PREDICTING NORMAL TISSUE RADIOSENSITIVITY

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ABSTRACT

Two methods of predicting normal cell radiosensitivity were investigated in different patient groups. Plasma transforming growth factor beta one (TGFβ1) levels were measured by ELISA, using a commercially available kit. Residual DNA double strand breaks were measured in normal epidermal fibroblasts following 150 Gy. After allowing 24 hours for repair, the DNA damage was assayed using pulsed field gel electrophoresis (PFGE). Pre-treatment plasma TGFβ1 levels were investigated retrospectively in patients with carcinoma of the cervix in relation to tumour control and late morbidity following radiotherapy. Plasma TGFβ1 levels increased with increasing disease stage. They also correlated with two other known measures of tumour burden i.e. plasma levels of carcinoma antigen 125 (CA125) and tissue polypeptide antigen (TPA). Elevated pre-treatment plasma TGFβ1 levels predicted for a poor outcome both in terms of local control and overall survival. Plasma TGFβ1 levels did not predict for the development of radiotherapy morbidity of any grade. In conclusion pre-treatment plasma TGFβ1 levels predict for tumour burden and tumour outcome in patients with carcinoma of the cervix. Changes in plasma TGFβ1 levels measured prospectively may predict for radiation morbidity and should be investigated. A prospective study was undertaken in patients with carcinoma of the head and neck region. Changes in plasma TGFβ1 levels between the start and the end of a course of radical radiotherapy were investigated in relation to the development of acute radiation toxicity. Patients were categorised according to the pattern of response of their TGFβ1 levels over the course of their treatment. Those patients whose TGFβ1 levels decreased, but did not normalise during radiotherapy were assigned to category 2. Category 2 predicted for a severe acute reaction, as measured using the LENT SOMA score, with a sensitivity of 33% and a specificity of 100%. The positive predictive value of was 100%. As part of the validation of the commercially available TGFβ1 kit, samples were obtained from sixty-six normal volunteers with a wide age distribution. This large series demonstrated an unexpected age-related rise in TGFβ1 levels that had not been previously demonstrated in the literature. In breast carcinoma patients, two assays were performed retrospectively. Both pre-treatment plasma TGFβ1 levels and residual DNA double strand breaks (measured using PFGE) were correlated with clinical outcome. Outcome was in the form of a total LENT SOMA score and late radiation fibrosis score, as measured by clinical palpation. No relationship was demonstrated between either pre-treatment TGFβ1 levels or residual DNA double strand breaks and late radiotherapy outcome. This failed to validate a similar series of patients investigated in the same department using the same technique. This work has shown that measurement of residual DNA double strand breaks using PFGE is not sufficiently robust to be used clinically as a predictor of normal tissue radioresponse. In conclusion, changes in TGFβ1 plasma levels occurring over time during a course of radical radiotherapy, hold promise for the development of a rapid test of intrinsic radiosensitivity.

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ABBREVIATIONS

ANOVA - analysis of variance

BME – basal medium–Eagle's

BMT – bone marrow transplantation

CA125 – carcinoma antigen 125

CLL – chronic lymphocytic leukaemia

CNI – cervical nodal irradiation

CT - computerised axial tomography

ddH_2O – double distilled water

DNA – deoxyribonucleic acid

dsb – double strand breaks

ECM – extra-cellular matrix

EDTA – ethylenediaminetetra-acetic acid

ELISA – enzyme-linked immunosorbent assay

EORTC – European Organisation for Radiation Therapy

EUA – examination under anaesthetic

FDR - fraction of damage released

FIGO – Federacione Internationale de Gynaecologie Oncologique

FOM – floor of mouth

GP – general practitioner

Gy – gray

HBSS – Hank's balanced salt solution without calcium and magnesium

HCC – hepatocellular cancer

HEPES – N - (2-hydroxyethyl) piperazine-N'- (2-ethanesulphonic acid)

HPV – human papilloma virus

IFD – interfield distance

IgG – immunoglobulin G

IgM – immunoglobulin M

kD – kilodalton

LAK – lymphocyte activated killer

LAP – latency associated peptide

LENT SOMA - late effects normal tissues: subjective, objective, management, analytic

LTBP – latent TGF binding protein

LSM – lymphocyte separation medium

MEM – minimum essential medium

MI – myocardial infarction

mRNA – messenger ribonucleic acid

NK – natural killer

NRK – normal rat kidney

OD – optical density

PFGE - pulsed-field gel electrophoresis

PR - per rectum

SF_2 – surviving fraction after 2 Gy

SCC – squamous cell carcinoma antigen

TBE – TRIS – borate – EDTA

TBI – total body irradiation

TCC – transitional cell carcinoma

TGFβ - Transforming growth factor beta

TGFβRI - TGFβ receptor type I

TGFβRII - TGFβ receptor type II

TPA – tissue polypeptide antigen

TRIS – tris (hydroxymethyl) - aminomethane

rcf – relative centrifugal force

rpm – revolutions per minute

WHO - world health organisation

WLE - wide local excision

CHAPTER 1: INTRODUCTION

1.1 Aetiology of cancers studied in this thesis

The incidence of cancer in the population is set to rise in the 21st century for two main reasons. First, as the size of the population increases, there is an absolute rise in the number of cancer cases diagnosed. Second, as the average age of the population increases, the incidence of cancer also rises. This occurs as most tumours show a significant age-related increase in incidence. At present, one in four people will develop cancer during their lifetime. By the middle of the century, this rate is expected to rise to one in three.

Cervical cancer

The incidence of cervical cancer in the UK is currently 10.4 per 100,000 of the female population (Factsheet 1.4, Cancer Research Campaign, 1998). For the year 1997, the mortality rate was 46 per 1,000,000 women (CRC CancerStats: Mortality – UK, 1999). Approximately 4,500 cases of cervical cancer are seen each year in the UK and this incidence is currently decreasing (Factsheet 12.1, Cancer Research Campaign, 1994)). It is thought that the widespread introduction of screening has been at least partly responsible for this (Gatta *et al*, 1999). Mortality rates vary greatly with disease stage at presentation: less than 10% of women with stage IV disease survive five years compared with between 80 and 90% of those women with stage I disease (Magos *et al*, 1991, Marcial and Marcial, 1993). There are a number of risk factors for the development of cervical cancer, of which

sexual factors are the strongest. Low age at first intercourse and multiple sexual partners are the greatest predictors of disease. The link between oral contraceptive pill use and cervical cancer has yet to be fully elucidated, but this may merely represent clustering of risk behaviour. A sexually transmitted factor has been proposed for many years, and recently attention has focused on the human papilloma virus (HPV), especially HPV types 16 and 18 (Wang *et al*, 1997). Smoking greatly increases the risk of developing cervical cancer, but is less strong than sexual factors. Cervical cancer is also commoner in women in lower socio-economic classes. In the past, more than 95% of cervical cancers were of squamous histology. In recent years, there has been a slight increase in the percentage of adenocarcinomas seen (Stockton *et al*, 1997).

Head and neck cancer

Overall there are approximately five thousand new cases of head and neck cancer registered in the UK every year (Factsheet 1.3, Cancer Research Campaign, 1998) and at least 2,500 disease-related deaths (CRC CancerStats: Mortality – UK, 1999)). Apart from tumour stage and nodal involvement, tumour site is also an important prognostic indicator. There is a male preponderance in the incidence of head and neck tumours, with a sex ratio of between 1.4 and 2 male cases for every female, in the various tumour types. These tumours occur predominantly in the lower socio-economic groups. Tumours of the head and neck region form a heterogeneous group. The aetiology, biological behaviour, treatment and prognosis of these cancers vary depending on their site of origin (DeVita *et al*, 1997; Wang, 1997b). In this thesis, cancers of the orbital tissues and central nervous system are not included in the definition of head and neck tumours. The tumours discussed

here comprise six groupings. The first group comprises tumours of the larynx. The second group constitutes tumours of the oral cavity. This comprises tumours of the lip, floor of mouth (FOM), anterior two-thirds of the tongue, buccal mucosa, hard palate, alveolar sulci and retromolar trigone. The third grouping is tumours of the pharynx. The fourth group of tumours comprises those of the nasal cavity and sinuses. The fifth group includes tumours of the ear. The sixth and final grouping constitute tumours of the salivary glands, both major and minor. The majority of these tumours are benign, but prone to local recurrence (Renehan *et al*, 1996).

The majority of head and neck tumours exhibit a squamous histology. Other tumour types are seen, but none is particularly common. Malignant mucosal melanomas are rare, accounting for less than 10% of all melanomas, but occur with similar frequencies in the head and neck, anal and genital regions (Pandey *et al*, 1998). Benign tumours of the salivary glands are generally pleomorphic salivary adenomas. Malignant salivary tumours are uncommon and comprise a number of pathologies e.g. adenoid cystic carcinoma, adenocarcinoma, muco-epidermoid carcinoma, but real rarities, such as small cell carcinoma do occur (Renehan *et al*, 1996).

The most potent risk factor in developing any form of head and neck cancer is tobacco. This applies to cigarette and pipe smoking, as well as chewing tobacco. Cigarette smoking is a greater risk factor than tobacco smoking. Ingestion of alcohol is the second factor. These risk factors are synergistic rather than additive in combination (Lewin *et al*, 1998). Other risk factors tend to be specific for the development of head and neck cancer in a given site. Adenocarcinomas of the paranasal sinuses are found more commonly in those

people who have worked with wood. Infection with the Epstein-Barr virus is a recognised risk factor in the development of nasopharyngeal cancer, especially in those patients who originate from South East Asia. Exposure to ultra-violet radiation is associated with an increased risk of carcinoma of the lip (Pogoda *et al*, 1995). Carcinoma of the oral cavity has been linked with long-term trauma from e.g. poor dentition. Oral cancer secondary to infection with syphilis is decreasing in incidence (Dickenson *et al*, 1998). Carcinoma of the hypopharynx is commoner in patients with iron-deficiency anaemia and upper oesophageal webs i.e. Brown-Kelly-Paterson (Plummer-Vinson) syndrome (Wahlberg *et al*, 1998).

Breast cancer

Women in the UK have a 1 in 10 risk of developing breast cancer during the course of their lifetimes. This risk has been increasing in recent years. With the advent of screening, cancer incidence has increased in the screened population. This is due to the increased detection of impalpable, asymptomatic tumours. The mortality from breast cancer has decreased in some patient groups over the past ten years. Currently breast cancer accounts for over 13,000 deaths per annum in the UK (CRC CancerStats: Mortality – UK, 1999).

Breast cancer is commonest in older age groups, with a marked age-related rise in incidence. As much as 5% of the total incidence of breast cancer may be directly related to genetic factors, such as the presence of germline mutations in the BRCA1 and 2 genes. These are likely to account for a disproportionately large amount of the breast cancer incidence in women under 40 years of age (Frank *et al*, 1999). The risk of breast cancer is greatest in women of higher socio-economic status. Women with a late menopause or early

menarche are also at increased risk of breast cancer. The risk of developing breast cancer is decreased in those women whose first full term pregnancy is before 17 years of age. Pregnancy has a protective effect on developing breast cancer unless the first full term pregnancy occurs after 35 years of age (Ghadirian *et al*, 1998). Women who take the oral contraceptive pill have an increased risk of breast cancer during and for the ten years following use. Their risk then drops to that of their non-pill taking contemporaries. The risk of breast cancer associated with the use of combined hormone replacement therapy is, as yet, unquantified. Finally, ionising radiation is an accepted risk factor in the development of breast cancer (Mattsson *et al*, 1995).

Adenocarcinoma is the most common histology. Most cancers arise in the upper outer quadrant of the breast. Sixty to seventy per cent of breast cancers are ductal adenocarcinomas of no special type. Twenty to thirty per cent of breast cancers are lobular adenocarcinomas. The remaining breast cancers are made up of tumours of special histological type that confer an improved prognosis. These include tubular and mucinous adenocarcinomas (Pereira *et al*, 1995).

1.2 Radiotherapy

At present, 50% of patients with a diagnosis of cancer receive radiotherapy at some stage in the course of their disease. Radiotherapy is estimated to be directly responsible for at least 40% of those who survive beyond 5 years (Sikora 1999). In a few tumour types, e.g. cervix, radiation is the mainstay of curative treatment. For some tumour types, e.g. head and neck cancer, radiotherapy offers similar cure rates to radical surgery, but with the added

advantage of functional organ preservation (DeVita *et al*, 1997). In other cancers, e.g. breast, adjuvant radiotherapy is given following surgery to decrease the rate of local recurrence. In a few tumour types, e.g. ovary and teratoma, the role of radiotherapy is limited mainly to the palliative setting.

Radiotherapy is a local treatment. The aim of radiotherapy is to deliver a tumouricidal dose of ionising radiation to a well-defined area, known as the planning target volume. The ionising radiation is most commonly delivered as photons or electrons. Access to proton therapy is severely limited in this country. Treatment with neutrons and heavy particles is reserved for trial settings. The planning target volume is comprised of three main parts. First, any gross macroscopic tumour, either in those patients for whom radiotherapy is the primary treatment modality, or following incomplete surgical resection. Second, the presumed extent of any microscopic tumour remaining. This varies between tumour types and is derived from the examination of surgical and post-mortem specimens. It may also include the rest of the organ at risk, e.g. all the breast tissue remaining in the ipsilateral breast following wide local excision (WLE) of the tumour in breast cancer. Finally, there is a margin allowed for physical factors such as patient movement during respiration and machine set-up variation (Dobbs *et al*, 1992; DeVita *et al*, 1997).

Cervical cancer

Treatment of stage I disease generally involves radical surgery, using Wertheim's hysterectomy. In those patients medically unfit for or who refuse surgery, treatment is with radical radiotherapy. Treatment of Stage II - IV disease involves radical radiotherapy.

Radiotherapy is administered with a mixture of external beam treatment and brachytherapy, depending on disease stage and local protocols. There is increasing evidence for the use of concurrent chemo-radiotherapy in the treatment of locally advanced disease (Morris *et al*, 1999; Rose *et al*, 1999). Overall, approximately 55% of women diagnosed as having cervical cancer survive 5 years or more, the bulk of these being due to radiation treatment (Marcial and Marcial, 1993).

Head and neck cancer

Treatment for carcinoma of the head and neck depends on the site and stage of the disease and may often involve combined modality therapy with surgery and radiotherapy. Concurrent chemo-radiotherapy is becoming increasingly popular, especially in those tumour types with a poor prognosis (reviewed in Haffty 1999). Small good prognosis tumours are generally treated with radical radiotherapy. This affords cure rates comparable with radical surgery, while allowing organ and hence voice preservation. This has a significant positive effect on a patient's quality of life. Curative surgical excision remains an option reserved for those patients who develop isolated local recurrences. Methods of surgical reconstruction have advanced greatly in recent years. Advanced tumours that respond poorly to primary radiotherapy can sometimes be surgically removed, with successful functional reconstruction. Radiotherapy can be given post-operatively to those at high risk of recurrence (Ravasz *et al*, 1993). This may involve radiotherapy to the primary tumour only, e.g. when there is lymphovascular invasion; or to the neck nodes only e.g. when there is extra-capsular nodal spread. Post-operative radiotherapy can be given to both these regions simultaneously. Treatment for cancers of the head and neck region are best

decided in the context of a multi-disciplinary team, with in-put of both clinical oncologists and surgeons who specialise in the treatment of tumours of this type. Following radiotherapy, five year local control rates for T_1 and T_2 carcinoma vary between 20 and 77% depending on tumour site and nodal status. For more locally advanced tumours this figure falls to 0 – 37% (Hong *et al*, 1990). The picture is further complicated by the fact that patients treated initially with either surgery or radiotherapy can sometimes be salvaged, at relapse, by the other treatment modality.

Breast cancer

It is now currently accepted that local recurrence rates are no different between those women who have a simple mastectomy and those who have a wide local excision of their tumour (lumpectomy) and radiotherapy to the remainder of the breast (Hess and Schmidberger, 1998). This has led to a trend toward breast conserving surgery. This is especially true of women with smaller tumours, or those with slightly larger tumours and more substantial breasts. Most cancers detected by screening are amenable to breast conserving surgery. Thus, with the advent of screening the number of patients who receive radiotherapy is increasing. The main impact from radiotherapy is the decrease in local relapse rates from approximately 30 - 40% (over all stages), to approximately 5%, in those patients receiving adjuvant radiotherapy. Recently, evidence is accruing for a survival benefit in patients with poor prognosis disease treated with chest wall radiotherapy following mastectomy (Overgaard *et al*, 1997). Current practice is to give appropriate systemic treatment, in addition to radiotherapy, to improve survival rates. This systemic

treatment may involve cytotoxic chemotherapy, hormonal manipulation to induce the menopause, the anti-oestrogen tamoxifen or a combination of all three (Cufer 1999).

When prescribing radiotherapy to the breast, the whole of the breast is treated via a tangential two-field arrangement. As the breast curves around the chest wall, it is impossible to treat the whole of the breast without treating some underlying lung tissue. In left-sided treatments, part of the heart may also be treated. In patients whose axillary contents have been removed (complete axillary clearance), further treatment is generally only given to the axilla if nodal involvement is heavy or there is residual disease. In patients with a negative, but adequate axillary sample (more than four nodes recovered), no further treatment is required to the axilla. In patients with a positive or inadequate sample, further treatment to the axilla may be required. This involves irradiation of the axillary contents and varies according to local protocols.

1.3 Radiation tolerance

The dose of radiation that can be delivered to a patient is limited by the sensitivity of the surrounding normal tissues. This intrinsic radiosensitivity varies from site to site within the body, but all normal tissues have a specific radiation tolerance. Thus, the site of a tumour has an impact on the radiation dose which can be delivered, which in turn affects the radiocurability of any given tumour (Tan *et al*, 1997). The radiation tolerance doses of different tissues were discovered initially by trial and error, but more recently complex mathematical models have been developed. For most tumours, doses are prescribed to cause a population incidence of severe late radiation side effects of 5% or less. In the case

of the spinal cord, where severe late radiation side effects must be avoided at all costs, the dose prescribed is reduced below the population threshold of late radiation side effects.

Most tumours show a dose response for the probability of obtaining local tumour control. This dose-response curve is sigmoidal in shape, with the steepest part of the curve occurring over the range of therapeutic doses delivered. Thus, for any small increase in radiotherapy dose delivered, the rate of tumour control rises rapidly. However, normal tissues have a dose response relationship between dose delivered and the risk of severe late radiation sequelae that is also sigmoidal in nature. This curve is shifted to the right of the tumour dose-response curve. This means that for any dose of radiation delivered, there is a risk of late radiation sequelae. Over the therapeutic range, any gains in local control made by increasing the radiation dose are offset by a sudden rapid rise in the incidence of late radiation side effects (Hall 1994; DeVita *et al*, 1997). This is illustrated in Figure 1.1.

