes No
go to Q35

33. What brand are you taking at the moment? Code:

34. When did you commence taking the contraceptives this time?

Year

Month

35. Have you ever taken oral or injected contraceptives in the past?

Yes No

go to Q38

36. If yes, what brands have you used in the past?

Code:

37. Please state how long you took the pill for. Months

38. Are you currently taking Hormone Replacement Therapy (HRT) by mouth, or skin patch or using any hormone cream?

Yes No

go to Q41

39. If yes, name the brand and how it is used.

Code:

40. When did you commence taking the HRT?

Year

Month

41. Have you ever taken HRT in the past?

Yes No

go to Q44

42. If yes, when did you commence taking the HRT?

Year

Month

43. Please name the brand, if known.

Code:

44. Have you had an ovary removed surgically? Yes No
go to Q47

45. Which one? a. left
 b. right
 c. both

46. When was the operation on your ovary? Year
Month

47. Have you had a hysterectomy? Yes No

48. If, yes, was it performed because of cancer? Yes No
go to Q50

49. Date of the operation Year Month

OBSTETRIC HISTORY

50. How many live children have you given birth to?
if zero go to Q54

51. Please state your age at your first full term pregnancy

52. Were your children breast fed? Yes No
go to Q54

53. How long did you breast feed each child for?

Child 1 _____ months
2 _____ months
3 _____ months
4 _____ months
5 _____ months

GENERAL HEALTH

54. Do you smoke cigarettes? Yes No
go to Q57

55. How many cigarettes per day?

56. How many years have you been smoking?
57. Have you smoked cigarettes in the past? Yes No
go to Q60
58. For how many years did you smoke?
59. How many cigarettes per day did you smoke?
60. Do you consume alcohol? Yes No
go to Q63
61. How often do you drink alcohol? daily*
 weekly
 monthly
 other—
62. Are you on any **prescribed** medication? Yes No
go to Q65
63. If yes, please provide details. (Contraceptives or HRT not required)
- a. central nervous system conditions/epilepsy, schizophrenia etc.
 - b. anti-depressants
 - c. diuretics/anti-hypertensives
 - d. cholesterol lowering drugs/other heart conditions
 - e. hormones/insulin, thyroid hormones etc
 - f. chemotherapy/cancer
 - g. painkillers
 - h. anti-inflammatories/arthritis, joint problems
 - i. gastrointestinal system/ulcer
 - j. respiratory/bronchodilators, anti-asthmatics etc
 - k. antibiotics/infection
 - l. reproductive system/fertility drugs, endometriosis etc
 - m. blood disorders/anti-coagulants etc.
 - n. herbal and homeopathic remedies (Give drug name and condition)

o. other (Give drug name and condition)

65. Do you take any non-prescription drugs? Yes No
go to Q67

66. If yes, please indicate

a. vitamins

b. herbal & homeopathic remedies

c. anti-histamines and anti-inflammatories

d. drugs for nausea, gastric upset

e. painkillers (panadol etc)

f. marijuana

g. heroin

h. amphetamines (speed)

i. other (Please name drug and condition)

PART 4: EXPOSURE TO CHEMICALS

IN THE GARDEN:

67. Do you use any weedkiller in the garden? Yes No
go to Q71
68. If so, please name the brand(s) Code:

69. How often do you use those weedkillers? (Times per year)
70. When handling the weedkillers, do you use gloves? Yes
 Sometimes
 Always
 No

IN THE HOME OR GARDEN

71. Do you use insecticides (pestkillers) in the house/garden? Yes
 Sometimes
 Always
 No go to Q75
72. If so, please name the brand(s) Code:

73. How often do you use those treatments? (Times per year)
74. When handling the insecticides, do you use gloves? Yes
 Sometimes
 Always
 No

OCCUPATIONAL EXPOSURE

75. Do you live or work, or have you ever lived or worked, on a farm where chemicals have been used for treating animals? Yes No
go to Q81
76. For how long? (years)
77. How often would these chemicals have been used? (Times/year)
78. If yes, have you handled these chemicals yourself? Yes No
79. When handling, did you use protective clothing? Yes
 sometimes
 always
 No
80. Did you change your clothes after handling chemicals? Yes
 immediately
 at the end of the day
81. Does any person living in your household handle chemicals used for treating animals? Yes No
go to Q84
82. Please name the brands of chemicals used, if known. Code:

83. Did you wash clothing which had been exposed to chemicals for treating animals? Yes No

84. Do you live or work, or have you ever lived or worked, on a farm where chemicals have been used for treating crops or buildings?

Yes No
go to Q90

85. For how long? (years)

86. How often would these chemicals have been used? (Times/year)

87. If yes, have you handled these chemicals yourself? Yes No

88. When handling, did you use protective clothing?
 Yes
 sometimes
 always
 No

89. Did you change your clothes after handling chemicals?
 Yes
 immediately
 at the end of the day

90. Does any person living in your household handle chemicals used for treating crops or buildings?

Yes No
go to Q93

91. Please name the brands used, if known. Code:

92. Did you wash clothing which had been exposed to chemicals for treating crops or buildings?

Yes

No

93. Have you ever lived in a house which has been treated with pesticides?
 Yes No
go to Q96

94. Why was the house treated?

95. Please name the brands used, if known.

96. How often does your local council spray herbicides or pesticides in your area?

PART 5: DIET (SHORT FAT QUESTIONNAIRE), adapted from Dobson, Blijevens, Alexander, Groce, Heller, Higginbotham, Pike, Plotnikoff, Russell and Walker, 1993. *Australian Journal of Public Health*, Vol 17, No 2).

INSTRUCTIONS

Tick the box which corresponds to your diet - please tick only one answer for each question. If you have changed your diet since your time of diagnosis, please answer these questions as of before you changed.

97. How often do you eat fried food, for example, chips, french fries or food that is cooked in batter or breadcrumbs?

Six or more times a week	<input type="checkbox"/>	4	
Three to five times a week	<input type="checkbox"/>	3	
Once or twice a week	<input type="checkbox"/>	2	
Less than once a week	<input type="checkbox"/>	1	
Never	<input type="checkbox"/>	0	_____

98. How often do you eat gravy, cream sauces or cheese sauces?

Six or more times a week	<input type="checkbox"/>	4	
Three to five times a week	<input type="checkbox"/>	3	
Once or twice a week	<input type="checkbox"/>	2	
Less than once a week	<input type="checkbox"/>	1	
Never	<input type="checkbox"/>	0	_____

99. How often do you add butter, margarine, oil or sour cream to vegetables, cooked rice or spaghetti?

Six or more times a week	<input type="checkbox"/>	4	
Three to five times a week	<input type="checkbox"/>	3	
Once or twice a week	<input type="checkbox"/>	2	
Less than once a week	<input type="checkbox"/>	1	
Never	<input type="checkbox"/>	0	_____

100. How often do you eat vegetables that are fried or roasted with fat or oil?

Six or more times a week	<input type="checkbox"/>	4	
Three to five times a week	<input type="checkbox"/>	3	
Once or twice a week	<input type="checkbox"/>	2	
Less than once a week	<input type="checkbox"/>	1	
Never	<input type="checkbox"/>	0	_____

101. How do you spread your butter/margarine on your bread?

Thickly	<input type="checkbox"/>	3	
Medium	<input type="checkbox"/>	2	
Thinly	<input type="checkbox"/>	1	
Don't use butter/margarine	<input type="checkbox"/>	0	

102. How often do you eat cream?

Six or more times a week	<input type="checkbox"/>	4	
Three to five times a week	<input type="checkbox"/>	3	
Once or twice a week	<input type="checkbox"/>	2	
Less than once a week	<input type="checkbox"/>	1	
Never	<input type="checkbox"/>	0	_____

103. How often do you eat ice cream?

Six or more times a week	<input type="checkbox"/>	4	
Three to five times a week	<input type="checkbox"/>	3	
Once or twice a week	<input type="checkbox"/>	2	
Less than once a week	<input type="checkbox"/>	1	
Never	<input type="checkbox"/>	0	_____

104. How many times a week do you eat cheese?

Six or more times a week

Three to five times a week

Once or twice a week

Less than once a week

Never

	4
	3
	2
	1
	0

105. How much cow's or goat's milk do you drink per day?

Six or more glassfuls per day

Three to five glassfuls per day

One or two glassfuls per day

Less than one glassful per day

Do not drink milk

	4
	3
	2
	1
	0

106. What type of milk do you drink or use in cooking or tea and coffee?

Condensed

Full cream

Full cream and reduced fat

Reduced fat

Skim or none

	4
	3
	2
	1
	0

107. How many times a week do you eat meat?

Six or more times a week

Three to five times a week

Once or twice a week

Less than once a week

Never

	4
	3
	2
	1
	0

108. How much of the fat on your meat do you eat?

Most or all of the fat

	2
--	---

Some of the fat

	1
--	---

None of the fat

	0
--	---

I am a vegetarian

	0
--	---

109. How much of the skin on your chicken do you eat?

Most or all of the skin

	2
--	---

Some of the skin

	1
--	---

None of the skin

	0
--	---

I am a vegetarian

	0
--	---

110. How is your meat usually cooked?

Fried

	4
--	---

Stewed or goulash

	3
--	---

Grilled or roasted with
added oil or fat

	2
--	---

Grilled or roasted without
added oil or fat

	1
--	---

Eat meat occasionally or
never

	0
--	---

111. How many times a week do you eat sausages, salamis, meat pies, hamburgers or bacon?

Six or more times a week	<input type="checkbox"/>	4	
Three to five times a week	<input type="checkbox"/>	3	
Once or twice a week	<input type="checkbox"/>	2	
Less than once a week	<input type="checkbox"/>	1	
Never	<input type="checkbox"/>	0	_____

112. How many times a week do you eat fish and other seafoods?

Six or more times a week	<input type="checkbox"/>	4	
Three to five times a week	<input type="checkbox"/>	3	
Once or twice a week	<input type="checkbox"/>	2	
Less than once a week	<input type="checkbox"/>	1	
Never	<input type="checkbox"/>	0	_____

113. How often do you eat pastries, cakes, sweet biscuits or croissants?

Six or more times a week	<input type="checkbox"/>	4	
Three to five times a week	<input type="checkbox"/>	3	
Once or twice a week	<input type="checkbox"/>	2	
Less than once a week	<input type="checkbox"/>	1	
Never	<input type="checkbox"/>	0	_____

114. How many times a week do you eat chocolate, chocolate biscuits or sweet snack bars?

Six or more times a week	<input type="checkbox"/>	4	
Three to five times a week	<input type="checkbox"/>	3	
Once or twice a week	<input type="checkbox"/>	2	
Less than once a week	<input type="checkbox"/>	1	
Never	<input type="checkbox"/>	0	_____

115. How many times a week do you eat potato crisps, corn chips or nuts?

Six or more times a week	<input type="checkbox"/>	4	
Three to five times a week	<input type="checkbox"/>	3	
Once or twice a week	<input type="checkbox"/>	2	
Less than once a week	<input type="checkbox"/>	1	
Never	<input type="checkbox"/>	0	_____

116. How many times a week do you eat whole-grain products?

Six or more times a week	<input type="checkbox"/>	4	
Three to five times a week	<input type="checkbox"/>	3	
Once or twice a week	<input type="checkbox"/>	2	
Less than once a week	<input type="checkbox"/>	1	
Never	<input type="checkbox"/>	0	_____

Thank you very much for your assistance.

APPENDIX B

MEMORANDUM

TO: Ms Cheryl Taylor
Biomedical Sciences
St Albans Campus

FROM: Dr John Allen
Director, Office for Research

DATE: 2 June, 1997

SUBJECT: Approval of application involving human subjects

At its meeting on Thursday, 22 May, 1997 the Human Research Ethics Committee considered an application for project *Environmental Factors Affecting the Risk of Breast Cancer*.

It was resolved to approve application HRETH 41/96 (HREC 97/148) from 22 May 1997 to 31 March 1999.

If you have any further queries, please do not hesitate to contact me on ext 4710.



Dr John Allen
for Secretary HREC

APPENDIX C

ST. VINCENT'S HOSPITAL MELBOURNE
HUMAN RESEARCH ETHICS COMMITTEE
APPLICATION FOR ETHICAL APPROVAL FOR HUMAN
EXPERIMENTATION

27/94

Application to be typewritten. Seventeen (17) copies to be forwarded to:

The Research Secretary
Medical Administration

This is a: NEW PROJECT
 MODIFICATION OF AN EXISTING PROJECT - Protocol No. _____
 RENEWAL OF AN EXISTING PROJECT - Protocol No. _____

PROJECT TITLE: *Environmental pesticide exposure and the risk of breast cancer*

PRINCIPAL INVESTIGATOR(S): *M A Henderson FRACS, W Probert BSc(Hons),
L Ngh Msc, B Fairclough PhD, A Alder BA,
G S Balasubramaniam FRCPA, P R Hayes FRACS,
P R B Kitchen FRACS*

DEPARTMENT/UNIT: *University of Melbourne, Department of Surgery
Victoria University of Technology*

CONTACT TELEPHONE NO: *M A Henderson 288 2545*

PROPOSED DATE OF COMMENCEMENT OF PROJECT: *1.6.94*

EXPECTED DURATION OF PROJECT:
*Patient accrual will occur over approximately 2 years. Patients
will be followed for at least a further 2 years.*

Received by Research Secretary: *Date: 30.5.94*

PROTOCOL NO: *27/94*

This project was granted full approval by the Human Research Ethics Committee at Meeting No: *3 194*, held on *Thursday 16 June 1994*. Approval is granted subject to the following conditions:

1. immediate notification to the committee of any adverse effects on subjects;
2. immediate notification of any unforeseen events that may affect the continuing ethical acceptability of the project;
3. the completion of an annual report on progress of the project;
4. committee approval of any proposed modification to the project.

Additional conditions/comments:

Period of Approval: *17.6.94* to: *17.6.98*
Date: *17.6.98* **Chairman:**



Principal investigator notified: *23.6.94.*

APPENDIX D



Friday, 9th August, 1996

Ms. Cheryl Taylor,
Centre for Bioprocessing and Food Technology (Science Faculty),
Victoria University of Technology,
McKechnie Street,
ST. ALBANS, Vic., 3021

25
YEARS

Dear Ms. Taylor,

**Re: Environmental Factors affecting the Risk of Breast Cancer (project
no. 96/5)**

*of caring for
Victoria's
women and
babies*

Following a recommendation by the Research and Ethics Committee on 16th April, 1996, this project was approved by the Board of Mercy Health and Aged Care on 7th May, 1996, subject to the following:

1. That the questionnaire include questions which ascertain whether participants have had their homes treated with pesticides, and the incidence of, or exposure to local council sprays, particularly in areas of low population density;
2. That documentation (e.g. Consent Form and Questionnaire) to be modified to refer to "Mercy Hospital for Women".

These changes have been noted and a letter of advice regarding the project has been forwarded to medical staff.

Could you please provide me with a progress report six months into your work?

Yours sincerely,



Meg Hawkins
Secretary, Research and Ethics Committee

taylorch:mmm

APPENDIX E

ST. VINCENT'S HOSPITAL, MERCY HOSPITAL FOR WOMEN AND
VICTORIA UNIVERSITY OF TECHNOLOGY

CONSENT OF PATIENT TO PARTICIPATE IN RESEARCH STUDY

PROTOCOL NUMBER. (SVH):

NAME OF PATIENT:

U.R. NO:

NAME OF INVESTIGATORS: C M Taylor, M A Henderson, W Probert, L Ngeh,
B Fairclough, A Alder, G S Balasubramaniam, P
R B Kitchen, P R Hayes, J Scurry

STUDY TITLE:

EXPLANATION TO PATIENT OF NATURE AND PURPOSE OF RESEARCH
PROCEDURE, EXACT PROCEDURES INVOLVED FOR PATIENT AND RISKS
AND DISCOMFORT INVOLVED, IF ANY:

(If insufficient space, please attach additional sheets)

Recently evidence has emerged that some cases of breast cancer may be due to exposure to various pesticides.

We are asking patients to participate in this study that aims to find out whether patients with breast cancer have higher levels of pesticides in their body compared to women undergoing surgery who do not have breast cancer. We plan to measure the level of pesticides in the breast tissue removed at the time of surgery, as well as test how these chemicals may affect certain genes in the body.

If you agree to participate in this study you will be asked to fill out a questionnaire on exposure to pesticides, diet and details of any pregnancy and hormone use. As part of our standard practice the specimen removed at the time of surgery of your prior operation has been sent to the pathology department for analysis. If you agree to participate in the study a small portion of this sample, approximately a quarter of an inch in size, will be assayed for pesticides and gene mutations.

All questionnaires and results will be treated with strict confidentiality. It will not be possible to provide results to patients as the tests for pesticides in the tissue will be performed without knowledge of the patient's identity.

I have read/had translated to me the above explanation and understand it.

Signed:.....

(Patient/guardian/next of kin/friend)

PATIENT DECLARATION

Dr. has explained the purpose and nature of the research, the research methods and procedures and risks and discomfort associated with them. I am willing to take part in this research and I consent to all of the procedures, and to the risks associated with them that have been explained to me. I understand that I am free to withdraw from this study at any time without jeopardising the management of my condition, and the future care and attention which I will receive.

Dated the day of 199

Signed

(Patient/Guardian/Next Friend)

Witness

Where applicable: I certify that I have translated the above explanation and declaration and assisted Dr. with the oral translation to the above patient in the language which the patient has indicated he/she understands.

Interpreter:

I certify that I have provided the above patient/the guardian of the patient/the next friend of the patient, with adequate information on the above research procedure which, according to my assessment of the patient's level of comprehension, he/she seemed fully to understand. I declare that the abovenamed patient freely gave consent to take part in this research study and investigation.

Investigator's signature

(Including title)

APPENDIX F

Cheryl Taylor
Victoria University of Technology
School of Life Sciences and Technology

(*date*)

Dear (*doctor's name*),

Re: Pesticides and Breast Cancer Study

Here are is a letter addressed to Ms (*patient's name*) which requires your signature before being sent.

If you feel that this patient is not suitable for this study, could you please return the letter to me. Otherwise, once you have signed the letter, could you please place it, along with the respective consent form, in the envelope addressed to the patient concerned and send it off.

Thanks for your cooperation

Regards,

Cheryl Taylor

Ph. 9365 2711

Fax. 9365 2717

APPENDIX G

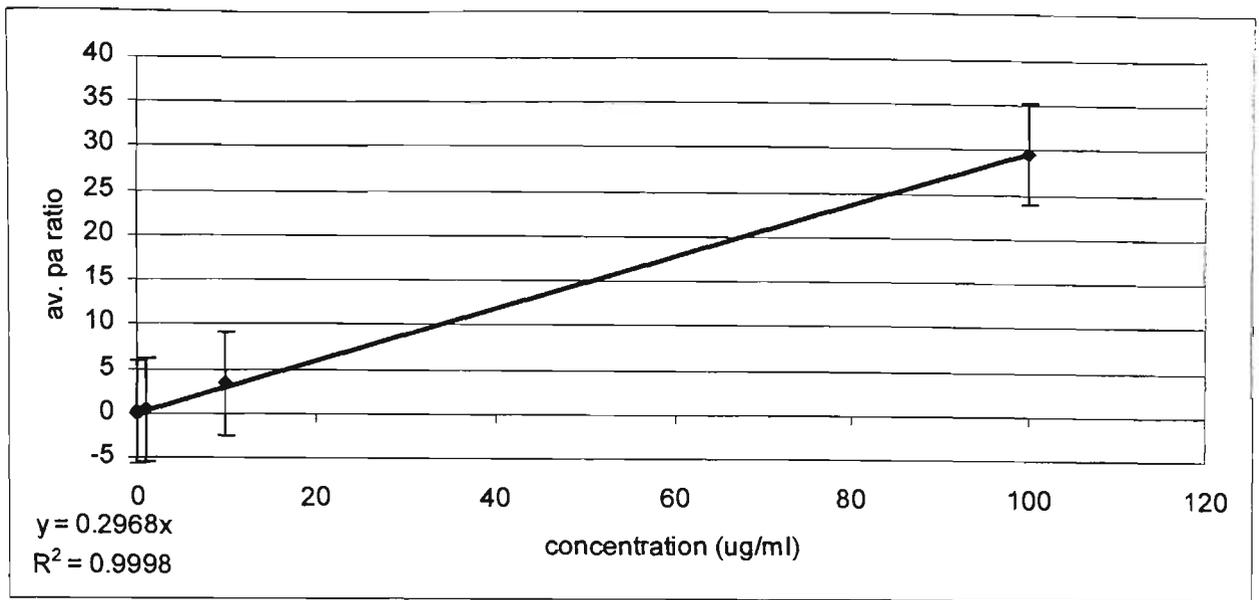


Figure A. Calibration curve for beta isomer of benzene hexachloride.