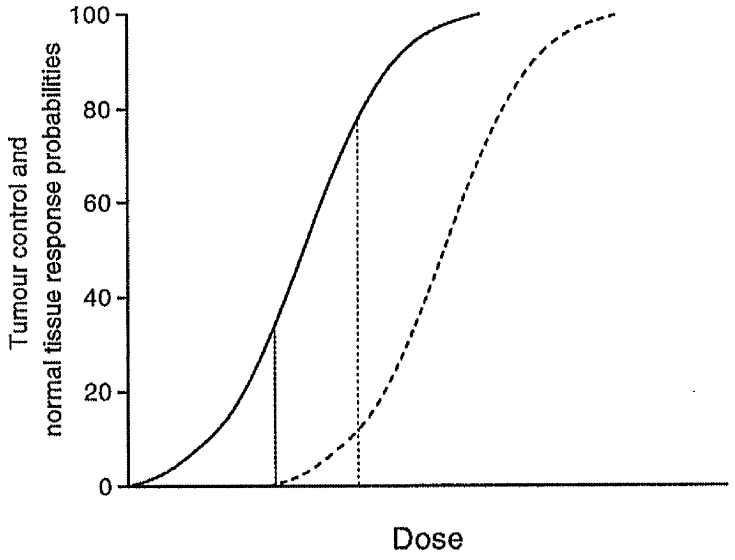


Figure 1.1: The relationship between tumour control probability (solid line) and normal tissue complication probability (broken line) (taken from Burnet *et al*, 1996)

1.4 Side effects of radiotherapy

Radiotherapy is a treatment that is directed locally at a specific area. Therefore most side effects experienced by patients are dependent on the area being treated. Local radiation side effects can be divided into two broad categories: acute and late. Acute side effects often include a systemic component, which in most patients is limited to a feeling of tiredness and lethargy during treatment and for the subsequent 4 - 8 weeks. Some patients also experience anorexia. This is thought to result from cytokines, released by dead and dying cells, leaking into the circulation and mediating systemic effects via an endocrine effect. Patients generally only experience severe nausea and vomiting if the liver or small intestine are irradiated (Fossa *et al*, 1999). As the number of people with cancer in the population rises, increasing numbers of patients will receive radiotherapy. Many of these patients will be elderly and have a significant burden of co-morbid disease. As a patient's performance status worsens, their ability to tolerate complex treatments with significant side effects is decreased (Pignon *et al*, 1998).

Acute side effects

Acute side effects start during treatment and can continue for up to six months following the completion of treatment. In many patients, they have fully resolved two months following the completion of treatment. The severity of acute side effects varies from person to person, but is unpredictable. Acute side effects are thought to be result from depletion of viable stem cells in the basal compartments of tissues with a high turnover (Wang, 1997). Thus, acute side effects most commonly affect the skin, haemopoietic tissues and mucosal

surfaces such as the gut, mouth and vagina. Table 1.1 summarises the histological features associated with acute radiation reactions in the skin and subcutaneous tissues.

For acute effects, the main determinants of side effect severity are the time course of the radiation and fraction size (Hall 1994; DeVita *et al*, 1997). Acute radiation side effects are mainly seen in those tissues with a high turnover. Thus, side effects are only seen when cell death in rapidly dividing tissues outweighs replacement of cells from the stem cell compartment. The longer the time allowed for repopulation, the less severe the side effects. Increased fraction size leads to an increase in cell kill per fraction. This puts increased pressure on the repopulation ability of the tissue, leading to increased severity of acute effects. Providing tissue tolerance is not exceeded and the stem cell compartment too depleted to recover, acute radiation side effects will settle, despite their severity. Severe prolonged acute side effects, e.g. mucosal ulceration, leaves the underlying soft tissues unprotected and vulnerable to pathogens. This can lead to increased risk of late side effects, but is not as a direct result of the acute side effects themselves. These are known as consequential late effects (Denham *et al*, 1999).

Late effects

Late radiation side effects are divided into two overlapping categories: consequential and generic. For many years it was thought that the severity of acute radiation side effects bore no relationship to the development of late radiation sequelae. This has recently been brought into dispute (Turesson *et al*, 1996; Denham *et al*, 1999). It is now accepted, that while the majority of late radiation sequelae do indeed occur de novo, some arise as a result

of damage consequent on severe acute reactions. This is most important where the skin or mucosal barrier has been breached allowing the entry of infection during treatment. Late radiation sequelae do not appear until at least six months following the completion of treatment, and may not appear for many years. Once established, they are progressive and generally irreversible (Turesson *et al*, 1996). They are therefore more serious than acute reactions. Histological features of late radiation sequelae in the skin and subcutaneous tissues are displayed in Table 1.1. Late radiation sequelae are manifest as functional loss. Although the histological changes seen in tissues with late radiation sequelae are well established, their aetiology is complex and poorly understood. Controversy exists as to the precise aetiological mechanisms underlying the observed changes. There are two main hypotheses on the aetiology of late radiation sequelae (discussed in Fajardo 1982). One school of thought believes that the depletion of endothelial cells is the crucial initiating step and that all subsequent damage is dependent on this (Hendry, 1987; Wang, 1997). Dysfunction of the endothelium occurs acutely during radiotherapy, when blood vessels become leaky. Damage to the endothelial cells becomes evident over a prolonged period of time, due to slow cell turnover in the endothelium. Depletion of the stem cell compartment occurs. As blood vessels are ubiquitous, late radiation sequelae occur in all tissue types. As the endothelial cells die, they are replaced by fibroblasts that migrate into the damaged area. Unfortunately, the fibroblasts cannot perform the specialised role of endothelial cells and the tissue becomes relatively hypoxic. This results in the death of the specialised cells that comprise the tissue, which in turn results in tissue atrophy and dysfunction. Further fibrosis is consequent on these changes, resulting in the establishment of a vicious circle, which continues to progress, leading to loss of tissue function. The alternative hypothesis of late radiation sequelae views the death of the specialised cells of the irradiated tissue as

the initial step. As occurs in acute reactions, stem cell depletion takes place in the irradiated tissue. These cells have a much slower cell turnover time than acutely reacting tissues and thus the loss of tissue function is delayed (Otsuka and Meistrich, 1990). This hypothesis is somewhat analogous to the aetiology of acute reactions, but with a longer lag time, due to differing cell kinetics. These cells are replaced by fibroblasts, which do not perform specialist functions. Collagen deposited by the fibroblasts disrupts the architecture of the tissue, leading to reduced oxygenation and subsequent increased cell death. The fibrosis is therefore progressive. In addition, endothelial stem cells are also depleted, leading to fibrosis and poorly functional blood vessels, with consequent fibrosis. As can be seen, both hypotheses fit the observed clinical pattern of late radiation sequelae: they have a latent period, they are progressive, they are characterised by histological changes in the architecture of the blood vessels, as well as widespread tissue atrophy and fibrosis. Both hypotheses are not mutually exclusive as they result in the same final common pathway. However, the relative importance of the role played by endothelial dysfunction has not yet been elucidated.

Late radiation side effects are dependent on the total dose received, the volume irradiated and the fraction size. The overall treatment time is less important in the development of late effects. Enough time must be left between fractions to allow for maximal repair of normal tissues. The optimal inter-fraction interval is still controversial, but generally accepted to be no less than six hours (Bentzen *et al*, 1999; Lee *et al*, 1999). If a treatment is accelerated, then the volume irradiated must be decreased or the fraction size reduced otherwise the risk of late reactions is increased.

Table1.1: Comparison of acute and late histological changes seen in the skin and subcutaneous tissues following radiation exposure (after Fajardo 1982).

Acute changes	Late changes
Often visible within one week	Visible by six months
Established by three weeks	Continues to progress
Resolves by three, or at most six, months	
	Absolute decrease in capillary number, with irregularly dilated superficial blood vessels (telangiectasia)
Capillary dilatation	
Fibrin thrombi blocking small arterioles	Persistent fibrin thrombi
	Myointimal proliferation in arterioles
Increased vascular permeability	Eccentric, enlarged endothelial cells
	Inflammatory cells absent unless there is infection present
	Persistent fibrinous exudate
	Stromal oedema
	Dense irregular collagen deposition forming rigid skeleton around other structures e.g. nerves
± Inflammatory exudate	
Oedema	
	Large, irregular, atypical fibroblasts
	Hypopigmentation
	Thin, atrophic skin
Increased pigmentation	Ulceration (occurs at high doses)
Ulceration (at high doses or with hypofractionation)	
	Some recovery of hair follicles (dose-dependent)
Epilation and loss of sebaceous glands	

Relationship between acute and late effects

A number of studies have investigated the link between acute and late reactions. These studies have generally concentrated on patients with breast cancer. The are a number of reasons for this. There are a large number of patients with breast cancer receiving radiotherapy each year, especially since breast conservation has become an accepted treatment modality. The advent of screening has increased the proportion of patients eligible for breast conservation surgery. Overall survival in breast cancer is good, meaning a large proportion of patients live long enough for their late radiation reactions to become manifest. Finally, the breast is an easy organ to assess from the standpoint of both acute and late reactions. Clinical photographs can be used for some end-points, reducing the number of clinicians seen by the patient.

The main evidence of a link between acute and late effects comes from an intensively studied group of patients from Gothenburg. These patients received post-mastectomy radiotherapy between 1974 and 1982 and have been monitored ever since. The latest analysis of these data (Turesson *et al*, 1996) has shown a positive relationship between severity of acute skin reaction (measured by reflectance spectrophotometry) and the lag period to the development of telangiectasia in the treatment field. A relationship was also demonstrated between acute skin reaction and telangiectasia progression i.e. a more severe acute skin reaction led to the development of earlier and more severe telangiectasia. There is no link demonstrable in this series of patients between severity of acute reaction and development of late radiation fibrosis. This is generally agreed on (Overgaard *et al*, 1987; Brock *et al*, 1995; Johansen *et al*, 1996). Probably the main reason for this is that all late

tissue responses are reported together. It is likely that different cell types mediate different late radiation end-points, e.g. fibroblasts are responsible for late radiation fibrosis, whereas telangiectasia is mediated by damage to endothelial cells.

Scoring of late radiation side effects

In the past, recording of late radiation effects has been poor in a number of ways and for a number of reasons. Most studies have not systematically included a measure of late radiation sequelae in their protocols. Reporting has been intermittent and incomplete. In those studies that have reported side effects, there has been a mixture of purely descriptive and graded reporting of reactions. Grading varies between treatment sites. The graded reports have been inconsistent from centre to centre and for the same treatment site within the same centre. Differences in recording practices have made direct comparisons between different centres and regimens virtually impossible. Some tumour sites, where late radiation toxicity is of critical importance, e.g. cervix have evolved complex validated tools for recording sequelae e.g. the Franco-Italian glossary (Sinistrero *et al*, 1993), but this is not applicable to most tumour sites.

LENT SOMA

In 1995, a joint group of the EORTC and RTOG published a consensus document on the nomenclature of late radiation reactions (Rubin *et al*, 1995a; Rubin *et al*, 1995b; Pavy *et al*, 1995). This was called the LENT (Late Effects Normal Tissues). All possible degrees of late radiation injury for all tumour sites were incorporated in a number of scales. These

were called the SOMA scales (Subjective, Objective, Management and Analytic). The LENT SOMA document was one of the first to allow patients to grade subjective perceptions of their symptoms. This included both intensity and duration of the symptoms. The late radiation side effects are rated by an oncologist, giving an objective account of the damage present, even if it is subclinical in nature. Simple, routinely available tests of damage e.g. haemoglobin and weight are also included in this category as raw data to allow as much access to unrefined source material as possible. The management aspects of the symptoms are recorded. This not only allows a description of sequelae at a defined time point following radiotherapy, but also allows the individual symptoms to be followed in a longitudinal fashion. This highlights the progressive nature of many late radiation sequelae, visible as patient management changes with time. The analytical category allows other, more sophisticated techniques to be involved in the grading of damage in a site appropriate manner. However, as the management scales form a distinct category, they are not mandatory to the function of the scale. This was a deliberate measure to allow all centres, even those relatively materiel poor, to use the scales. Thus the clinical utility of the scales would be available to the largest number of clinicians possible. The number of centres that could participate in any trial using the SOMA scales was also maximised. The LENT SOMA should be administered prior to the start of any treatment, to obtain a baseline value and delineate any inter-individual variations in organ function. This is particularly important in older patient age groups for two main reasons. First, many elderly patients receive suboptimal treatment, as there is a widespread belief that they are unable to tolerate conventional treatment schedules. Adequate use of the LENT SOMA would answer this question decisively. Second, proper use of the LENT SOMA would highlight a treatment option with unacceptable morbidity in a population with poor baseline function. For the

LENT SOMA to become an accepted and valuable tool for describing and recording late radiation reactions, it must undergo validation. This is more easily achievable in those tumour sites with an existing validated tool e.g. cervical cancer. Once the validation process is completed, it is hoped that the LENT SOMA will become adopted as the gold standard tool for scoring late radiation morbidity worldwide.

1.5 Radiotherapy side effects in the cancers studied in this thesis

Cervical cancer

In cervical cancer treatment acute systemic side effects generally include non-specific weakness and lethargy. Nausea is uncommon, although not unknown. The skin of the groin becomes erythematous, and dry desquamation, sometimes accompanied by hyperpigmentation occurs. This may progress to moist desquamation, especially in skin creases, due to increased friction and radiation dose build-up over curved surfaces (DeVita *et al*, 1997). Groin hair is lost after 2 -3 weeks treatment, but generally some regrowth occurs following the completion of treatment. Radiation cystitis can occur, with frequency, urgency, and strangury. Haematuria may also develop. Radiation proctitis can occur, with increased stool frequency and decreased stool consistency. Urgency and tenesmus are also features. Fresh spotting of blood *per rectum* (PR) is also a relatively common occurrence, especially where haemorrhoids are present prior to radiation treatment.

Late radiation side effects develop more than six months following the end of treatment, and may not be manifest for years. They include subcutaneous fibrosis in the treated area.

This is generally asymptomatic and of no great consequence. Late radiation cystitis can occur due to fibrosis of the bladder wall muscle. This leaves a shrunken bladder that is poorly functional and may be excessively irritable. Symptomatically, this can lead to persistent urgency, frequency and, in some cases, incontinence. The incidence of urinary infections may increase. Damage to the blood vessels in the bladder wall can cause them to become telangiectatic and fragile, leading to haematuria, which may be both sudden and severe. The vagina becomes shortened and stenosed. This may be prevented, in some cases, by diligent use of dilators in the immediate post-treatment period. Late radiation damage to the small and large bowel can occur. In the small bowel, fibrosis can lead to subacute obstruction, with subsequent cramping abdominal pains, constipation and severe diarrhoea, and nausea and vomiting. In extreme cases, surgical resection of the affected segment is necessary. In the large bowel late radiation damage is generally manifest as lower abdominal pain, stool frequency and urgency, and faecal incontinence. Severe PR bleeding may occur due to telangiectatic blood vessels in the bowel mucosa. Surgical resection of affected areas may again be necessary. In the most severe cases, fistulae may develop between the bladder, bowel, vagina or abdominal wall. Due to the poor healing of irradiated tissues, defunctioning urostomies or colostomies may be the only viable treatment option. Patients with late radiation sequelae can suffer pain of varying intensities, but may require opioid analgesia. All late radiation sequelae have a tremendous negative impact on quality of life in these patients, who may be cured of their disease, but severely impaired in their activities of daily living by their radiation-induced side effects. Finally, the incidence of second malignancies is increased in those patients who have received radiotherapy.

Systemic acute side effects of radiotherapy include lethargy and often anorexia. Other side effects are dependent on the area treated. Acute skin reactions include erythema, dry and, sometimes, moist desquamation. In tumours of the nasal cavity, nasal sinuses, nasopharynx, oral cavity, salivary glands and oropharynx this occurs on the skin of the face. In tumours of the larynx and hypopharynx, the reaction is seen on the skin of the neck. This reaction is worsened by friction, and therefore wet shaving is discouraged in those patients undergoing treatment (Neal and Hoskin, 1997). Loss of beard hair in treated areas begins within two weeks of starting treatment. This generally recovers in the longer term, providing doses of less than 50-55 Gy are given, but beard growth may always remain sparse in the treated area (Dobbs *et al*, 1992; Wang, 1997). Acutely, the nasal cavity mucosa is damaged, leading to dryness, discomfort, nasal blockage or running and crusting. Small epistaxis is a common phenomenon. Acute mucosal damage in the mouth leads to loss of the mucosal barrier. This may be patchy or confluent. This results in mouth ulcers, which can be acutely painful for the patient leading to a decreased oral intake, with consequential dehydration and malnourishment. These mouth ulcers may bleed. At doses below 26 Gy, reversible transitory impairment of saliva production is seen (Eisbruch *et al*, 1999). This leads to a dry mouth, which is distressing for the patient. If the posterior third of the tongue is irradiated, the sensation of taste is altered or completely lost. At doses above 55 - 60 Gy, return of this sensation is very slow (may take years) and can be incomplete. Food is cleared inefficiently from the mouth leading to stagnation and often low-grade infection with *Candida albicans*. Mucositis of the throat leads initially to the feeling of "something being present" at the back of the throat. This can progress through

discomfort to frank pain and dysphagia requiring strong opioid analgesia and, in severe cases, institution of enteral feeding to maintain weight.

Late radiation damage can occur at all sites in the head and neck. Subcutaneous fibrosis, leading to palpable thickening of the skin is common. It is generally asymptomatic, save where it affects large areas of the skin beneath the chin (woody induration - the radiotherapy "dewlap"). This is most commonly seen in patients treated for cancer of the floor of mouth. Permanent ulceration of the skin is very rare. Telangiectasia are uncommon, but most often seen in patients treated for laryngeal cancer, where a strip of bolus material has been added to the shell over the midline. When treating carcinoma of the nasopharynx, care must be taken to limit the dose to the optic chiasm and auditory nerves, to less than 55 Gy (in 2 Gy fractions), as damage to these structures leads to blindness and deafness (Dobbs *et al*, 1992). When the paranasal sinuses, in particular the maxillary sinus, are treated, scattered dose to the lens of the eye should be kept below 6-8 Gy, as higher doses may result in the development of a radiation cataract. High, but therapeutic, doses of radiation to the nasal cavity can result in permanent damage to the mucosal tissues with epilation of nasal hairs. This results in permanent nasal running and crusting. Epistaxis can become a long-term problem. A damaged nasal mucosa can leave the patient prone to infections, with subsequent consequential damage to the mucosa. The most frequent late radiation complication in the oral cavity is loss of function of one or more salivary glands. Loss of function of one salivary gland leads to a decreased amount of saliva being present. If both parotid glands are affected, the mouth may produce saliva with an altered consistency or may be completely dry. This is very distressing for the patient. It can also lead to poor dental hygiene. Dental caries can progress very quickly in a mouth with

inadequate saliva. Dental extractions in those patients who have had their mandible irradiated can be very dangerous. Irradiated bone is less able to repair itself than normal bone. Any dental extraction has the potential to become infected. In irradiated bone, infection is not as easily cleared and may lead to chronic osteomyelitis. Even in the absence of infection, the mandible can suffer from osteoradionecrosis. The mandible becomes necrotic due to late radiation damage. This has a severe impact on the patient's ability to eat and consequent quality of life. Damage to the vocal cords may result in permanent hoarseness following radiotherapy. In the larynx, late radiation damage can cause swelling. This may be of no functional consequence, or may threaten the patient's airway (DeVita *et al*, 1997). In the latter case, a permanent tracheostomy may be required, even in the absence of disease recurrence. The larynx itself may develop cartilaginous radionecrosis, requiring removal. This condition can result in the death of the patient. Again, there is an increased incidence of second malignancies in those patients who have received radiotherapy. The increase in second tumours is at a maximum 5 – 7 years following treatment.