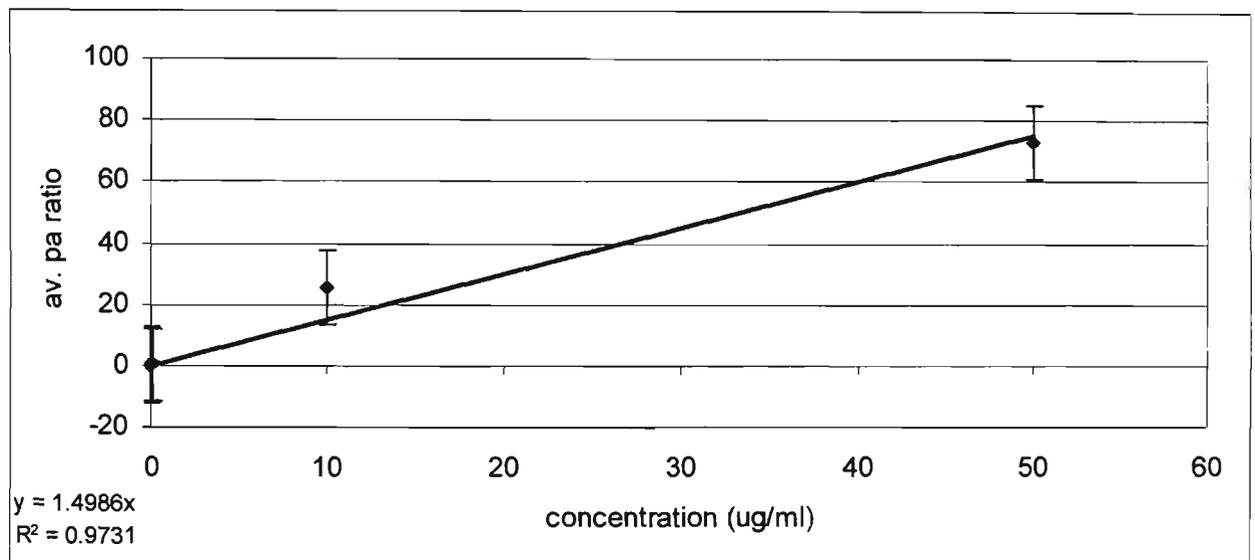


Figure B. Calibration curve for heptachlor epoxide

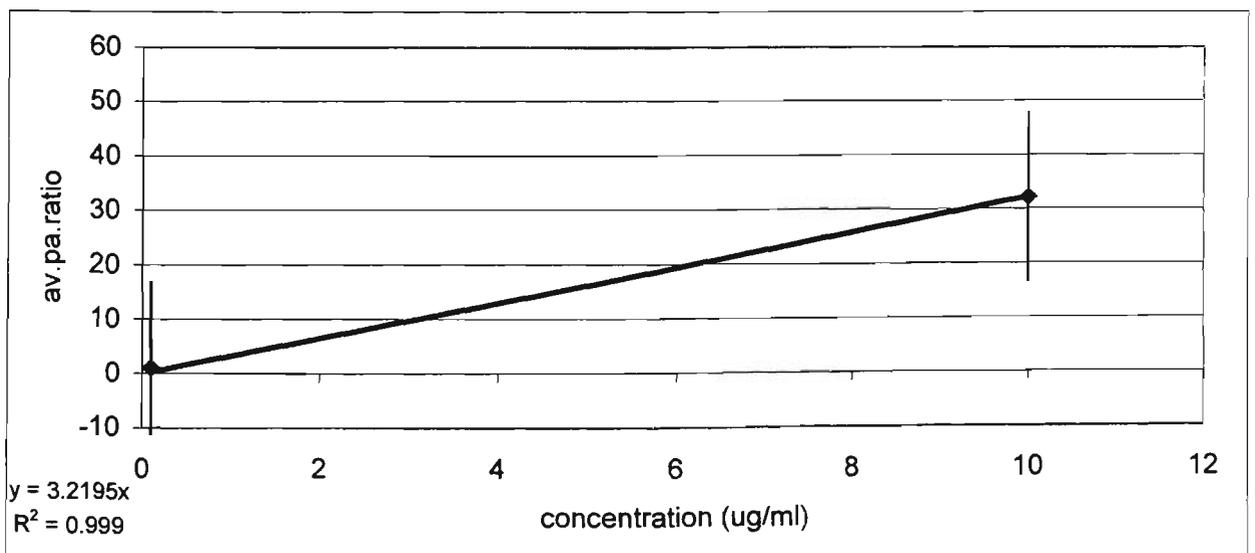


Figure C. Calibration curve for trans isomer of chlordane

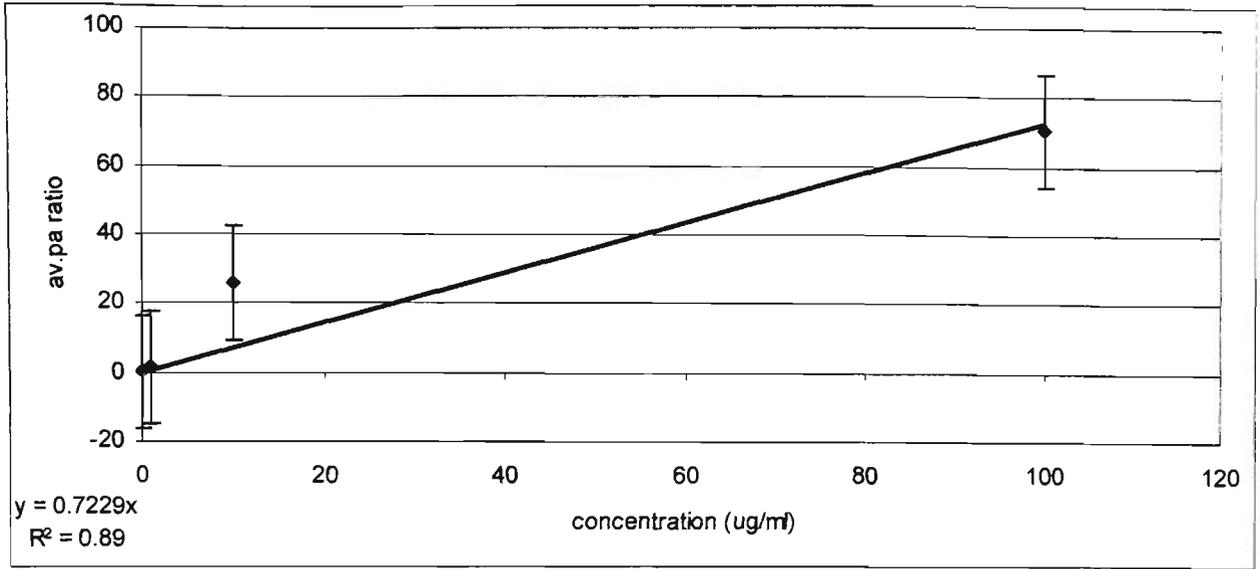


Figure D. Calibration curve for cis isomer of chlordane.

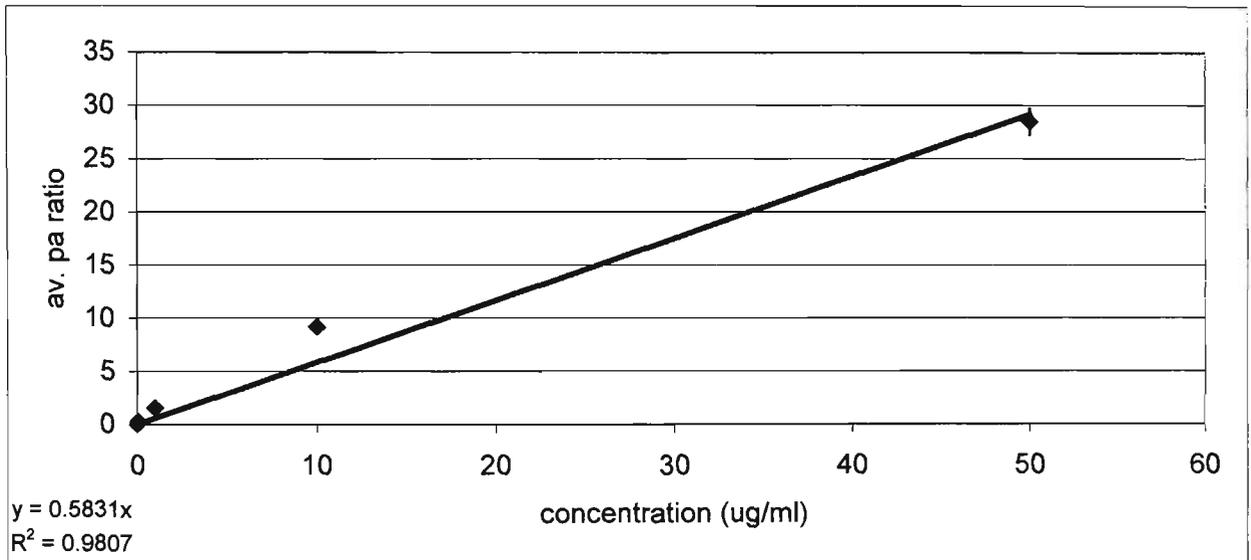


Figure E. Calibration curve for DDE.

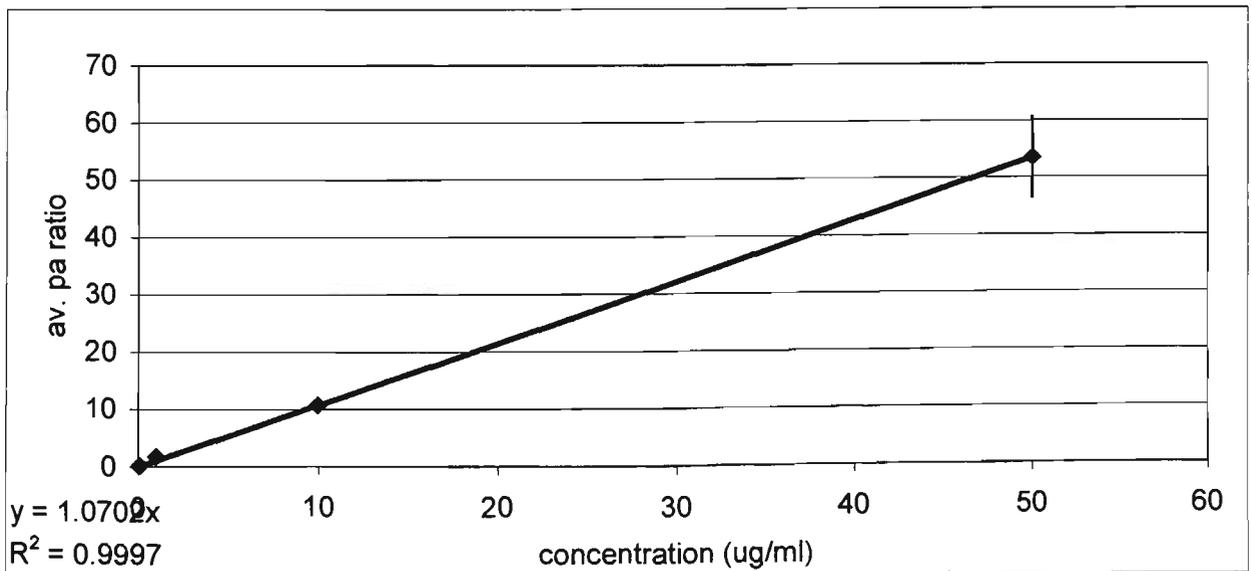


Figure F. Calibration curve for dieldrin.

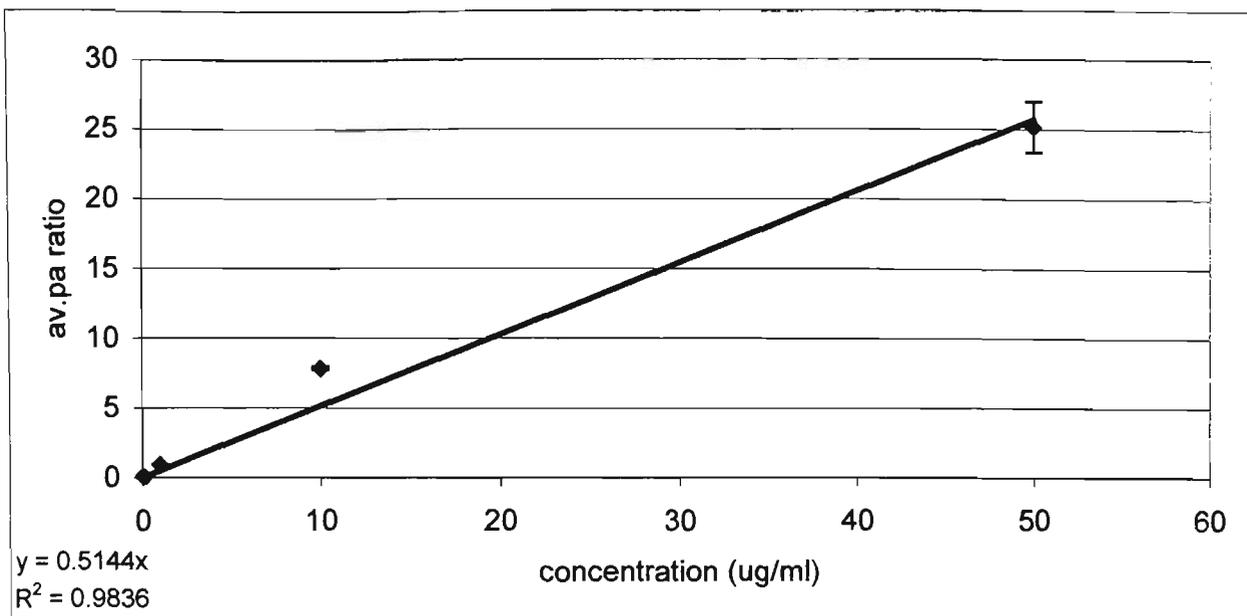


Figure G. Calibration curve for DDD.

APPENDIX H

Coding Scheme For The Questionnaire Responses

Topic	Response	Code
Country of Birth	Australia	1
	Foreign	2
Industry Close to Residence	Residential	1
	Potential Low Exposure to OCs. Business, Commercial, Transport, Clothing Manufacture, Metalwork, Health & Welfare, Education, Public Service.	2
	Potential High Exposure to OCs. Agriculture, Chemical Manufacture, Cleaning, Dry Cleaning, Wood Production, Food Processing, Electrical Components, Textile Production.	3
Occupation	Professional	1
	Retired/Unemployed/Home Duties	2
	Administrative/Clerical/Sales	3
	Trades/Labour	4
Industry You Work In	Potential Low Exposure Business, Commercial, Transport, Clothing Manufacture, Metalwork, Health & Welfare, Education, Public Service.	1
	Potential High Exposure Agriculture, Chemical Manufacture, Cleaning, Dry Cleaning, Wood Production, Food Processing, Electrical Components, Textile Production.	2
Partner's Occupation	Professional	1
	Retired/Unemployed/Home Duties	2
	Administrative/Clerical/Sales	3
	Trades/Labour	4
Industry Your Partner Works In	Potential Low Exposure Business, Commercial, Transport, Clothing Manufacture, Metalwork, Health & Welfare, Education, Public Service.	1
	Potential High Exposure Agriculture, Chemical Manufacture, Cleaning, Dry Cleaning, Wood Production, Food Processing, Electrical Components, Textile Production.	2

Family History of Breast Cancer	None	0
	1st Degree (mother/sister)	1
	2nd Degree (grandmother/aunt)	2
Liver Disease	No	0
	Yes	1
Condition	Hepatitis A	1
	Scarring	2
Treatment	None	0
	Surgery	1
	Medication	2
	Diet	3
	Hospitalisation	4
	Other	5
Other Breast Diseases	No	0
	Yes	1
Condition	Cyst	1
	Abscess	2
	Benign Lump	3
	Breast Cancer	4
	Sarcoidosis	5
	Nipple Discharge	6
Which Breast	Left	1
	Right	2
	Both	3
	Unknown	4
Treatment	None	0
	Surgery	1
	Medication	2
	Vitamin Therapy	3
	Natural Therapies	4
	Drain	5
Postmenopausal	No	0
	Yes	1
Regular Periods in Past Year	No	0
	Yes	1
Taking the Pill Currently	No	0
	Yes	1

Taken the Pill in the Past	No	0
	Yes	1
Which Brand	Unknown	1
	Progesterone Only	2
	Estrogen Only	3
	Combined	4
<hr/>		
Taking HRT Currently	No	0
	Yes	1
Which Brand	Unknown	1
	Progesterone Only	2
	Estrogen Only	3
	Combined	4
<hr/>		
Taken HRT in the Past	No	0
	Yes	1
Which Brand	Unknown	1
	Progesterone Only	2
	Estrogen Only	3
	Combined	4
<hr/>		
Ovary Removed Surgically	No	0
	Yes	1
Which Ovary	Left	1
	Right	2
	Both	3
<hr/>		
Hysterectomy	No	0
	Yes	1
Was it Performed Due to Cancer	No	0
	Yes	1
	Unknown	2
<hr/>		
Children Breastfed	No	0
	Yes	1
<hr/>		
Smoke Cigarettes	No	0
	Yes	1
<hr/>		
Smoked in the Past	No	0
	Yes	1
<hr/>		

Consume Alcohol	No	0
	Yes	1
How Often	Daily	1
	Weekly	2
	Monthly	3
	Other	4
<hr/>		
Prescribed Medication	No	0
	Yes	1
Details	Central Nervous System Conditions/ Epilepsy/Schizophrenia	1
	Anti-Depressants	2
	Diuretics/Anti-Hypertensives	3
	Cholesterol Lowering Drugs/Other	
	Heart Conditions	4
	Hormones/Insulin/Thyroid Hormones	5
	Chemotherapy/Cancer	6
	Painkillers	7
	Anti-Inflammatories/Arthritis/Joint Problems	8
	Gastrointestinal System/Ulcer	9
	Respiratory/Bronchodilators/Anti- Asthmatics	10
	Antibiotics/Infection	11
	Reproductive System/Fertility Drugs/ Endometriosis	12
	Blood Disorders/Anti-Coagulants	13
	Herbal/Homeopathic Remedies	14
	Osteoporosis	15
Renal	16	
<hr/>		
Non-Prescribed Drugs	No	0
	Yes	1
Details	Vitamins	1
	Herbal/Homeopathic Remedies	2
	Anti-Histamines/Anti-Inflammatories	3
	Drugs for Nausea/Gastric Upset	4
	Painkillers	5
	Marijuana	6
	Heroin	7
	Amphetamines	8
	Other	9
<hr/>		

Weedkiller in the Garden	No	0
	Yes	1
Brand Names	Unknown	0
	Pyrethroids	1
	Carbamates	2
	Organophosphates	3
	Organochlorines	4
	Other	5
Gloves Used	No	0
	Sometimes	1
	Always	2

Insecticide Use in House or Garden	No	0
	Yes	1
Brand Names	Unknown	0
	Pyrethroids	1
	Carbamates	2
	Organophosphates	3
	Organochlorines	4
	Other	5
Gloves Used	No	0
	Sometimes	1
	Always	2

Lived or Worked on Farm Where Chemicals Used on Animals	No	0
	Yes	1
Handled Chemicals Yourself	No	0
	Yes	1
Any Other Person Handle Chemicals	No	0
	Yes	1
Brand Names	Unknown	0
	Pyrethroids	1
	Carbamates	2
	Organophosphates	3
	Organochlorines	4
	Other	5
Washed Exposed Clothing	No	0
	Yes	1

Lived or Worked on Farm Where Chemicals Used on Crops or Buildings	No	0
	Yes	1
Handled Chemicals Yourself	No	0
	Yes	1
Any Other Person Handled Chemicals	No	0
	Yes	1
Brand Names	Unknown	0
	Pyrethroids	1
	Carbamates	2
	Organophosphates	3
	Organochlorines	4
	Other	5
Washed Exposed Clothing	No	0
	Yes	1
House Treated With Pesticides	No	0
	Yes	1
	Unanswered	2
Why Treated	Termites	1
	Mice	2
	Spiders	3
	Ants	4
	Bees	5
	Cockroaches	6
	Mosquitoes	7
	Fleas	8
	Birds	9
	Silverfish	10
	Rats	11
Brand Names	Unknown	0
	Pyrethroids	1
	Carbamates	2
	Organophosphates	3
	Organochlorines	4
	Other	5
When the Local Council Sprays	Never Seen in the Area	0
	1-2 Times per Year	1
	3-4 Times per Year	2
	Did Not Respond to Ques.	3

APPENDIX I

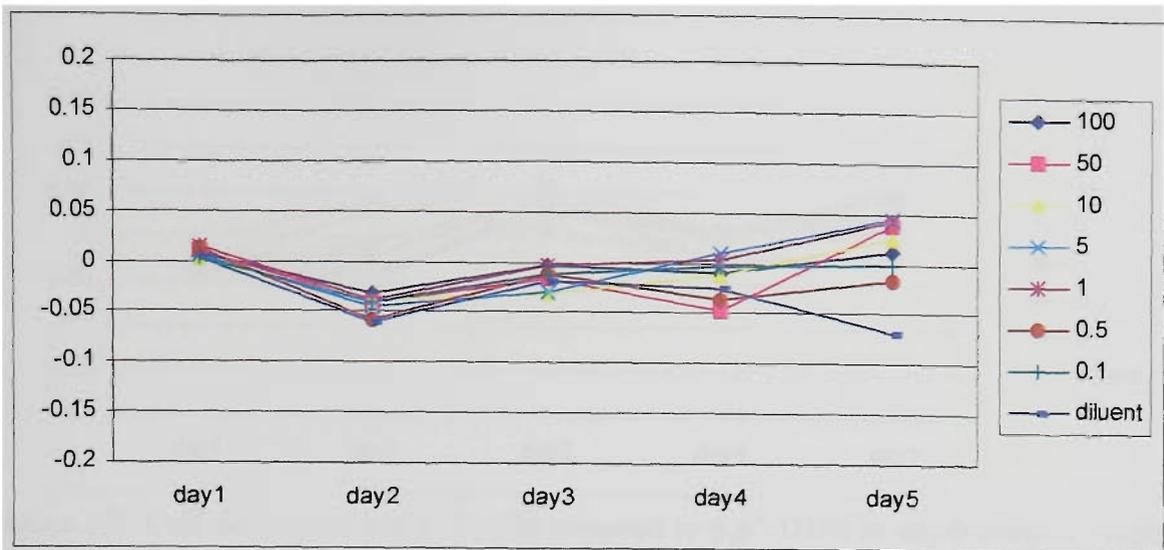


Figure 1A. Cell density of MCF-7 cells exposed to o,p'-DDE in growth media.

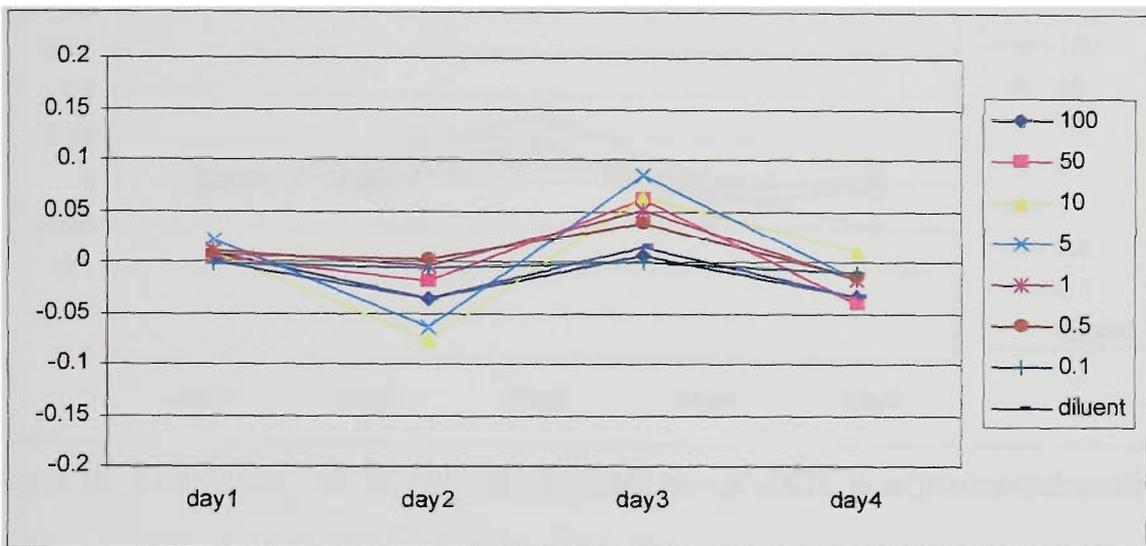


Figure 1B. Cell density of MCF-7 cells exposed to p,p'-DDT in growth media.

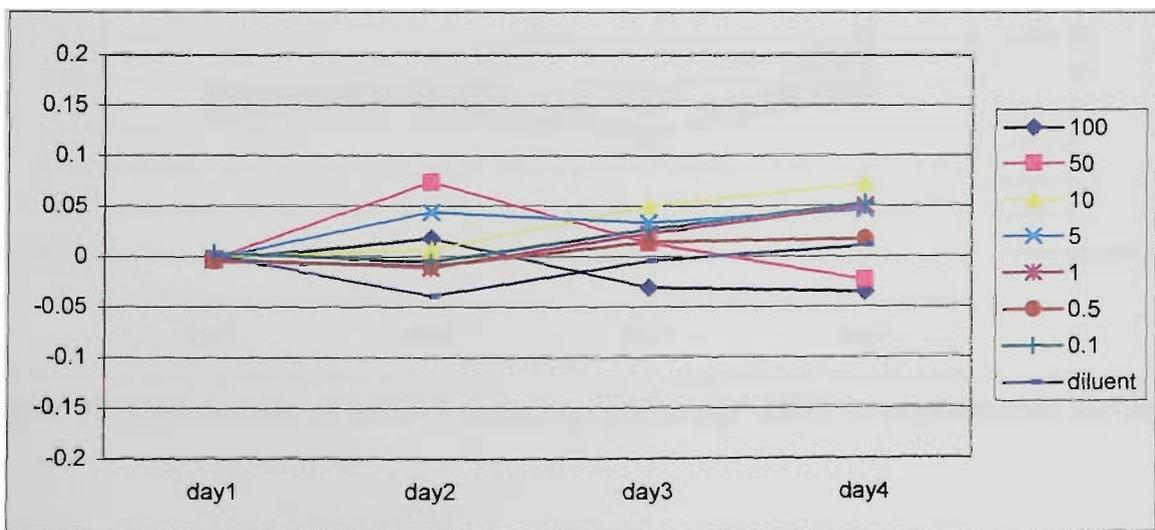


Figure 1C. Cell density of MCF-7 cells exposed to o,p'-DDT in growth media.

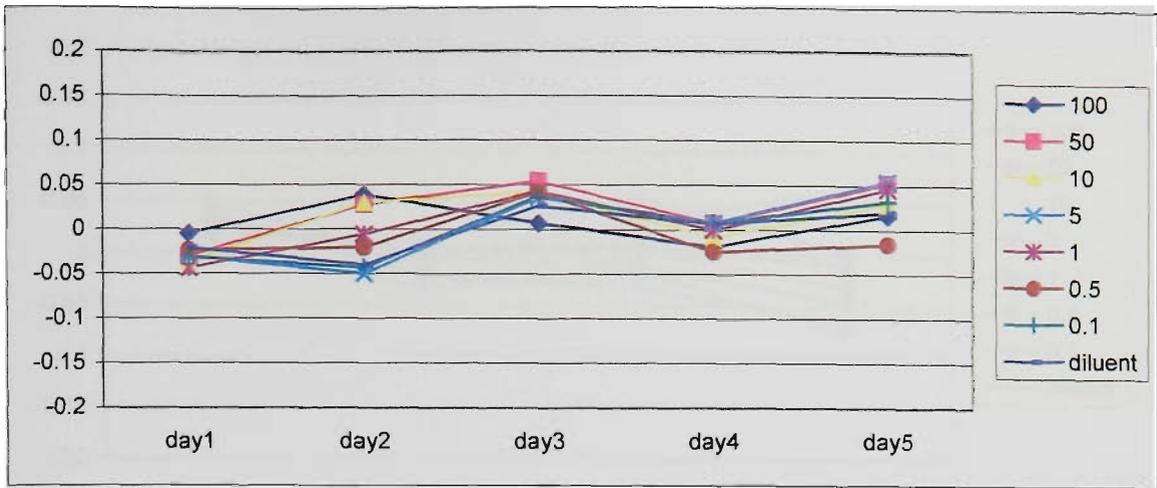


Figure 1D. Cell density of MCF-7 cells exposed to p,p'-DDE in experimental media.