Breast cancer

Patients who receive chemotherapy in addition to radiotherapy are at increased risk of acute and late radiation side effects (Dubey *et al*, 1998). Acute systemic side effects include tiredness and lethargy. Locally, the skin of the breast becomes erythematous after approximately one weeks treatment. Dry desquamation and hyperpigmentation often occur in the next few weeks. Moist desquamation is common, especially in women with heavier busts, and especially in the inframammary and axillary folds. The breast becomes swollen

and often tender, with the skin resembling peau d'orange. In those patients, whose supraclavicular nodes are being treated, there is often a mild radiation oesophagitis, resulting in dysphagia. In patients who have had a large volume of lung treated, symptomatic radiation pneumonitis may develop. This is manifest by a dry cough, low-grade fever and inflammatory infiltrates visible on the chest X-ray. In patients with impaired respiratory function, a feeling of breathlessness may occur.

In the longer term, most women who have undergone breast irradiation notice a persistent alteration in the texture of the breast. This generally is of no clinical or symptomatic importance. The treated breast may visibly and palpably more swollen than the contralateral breast. This is again generally asymptomatic, but may cause a feeling of heaviness or a general "ache", which may be refractory to analgesia. Fibrosis and retraction of the soft tissues can lead to shrinkage and distortion of the breast. This may be very painful, as well as cosmetically unattractive. Fat necrosis is a rare event, which leads to atrophy of segments of the breast in an uneven fashion, again disrupting cosmesis. Persistent, non-healing skin ulcers are a rare, but acknowledged occurrence. The skin of the breast may develop telangiectasia from damaged blood vessels. These can continue to progress for at least 8 years (Turesson *et al*, 1996) and have no known treatment. The lung underlying the breast, in the longer term, becomes fibrotic and non-functioning. This has no clinical impact unless the patient already has impaired lung function, when it is manifest as a deterioration in breathlessness. Late radiation reaction in the costal cartilages can lead to symptomatic costochondritis. This can be painful on both inspiration and direct palpation, and can be very debilitating to the patient. Rib fractures can also occur. Late radiation damage incurred by the heart is still a matter of controversy. In patients treated

with orthovoltage machines (where the absorbed cardiac dose was higher), there is evidence of an excess of non-cancer, cardiac-related deaths in those treated for left sided tumours (Gyenes *et al*, 1998). This has not been shown in those treated with more modern megavoltage equipment, but a small excess of cardiac deaths cannot yet be ruled out (Gustavsson *et al*, 1999). In those patients who have had axillary radiotherapy or surgery alone, the rate of lymphoedema in the affected arm is between 5 and 10% (Hoe *et al*, 1992; Stotter and Chandler, 1999). In those patients treated with surgery and radiotherapy, the risk of lymphoedema can reach 80% in some series (Yeoh *et al*, 1986). This is due to damage to lymphatic drainage channels by both treatment modalities. Probably, the most feared late radiation complication is that of brachial plexus neuropathy. This results in a painful, non-functioning arm. In the past, this has most commonly been caused by overdosage of the brachial plexus due to repositioning of the arm between treatment of the breast and axilla (Royal College of Radiologists, 1996). Brachial plexus neuropathy can occur rarely in those given less than tolerance doses of radiotherapy.

1.6 Molecular mechanisms of radiotherapy damage

Ionising radiation causes damage at the molecular level that is then reflected in the overall function of the tissue and/or organ irradiated. When the body is irradiated, the energy of the radiation is deposited in the tissues, leading to the formation of free radicals. It is these molecules, which are formed as a result of ionisation, that are responsible for the damage caused by radiation. Damage due to free radicals occurs in all components of the cell. It is now accepted that damage to the DNA is responsible for the observed long-term effects of radiation (Dahm-Daphi *et al*, 1998; Ross, 1999). One gray of radiation results in

approximately 150,000 nuclear ionisation events per cell. The free radicals produced have a short half-life, which is terminated by oxidation reactions. Some of the radicals are harmlessly cleared by the tissue itself, using molecules which easily donate electron pairs e.g. superoxide dismutase. Those radicals that are responsible for long-term damage react with protein in general and DNA especially. The damage is generally divided into two types: direct and indirect. Direct damage is the rarer of the two. This occurs when the radiation interacts directly with the DNA molecule to create a DNA free radical. This is an oxidative reaction, which changes the chemical structure of the DNA, with a subsequent alteration in DNA function. Indirect damage is the more common type of damage seen, accounting for up to 80% of reactions. Here, the radiation interacts with an intracellular molecule (water at least two-thirds of the time). A free radical is formed (in the case of water, this is the hydroxyl radical). This radical then diffuses to the DNA, with which it interacts, breaking chemical bonds and leading to alterations in DNA structure. These alterations lead to functional changes that are at the heart of radiation damage. This molecular damage is manifest in a number of different ways. Adjacent pyrimidine bases on the DNA can dimerise. DNA-DNA and DNA-protein cross-linkages and single and double strand DNA breaks can occur. In areas where large amounts of energy are deposited complex multiply damaged sites of DNA are generated. DNA double strand breaks (dsb) and multiply damaged sites are thought to be the main lesions resulting in lethal damage to any cell, either tumour or normal (Jenner *et al*, 1993; Olive, 1998). All cells have mechanisms whereby the repair of these lesions is attempted. Some lesions are more efficiently repaired than others. It is this difference in repair capacity between tissues that gives rise to the therapeutic ratio observed in patients.

1.7 Determinants of radiation-induced side effects

Individuals vary in their response to radiotherapy. This variation is governed by medical, physical and dosimetric confounding factors and intrinsic normal cell radiosensitivity.

Physical confounding factors

Physical confounding factors are patient-dependent. Some are related to the patient's behaviour during treatment, while others are related to the physical nature of the patient. Behavioural factors may impact on the severity of acute radiation reactions. Patients who smoke during treatment tend to have more severe acute mucosal reactions (Rugg *et al*, 1990). They also have poorer lung function after 3 years than their non-smoking counterparts (Smith *et al*, 1989). Patients who are incontinent tend to develop more severe acute skin reactions during pelvic treatments due to friction and constant moistness of the skin in the treated area. Factors that are patient-dependent, but are not affected by patient behaviour include patient build. Patients with larger breasts are at increased risk of suffering severe acute radiation reactions (Brierley *et al*, 1991).

Medical confounding factors

Certain conditions increase the likelihood of developing a severe late radiation reaction. These conditions generally affect the vascular system and include diabetes and hypertension (Gragoudas *et al*, 1999; Zelefsky *et al*, 1999). Certain vasculo-connective tissue disorders have been reported as causing an increased risk of developing severe late

sequelae (Morris *et al*, 1997). These reports are generally anecdotal or contain very small numbers of patients. There are no large randomised studies covering any of the conditions. This is partly due to the small numbers of patients with these diseases and also the fact that only a small proportion of them will receive radiation treatment at any one centre (De Naeyer *et al*, 1999). Multi-modality treatment leads to increased severity of acute radiation effects (Bosset *et al*, 2000). The late radiation sequelae following multi-modality treatments are unquantified for most tumour sites, but use of multiple modalities is likely to increase the severity of reactions seen (Robert *et al*, 1999).

Dosimetric confounding factors

The aim of radiation therapy is to irradiate the planning target volume (PTV) to the prescribed dose in a uniform fashion, while limiting the dose delivered to surrounding tissues (Dobbs *et al*, 1992; DeVita *et al*, 1997). Due to the constraints of planning the PTV is generally rhomboidal in shape. This is not the shape of most tumours. In the case of breast cancer, the entire ipsilateral breast tissue remaining following surgery is irradiated. Even if there is no post-surgical distortion of the breast, this volume presents a number of problems for treatment planning. The breast has a different three-dimensional shape, both in a cranio-caudal and right to left lateral direction. It also curves around the chest wall, meaning the posterior border of the treatment volume (over the ribcage) is concave. This presents great technical difficulties in producing a plan where the dose delivered does not fluctuate. ICRU Report 29 states it is usually unacceptable for the dose of radiation delivered to vary by more than ± 5% over the PTV. In practice, this is virtually impossible to achieve for a breast PTV. Most centres compromise in some fashion and accept a dose

variation of ± 10% in breast treatment plans. The main areas of the breast that become overdosed are the inferior lateral corners of the breast, which may receive up to 110% of the prescribed dose. This overdosage poses a problem for all women, but especially those with larger breasts or with wide inter-field distances. These women may exhibit an excess of severe late radiation reactions due to an accepted overdosage in their treatments.

Normal cell radiosensitivity

There is significant inter-individual variation in both acute and late responses of normal tissues to radiotherapy (Tucker *et al*, 1992). It has been estimated that between 80 and 90% of this variation is genetic in origin (Geara *et al*, 1993; Turesson *et al*, 1996). The main support for this comes from a well-defined cohort of patients treated for early breast cancer in Gothenburg between 1972 and 1985 and subsequently followed intensively (Turesson *et al*, 1986; Tucker *et al*, 1992; Burnet *et al*, 1992; Burnet *et al*, 1994; Brock *et al*, 1995; Turesson *et al*, 1996). There were significant inter-individual variations in the onset and rate of progression of telangiectasia in these patients (Turesson, 1990; Tucker *et al*, 1992; Turesson *et al*, 1996). Despite different fractionation schedules, however, marked correlations were seen between acute reactions in the right and left internal mammary node fields of the same patient (Tucker *et al*, 1992). As intrinsic cellular radiosensitivity plays a significant role in the determination of normal tissue radioresponse, there is considerable interest in developing a robust radiosensitivity assay that can be used to individualise patient treatment schedules in a clinical setting.

1.8 Measuring normal cell radiosensitivity

Taylor *et al* (1975) first demonstrated that fibroblasts cultured from individuals with ataxia telangiectasia (A-T) have a significantly higher radiosensitivity than normal fibroblasts. This was confirmed (Little and Nove, 1990) and extended to other genetic disorders (Deschavanne *et al*, 1986; Little and Nove, 1990). Significant inter-individual differences were subsequently demonstrated in the radiosensitivity of fibroblasts cultured from normal individuals (Little *et al*, 1988; Malaise *et al*, 1994). Malaise *et al* (1994) also demonstrated that the best discrimination between cells was obtained using parameters that describe the initial part of radiation survival curves (i.e. doses below 3.5 Gy).

A number of retrospective series have examined the intrinsic radiosensitivity of fibroblasts from patients with an atypically severe reaction to radiotherapy (e.g. Woods *et al*, 1988; Loeffler *et al*, 1990). These have demonstrated a fibroblast radiosensitivity that is significantly greater than in normal patients. These non-syndromic radiosensitive individuals are rare. As differences in fibroblast radiosensitivity existed between individuals and could be detected, studies were undertaken to investigate the relationship between fibroblast radiosensitivity and clinical endpoints in the form of acute and late radiation sequelae. In a selected series of Gothenburg patients, Burnet *et al* (1992) demonstrated a positive correlation between fibroblast radiosensitivity and the degree of both maximal erythema (acute) and telangiectasia (late). The relationship between measured fibroblast radiosensitivity and acute radiation reaction was not confirmed in a reanalysis of the same data and in other independent studies (Begg *et al*, 1993; Geara *et al*, 1993; Burnet *et al*, 1994). Although a number of studies have correlated fibroblast

radiosensitivity with late radiation reactions, including fibrosis (Geara *et al*, 1993; Johansen *et al*, 1994) and telangiectasia (Burnet *et al*, 1994; Brock *et al*, 1995), more recent studies have not produced significant correlations with late clinical endpoints (Russell *et al*, 1998; Rudat *et al*, 1999; Carlomango *et al*, 2000; Peacock *et al*, 2000).

All of these studies were carried out on fibroblasts using clonogenic assays. Although clonogenic assays are the acknowledged "gold standard", at least 8 weeks are required to generate results using fibroblasts. Results using lymphoblastoid cell lines derived from patient lymphocytes are quicker but less robust (Ramsay *et al*, 1995). There is also interest in using peripheral blood lymphocytes because they are easily available and in sufficiently large numbers to perform assays. Lymphocytes from patients with A-T demonstrate hypersensitivity to radiation (West *et al*, 1994). Significant inter-individual differences are also seen in lymphocytes from normal donors (Geara *et al*, 1992; Elyan *et al*, 1993). However, lymphocyte intrinsic radiosensitivity correlates with clinical endpoints in some studies (West *et al*, 1995; West *et al*, 1998), but not in others (Geara 1993).

Both lymphocytes and fibroblasts have been used in studies aimed at evaluating potential rapid predictive assays for measuring normal cell radiosensitivity. These have involved a number of non-clonogenic endpoints. Assays of chromosome damage include using premature chromosomal condensation (Johnson *et al*, 1970) or fluorescent *in situ* hybridisation (FISH) (Russell *et al*, 1995) to score chromosomal aberrations. Aberrations can also be scored directly in both G_0 (Jones *et al*, 1995) and G_2 (Mitchell and Scott, 1997). The micronucleus assay is also of interest as a potentially rapid method for scoring chromosomal damage (Huber *et al*, 1989; Mill *et al*, 1996; Scott *et al*, 1996). It has also

been used on fibroblast lines but studies of the relationship with clonogenic data have produced conflicting results (O'Driscoll *et al*, 1998; Johansen *et al*, 1998). However, chromosome damage assays are predominantly performed on lymphocytes for a number of reasons. First, large numbers of cells can be obtained easily. Second, synchronisation of the cell cycle and induction of mitosis can be controlled using drugs. Some studies have shown a relationship between lymphocyte radiosensitivity, measured using chromosome damage as an endpoint and normal tissue response (Jones *et al*, 1995; Slonina and Gasinska, 1997; Barber *et al*, 2000). However, others have shown no relationship (Rached *et al*, 1998).

Radiation can affect the differentiation state of fibroblasts and this has been exploited in the development of a differentiation assay that may predict for late radiotherapy reactions (Rodemann *et al*, 1996; Lara *et al*, 1996; Herskind *et al*, 1998). Measuring radiation-induced apoptosis has also formed the basis of assay development (Crompton *et al*, 1999; Bedi *et al*, 1995). Defects in DNA repair can lead to severe reactions to radiotherapy in syndromic individuals (Little and Nove, 1990). Polymorphisms in the DNA repair genes XRCC1 and XRCC3 have been assessed as the basis for a rapid predictive assay of normal cellular sensitivity (Price *et al*, 1997). Recently, there has been increased interest in the potential clinical utility of assays measuring the plasma levels of TGFβ1, a cytokine intimately linked with radiation response (see Section 1.11).

Radiation toxicity is manifest because of DNA damage. Patients with a recognised genetic syndrome that leaves them hypersensitive to radiation, often have defective DNA damage repair mechanisms. Therefore, interest has centred on DNA damage assays in the search for a robust measure of intrinsic radiosensitivity. Assays performed on single cells are

attractive, as it is easier to obtain sufficient cells to perform a test. Single cell gel electrophoresis ("comet" assay) can measure both double and single strand DNA breaks, depending on experimental conditions (Olive *et al*, 1991). This assay can measure significant inter-individual differences in radiation sensitivity using either lymphocytes or fibroblasts (Sarkaria *et al*, 1998). However, the sensitivity of the assay in detecting differences between cell lines of differing radiosensitivities is poor (Olive *et al*, 1994). No correlation was found between clonogenic survival and radiosensitivity measured in lymphocytes using the comet assay (Sarkaria *et al*, 1998), but a correlation between clonogenic parameters and fibroblast radiosensitivity has been seen (Eastham *et al*, 1999). Measurements of DNA damage in large numbers of cells can be investigated using a variety of electrophoretic methods. These include clamped field gel electrophoresis (CFGE) (Chukhlovin *et al*, 1995; Dikomey *et al*, 2000), graded field gel electrophoresis (GFGE) (Dahm-Daphi *et al*, 1995; Dahm-Daphi *et al*, 1998), as well as pulsed field gel electrophoresis (PFGE) (Wurm *et al*, 1994; Kiltie *et al*, 1997).

1.9 Pulsed-field gel electrophoresis (PFGE)

In PFGE, the sample to be studied is loaded into a gel and subjected to electrophoresis for a given period of time, depending on the protocol used. By varying the electrophoretic conditions, different sized DNA fragments migrate for different distances into the gel. Both single and double DNA strand breaks can be measured by varying the pH of the buffer solution. However, in the field of predictive assay testing, double strand breaks are thought to be the more important lesion and, in this thesis, only work concerned with these will be

discussed. The amount of DNA that migrates into the gel is measured and expressed as the fraction of damage released (FDR) from the sample.

In fibroblasts, measurements of initial and residual (i.e. following a period for repair) DNA damage have been investigated, as well as rate of repair. In most studies correlation with clonogenic endpoints has been undertaken, but in a small number of studies correlations with clinical endpoints have been assessed.

No relationship has been demonstrated in normal cells between initial damage and clonogenic radiosensitivity. Correlation between the rate of repair of radiation-induced DNA damage and clonogenic radiosensitivity has been demonstrated using fibroblasts for both non-syndromic (Blocher *et al*, 1990; Kiltie *et al*, 1997; Sarkaria *et al*, 1998) and syndromic (Wurm *et al*, 1994; Foray *et al*, 1995) individuals. A number of studies have shown that clonogenic parameters are also correlated with the levels of residual DNA damage (Wurm *et al*, 1994; Foray *et al*, 1995; Kiltie *et al*, 1997). This is advantageous as measures of rate of repair are timing consuming to perform. Controversy exists as to the optimum time point at which to measure residual damage. Wurm *et al* (1994) found a correlation between clonogenic radiosensitivity and residual damage measured after 4 hours repair. Foray *et al* (1995) showed that A-T homozygotes had a more rapid initial repair phase (first 4 hours) than normals, but after this, repair was greater in normals. He found no further repair after 24 hours. Kiltie *et al* (1997) found residual DNA double strand breaks at 24 hours correlated with clonogenic radiosensitivity using vaginal fibroblasts obtained from patients with cervical cancer prior to radiotherapy with a small range of SF2 values.

Subsequently, Kiltie *et al* (1999) correlated residual DNA double strand breaks at 24 hours with clinical outcome in a group of 39 patients treated more than ten years previously for breast cancer. Normal fibroblasts were cultured from a 5 mm skin punch biopsy. Clinical endpoints included fibrosis and oedema (measured clinically) and retraction, atrophy and telangiectasia (scored independently, in a blinded fashion, by three experienced clinicians, from a clinical photograph). A significant correlation was demonstrated between the degree of fibrosis and the level of residual radiation-induced DNA damage at 24 hours ($r = 0.46$, $p = 0.003$).