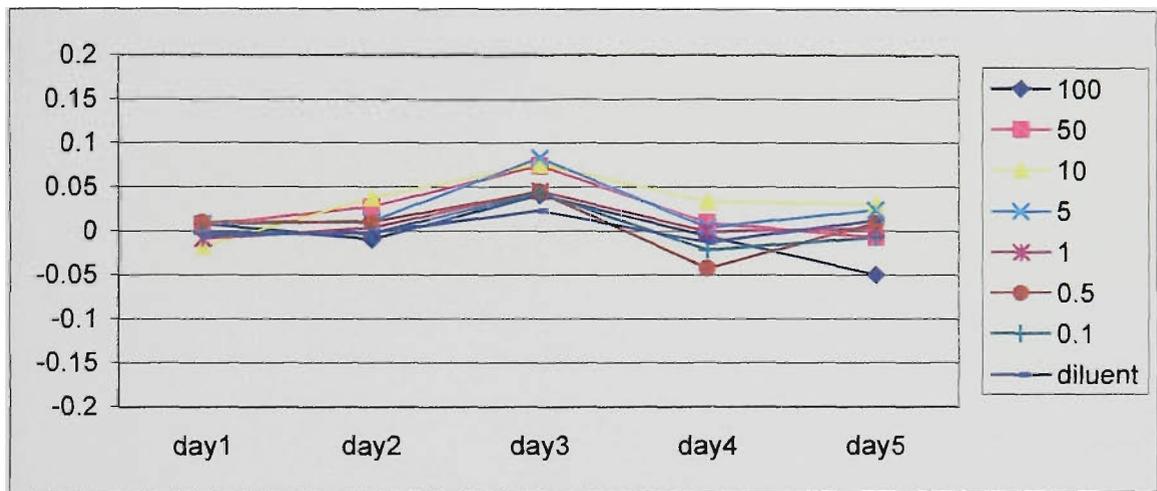


Figure 1E. Cell density of MCF-7 cells exposed to o,p'-DDE in experimental media.

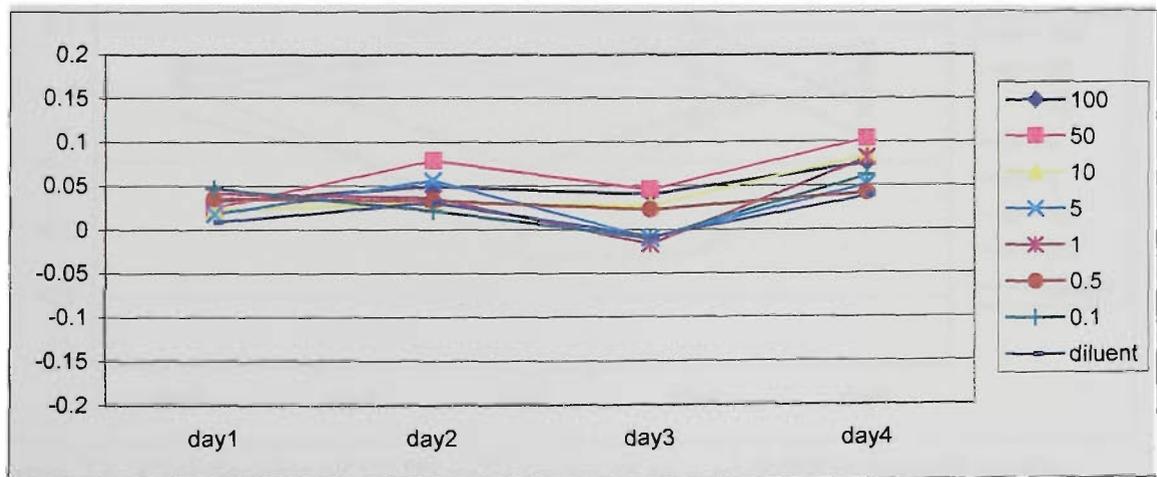


Figure 1F. Cell density of MCF-7 cells exposed to p,p'-DDT in experimental media.

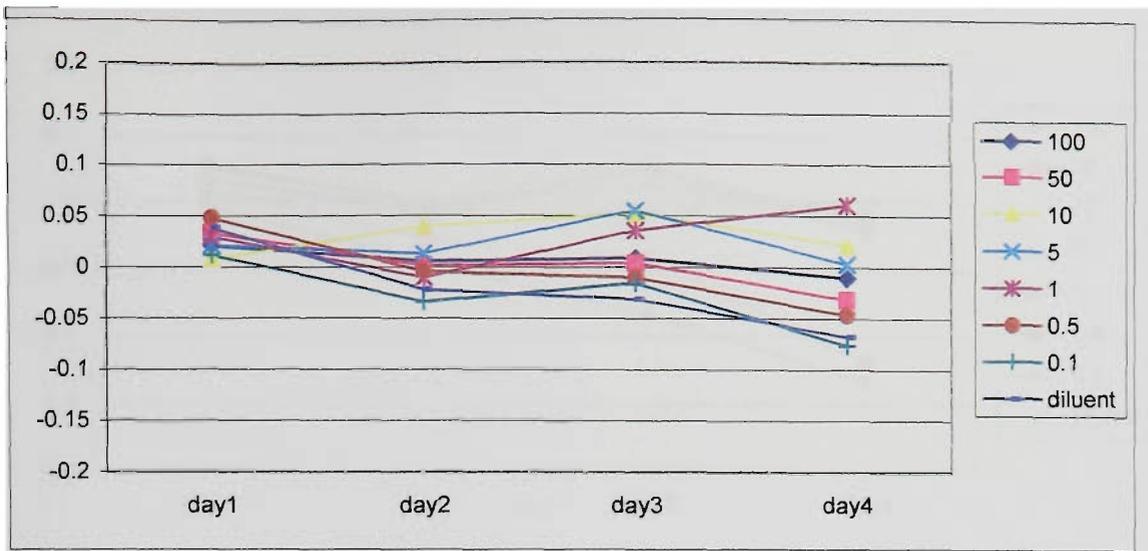


Figure 1G. Cell density of MCF-7 cells exposed to o,p'-DDT in experimental media.

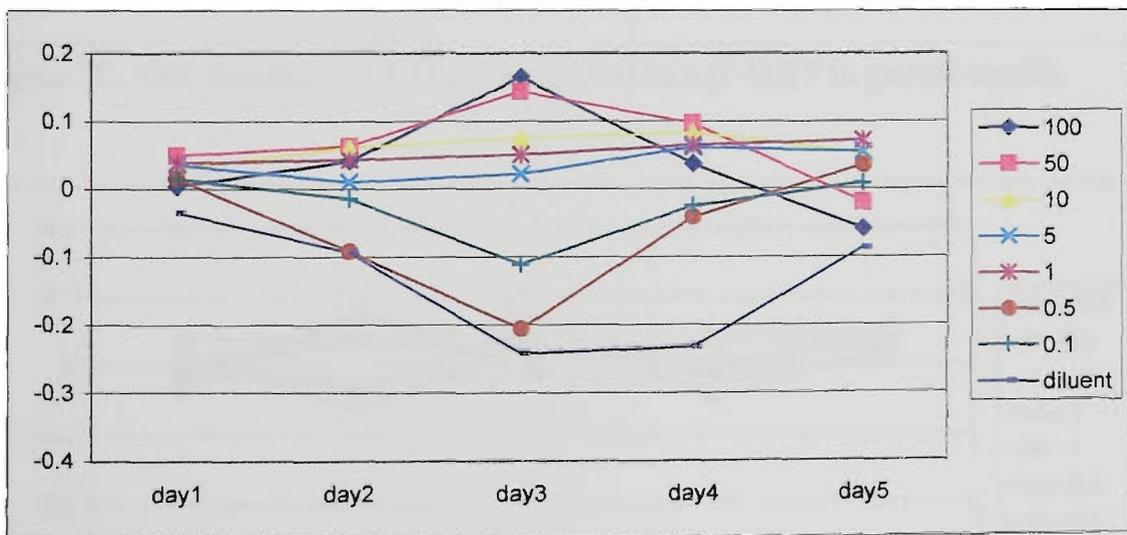


Figure 2A. Cell density of T47D cells exposed to o,p'-DDE in growth media.

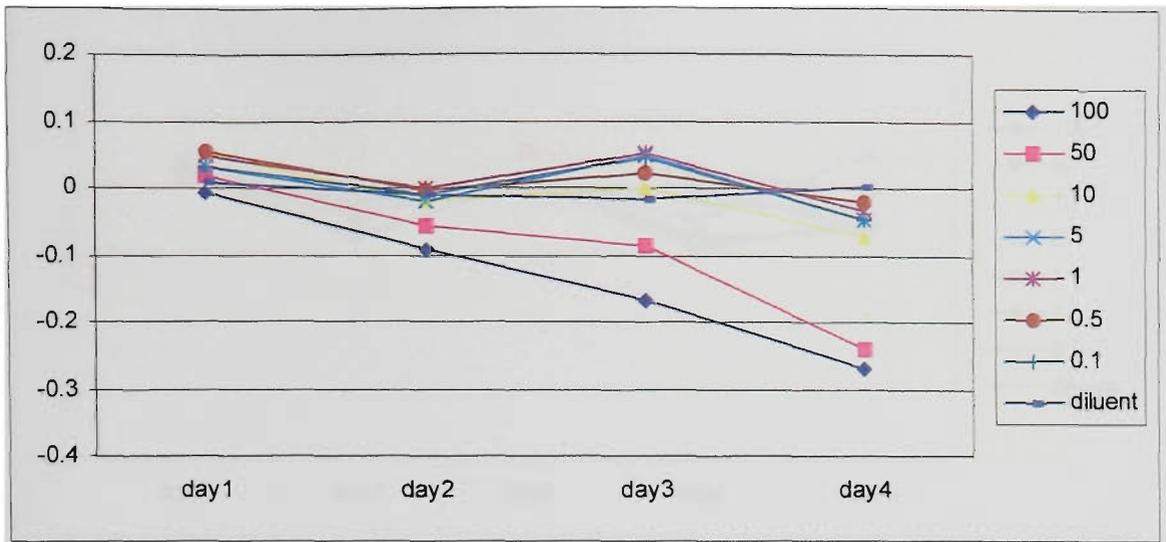


Figure 2B. Cell density of T47D cells exposed to p,p'-DDT in growth media.

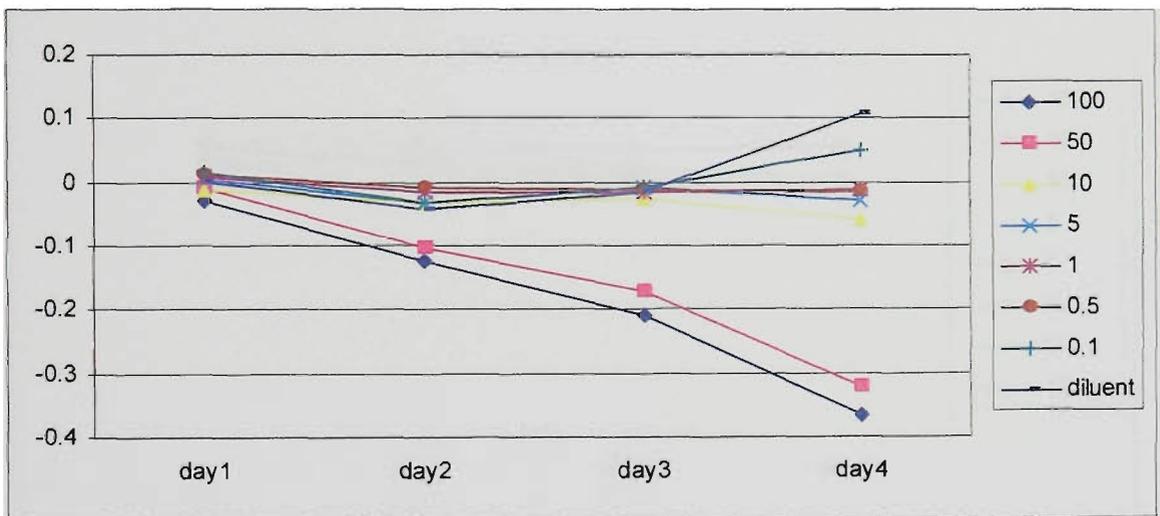


Figure 2C. Cell density of T47D cells exposed to o,p'-DDT in growth media.

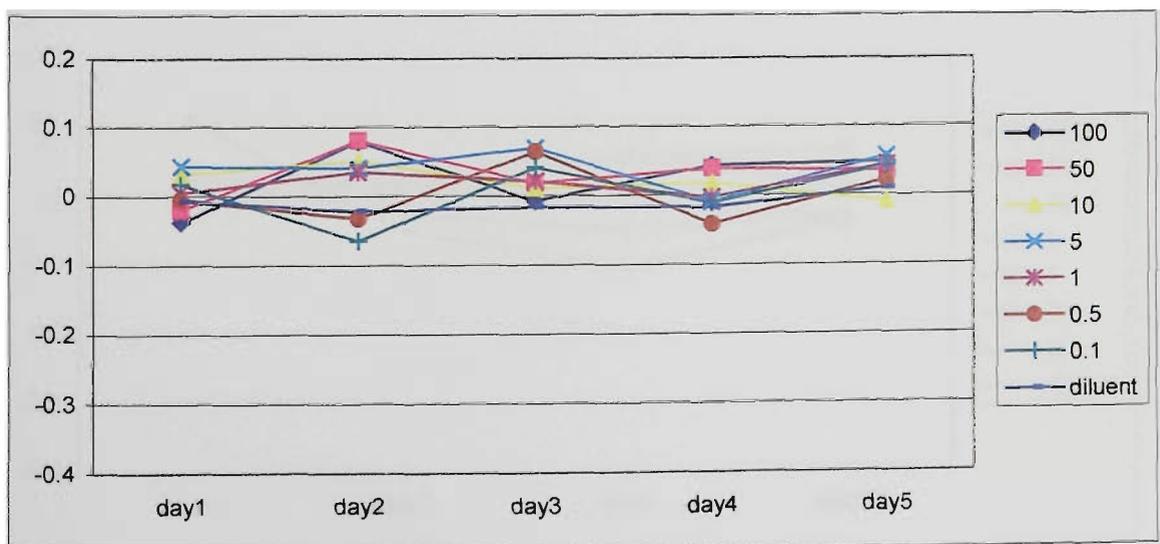


Figure 2D. Cell density of T47D cells exposed to p,p'-DDE in experimental media.

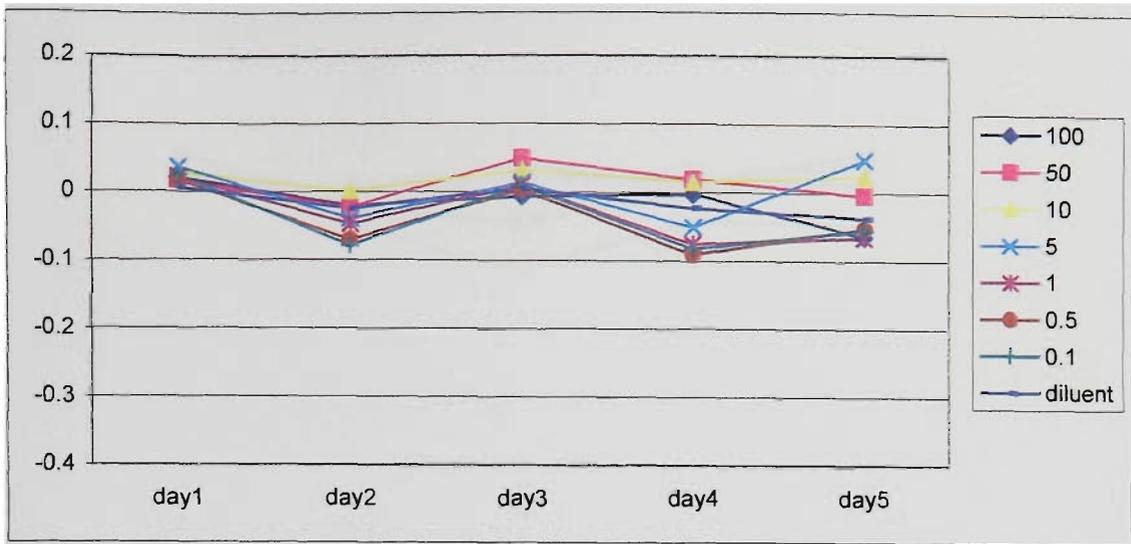


Figure 2E. Cell density of T47D cells exposed to o,p'-DDE in experimental media.

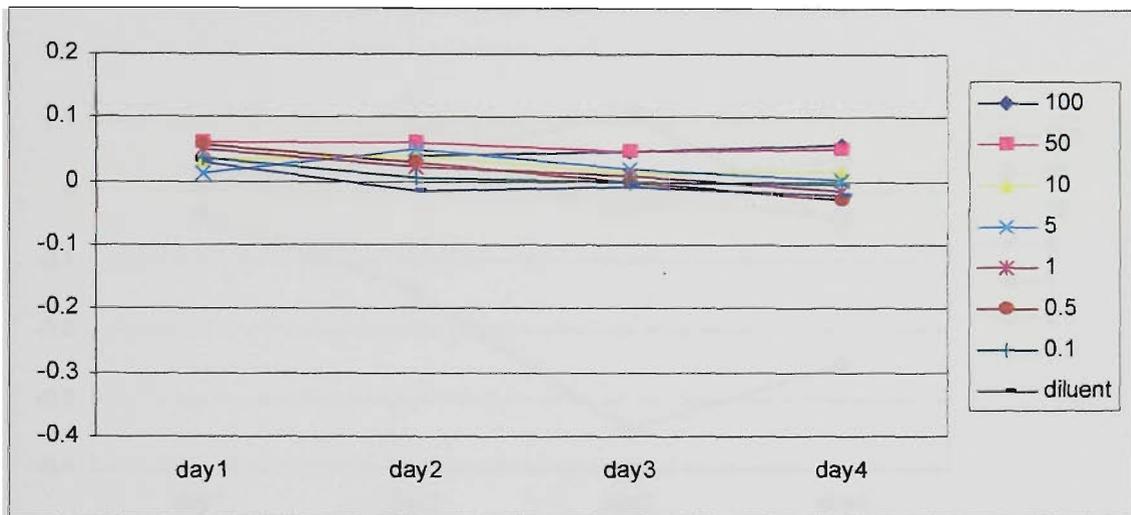


Figure 2F. Cell density of T47D cells exposed to p,p'-DDT in experimental media.

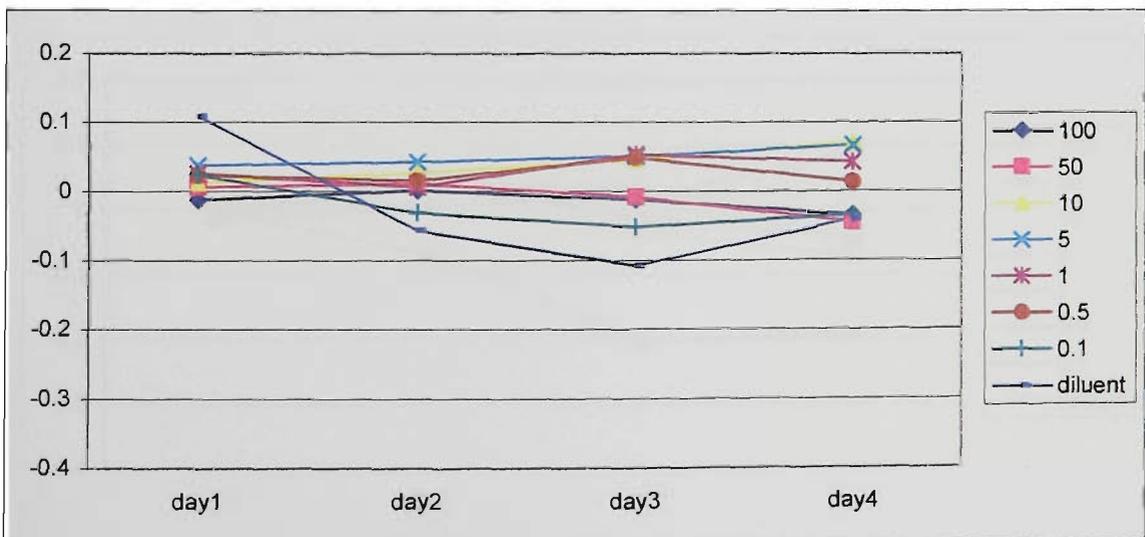


Figure 2G. Cell density of T47D cells exposed to o,p'-DDT in experimental media.

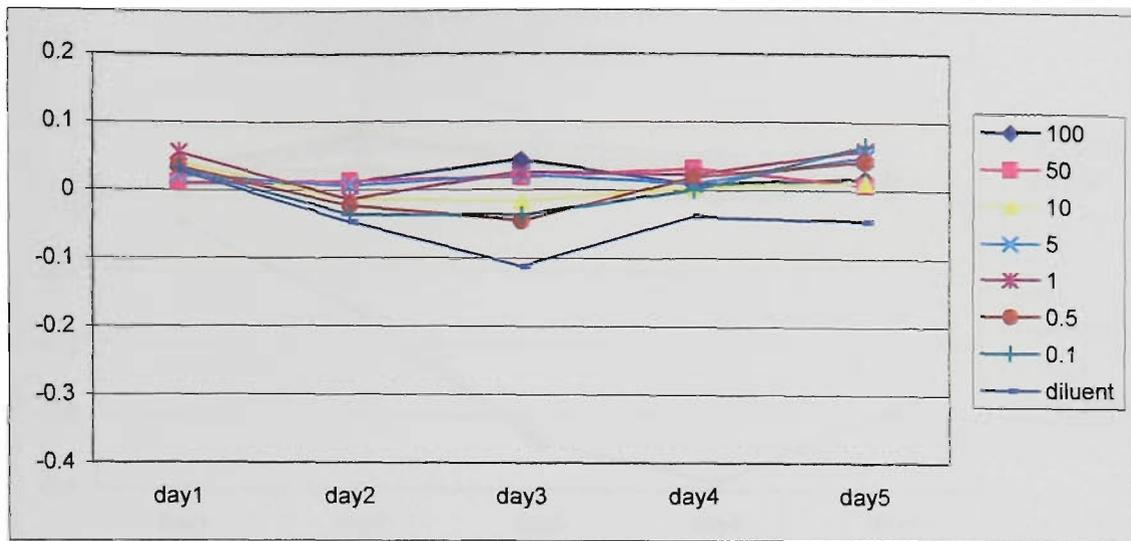


Figure 3A. Cell density of MDA-MB-231 cells exposed to o,p'-DDE in growth media.

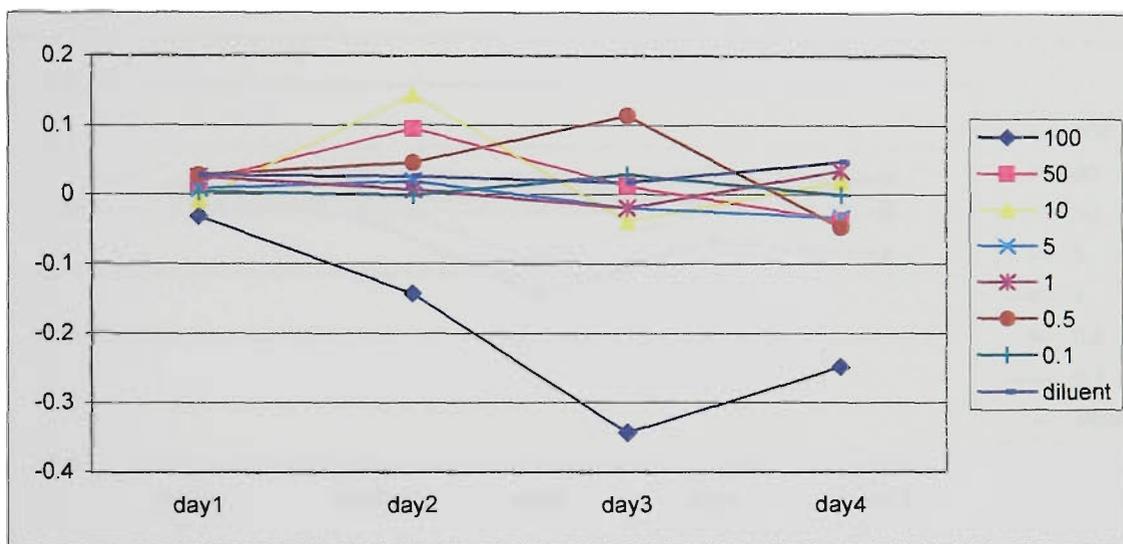


Figure 3B. Cell density of MDA-MB-231 cells exposed to p,p'-DDT in growth media.