1.10 Transforming growth factor beta one (TGFβ1)

The transforming growth factor beta (TGFβ) group of molecules was discovered in 1981. They were named for their observed ability to cause anchorage-independent growth in normal rat kidney (NRK) fibroblasts in culture. Anchorage-independent growth in non-haemopoietic cells is generally limited to transformed cell lines. On removal of TGFβ, NRK cells reverted to their normal phenotype; i.e. TGFβ is not a transforming agent *per se*. TGFβ belong to a large superfamily of related proteins that appear in many animal species ranging from *Drosophila* and *Xenopus*, through to man. The superfamily includes activins, inhibins, bone morphogenic proteins and Mullerian inhibitory substance (Roberts *et al*, 1988). Subsequently, TGFβ was discovered to exist in a number of isoforms. TGFβ1-3 are found in man, TGFβ4 in chickens and TGFβ5 in *Xenopus* (Lawrence, 1996). In this thesis only TGFβ1 will be discussed in detail.

TGFβ1 is a multi-functional cytokine, the full roles of which, despite intensive study, have not been completely elucidated. The isoforms of TGFβ are coded for by independent genes, which have different promoter regions. The gene that codes for TGFβ1 is located on chromosome 9q13, has 6 introns and encodes for an mRNA molecule of 2.5 kb (Lawrence, 1996). The active TGFβ1 molecule is the C-terminal of the protein product. TGFβ1 is a homodimer of two 112 amino acid chains (Lawrence, 1996). Trace quantities only of heterodimers of β1/β2, β2/β3 and β1/β3 have been found. Conservation of the isoforms of TGFβ is high, with homology between the human isoforms of TGFβ ranging from 72 - 84% (Lawrence, 1996). The amino acid chains are linked by one interchain disulphide bond occurring between cysteine residues. Each TGFβ1 molecule has eight further cysteine residues that take part in forming the protein's conformal structure (Lawrence, 1996).

TGFβ1 is secreted initially as an inactive latent precursor (known as proTGFβ1). This molecule comprises the active C-terminus end of the TGFβ1 protein product in non-covalent association with the non-functional N-terminal end. The N-terminus is known as the latency associated peptide (LAP) and the complex of LAP and TGFβ as the small latent complex (Lawrence, 1996; Gleizes *et al*, 1997). Binding to LAP is thought to mask the TGFβ1 active molecule from intracellular lysosomes, preventing degradation of the protein. The plasma half-life of latent TGFβ1 is greater than 100 minutes, as compared with a few minutes for the active form (Lawrence, 1996). The existence of a latent form is unusual for a cytokine and this implies that activation rather than genetic up-regulation is the most important area of TGFβ1 control. It also means that increased TGFβ1 levels can be achieved quickly, with no lag time needed for increased protein transcription. The small

latent complex can become covalently associated with a number of homologous latent TGF binding proteins (LTBP). Large LTBP is released from platelets along with the small latent complex. Small LTBP is similar in structure to the fibrillin proteins of the extracellular matrix (ECM) and may become covalently incorporated into the ECM. There is no covalent link between the TGFβ1 molecule and the small LTBP (Lawrence, 1996). A stable pool of activatable TGFβ1 is therefore available in the stroma of most tissues. The structures of the small and large latent complexes are illustrated in Figure 1.2.

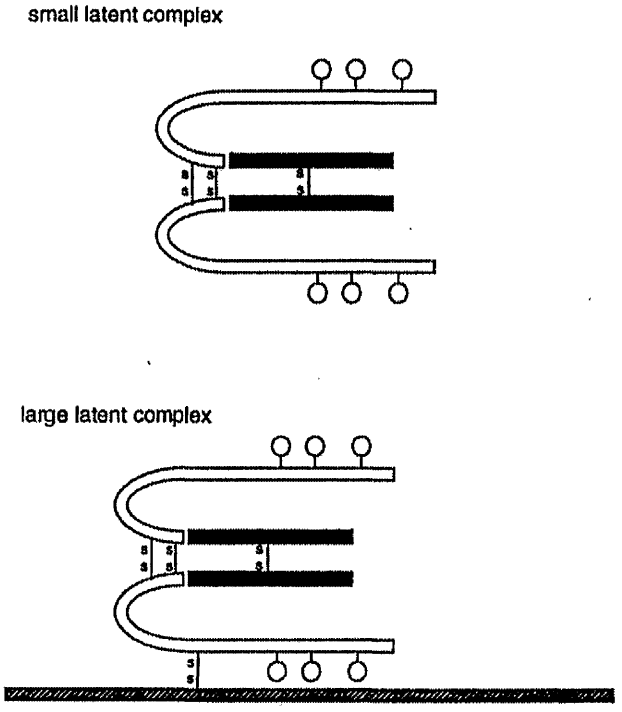


Figure 1.2: Diagram of the structure of the small and large latent complexes of TGFβ1 (black area represents TGFβ1 molecule; white area represents latency associated peptide; hatched area represents latent TGFβ binding protein; s-s represents disulphide bonds) (taken from Lawrence 1996)

There are a number of mechanisms whereby TGFβ1 can be activated. *In vitro* TGFβ1 can be activated by acidification, alkalinisation, heat, redox reactions and chaotropic agents e.g. urea. *In vivo* the exact mechanism of TGFβ1 activation is unclear and indeed may differ between different tissue types (Roberts *et al*, 1988; Lawrence, 1996). TGFβ1 plays an important role in controlling bone formation and the microenvironment surrounding osteoblasts can be as low as pH 3.0, implying activation by acidification is important in this site. Plasmin can activate TGFβ1, but thrombospondin, a glycoprotein found in blood platelets can activate TGFβ1 via enzyme-independent mechanisms. Active TGFβ1 can be found in mammary tissue one hour following exposure to ionising radiation (Barcellos-Hoff *et al*, 1994). It is unclear whether radiation directly activates TGFβ1 or whether activation takes place via redox reactions produced by ionising radiation. Active TGFβ1 can persist in the stroma of irradiated tissues for many weeks (Canney and Dean, 1990; Langberg *et al*, 1994; Richter *et al*, 1997).

TGFβ1 is produced by many different cell types, with the highest concentration per dry cell weight found in blood platelets (Roberts *et al*, 1988). All normal mammalian cells have receptors for TGFβ and these receptors occur in three main types (Lawrence, 1996). Receptor types I and II are both serine/threonine protein kinases. Receptor type II (70 -80 kD) is constitutively active (Wrana *et al*, 1994). When TGFβ is bound, receptor type II transphosphorylates receptor I (Wrana *et al*, 1994; Massague *et al*, 1996). A heterotetramer is the functional unit (Attisano and Wrana, 1996) and the intracellular domains of both receptors are required for signal transduction (Lawrence, 1996; Massague *et al*, 1996). There is relatively little homology between both receptor types. Both type I and II receptors

exist in a number of forms. Different TGFβ isoforms have different affinities for different receptor forms. The relative abundance of receptor types varies between different tissues and this is thought to partly account for the different effects of TGFβ isoforms in different tissues. There can be up to 4000 receptors per cell and signal transduction to the nucleus is via the Smad pathway (Attisano and Wrana, 1996; Massague *et al*, 1996). Receptor type III is betaglycan, a 300 kD protein. It is not thought to be involved in signal transduction, but instead acts by assisting ligand binding and possibly acting as a reservoir of active TGFβ1. It is not found in epithelial, endothelial or lymphoid cells. Endoglin, a circulating betaglycan analogue, is known to bind TGFβ1, as is α-2 macroglobulin. This latter molecule is known to undertake a scavenging role for other active proteases. TGFβ1 bound to α-2 macroglobulin is inactive, which lends supportive evidence to a scavenging role (O'Connor-McCourt and Wakefield, 1987; Lawrence 1996).

Most cytokines have paracrine and autocrine functions only. TGFβ can be identified in the plasma of humans, implying an additional endocrine role for this molecule. Circulating TGFβ levels can be measured in serum or plasma by a number of different protocols (Anscher *et al*, 1994; Reinhold *et al*, 1997; Philips *et al*, 1995; Abe *et al*, 1994). Generally, levels are measured in platelet free plasma as the high concentration of TGFβ released from degranulated platelets gives misleading results in serum. Normal plasma levels in the region of 2 – 6 ng/ml are most commonly quoted. In general, most of the studies have shown no age-related increase in TGFβ1 levels (Wakefield *et al*, 1995). The vast majority of circulating TGFβ is in the form of TGFβ1. Less than 0.2 ng/ml is TGFβ2 (Grainger *et al*, 1999; Wakefield *et al*, 1995). TGFβ3 has been isolated in trace amounts only from 20%

of individuals studied (Grainger *et al*, 1999). Grainger *et al* (1999) investigated 170 pairs of female twins (84 monozygotic and 86 dizygotic) and found that around 54% of the variation in plasma levels of TGFβ1 had a heritable basis. He also identified some common allele polymorphisms occurring in the promoter region of the TGFβ1 gene. Thus genetic factors play a part in the control of circulating TGFβ1 levels.

1.11 Functions of TGFβ1

TGFβ1 has multiple functions that differ between cell types. Despite intensive study, many of these functions are poorly understood especially where interactions with other cytokines occur. The distinct actions of TGFβ1 are summarised in Table 1.2.

TGFβ1 is necessary for ordered embryogenesis. Fifty percent of TGFβ1 null mice die *in utero* due to impaired haemopoiesis and vasculogenesis of the yolk sac (Bonyadi *et al*, 1997; Martin *et al*, 1995). The remaining mice die at 2 – 3 weeks *post-partum* due to a systemic wasting inflammatory disorder that results in organ necrosis (Kulkarmi *et al*, 1993; McCartney-Francis and Wahl, 1994). Transgenic fusion mice, who produce a constitutively active form of TGFβ1, die rapidly after birth due to a progressive systemic inflammatory/ fibrotic illness (McCartney-Francis and Wahl, 1994; Sanderson *et al*, 1995). Exogenous TGFβ1, when administered parenterally also gives rise to a similar systemic fibrotic disorder (Zugmaier *et al*, 1991). This can be successfully reversed by administration of the latency associated peptide (Böttinger *et al*, 1996). Thus TGFβ1 plays an important role in the normal embryonic development, as well as regulation of fibrotic

tissue production. Mice who lack the gene for the TGFβ1RII also die *in utero* from impaired vasculogenesis and haemopoiesis (Oshima *et al*, 1996).

Table 1.2: Individual functions of TGFβ1 *in vivo*

Action	Cell type affected	Reference
Growth induction	Mesenchymal cells e.g. fibroblasts, myoblasts Malignant cells (late in progression)	Roberts *et al*, 1992 Lawrence 1996
Growth suppression	Epithelial and endothelial cells T and B lymphocytes Malignant cells (early in progression)	Roberts *et al*, 1992 Lawrence 1996
Immune suppression	IgG and IgM production decreased NK and LAK cell activity decreased	Roberts *et al*, 1992 Lawrence 1996
Inflammatory mediator	Chemo-attractant for neutrophils, macrophages, monocytes and mast cells (strongest known)	Roberts *et al*, 1992 Richter *et al*, 1997
Apoptosis	Demonstrated in cells in culture	Raynal *et al*, 1997
Prevention of differentiation	Adipocytes and myoblasts	Roberts *et al*, 1992

The individual effects of TGFβ1 in different cell types do not occur in isolation in the body. Body tissues are made up of multiple cellular types, which are diversely affected by TGFβ1. The observed action of TGFβ1 is a composite of many effects, some of which are mutually exclusive. This is illustrated in Table 1.3.for tissue healing and extra-cellular matrix production.

Table 1.3: Tissue associated properties of TGFβ1

Tissue	TGFβ1 associated properties	Reference
Extra-cellular matrix	↑ production of ECM components ↓ breakdown of the ECM ↑ production of ECM breakdown inhibitors ↑ collagen production indirect promoter of angiogenesis	Roberts *et al*, 1986 Roberts *et al*, 1988 Lawrence, 1996
Tissue healing (wounds, post-MI and hepatectomy, bony fractures)	leucocyte attraction and activation angiogenesis fibrosis	Roberts *et al*, 1986 Roberts *et al*, 1988 McCartney-Francis and Wahl, 1994

1.12 TGFβ1 in oncology

Marker of tumour progression

Cervical cancer has been used as a model to study the role of TGFβ1 in tumour progression. TGFβ1 is the strongest known inhibitor of epithelial cell growth and acts early in malignant progression as a tumour suppressor (Reiss, 1997). For example, cell lines derived from the normal ectocervix are growth inhibited by exogenously administered TGFβ1 (De Geest *et al*, 1994). Malignant transformation is associated with loss of sensitivity to the growth inhibition of TGFβ1. In carcinoma of the cervix, this is independent of HPV status (Braun *et al*, 1990; De Geest *et al*, 1994; Rorke and Jacobberger, 1995), suggesting that acquired TGFβ1 insensitivity is a late occurrence in carcinogenesis. Braun *et al* (1990) demonstrated that HPV mRNA production also decreased in response to TGFβ1 administration, but only in lines retaining sensitivity to the growth inhibitory effects of TGFβ1. Woodworth *et al* (1996), however, found that five out of seven HPV immortalised cell lines were growth promoted by the addition of exogenous TGFβ1.

Immunohistochemical studies have shown that the distribution of TGFβ1 changes with disease progression (Comerci *et al*, 1996; Xu *et al*, 1999). Normal cervical epithelium stains strongly positive for intracellular TGFβ1, with minimal staining in the stroma. In malignant disease, TGFβ1 staining is concentrated in the extracellular compartment of the stroma adjacent to the neoplastic epithelium, with no staining remaining in the epithelium,

or in adjacent stroma underneath normal epithelium. Sections from patients with *in situ* disease fell between the two extremes of TGFβ1 expression, giving a distinct spectrum of change in TGFβ1 expression as cervical carcinogenesis progresses. Although de Gruijl *et al* (1999) showed TGFβ1 mRNA is present in both malignant and benign cervical disease, no quantitation of TGFβ1 mRNA or staining for the active TGFβ1 molecule was undertaken. In contrast, a study in breast cancer showed increased TGFβ1 mRNA and activity in cancer stroma and an inverse correlation between TGFβ1 expression and tumour differentiation (Cardillo *et al*, 1997). This latter finding was confirmed in a study showing increased TGFβ1 in conditioned medium from fibroblasts derived from the stroma surrounding malignant breast lesions, but not from fibroblasts obtained from nearby areas with a normal overlying epithelium (Chakravarthy *et al*, 1999).

Different mechanisms have been proposed for the loss of TGFβ1 growth inhibitory effects during malignant transformation. These include decreased TGFβ1 production, inability to activate TGFβ1 and changes in receptor signaling mechanisms (Reiss, 1997). The latter two have been the most intensely studied. Signaling changes can occur due to receptor mutation (Chen *et al*, 1999), altered distribution (Muro-Cacho *et al*, 1999, Anderson *et al*, 1999, Chakravarthy *et al*, 1999), or decreased expression (Tokunaga *et al*, 1999). Temporally initiated up-regulation of TGFβ1 production, sequentially followed by TGFβ1RI and II production occurred in mice mammary cancers treated with perillyl alcohol, a recognised differentiation-inducing anti-cancer agent. This increase in TGFβ1 corresponded with tumour regression (Ariazi *et al*, 1999). However, this has not been demonstrated in all tumour types. Friedenberg *et al* (1999) demonstrated no change in

TGFβ1 receptor density on malignant cells from patients suffering from chronic lymphocytic leukaemia (CLL) compared with normals. Nørgaard *et al* (1996) established and characterised three cell lines from the same patient with small cell carcinoma, at different stages during their disease. In this patient, disease progression was linked to the reappearance of TGFβ1RI and II proteins and increasing sensitivity to TGFβ1 growth inhibition. The initial cell line derived from this patient, prior to any treatment, possessed neither of the above. It may be that treatment, in the form of ionising radiation and polychemotherapy, selected clonally for both of these traits for, as yet, unknown survival gains.

A number of studies have shown that TGFβ1 production continues and is increased in tumours that are resistant to the negative growth effects of TGFβ1. The TGFβ1 is deposited extracellularly in increasing amounts in the peri-tumoural stroma. The selection advantage this accrues is not precisely known, but it is likely that severe local immune suppression (Young *et al*, 1996; Spellman *et al*, 1996) and increased angiogenesis (Taipale *et al*, 1998; Saito *et al*, 1999) both play a part.

Tumour marker

As described above, excessive deposition of TGFβ1 can occur extracellularly in the stroma of patients with malignant disease. This is thought to provide a pool from which increased circulating TGFβ1 is derived. Circulating TGFβ1 has been investigated as a tumour marker in a wide range of malignancies. When a normal range is quoted, it has been generated using methodology identical to that of the study population. As such, the normal ranges

vary from study to study. Elevated serum levels of TGFβ1 have been found in patients with transitional cell carcinoma (TCC) of the bladder (Eder *et al*, 1997), nasopharyngeal cancer (Xu *et al*, 1999) and cervical cancer (Chopra *et al*, 1998). Elevated plasma levels of TGFβ1 have been demonstrated in patients with malignant melanoma (Krasagakis *et al*, 1998), renal cell carcinoma (Wunderlich *et al*, 1998; Reinhold *et al*, 1997), colorectal carcinoma (Tsushima *et al*, 1996), a variety of intracerebral malignancies (Gridley *et al*, 1998), squamous cell carcinoma of the lung (Kong *et al*, 1996) and breast cancer prior to surgery (Sminia *et al*, 1998; Kong *et al*, 1995). Wakefield *et al* (1995) found elevated plasma TGFβ1 levels in only 2/28 patients with metastatic breast cancer. Perry *et al* (1997) showed no significant elevation in plasma levels of TGFβ1 in patients with prostate cancer, however urinary levels of TGFβ1 were significantly elevated. Tsai *et al* (1997) also demonstrated this finding in hepatocellular carcinoma (HCC). Finally, Friedenberg *et al* (1999) demonstrated a significant decrease in plasma TGFβ1 levels correlated with advancing disease stage in patients with chronic lymphocytic leukaemia.

Prognostic marker

A number of studies have correlated elevated TGFβ1 levels with poor prognosis in a variety of tumours. These studies comprise a mixture of ELISA-based blood and urine and immunohistochemical studies. Increased expression of TGFβ1 in tumours has been correlated with poor prognosis in colorectal tumours (Robson *et al*, 1996) and gastric cancer (Maehara *et al*, 1999). Increased urinary excretion of TGFβ1 correlated with worsening prognosis in hepatocellular carcinoma. Elevated serum levels of TGFβ1

correlated with poorer prognosis in nasopharyngeal cancer (Xu *et al*, 1999). In breast cancer the picture is more confused due to a greater volume of published work. Kong *et al* (1995) demonstrated that persistently elevated plasma levels of TGFβ1 in the post-operative period were due either to the presence of residual disease or axillary node positive status. However, Sminia *et al* (1998) and Li *et al* (1998) found no correlation between TGFβ1 plasma levels and poorer prognostic factors in breast cancer patients. Finally, Decensi *et al* (1998) correlated significantly increased plasma levels of TGFβ1 after twelve months treatment with a retinoid compound (fenretinide), to shorter survival free from the development of a second primary breast cancer.

Effect of radiation on TGFβ1 in tissues

Immunohistochemically, active TGFβ1 in normal rodents is limited to the epithelium and peri-epithelial stroma. Following irradiation of the mouse mammary gland, active TGFβ1 can be seen for the first time in adipose stromal tissues within one hour (Barcellos-Hoff *et al*, 1994). This increased active TGFβ1 persists for at least seven days in the mouse mammary gland (Barcellos-Hoff *et al*, 1994) and 26 weeks in rat small intestine (Langberg *et al*, 1994; Richter *et al*, 1997). Mice irradiated with strontium (Sr-90) to varying doses (ranging from 1 – 50 Gy), showed an initial decrease in TGFβ1 mRNA activity for the first three hours following exposure. By 48 hours post-irradiation the TGFβ1 mRNA activity had increased by 200% over control levels and remained persistently elevated for at least 8 weeks (Randall and Coggle, 1995). Immunohistochemical analysis of TGFβ1 and quantitative measurement of TGFβ1 mRNA activity in irradiated pig skin gave comparable

results (Martin *et al*, 1993). The staining for TGFβ1 was increased in the dermis from 3 weeks (first measurement) and persisted for at least one year. There was a corresponding significant elevation in the activity of TGFβ1 mRNA levels in the same areas. In humans, 4/6 patients who received pre-operative radiotherapy for rectal cancer had significantly increased TGFβ1 immunoreactivity in normal tissues within the irradiated field. All six patients also had significantly increased TGFβ1 in tumour bearing tissue. Three matched patients, who had initial surgical management, but no radiotherapy, also had elevated TGFβ1 in the tumour tissue. These patients had no TGFβ1 immunoreactivity in any unirradiated tissues (Canney and Dean, 1990). The irradiated tissues were examined between 7 and 40 weeks post-radiotherapy. Thus, increased TGFβ1 immunoreactivity can be seen within one hour of irradiation and persists for prolonged periods (up to 40 weeks) depending on the model used. This implies that molecular events are set in motion at the time of radiotherapy, which persist for a prolonged period, often in the absence of symptoms.

Response to radiotherapy

Changes in plasma levels of TGFβ1 following treatment have been investigated in a small number of studies. At diagnosis, patients with intracerebral malignancy had increased plasma TGFβ1 levels (Gridley *et al*, 1998). Following radiotherapy, two patients had persistently elevated TGFβ1 levels. The malignancy in both of these patients was uncontrolled by radiotherapy. In a cohort of surviving lung cancer patients at one year follow-up, those patients who had no evidence of disease had significantly lower plasma

TGFβ1 levels than those patients with demonstrable recurrent disease (Kong *et al*, 1996). This finding was confirmed by Groen *et al* (1997). The former study showed the clinical utility of changes in plasma TGFβ1 as a predictor of response was restricted to those patients initially presenting with an elevated TGFβ1 level.

Prediction of normal tissue radioresponse

In tissues the distribution of TGFβ1 changes in response to disease progression or therapy. In the field of radiotherapy, there is interest in assessing whether these changes can be used to predict normal tissue response. This interest is in the area of both acute (symptomatic radiation pneumonitis) and late (fibrosis) reactions.

In vitro and *in vivo* studies have implicated TGFβ1 as a mediator of radiation damage in normal cells. Exogenous TGFβ1, when added to a mink lung epithelioid cell line in culture caused reversible growth impairment. When the colony-forming ability of the cell line was investigated using a combination of DNA damaging agents (including radiation) and TGFβ1, the lethal effects of the agents were enhanced (Raynal *et al* 1997). This increased lethality was mediated via TGFβ1-induced apoptosis in this wild-type p53 cell line. In mice, exogenous TGFβ1 administered one hour prior to total body irradiation (TBI) enhances the lethality of a given radiation dose. This increased radiosensitivity may be secondary to a G_1 phase arrest of the cell cycle (Neta, 1997).

Following radiotherapy, increased TGFβ1 deposition co-localised with *de novo* expression of collagen type III in mice (Barcellos-Hoff, 1993) and with fibrosis, mucosal ulceration and connective tissue mast cell hyperplasia in rats (Langberg *et al*, 1994; Richter *et al*, 1997). TGFβ1 immunoreactivity was also significantly correlated with an overall radiation injury score as well as intestinal wall fibrosis alone. Increasing TGFβ1 immunoreactivity significantly correlated with increasing fraction size as well as decreased overall treatment time (Langberg *et al*, 1994). The former implies increased TGFβ1 activity following irradiation is linked to the development of *de novo* late radiation sequelae in the form of fibrosis. The latter implies that increased TGFβ1 activity is responsible for the development of consequential radiation injury. Acute radiation injury, in the form of ulceration, increases with decreasing overall treatment time, if other parameters remain unchanged. Prolonged severe ulceration is the initiating lesion of consequential late radiation damage. Increased TGFβ1 immunoreactivity was seen in relation to areas of ulceration. TGFβ1 immunoreactivity as well as TGFβ1 mRNA activity both increased in a dose dependent manner in irradiated pig skin. This increase co-localised with increased dermal fibrosis (Martin *et al*, 1993). In rats who underwent hemi-thoracic radiotherapy, increased TGFβ1 activity was demonstrable in the broncho-alveolar lavage fluid and increased TGFβ1 mRNA activity in lung tissue (Yi *et al*, 1996). Histological analysis revealed increased TGFβ1 in areas of fibroblastic proliferation in the alveolar septae. Plasma levels of TGFβ1 increased in rats given hemi-thoracic radiotherapy from 18 weeks following the insult. They remained elevated for 6 –8 weeks and this coincided with the period of maximal symptoms (Vujaskovic *et al*, 1997). In irradiated rat liver, TGFβ1

immunoreactivity in hepatocytes increased in a dose dependent manner and the intensity of staining significantly correlated with the extent of fibrosis (Anscher *et al*, 1990).

Increased TGFβ1 staining was seen in the irradiated tissues of 4/6 patients treated with pre-operative radiotherapy for rectal cancer (Canney and Dean, 1990). This was present at 7 weeks and persisted for at least 40 weeks. Patients who receive pre-operative radiotherapy are known to develop fibrosis in the treated area (Napoleon *et al*, 1991). Thus it seems likely that TGFβ1 plays an important role in the development of radiation induced fibrosis.

Interstitial pneumonitis and hepatic veno-occlusive disease are major causes of morbidity and mortality following bone marrow transplantation (BMT). Both diseases are characterised by increased fibrosis. Forty-one patients who underwent BMT for poor prognosis breast cancer had their plasma TGFβ1 levels measured prior to treatment, after completion of induction chemotherapy and following transplantation. Those patients, in whom TGFβ1 levels were elevated at the end of induction chemotherapy, had an increased risk of developing a fibrotic complication. The positive predictive value of a plasma TGFβ1 level greater than two standard deviations above the mean control level, for fibrotic complications was 90% (Anscher *et al*, 1993). Changes in plasma TGFβ1 levels have been shown to be predictive of the development of symptomatic radiation pneumonitis in patients receiving thoracic radiotherapy, with curative intent, for lung cancer (Anscher *et al*, 1994; Anscher *et al*, 1998a; Groen *et al*, 1997) or a variety of malignancies (Anscher *et al*, 1997). These patients can have either normal or elevated pre-treatment plasma TGFβ1

levels. During radiotherapy, the plasma TGFβ1 level can remain the same (either normal or elevated), or can become elevated (if initially normal) or normalise (if initially elevated).

The TGFβ1 ratio is defined as below: -

$$\text{TGF}\beta1 \text{ ratio} = \frac{\text{end of treatment plasma TGF}\beta1 \text{ level}}{\text{pre-treatment plasma TGF}\beta1 \text{ level}}$$

In order for the TGFβ1 level to be fully normalised, it must not only be less than the pre-treatment value, it also must be lower than the normal population value (defined as the mean plus two standard deviations of normal controls). In patients with lung cancer, the TGFβ1 ratio has a 90% positive predictive value for freedom from symptomatic pneumonitis (Anscher *et al*, 1998a). In patients with intra-thoracic malignancies, the positive predictive value of developing pneumonitis is 75% (Anscher *et al*, 1997). For those patients with initial elevated pre-treatment plasma TGFβ1 levels, the comparable predictive values are 90% and 83% respectively. Elevated plasma TGFβ1 levels in post-operative breast cancer patients have also been found to correlate with the development of fibrosis following radiotherapy (Li *et al*, 1999).

1.13 Aims

The aims of this study were: -

(a) To investigate the relationship between pre-treatment plasma TGFβ1 levels and outcome in a cohort of radically treated patients with cervical cancer

(b) To establish the reliability of a commercially available kit for measuring plasma TGFβ1

(c) To correlate changes in plasma TGFβ1 levels with outcome in a group of patients undergoing radical radiotherapy to the head and neck region

(d) To investigate the link between plasma TGFβ1 levels and late radiation outcome in a group of patients treated 4 – 6 years ago for early stage breast cancer

(e) To validate the use of an established PFGE assay in the prediction of normal tissue radioresponse in a group of patients treated 4 – 6 years ago for early stage breast cancer

Study Design

The cervical and breast carcinoma studies were retrospective in nature. The head and neck carcinoma study was prospective. All outcome data were collected in a blinded fashion. No interim analyses of data sets were carried out. All correlations were sought in a blinded fashion.

CHAPTER 2: MATERIALS AND METHODS

2.1 Patients

Ethical approval was granted for all patient groups by the South Manchester Local Regional Ethics Committee. All patients received relevant information sheets, approved by the Committee (Appendices 1&2). All samples were obtained following informed written consent.

Cervical carcinoma

Patient samples were available from those patients referred to the Christie Hospital between June 1990 and December 1993. Prior to the start of treatment, an EUA (examination under anaesthetic) was performed on all patients. During this procedure tumour stage, according to the FIGO classification was confirmed, and fresh biopsy material was obtained for independent histological analysis. All patients were treated according to strict protocols, depending on the stage of disease at presentation. Patients with small stage Ia tumours were treated mostly by radical surgery. However, in those who were medically unfit or who declined surgical treatment, radical radiotherapy consisted of two intracavitary insertions a week apart, giving a total prescribed dose of 65 Gy. Patients with higher stage disease, but without enlarged pelvic lymph nodes on CT scan, received 45 Gy in 20 fractions, external beam radiotherapy, in a four field pelvic "brick". This was followed, after one week, by a single intracavitary insertion, bringing the total dose received to 67.5 Gy. Patients with enlarged pelvic nodes were also treated initially by

external beam therapy, to a dose of 40 Gy in 20 fractions. The pelvic field was larger and irregular. A single intracavitary insertion was performed one week following the end of external beam treatment, giving a total dose of 62.5 Gy. A small number of patients received initial treatment in the form of surgery or chemotherapy. Only patients treated with radical intent were included in the study.

Head and neck carcinoma

Between August 1998 and February 2000, consecutive new patient referrals to the specialist head and neck clinic at the Christie Hospital, Manchester were invited to take part in the study. All patients approached were eligible to receive radical radiotherapy and none had a second, uncontrolled malignancy. The dose prescribed for radical radiotherapy in the head and neck region varied according to tumour site, tumour size, whether or not the patient had undergone surgery and whether or not the draining lymph nodes in the neck were treated. Patients with small volume disease present in the larynx received 52.5 Gy in 15 fractions, by means of a wedged pair. Patients, whose primary tumour had not been resected surgically, received between 50 and 52.5 Gy in 16 fractions. The field arrangements used depended on the site of the primary tumour. Patients who had surgical resection of their primary tumour, or whose parotid gland was treated received 50 Gy in 20 fractions. The same protocol applied to patients whose treatment fields covered the temporal lobe, e.g. carcinoma of the middle ear. In addition to having treatment to the area of the primary tumour, patients at high risk of nodal involvement received 47.5 Gy in 15 fractions to the neck prophylactically. Those patients with proven nodal involvement received 50 Gy in 15 fractions to the neck.

Patients were approached either during treatment preparation (a process taking up to three weeks) or in the early stages of treatment (when less than a fifth of the dose prescribed had been delivered). Eighty-six patients entered the study and eleven patients withdrew at their own request. All patients had a histologically proven malignancy of the head and neck region.

Breast carcinoma

Patients were approached at routine follow up appointments. Those patients who agreed to take part in the study were given a further appointment. This second appointment was for more than two weeks in advance to allow the patients to discuss the trial with their family and withdraw consent, if they so desired. Patients were recruited from a cohort of 190 women with early stage breast cancer treated at the Christie Hospital, Manchester between 1993 and 1994. All patients received external beam radiotherapy to the remaining breast tissue, following lumpectomy. A dose of 40 Gy in 15 fractions, prescribed to the isocentre, was given via a tangential wedged pair. No patient received a boost to the tumour bed. Patients received either axillary surgery or adjuvant nodal irradiation. Nodal irradiation was prescribed as a single anterior field, and the dose received was 40 Gy in 15 fractions. This cohort of patients had already taken part in a clinical study, at the time of their radiotherapy. All patients had a clinical photograph available (taken post-surgery and prior to any radiotherapy) as well as stored plasma samples taken prior to radiotherapy.

Fifty patients were recruited. Their ages ranged from 45 - 75 years (median 62 years). Patients who received any adjuvant medical treatment, other than tamoxifen, were not

recruited. Other exclusion criteria were: an inability to give fully informed consent, further surgery to either breast, development of a second primary tumour, and development of metastatic disease.

2.2 Normal volunteers

Ethical approval was granted by the South Manchester Local Regional Ethics Committee to obtain blood from normal volunteers. People with no history of cancer were recruited. This provided a large group of normal control samples with which to compare the patient samples. There were 22 men and 44 women. Their ages ranged from 20 - 84 years (median 62 years).

2.3 Sample processing

Plasma processing

Heparin method: Ten millilitres of blood was added to a 20 ml sterile Universal (Bibby Sterilin Ltd., Stone, England) containing 600 µl Heparin (Monoparin, CP Pharmaceuticals, England). The sample was left overnight at room temperature and then centrifuged at 240 rcf for 30 mins. The upper 50% of the plasma supernatant was divided into 350 µl aliquots, frozen and stored at -80°C. This yielded typically between four and six aliquots for experimentation.

EDTA method: Ten millilitres of blood was added to a Universal containing 15 mg of Tri-K EDTA, and placed immediately on ice. Centrifugation at 1,000 rcf for 30 mins took place within 30 mins, in a centrifuge pre-cooled to between 2 and 8°C. The plasma supernatant was then divided into 1 ml aliquots. Each aliquot was subjected to a further centrifugation at 10,000 rcf for 10 mins, in a centrifuge pre-cooled to between 2 and 8°C. The upper 350 µl of each aliquot was frozen individually and stored at -80°C. This yielded typically 3 or 4 aliquots for experimentation.

In a random selection of patients, using the EDTA method, the aliquots were numbered sequentially in the order in which they were obtained from the plasma with 1 being the topmost aliquot and 6 the lowest. As defrosting and refreezing cycles can lead to degradation of TGFβ1 molecules and therefore to spuriously low results, aliquots were frozen individually to allow multiple assays of TGFβ1 without repeated defrosting of plasma samples.

Tourniquet usage

A tourniquet was used in all patients. Ideally, to fully minimise platelet degranulation, venepuncture should be performed without the aid of a tourniquet. Prior to the start of this study a decision was made that all blood samples (patients and volunteers) would be taken using a tourniquet. There were a number of reasons for this: first, patients with head and neck malignancies are often chronically malnourished, making venepuncture difficult without the aid of a tourniquet. Second, all patients were donating 20 ml of blood, on two separate occasions. It was thought that the chances of obtaining enough blood, from patients who were often unwell, with the minimum of discomfort to the patient, would

require the use of a tourniquet. Patients who have had a traumatic single venepuncture, or who have multiple attempts at venepuncture, are understandably more reluctant to have a further venepuncture which will be of no direct impact on their health. None of the patients who withdrew from the study cited traumatic venepuncture specifically as a reason. Less than 3% of samples required more than 1 venepuncture to obtain a blood sample. Finally, the more easily blood is withdrawn at venepuncture, the less likelihood of spontaneous coagulation or turbulence resulting in platelet degranulation occurring. Thus, for a larger volume of blood to be drawn quickly, use of a tourniquet is a sensible compromise. Therefore, to distribute the risk of a systematic increase in TGFβ1 levels secondary to tourniquet use evenly, a tourniquet was used when taking samples from all volunteers.

2.4 Patient samples

Cervical carcinoma

Prior to the start of treatment, a venous blood sample was obtained from each patient and processed according to the heparin method outlined above. The lymphocytes were separated using Cappel Lymphocyte Separation Medium (LSM) (ICN Pharmaceuticals Ltd., Basingstoke, England) and stored in liquid nitrogen. In 1998, 127 samples were identified and defrosted into 350 μl aliquots.

Head and neck carcinoma

Blood samples for the measurement of plasma TGFβ1 were obtained by venepuncture of all subjects at the time of initial recruitment. A further sample was obtained in the final week of treatment, as near to the completion of radiotherapy as possible. In a randomly picked group of nine patients weekly venepunctures were performed, at the same time each week. Using a 21F-gauge needle (Becton Dickinson UK Ltd., Cowley, England) and a 20 ml syringe (Kendall Monoject, Gosport, England), 20 ml of venous blood was obtained from each patient, and divided into two 10 ml aliquots. The blood was processed according to the EDTA method outlined above.

Breast carcinoma

All patients had a 10 ml venous blood sample taken and processed according to the heparin method outlined above.

Skin samples

Patients, who had been treated previously for breast carcinoma, answered a questionnaire on risk factors pertaining to local anaesthetic use and the presence of a bleeding diathesis. The right or left buttock was chosen for the procedure according to patient preference. The skin was exposed and draped using materials from a NHS standard sterile supply pack (Readyfield option II+, NHS Supplies Authority, England). Sterets Tisept antiseptic (Seton Prebbles, Oldham, England) was used to clean the skin. One percent plain lignocaine

(Astra Pharmaceuticals Ltd., Kings Langley, England) was infiltrated into the chosen area of skin, using a 23F-gauge needle (Becton Dickinson) and a 10 ml disposable plastic syringe (Becton Dickinson). The local anaesthetic was left for a few minutes to take effect and the area of skin then tested for pinprick sensation using the 23F-gauge needle. When the skin was anaesthetised, a 5mm diameter punch biopsy of full dermal thickness was taken using a sterile disposable punch biopsy needle (Stiefel Laboratories (UK) Ltd., Wooburn Green, England).