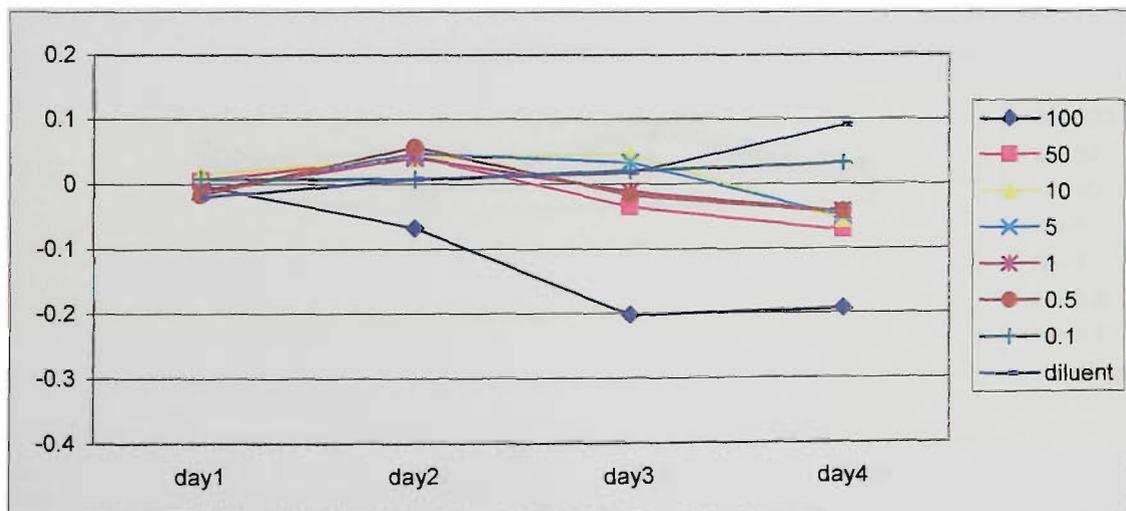


Figure 3C. Cell density of MDA-MB-231 cells exposed to o,p'-DDT in growth media.

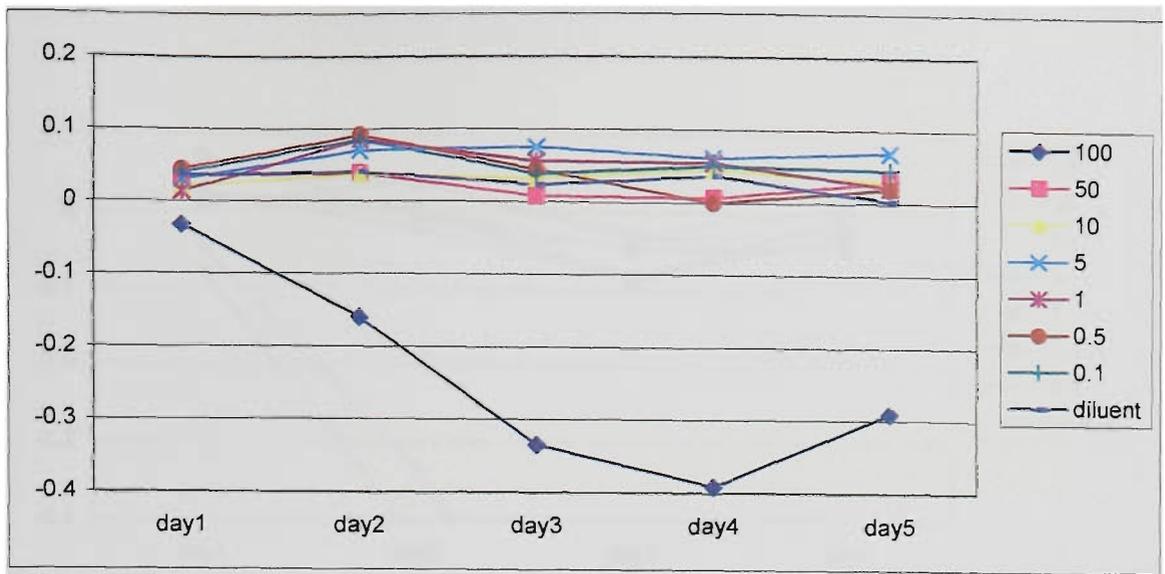


Figure 3D. Cell density of MDA-MB-231 cells exposed to p,p'-DDE in experimental media.

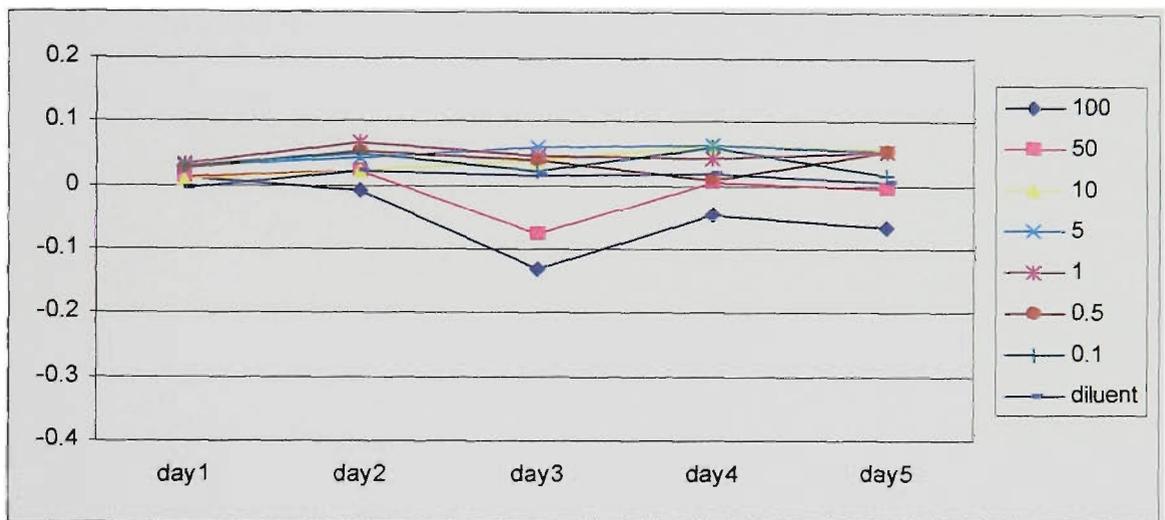


Figure 3E. Cell density of MDA-MB-231 cells exposed to o,p'-DDE in experimental media.

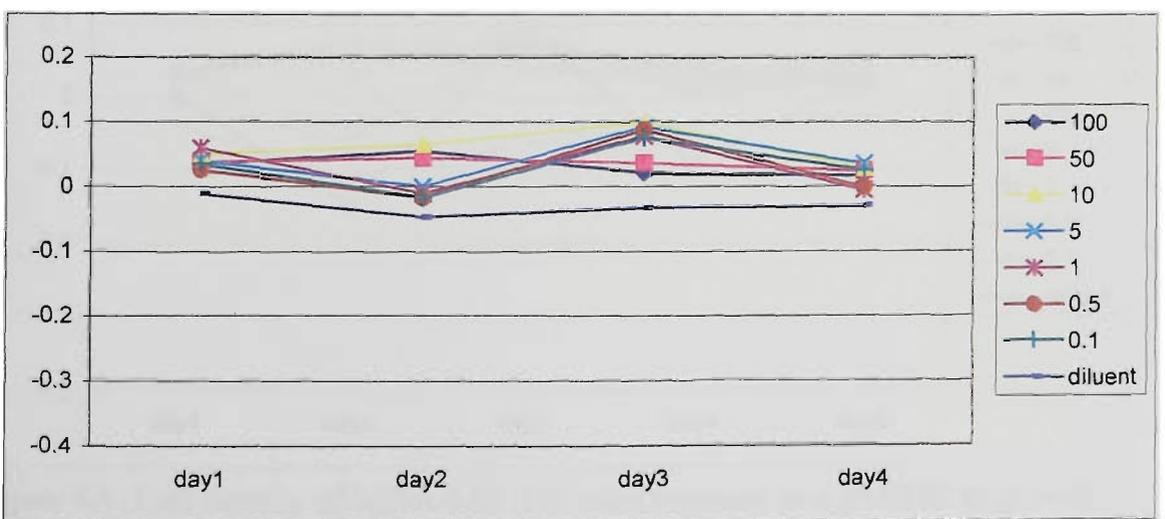


Figure 3F. Cell density of MDA-MB-231 cells exposed to p,p'-DDT in experimental media.

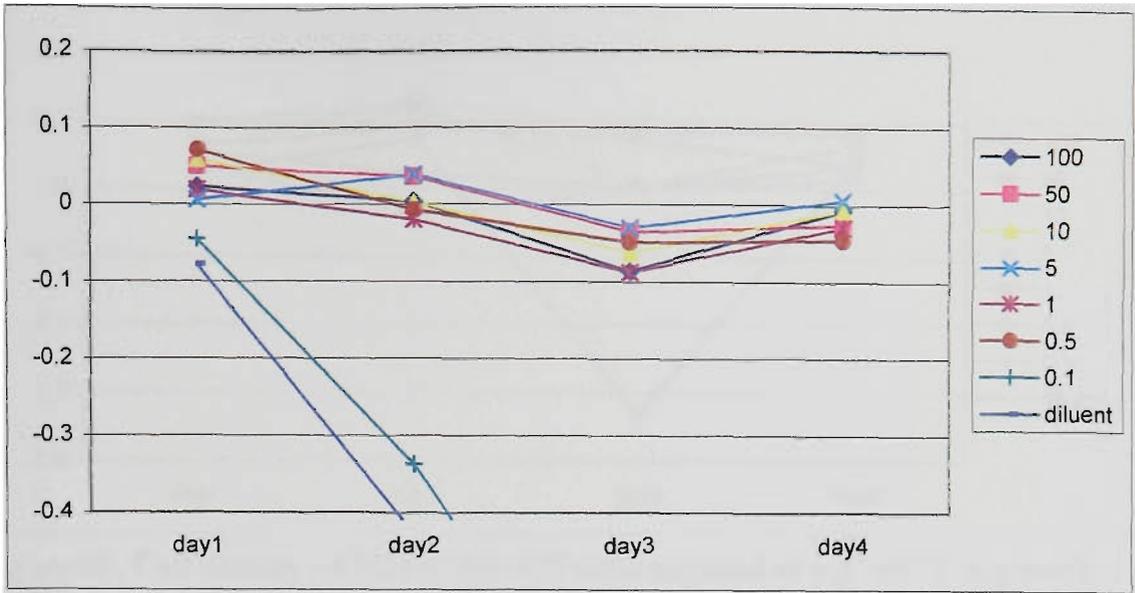


Figure 3G. Cell density of MDA-MB-231 cells exposed to o,p'-DDT in experimental media.

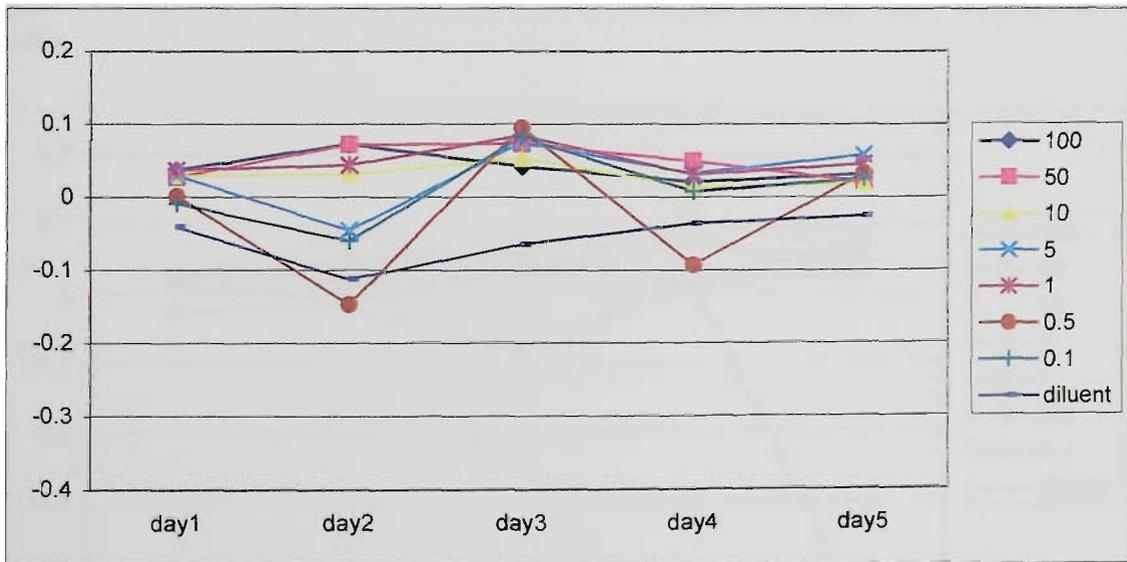


Figure 4A. Cell density of MDA-MB-435 cells exposed to o,p'-DDE in growth media.

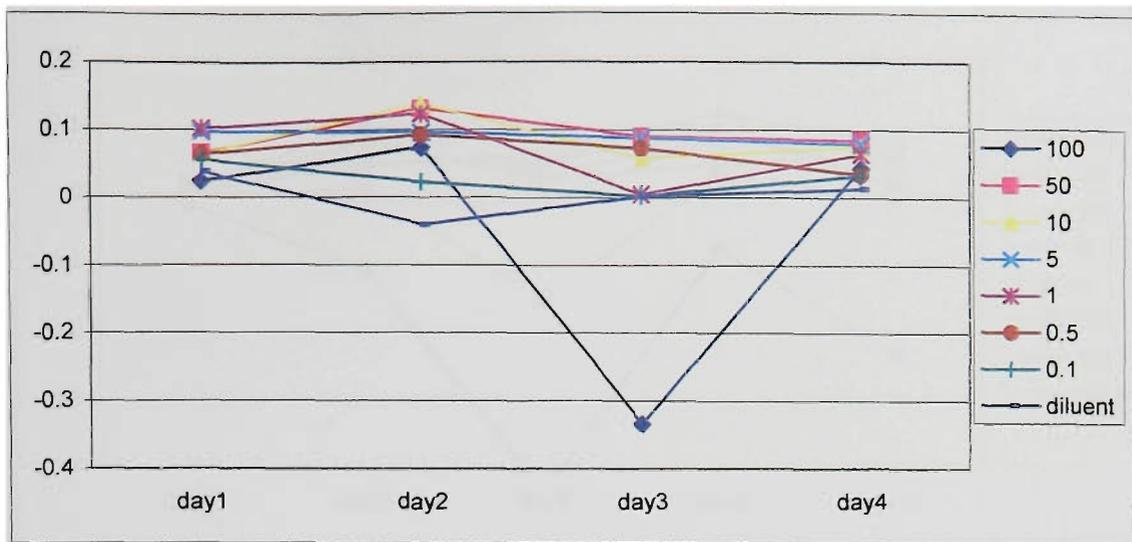


Figure 4B. Cell density of MDA-MB-435 cells exposed to p,p'-DDT in growth media.

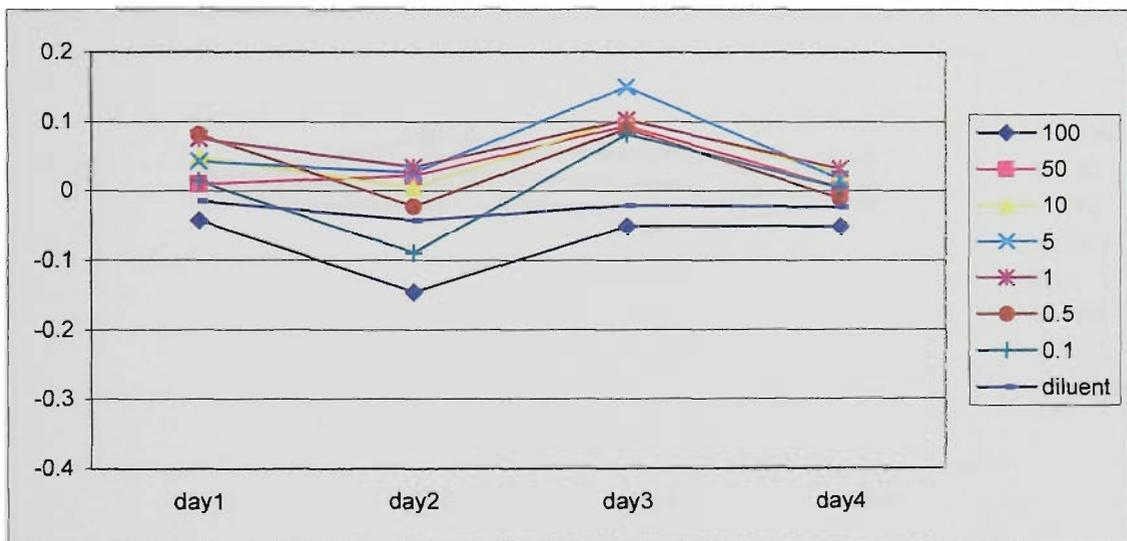


Figure 4C. Cell density of MDA-MB-435 cells exposed to o,p'-DDT in growth media.

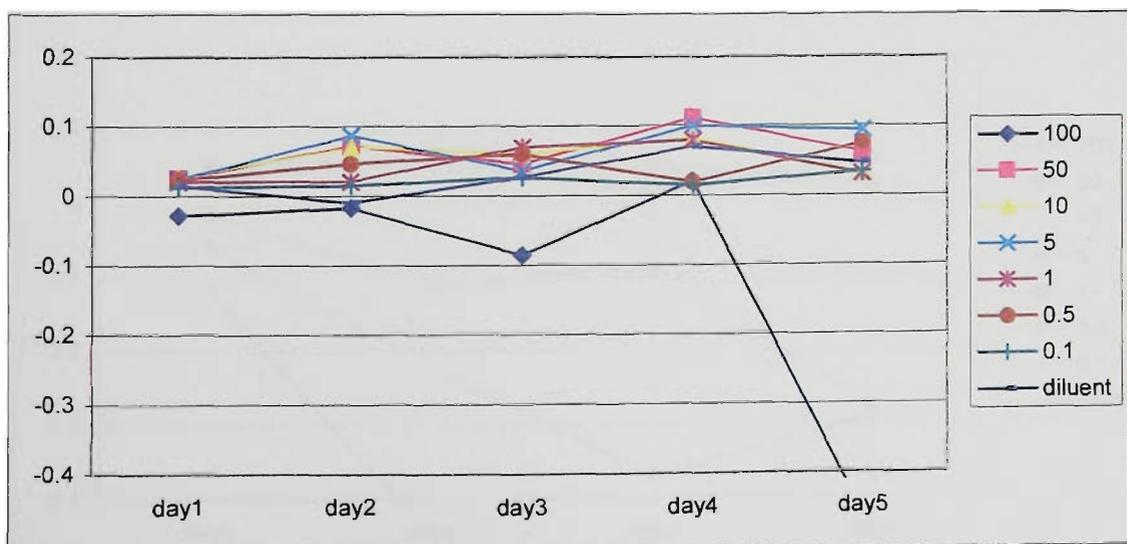


Figure 4D. Cell density of MDA-MB-435 cells exposed to p,p'-DDE in experimental media.

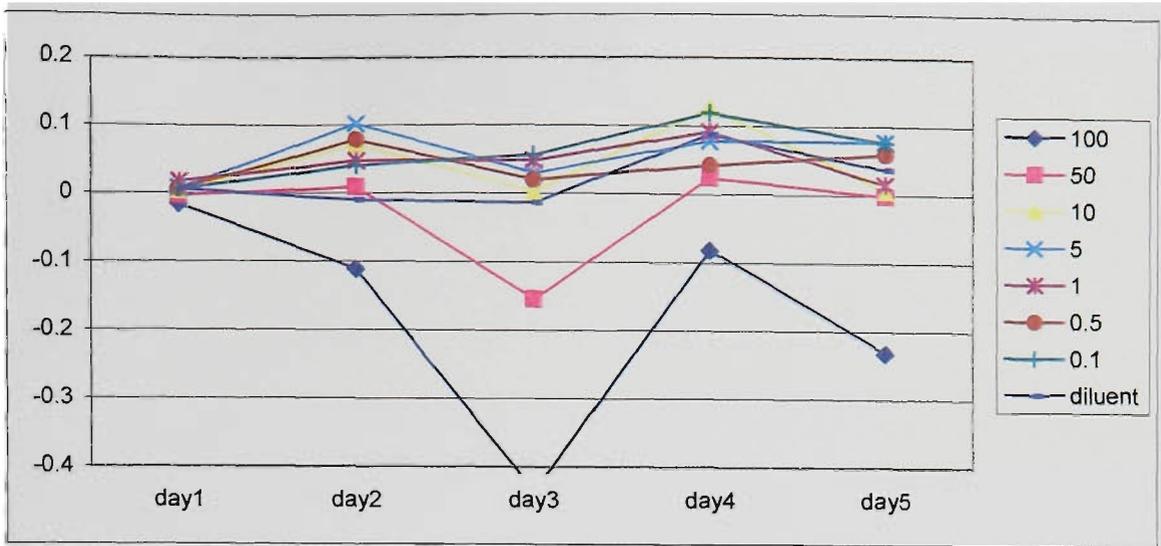


Figure 4E. Cell density of MDA-MB-435 cells exposed to o,p'-DDE in experimental media.

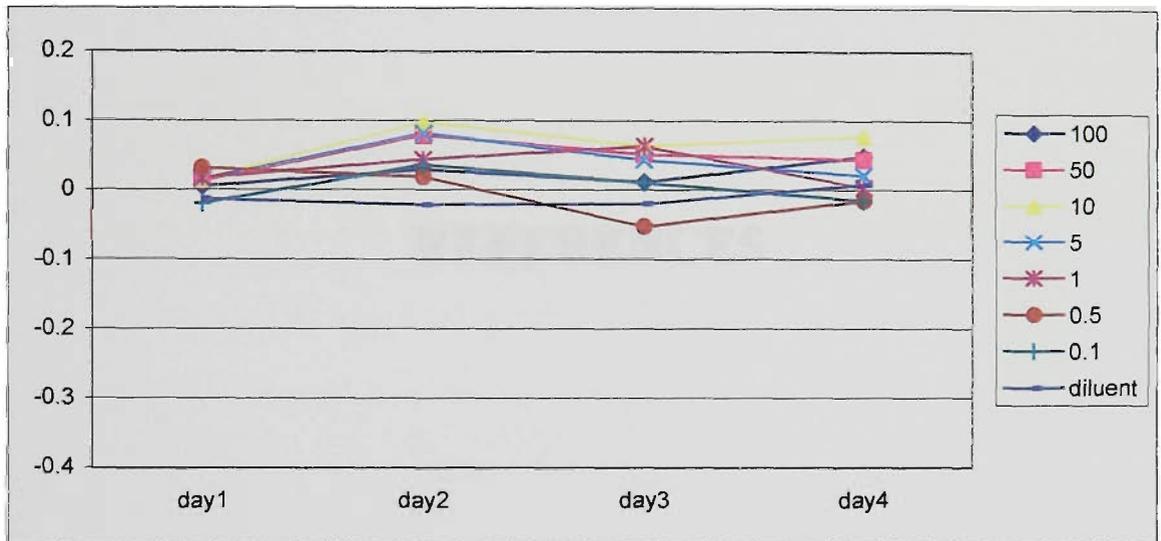


Figure 4F. Cell density of MDA-MB-435 cells exposed to p,p'-DDT in experimental media.

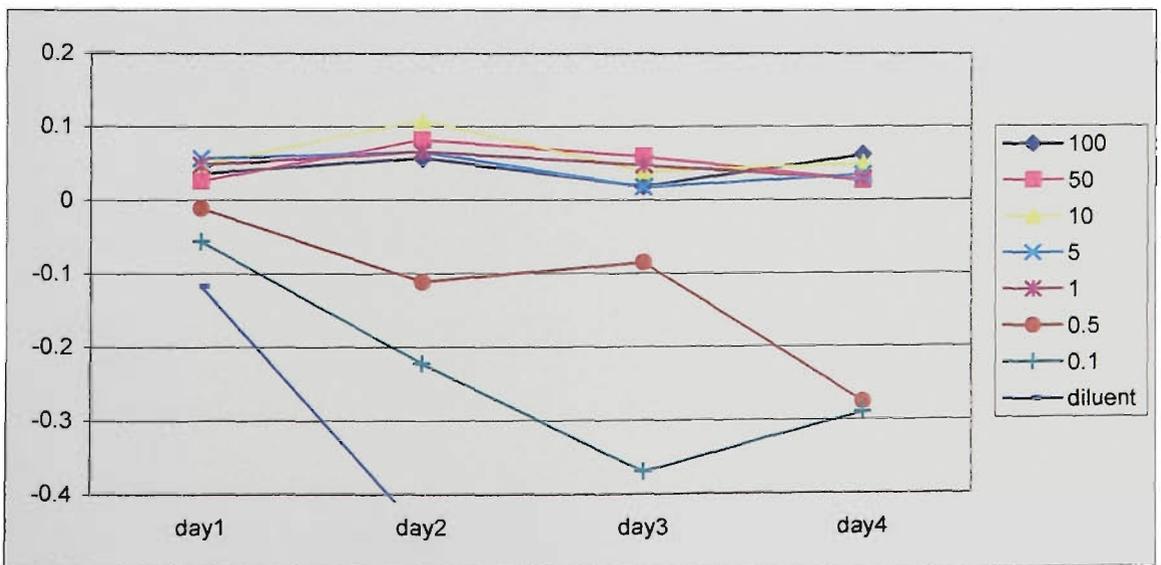


Figure 4G. Cell density of MDA-MB-435 cells exposed to o,p'-DDT in experimental media.

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