Pressure was applied to the wound until haemostasis was achieved. The edges of the wound were then approximated using Steristrips (3M Health Care, MN, USA); and the area covered with gauze swabs (Vernaid, Vernon-Carous Ltd., Preston, England) and secured with transpore tape (3M Health Care). Patients were given instructions as to skin care and then allowed home. A letter, detailing the procedure was sent to the patient's GP, both as courtesy and in case infectious complications developed.

2.5 Volunteer samples

Volunteers had a 20 ml sample of blood taken. A number of volunteers donated blood on more than one occasion. Blood was processed according to the heparin and EDTA methods outlined above.

2.6 Transforming growth factor beta one (TGFβ1) assay

Transforming Growth Factor Beta 1 (TGFβ1) levels were measured using a commercially available Quantikine Kit (R&D Systems Abingdon, Oxon, UK). Instructions for processing blood samples to obtain platelet free plasma for assay have been outlined above. Only those samples prepared according to the EDTA method were as per kit instructions. Previously stored samples had been prepared according to the heparin method. All samples were assayed in a blinded fashion.

Sample preparation

All samples to be measured on the same kit run were defrosted simultaneously. Each aliquot contained 350 µl of platelet free plasma. This meant multiple assays, at differing dilutions, could be performed on one sample during the same kit run. It also meant that preparation errors could be corrected for without defrosting another sample aliquot. One hundred microlitres of each sample were placed in individual 1.5 ml eppendorfs (Anachem Ltd., Luton, England). Thorough mixing of each sample with an equal volume of 2.5 N acetic acid/10 M urea was performed, prior to incubation at room temperature. After 10 mins, 100 µl of 2.7 N NaOH/1 M HEPES was added and the contents of each eppendorf mixed. Both of these reagents cause activation of latent TGFβ1. Therefore, in all samples, total TGFβ1 levels were measured. The resultant pH of the final mixture was checked on three randomly picked samples regularly, and confirmed to be between pH 7.0 and 7.5.

Prior to plating, the sample mixture was diluted to an appropriate concentration with the kit-specified diluent. In the case of the EDTA samples this was a 12-fold dilution. The stored samples taken using heparin as an anti-coagulant were measured using a 24-fold dilution. If the sample results were higher than the upper limit of the standard curve, the sample was assayed again, using a fresh aliquot and an appropriate dilution, at a later date. Any defrosted plasma not used was discarded safely.

The 96 wells (which comprised the plate) were supplied pre-coated with recombinant human TGFβ receptor type II (TGFβRII). For each kit run, a standard curve was generated. Recombinant human TGFβ1 was prepared to a known standard concentration using the kit-specified diluent. A serial dilution was then performed to produce decreasing concentrations of TGFβ1, using the same kit-specified diluent. Two hundred microlitres of diluent alone, and then each successive concentration of TGFβ1, were added to three successive wells. Thus 24 wells in every kit were used to generate a standard curve. The remaining 72 wells were each filled with 200 μl of the sample mixture, either in duplicate (cervix samples) or triplicate (all other samples). The plate was then covered with an airtight adhesive strip and left to incubate at room temperature. After three hours, the adhesive strip was removed and the contents of each well aspirated and discarded. The wells were then washed three times with 300 μl of kit supplied wash buffer. Each well then received 200 μl of TGFβ1 conjugate; this consisted of a polyclonal anti-TGFβ1 antibody conjugated to horseradish peroxidase. The plate was covered again with an airtight adhesive strip and left to incubate at room temperature.

After 1.5 hours the strip was removed, the wells aspirated and the wash step repeated. Two hundred microlitres of the substrate solution (which consisted of equal volumes of hydrogen peroxide and a stabilised chromogen solution, added together within 15 minutes of use) was added to each well and the plate left to incubate, this time uncovered. A blue colour developed in each well. After 20 mins, 50 µl of 2 N sulphuric acid was added to each well to halt the colour reaction. With the addition of the acid the mixture in the wells turned yellow.

The optical density (OD) of the well contents was measured using a dual wavelength spectrophotometer (Titertek Multiskan, MCC/340 mark II, Labsystems, UK) within 30 mins. The filter wavelengths used were 450nm and 540nm. The difference in OD between these filters gave the final OD from which all results were derived. In practice, the plate was read twice within a thirty-minute period for each plate run. This established that there was no significant colour degradation over time.

Processing of results

The individual ODs were input into an Excel spreadsheet and the mean OD value was calculated. The corrected OD was determined, for all samples, using the formula:

$$\text{corrected OD} = \text{mean OD} - \text{background OD}$$

The background OD was the mean OD of the three wells containing diluent only. The concentration of the standards ranged from 0-2000 pg/ml. The corrected ODs typically

ranged from 0.03 - 1.5. The standard curve data were transferred to Origin, and a scatter plot generated. A linear fit of the data was then performed. Logarithmic transformation of the data was not required to produce a straight line. The equation of the line was then transferred back to the Excel spreadsheet to derive the concentrations of all test samples.

2.7 Pulsed-field gel electrophoresis (PFGE) assay

Tissue disaggregation

Skin biopsies were placed in 10 ml of filtered HBSS (GIBCO, Paisley, Scotland) on ice and transported to the laboratory. All biopsies were processed within 2 hours of being taken. All manipulations of the skin biopsies, and cells subsequently grown were carried out in a Class 2 microbiological containment cabinet. Each skin biopsy was disaggregated in 50ml high anti-biotic medium. High anti-biotic medium comprised BME (GIBCO) without serum, supplemented with 20 μg/ml amphotericin (Sigma, Dorset, England), 20 μg/ml gentamicin (Sigma) and 50 mM HEPES (GIBCO). In addition collagenase type I (Sigma), DNAse type I (Sigma) and pronase (Boehringer Mannheim, GmbH, Germany) were dissolved in the medium to concentrations of 0.5 mg/ml, 0.5 mg/ml and 0.4 mg/ml, respectively.

The biopsy was trimmed of subcutaneous fat and then divided into six or eight equal pieces using two sterile scalpels (Swann-Morton Ltd., Sheffield, England). The samples were distributed between two 100 ml capacity durans each containing 25 ml of the high anti-biotic/enzyme cocktail. The neck of each duran was covered with adhesive tape (Scientific Marketing Associates, Hertfordshire, England). The durans were then placed in a preheated

shaking waterbath at 37°C. After 1.5 hours, the high anti-biotic/enzyme cocktail mixture was aspirated using 25ml sterile disposable pipettes (Falcon, Fred Baker Scientific, Cheshire, England), leaving the tissue samples in the duran. Ten ml of a 0.5% (w/v) trypsin (Lorne Laboratories, Twyford, England) solution was added to each duran. The bottles were resealed and replaced in the shaking waterbath.

After a further 30 mins, the bottles were removed and 10 ml medium added to each duran to neutralise the trypsin. The neutralising medium consisted of MEM (GIBCO) plus 15% FCS (Biowhittaker, Wokingham, England), 2 mM glutamine, 100 U/ml penicillin and 100 µl/ml streptomycin (all from GIBCO). This medium was also used for feeding and cryopreserving all fibroblast lines grown subsequently. The contents of each duran were individually filtered, first through a 100µm (Falcon) and then 40µm (Falcon) bottle top filter into a 50 ml conical bottomed centrifuge tube (Falcon). Both tubes were then centrifuged at 1800 rpm for 10 mins. Following centrifugation, the supernatant was removed and the cells resuspended in 2 ml of fresh medium. One millilitre of the cell suspension was added to a vented T12.5 sterile tissue culture flask (Becton Dickinson) i.e. four T12.5 flasks were derived from one tissue sample. These flasks were then placed in an incubator at 37°C, in a 5% CO_2 in air atmosphere and left undisturbed for seven days.

Tissue culture

At seven days post-biopsy, the T12.5 flasks were examined for signs of growth. All cell lines became established. Fresh medium, 1ml, was then added to each flask. Thereafter, the cells in each flask were fed at least once per week, and often twice, depending on their requirements. Most flasks initially grew a mixture of fibroblasts and keratinocytes,

reflecting the heterogeneous cell population released from the tissue biopsy. Keratinocytes are more difficult than fibroblasts to maintain in culture, requiring special media. By passage 2 all flasks contained a morphologically pure fibroblast culture.

Once the cells in each T12.5 flask became confluent, they were passaged into larger (T25) flasks. This occurred at a mean of 17 days post-biopsy (range 12 – 33 days). The cells in each of the four flasks grew at different rates and the range quoted is that of the quickest growing cells. When cells were ready for passaging, the medium was removed and 1-5 ml of 0.01% EDTA (BDH, Chemicals, Poole, England) was added, denaturing all non-specific serum based anti-trypsin activity. Between 1 and 5 ml of 0.01% (w/v) trypsin (Lorne Laboratories) was added to each flask to digest the collagen anchoring the cells to the plastic flask. The flask was then agitated to ensure all cells were in suspension. In general, this took around 5mins. The cells were exposed to trypsin for no longer than 15 mins, in order to prevent cell damage.

The cell suspension was added to an equal volume of medium in a sterile Universal container. If a cell count was required, 50 µl of the cell suspension was mixed with an equal volume of 0.03% trypan blue (ICN Pharmaceuticals) solution, and added to a haemocytometer slide (H12-158 double modified, Fuchs Rosenthal, Philip Harris, Trafford Park, Manchester). The total number of cells present was then calculated. The cell suspension was centrifuged at 1000 rpm for 5 mins, to produce a cell pellet. The supernatant was aspirated and the cells resuspended in the required volume of fresh medium and added to the larger flask. This was returned to the incubator. Generally, once the cell lines became established, they were passaged on a 7 - 14 day cycle.

Cell cryopreservation

Each tissue biopsy generated four T75 flasks of cells. Only one of these was needed for initial experiments. In culture non-transformed fibroblasts have a limited life span of 15-20 passages. The longer a cell line is kept in a flask, the greater the likelihood of infection occurring, resulting in loss of unique cell line material. For both these reasons, as well as to preserve stocks for future and repeat experiments, it was necessary to store the cells. This was accomplished by cryopreservation and storage of the cell lines in liquid nitrogen.

Cells were cryopreserved when they reached confluence in a T75 flask. Twenty-four hours prior to cryopreservation, fresh medium was added to the flask. On the day of cryopreservation, the cells were suspended in the flask using EDTA and trypsin and counted as outlined above. The cell suspension was then spun at 1000 rpm for 5 mins to form a cell pellet, which was resuspended in an appropriate amount of fresh medium. Cells were cryopreserved at a concentration of 1-2 x 10^6 per ml medium. To prevent lethal intracellular ice crystals forming during cryopreservation, DMSO (Sigma) was added to the cell suspension to a final concentration of 10%, i.e. a maximum of 2 x 10^6 cells were frozen in 0.9 ml fresh medium plus 0.1 ml of DMSO. This mixture was placed in 2ml capacity cryotubes (Nalgene Nunc, Techmate Ltd., Milton Keynes, England) that were transferred to the liquid nitrogen freezer for storage.

Defrosting cells

Cell lines were defrosted, as required, for further experiments. The cryotube containing the desired cell line was removed from liquid nitrogen storage, and defrosted rapidly in a water-bath at 37°C. The contents of the tube were then added to a Universal container, and

10 ml of previously warmed fresh medium was added dropwise. The resulting mixture was spun at 1000 rpm for 5 mins, to form a cell pellet. On removal from the centrifuge, the supernatant was aspirated and the cell pellet resuspended in 1 ml of warmed medium. This was added to 2 ml of warmed medium in a vented T12.5 flask and placed in the incubator. As most of the DMSO was removed by the initial centrifuge step, the medium did not require changing after 24 hrs. The cells were passaged into larger flasks once they had reached confluence (usually after 2-3 days), until enough cells were available for an experiment.

Cell preparation

For enough cells to be available, a minimum of one confluent T25 flask was required. In practice, a confluent T75 flask was most commonly used. Cells were disaggregated, using trypsin as described above and, while in suspension, were counted. A cell pellet was then formed by centrifugation at 1000 rpm for 5 mins. The cell pellet was resuspended in an appropriate volume of medium to give $1x10^6$ cells/ml. Cells were seeded simultaneously into two T12.5 flasks (one for irradiation and one as a control), at a density ranging from $2x10^4$ to $2x10^5$ cells, depending on the previously observed growth characteristics of that cell line. These flasks were placed side by side in the incubator until confluence was reached. This generally took 10-14 days, but ranged from 6-28 days.

Irradiation

When the cells reached confluence, the experimental flask was irradiated at room temperature to 150 Gy. A ^{137}Cs γ-ray source, at a dose rate of 3.11 Gy/min, was used for all

irradiations. Following irradiation, the flasks were replaced in the incubator for 24 hrs to allow repair to take place.

Plug preparation

After 24 hrs, the cells were disaggregated using 0.01% trypsin, neutralised with an equal volume of medium and counted. Flasks with less than $1.7x10^5$ or greater than $8x10^5$ cells were discarded. A cell pellet was formed by centrifugation at 1000 rpm for 5 mins. The supernatant was removed and the pellet resuspended by gentle pipetting with an appropriate amount of 0.7% Sea Plaque low gelling point agarose (FMC BioProducts, Flowgen, Staffordshire, England). The final concentration of cells was 2 x 10^5/75 µl of agarose. Aliquots of 75 µl were pipetted into 1.5 x 5 x 10 mm plug moulds (Bio-Rad Laboratories, Hemel Hempstead, England) which were placed in the fridge for 10 mins to set.

Control plugs containing lambda DNA (Promega, Southampton, England) were made in a similar fashion. Five microlitres of lambda DNA stock solution (428µg/ml) was mixed with 75.3 µl of ddH_2O. This solution was added subsequently to 320 µl of 0.7% low melting agarose and mixed thoroughly. The final mixture was then pipetted in 75 µl aliquots into plug moulds and allowed to set. This made a double plug. The double plugs were stored in 0.5 M EDTA prior to use. Ten double plugs required 4 ml of EDTA solution for storage. The double lambda plugs were stored at 4°C and used within two months.

Cell lysis

Control and irradiated double plugs were lysed in separate bijou bottles (Bibby Sterilin Ltd.). Lysis solution consisted of 2% sodium lauroyl sarcosinate "Sarkosyl" NL30 (BDH), 5 μg/ml proteinase K (Sigma) and 0.47 M EDTA (BDH). The lysis solution was made up less than one hour prior to use and stored at 4°C. Proteinase K was made up as a 10 mg/ml solution in ddH_2O. It was stored in 1 ml aliquots at -20°C and defrosted for each use. Each double plug was placed in a minimum of 1 ml lysis solution and returned to the fridge for a minimum of 30 mins. The plug/lysis mixture was then placed in the incubator overnight for a minimum of 17 hours. The double plugs were then stored at 4°C until electrophoresis. This took place within 4 weeks of irradiation.

PFGE

Prior to loading onto a gel, each double plug was cut in two transversely, giving two identical plugs. Each plug was loaded onto a separate gel. Two gels were loaded identically and run simultaneously. Each gel contained 0.95g of molecular specification agarose (Kodak Scientific Imaging Systems, Anachem, Luton, England) dissolved in 135 ml of x0.5 TBE. TBE was made up as a stock solution of x5 strength comprising 54 g TRIS base (Boehringer Mannheim, GmbH, Germany), 27.5 g boric acid (Sigma) and 20ml 0.5 M EDTA (pH 8.0), in 1000 ml ddH_2O. This stock solution was stored at room temperature and diluted with ddH_2O, as required. The gels were 15 x 15 x 0.6 cm in size and comprised 0.7% agarose. They were cast with a comb containing sixteen 5 x 1.5 mm teeth set approximately 2.5 cm from the anode end. Each plug was placed in the corresponding lane of each gel. A lambda control plug was placed in one lane of each gel. One to three lanes in

each gel were left empty to provide realistic background measurements when the gels were analysed. The plugs were sealed by addition of a small amount of 0.7% low melting point agarose once all lanes were filled.

The PFGE apparatus was a complex unit containing: two interconnected gel tanks (Pharmacia Biotech, St. Albans, Hertfordshire, England), a cooling unit, a power pack, and a control unit (Pulsaphor, Pharmacia Biotech). The gel tanks each contained 2.5 l of x0.5 TBE, which was circulated around the tank constantly. The cooling unit was set at 9°C, which was the equivalent of 14°C in the gel tank. Each gel was placed in a separate tank and both were subjected simultaneously to a 40 hour electrophoresis run at 47.5 V (1.7 V/cm), with a 75 min switch interval. This was followed by a 2 hour run at 100 V, with a switch time of 30 mins.

Gel drying

Both gels were dried at the end of each run. This facilitated storage and staining of the gels. Each gel was removed individually from the gel tank and carefully placed flat. All save 8 cm of gel was trimmed from the cathode end. The bottom left hand corner of the gel was cut on the diagonal to allow orientation when dried. The cathode end of the gel was cut approximately 2 mm from the plugs. A corresponding cut was made approximately 2 mm to the anode side of the plugs. The piece of gel containing the plugs was then flipped through 90° to display the largest surface area of the plugs in series with the lanes. The cut edges were then approximated and the gel transferred to a piece of 3mm chromatography paper (3mmChr, Whatman, Maidstone, England) on the gel dryer (Gel-Vac, Hybaid, Middlesex, England) and covered with Saran wrap (Jencons, Leighton Buzzard, England).

The gel dryer was run at 30°C for 30 mins prior to use, to clear any moisture in the trap. Once the gels were in place, the dryer was run at 30°C for 30 mins, then turned up to 60°C for 60 mins. The gels were then removed and the dryer run at 30°C for a further 30 mins to clear the trap. The gels could then be stored until staining took place.

Gel staining

The gels were rehydrated prior to staining. The Saran wrap was peeled carefully away from the anterior of the gel, and the blotting paper/gel complex placed in 500 ml of ddH_2O for 10 mins. The blotting paper was then peeled away from the gel, leaving it floating in the water. Using gentle movements, any remaining fibres of blotting paper were removed from the gel. Each gel was then placed in a separate sealed plastic container to be stained. Each container was filled with 200 ml x0.5 TBE at pH 8.0. Twenty microlitres of SYBR green I stain (Molecular Probes, Eugene, USA) were added to each container and mixed well. As SYBR green I is a photosensitive compound, all mixing steps were performed rapidly and the containers covered with aluminium foil. The gels were submerged fully in the liquid and the containers transferred to a prewarmed oven (50°C) for three hours.

Imaging with the STORM optical imager

After three hours incubation in the oven, the containers (still covered to exclude light) were transferred to the STORM optical imager. The gels were thoroughly rinsed in ddH_2O and placed on the glass screen of the STORM machine. The orientation was determined by means of the cut bottom left hand corner. In addition, all gels were loaded asymmetrically

so that any accidental change in orientation would be easily identifiable. The gels were imaged using the blue fluorescence screen of the STORM machine, and ImageQuant software. Rectangles were drawn around each plug and lane individually, as well as the equivalent areas for those plugs and lanes that had been left blank. The empty plug and lane were used to correct for incidental background fluorescence. The fluorescence in any one lane was divided by the total fluorescence in that lane plus the corresponding plug. This gave a measure of the fraction of damaged DNA released (FDR) into the lane from the plug. This corresponded to the residual DNA damage left after 24 hours repair. In addition, the same calculation was performed for the lambda plug that acted as a positive control. The FDR was calculated for all lanes in all gels using an Excel spreadsheet.

Normalising FDR results

The spreadsheet provided results for all plugs run on any one gel. Two identical gels ran simultaneously. The mean FDR of the control, i.e. unirradiated plugs, was obtained and subtracted from the mean FDR of the irradiated plugs of the same cell line. This gave the normalised FDR for that experiment. In forty-four cell lines, a minimum of three, independent normalised FDRs were obtained. In five cell lines, two independent FDRs were obtained. From one cell line, no useful results were obtained, due to high FDR values in the control plugs. The average of all valid normalised FDRs for a particular cell line was obtained and used in the final analysis of data.

2.8 Assessment of clinical outcome

Cervical carcinoma

In this group of patients survival was monitored prospectively by medical staff at routine follow-up appointments. Results of routine physical examinations were obtained also. The incidence of loco-regional and distant failure was recorded, as was cause of death. For those patients who defaulted from follow-up, regular letters inquiring about patient survival and recurrence were sent to the named GP. Morbidity was monitored retrospectively by means of the Franco-Italian glossary. Morbidity at all possible sites was recorded on a pro forma and entered on a password-controlled database. Grade of morbidity was recorded using WHO criteria, taking into account recorded patient symptoms, investigations and in-patient stays, if applicable.

Head and neck carcinoma

Following informed consent, a LENT SOMA questionnaire was completed by one of two dedicated Research Sisters. The same questionnaire was completed in the final week of radiotherapy treatment, six weeks following the end of treatment and six monthly thereafter for a total of three years.

Due to the geographically dispersed nature of the treated patient population, only those willing and able to attend the Christie Hospital for follow up visits were recruited initially. Consequently, initial recruitment was slow. It was realised that, in order to recruit enough patients to gain meaningful long term follow up data, patient accrual into the study would

have to be increased. Therefore, the LENT SOMA questionnaire was modified into a postal format. Once the postal questionnaire was available, accrual continued at an increased and satisfactory rate.

Breast carcinoma

When the patients returned for their second visit, the trial was again explained and any questions answered. Written informed consent was then obtained. One of two dedicated Research Sisters then completed a LENT SOMA questionnaire which dealt with subjective data. Objective data on fibrosis and oedema as late normal tissue end-points were obtained by breast examination. This was carried out in all patients by myself. A clinical photograph was taken to allow assessment of retraction, telangiectasia, breast and body size, by three independent clinicians, experienced in the field of late radiation reactions. Each photograph was scored, using a pro forma in a convenient A4 format (Appendix 3). At the time of scoring, the clinicians had access to a previously agreed library of clinical photographs for comparison. None of the patients being scored in this study appeared in the standard library of photographs.

2.9 Data collection and analysis

Cervical carcinoma

At the time of the initial experiments, all demographic and tumour-specific data were collected on a customised database. The database was password secured, and has been maintained by the regular addition of follow-up data. The measured plasma levels of TGFβ1 were added to this database, and an SPSS statistical package used to analyse the data. Plasma TGFβ1 levels were correlated with other parameters using nonparametric tests. Log-rank analysis of plasma TGFβ1 levels was performed in relation to morbidity, local control and survival. For measures of sensitivity a 2 x 2 table was constructed (see example below) and the patients assigned to the appropriate category.

	Disease factor positive i.e. patient alive	**Disease factor negative i.e. patient dead**
Test factor positive i.e. low TGFβ1 level	A	B
Test factor negative i.e. high TGFβ1 level	C	D

The sensitivity and specificity were then determined using the following formulae:-

Sensitivity = A / (A + C) and

Specificity = D / (B + D)

Head and neck carcinoma

Demographic and tumour details from all patients were prospectively loaded onto a customised database, which was password controlled. Plasma TGFβ1 levels were added to this database as soon as they became available. Data were analysed using an SPSS software package. Correlations between TGFβ1 levels and clinical endpoints were sought using nonparametric tests. Sensitivity and specificity were determined as described above. The positive predictive value was calculated as:-

$$\text{Positive predictive value} = A / (A + B)$$

Breast carcinoma

Demographic details obtained from all patients were loaded into a specially created, password secured database. Scores relating to cosmesis, derived from clinical photographs and physical examination, were added to the database, as were the mean normalised FDRs from all cell lines. Pre-treatment and delayed plasma TGFβ1 levels were also added. Correlations were sought between all aspects of cosmesis scored from the clinical photographs: subjective and objective measures obtained from the LENT SOMA questionnaires, plasma TGFβ1 levels, and normalised FDRs from each patient. The data were analysed using an SPSS software package, and nonparametric tests.

CHAPTER 3: CERVICAL CARCINOMA

3.1 Introduction

In normal tissues TGFβ1 is the most potent known inhibitor of epithelial growth (Moses *et al*, 1991). In response to injury, it stimulates fibroblast migration, proliferation and collagen production (Shah *et al*, 1999). TGFβ1 acts early in carcinogenesis as a tumour suppressor. Later in carcinogenesis it acts as a tumour promoter by stimulating tumour angiogenesis and proliferation and inhibiting host immune function (Roberts *et al*, 1988; Reiss, 1999). Evidence is accumulating that this switch may occur as a consequence of changes in TGFβ signaling, in particular mutations of the TGFβRII gene, which is prone to genomic instability (Lu *et al*, 1995; Simms *et al*, 1997).

In radiation oncology, interest in TGFβ1 lies in its role in the pathogenesis of both acute and late radiation sequelae. Changes in plasma TGFβ1 levels have been shown to have potential clinical usefulness as a predictor of acute radiation morbidity in patients receiving thoracic radiotherapy (see Section 1.12). Pre-treatment plasma levels have also been shown to identify breast cancer patients with an elevated risk of developing late fibrosis following radiotherapy (Li *et al*, 1999).

To date no studies have examined the prognostic significance of circulating TGFβ1 levels in relation to outcome in carcinoma of the cervix. Therefore, the following study was carried out to examine the prognostic significance of measurements of plasma TGFβ1 for

tumour control and morbidity in a group of patients treated with radical intent for carcinoma of the cervix.

3.2 Previous data available

Blood samples were taken from patients prior to the start of radical radiotherapy between 1990 and 1993. Plasma from the samples was frozen at -80°C and was available for study. The plasma samples had been assayed previously in a blinded fashion, for three tumour markers: carcinoma antigen 125 (CA125), tissue polypeptide antigen (TPA) and squamous cell carcinoma antigen (SCC). This work has been published elsewhere (Sproston *et al*, 1995). The intrinsic radiosensitivity of peripheral blood lymphocytes was determined using a limiting dilution clonogenic assay (West *et al*, 1998). All previous data are listed in Table 3.1.

Table 3.1: Data for patients with carcinoma of the cervix

Lab. Number	Age (years)	Stage[1]	Histology[2]	Grade[3]	Plasma TGFβ1 levels (ng/ml)	Plasma CA125 (U/ml)	Plasma SCC (ng/ml)	Plasma TPA (U/ml)	Lymph. SF_2[4]
1	30	2	1	2	12.01	U	U	U	0.23
2	38	1	1	2	9.08	U	U	U	0.42
4	59	2	1	2	7.12	8.7	0.52	24.1	0.49
5	50	1	1	2	4.61	18.6	6.73	77.2	U
6	70	2	2	3	6.98	22.4	0.12	68.4	0.39
7	68	2	1	3	5.82	35.4	0.16	22.8	0.43
8	U	U	1	2	11.64	64.7	3.69	81.6	U
9	45	3	1	2	12.92	17.9	23.5	370.7	0.37
10	33	2	1	U	7.1	32.9	3.29	26.6	0.25
12	54	1	1	U	15.91	U	U	U	0.18
13	45	2	1	2	4.76	15	1.18	48.4	0.48
15	81	1	1	1	3.95	24.5	0.11	92.7	0.46
16	77	3	2	U	6.8	25.5	0.24	109.9	0.37
18	60	1	1	U	5.05	U	1.99	69.8	0.27
19	81	1	1	3	9.32	12	24.7	105.7	0.21
21	69	2	1	U	4.81	15	3.12	62.7	0.53
22	53	1	2	2	4.93	10.5	0.48	15.3	0.54
23	60	1	1	U	6.02	25.7	0.47	47.5	0.24
24	60	1	2	3	7.09	8.3	0.24	26.3	0.3
26	69	1	1	2	5.88	U	U	U	0.3
27	64	2	1	2	3.96	18.7	4.02	72.3	0.18
30	67	1	1	U	3.06	12	0.48	20.1	0.32
31	32	3	1	U	21.85	114.6	140.2	682.6	0.09
32	64	2	1	U	9.6	40.9	0.01	89.9	0.2
33	47	2	1	3	8.52	12.7	0.41	372.8	U
35	83	2	1	2	6.19	6.57	25.8	61.6	0.34
37	66	2	1	2	4.99	11.1	0.59	36.3	0.31
38	50	2	1	U	6.97	19.1	16.22	51.9	0.3
39	41	2	1	2	11	20.6	9.59	246.5	0.3
42	61	1	1	U	5.28	21	0.89	21.8	0.37
43	70	1	1	U	8.2	11.2	1.17	51.7	0.39
44	43	1	1	U	7.01	11.8	3.33	106.5	0.3
45	56	1	1	2	4.41	3.5	0.21	23.2	U
46	55	1	1	U	3.1	19	0.09	21.9	0.27
47	60	2	1	2	5.94	12.3	1.14	19.9	0.32
48	59	2	1	1	5.32	18	5.81	76.5	U
52	51	1	1	U	4.87	32.7	0.49	67.4	0.36
56	40	1	1	3	2.46	11.3	1.58	54.1	U
58	63	2	1	3	5.7	24.2	2.14	182.4	0.35
59	50	1	1	U	4.11	19.7	0.57	49.6	0.33
60	74	3	1	2	8.95	13	5	82.5	0.23
61	73	3	1	1	5.2	30.3	1.43	134.5	0.23
62	64	3	1	2	6.74	101.4	20.6	180.7	0.34
64	49	2	2	3	6.2	29	1.18	113.8	0.3
65	68	1	1	1	6.63	25.2	1.06	31.9	0.22

Lab. Number	Age (years)	Stage[1]	Histology[2]	Grade[3]	Plasma TGFβ1 levels (ng/ml)	Plasma CA125 (U/ml)	Plasma SCC (ng/ml)	Plasma TPA (U/ml)	Lymph. SF_2[4]
68	29	2	U	U	10.46	24.7	1.37	99.1	0.25
71	54	1	1	U	3.86	12.8	1.26	84.3	0.22
73	70	2	1	2	6.68	17.6	0.54	47.7	0.27
75	62	2	1	2	4.75	2.9	3.78	108.1	0.65
81	70	1	1	2	3.5	10	9	266.5	0.33
84	48	2	1	2	7.13	12.5	2.86	51	0.21
86	56	1	1	U	5.53	4.6	0.49	45.7	0.42
87	52	3	1	3	9.59	458.8	43.8	327.8	0.28
90	61	3	1	2	7.48	23.4	2.12	62.2	0.28
91	68	3	1	2	3.74	3.8	108.7	595.3	0.21
92	53	1	1	U	3.51	6.9	0.3	25.2	0.42
96	50	1	1	2	2.38	11.1	1.86	41.6	0.36
102	60	1	2	1	3.88	0	0.1	29.6	0.38
103	61	1	U	U	4.96	7.51	0.07	36.5	0.4
105	23	1	1	2	4.31	11.2	64.8	41.4	0.33
107	66	1	1	2	6.67	32.8	0.09	27.8	0.23
108	36	3	1	2	5.57	19.4	18.07	24.1	0.26
109	70	1	1	3	2.55	0	0	43.1	0.2
110	38	3	1	2	7.36	45	7.02	100.5	0.27
111	68	1	1	3	12.04	20.7	0.52	67	0.44
112	69	2	1	3	4.99	22.4	0.36	48.4	0.33
114	61	2	1	2	5.53	5.7	17.3	189	0.35
118	60	1	1	U	2.91	6.2	7.23	31.7	0.36
120	54	1	1	2	1.95	8.2	1.31	50.9	0.4
125	38	1	1	U	6.44	12.2	0.22	30.7	0.27
126	38	2	2	2	1.87	10.1	1.22	56.9	0.32
127	33	2	1	2	6.02	19.7	3.46	37.9	0.44
140	48	2	1	U	3.07	U	4.28	51.2	0.24
141	76	3	1	2	3.7	U	3.16	38.1	0.34
186	69	2	1	2	9.69	U	7.58	22.9	0.33
230	65	3	1	1	4.61	9.4	41.1	59.6	0.33
232	71	3	1	U	7.46	10.5	3.2	77.5	0.26
242	45	2	1	2	5.75	32.7	41	146.3	U
275	67	3	1	1	4.99	9.2	20.4	25.1	0.28
276	38	2	1	U	4.2	47.1	11.4	33.9	0.11
277	44	2	1	1	3.48	15.9	0.1	33.9	0.45

[1] – U indicates unknown

[2] – Histology – 1 = squamous carcinoma, 2 = adenocarcinoma

[3] – Grade – 1 = well differentiated, 2 = moderately differentiated, 3 = poorly differentiated

[4] – Lymph. - lymphocyte

3.3 Plasma TGFβ1 results

The samples were processed and centrifuged as per the Heparin protocol described in Section 2.3, prior to storage at -80°C. In 1998, 127 samples were identified and defrosted into 350 μl aliquots. Plasma TGFβ1 levels were estimated using the Quantikine kit and the values obtained ranged from 1.87-21.85 ng/ml (Figure 3.1). The mean with one standard deviation and median values were 6.30 ± 3.20 ng/ml and 5.70 ng/ml, respectively. Significant differences were detected between patient samples using one-way analysis of variance (ANOVA) ($p < 0.001$). Assay reproducibility was examined using 43 volunteers measured in triplicate. Intra-individual variability was ascertained on 9 volunteers measured on two or three separate occasions. Table 3.2 summarises the comparison of assay and donor variability.

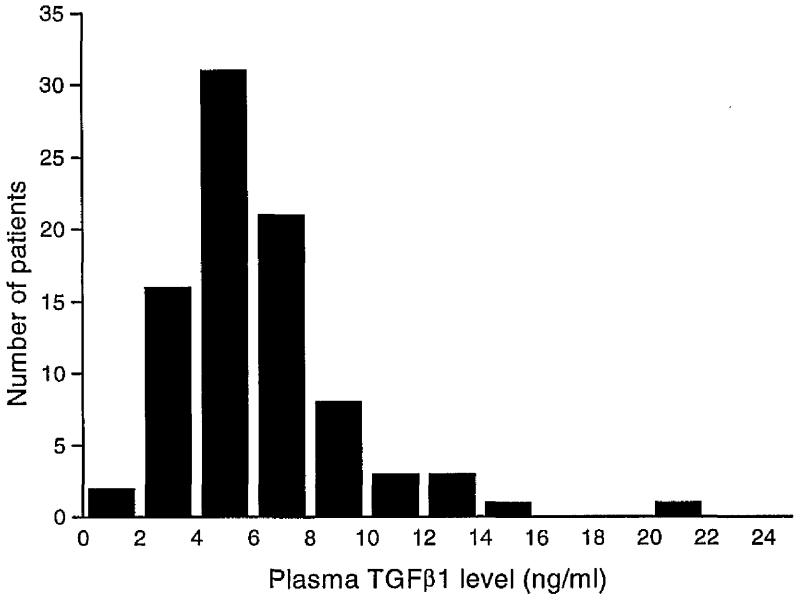


Figure 3.1: Distribution of TGFβ1 levels in samples from patients with carcinoma of the cervix

Table 3.2: Summary of assay reproducibility

Variability	N	Coefficient of variation (CV)
Assay	43	4%
Intra-volunteer	9	13%
Inter-patient	79	51%

3.4 Comparison of biological and clinical parameters

Spearman's non-parametric regression analysis was used to examine correlations between TGFβ1 levels and patient age, tumour stage and grade (Table 3.3). There was a weak significant correlation between stage and TGFβ1 levels ($r = 0.30$, $p = 0.006$). The distribution of TGFβ1 across the disease stages is illustrated in Figure 3.2. None of the other patient parameters showed significant correlation with plasma TGFβ1 levels. The relationship between TGFβ1 levels and histology was investigated using the Mann-Whitney u-test. The mean plasma levels of TGFβ1 in 72 patients with squamous carcinoma and 7 patients with adenocarcinoma were 6.35 and 5.39 ng/ml respectively. There was no significant differences between these values ($p = 0.28$). Using Spearman's regression analysis, there was a significant positive relationship between plasma TGFβ1 levels and the levels of previously measured circulating tumour markers, CA125 and TPA. There was no relationship between TGFβ1 levels and SCC. These correlations are displayed in Figure 3.3.

Table 3.3: Correlation of TGFβ1 levels with patient parameters

Patient parameter	n	r	p*
Age	80	-0.047	0.68
Stage	80	0.31	0.0060
Differentiation	55	0.26	0.055

* 2 tailed

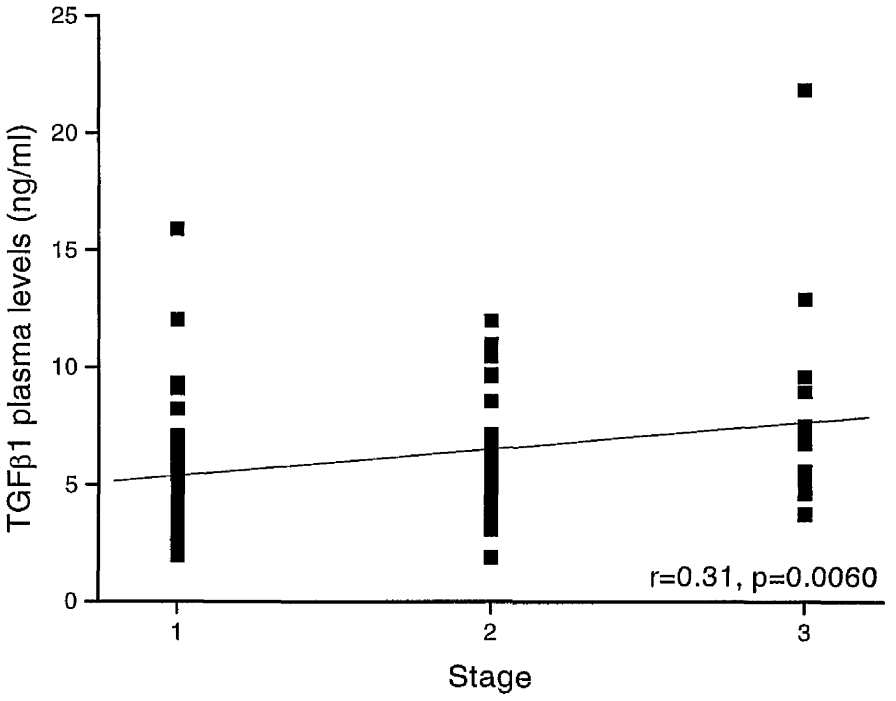


Figure 3.2: Correlation of TGFβ1 levels with disease stage

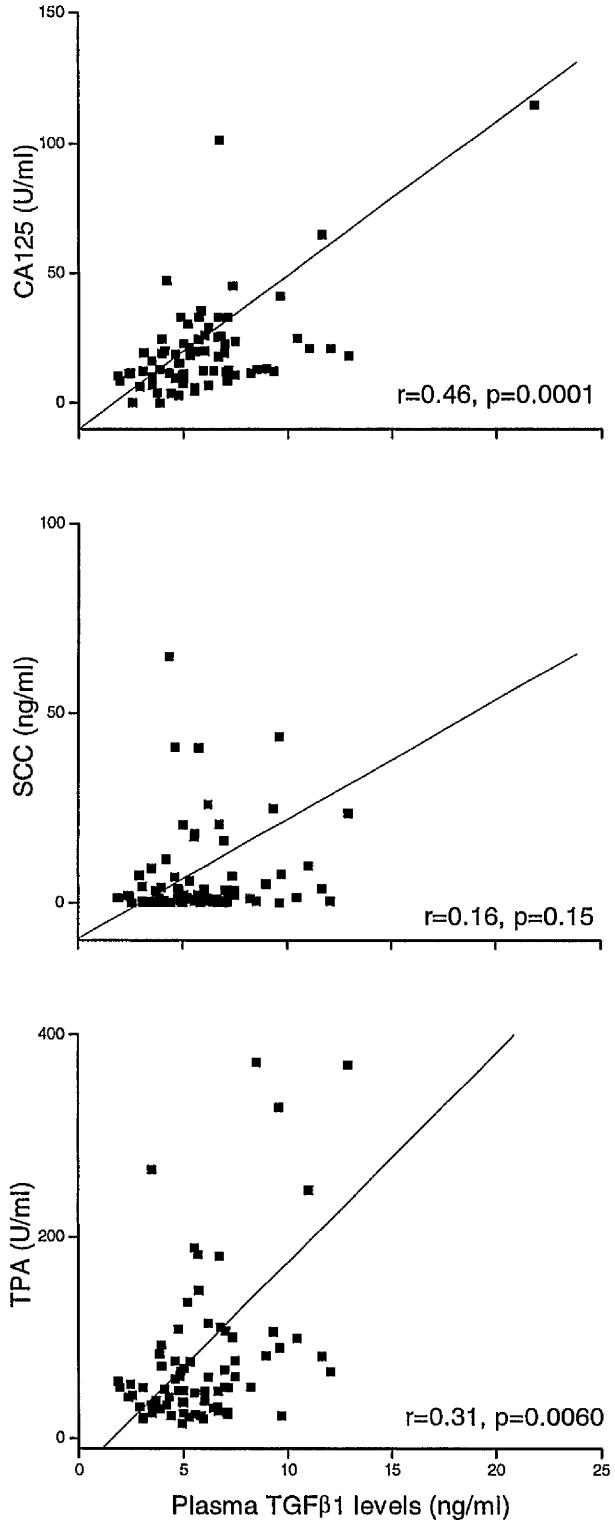


Figure 3.3: Relationship between plasma levels of TGFβ1, CA125, SCC and TPA

(patient numbers 73, 77 and 77 respectively)

3.5 Tumour control

The median follow-up time was 66 months. The level of TGFβ1 used for stratification was decided prior to any analyses being performed. There were no contemporary controls available. For the analyses of survival and local control, a level of 4.28 ng/ml was selected as it corresponded with the first quartile and the upper limit of the normal population, as quoted in the kit literature.

Patients with elevated plasma TGFβ1 levels had a significantly decreased probability of survival (Figure 3.4). There was a 90% survival rate for patients with levels in the lowest quartile compared with a rate of 59% for the remaining patients. The sensitivity of the assay as a prognostic factor for survival was 92% and the specificity was 34%. A significant difference was also seen in local control (Figure 3.4). The local control rate was 95% for patients with low pre-treatment plasma TGFβ1 levels versus a rate of 71% for the remaining patients. Table 3.4 summarises the survival and local control data for disease stage and patient age. As disease stage was a highly significant prognostic factor and because of the association seen between stage and TGFβ1 levels, a bivariable log-rank analysis was carried out including these two factors. After allowing for stage, TGFβ1 levels showed borderline significance as a prognostic factor for both survival ($p=0.065$) and local control ($p=0.073$).

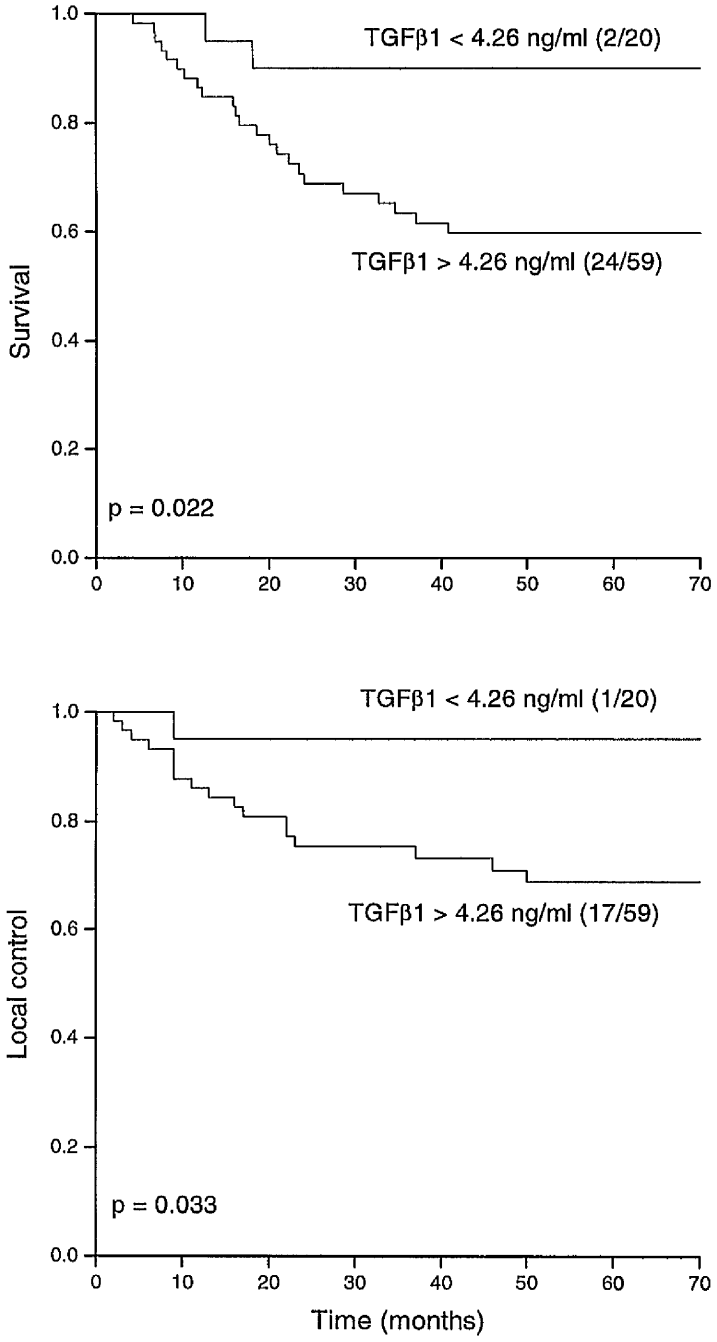


Figure 3.4: Kaplan Meier plot of overall survival (upper) and local control (lower) for patients with TGFβ1 levels in the lowest versus the highest three quartiles. Values in parentheses indicate the number of deaths/local recurrences and the total number of patients in the groups.

Table 3.4: Summary of local control and survival data

Clinical parameter		N	Survival	Local control
Age	<60 years	38	p = 0.57	p= 0.68
	≥60 years	41		
Stage	I	34	p= 0.0098	p= 0.011
	II	30		
	III	15		
TGFβ1	<4.26 ng/ml	20	p= 0.022	p= 0.033
	>4.26 ng/ml	59		

3.6 Morbidity

The median follow-up time was 66 months. The level of TGFβ1 used for stratification was again decided prior to any analyses being performed. A level of 7.12 ng/ml was chosen as this corresponded to the third quartile and the level most often quoted in the published literature (Anscher *et al*, 1998).

The incidence of morbidity of all types and grades was 42% for this cohort of patients. There were no fatalities due to radiation side effects. Severe morbidity (Franco-Italian glossary grade III) was seen in less than 2% of patients. Figure 3.5 displays the incidence of all morbidities (upper graph) and grade III morbidity only (lower graph). In neither case

was there a significant relationship with TGFβ1 levels. Figure 3.6 displays the weak, but significant inverse correlation of plasma TGFβ1 levels and lymphocyte (i.e. normal tissue) radiosensitivity, as measured by Spearman's test. Thus, despite there being no relationship between plasma TGFβ1 levels and morbidity, patients with radiosensitive normal tissues had increased levels of circulating plasma TGFβ1.

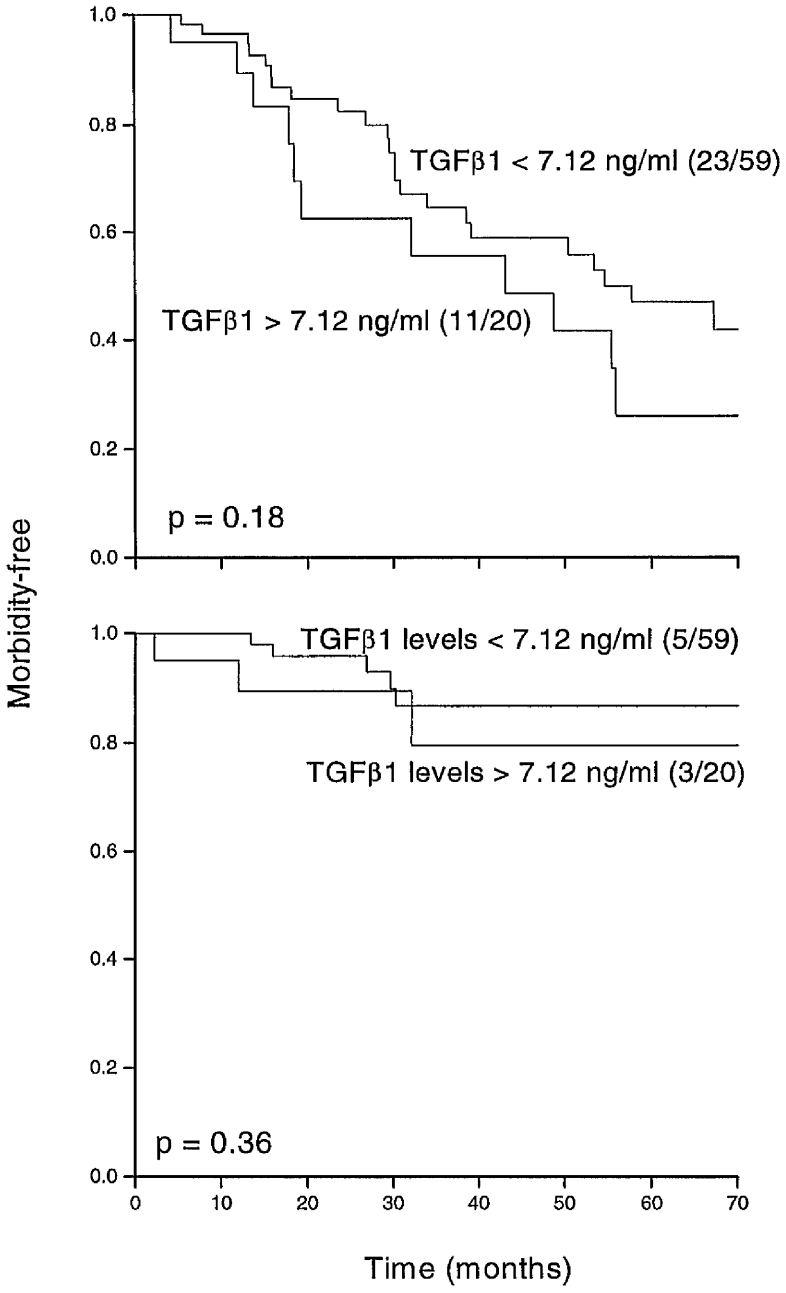


Figure 3.5: Pre-treatment plasma TGFβ1 levels in relation to all morbidity (upper graph) and grade III morbidity only (lower graph) for those patients with plasma TGFβ1 levels in the highest versus the lowest three quartiles. Values in parentheses indicate number of events in each group and the total number of patients in the group.

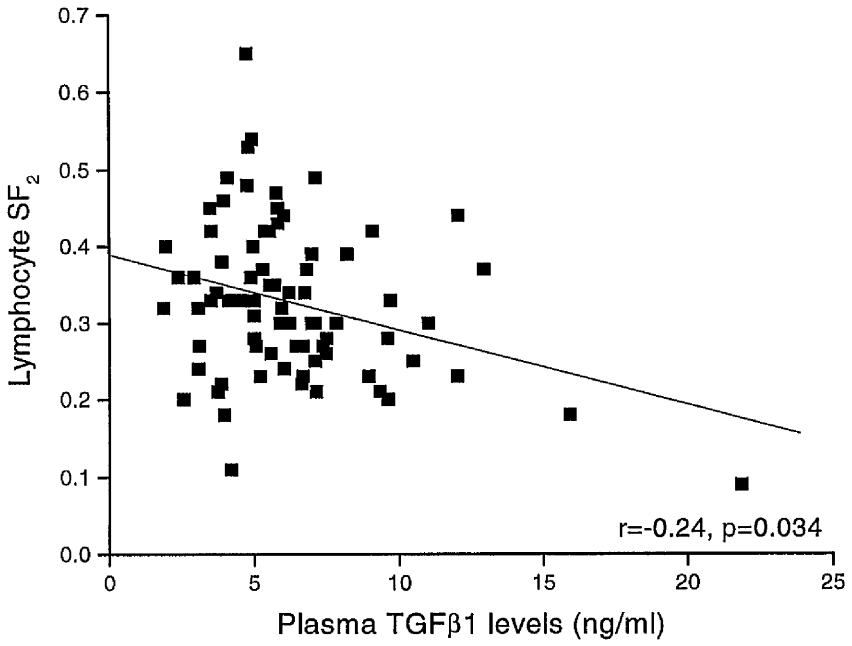


Figure 3.6: Relationship between lymphocyte radiosensitivity and plasma TGFβ1 levels

3.7 Discussion

Despite intensive study over the past 17 years, the multiple roles played by TGFβ1 in malignant disease have yet to be fully understood. It is likely that the predominant function of TGFβ1 varies at differing stages in the development and progression of any malignancy. As cervical epithelial cells become invasive, they lose sensitivity to the inhibitory effects of TGFβ1. This is independent of human papillomavirus (HPV) genome expression (Braun *et al*, 1990). In response to this negative feedback loop, increased TGFβ1 is deposited

extracellularly in the tumour stroma (Comerci *et al*, 1996). It is likely that increased circulating TGFβ1 is derived from this pool.

Plasma TGFβ1 has been put forward as a marker of tumour burden in biologically and histologically very different tumours, including colorectal (Tsushima *et al*, 1996), renal (Wunderlich *et al*, 1998), nasopharyngeal (Xu *et al*, 1999) and hepatocellular carcinoma (Shirai *et al*, 1994). In this study, pretreatment plasma TGFβ1 levels were significantly correlated with disease stage, and hence tumour burden. In support of this we also found correlations with plasma levels of circulating serum markers known to be associated with burden of disease (Sproston *et al*, 1995; Ngan *et al*, 1996). Our findings are also in agreement with published data showing significantly increased circulating levels of TGFβ1 in patients with stages II - IV carcinoma of the cervix versus normal controls or patients with stage I disease (Chopra *et al*, 1998).

There is evidence that measurements of TGFβ1 can provide prognostic information with elevated levels predicting poor outcome. This has been demonstrated using urine samples from patients with hepatocellular carcinoma (Tsai *et al*, 1997), serum samples in nasopharyngeal carcinoma (Xu *et al*, 1999), and tumour sections from gastric (Saito *et al*, 1999) and colorectal (Robson *et al*, 1996) cancers. In this study we have shown that pre-treatment plasma levels of TGFβ1 can provide prognostic information in carcinoma of the cervix. There is also some suggestion from our work that this may prove to be independent of disease stage. There was a borderline level of significance in bivariable analysis after allowing for disease stage that would probably become significant with a larger patient population.

In contrast, we found no relationship between pre-treatment plasma levels of TGFβ1 and morbidity in carcinoma of the cervix. For patients receiving radical thoracic radiotherapy, the TGFβ1 ratio (the plasma TGFβ1 level in the final week of treatment divided by the pretreatment value) significantly predicted the development of acute radiation pneumonitis (Groen *et al*, 1997; Anscher *et al*, 1998a). In patients treated with high dose chemotherapy and autologous bone marrow transplant for locally advanced breast cancer, plasma TGFβ1 significantly predicted the development of fibrotic complications in both liver and lung (Anscher *et al*, 1993). Also, pre-treatment levels in breast cancer have been shown to correlate with the development of late radiation-induced fibrosis (Li *et al*, 1999). In our study, the lack of correlation may be because of a confounding influence of tumour burden. Elevated plasma TGFβ1 levels can decrease after surgical removal of a tumour. This has been shown in both breast (Kong *et al*, 1995) and colorectal (Tsushima *et al*, 1996) cancers. Therefore, our finding does not rule out the possible utility of changes in TGFβ1 levels during radiotherapy predicting morbidity. In support of this we found a weak association between pre-treatment plasma TGFβ1 levels and intrinsic radiosensitivity measured in lymphocytes as SF2.

In conclusion, pretreatment plasma TGFβ1 levels are a significant prognostic factor for survival and local control in patients with cervical carcinoma. Although, no relationship was found with late radiation-induced morbidity, it might be necessary to evaluate measurements towards the end of treatment once tumour burden is reduced. The evidence in the literature points to this being a worthwhile study for future consideration.

CHAPTER 4: HEAD AND NECK CARCINOMA

4.1 Introduction

TGFβ1 is a multi-functional cytokine. Generally its function is that of a tumour suppressor (Reiss, 1999), but as carcinogenesis progresses it acts as a tumour promoter (De Geest *et al*, 1994; Woodworth *et al*, 1996). Plasma TGFβ1 levels are a measure of tumour burden in cervical (Chopra *et al*, 1998), colorectal (Tsushima *et al*, 1996) and breast (Kong *et al*, 1995; Sminia *et al*, 1998) cancer. Persistently elevated plasma TGFβ1 levels following radiotherapy indicate the presence of residual tumour in lung (Kong *et al*, 1996; Groen *et al*, 1997) and brain tumours (Gridley *et al*, 1998). Changes in plasma TGFβ1 levels have been shown to accurately predict the occurrence of symptomatic radiation pneumonitis in patients receiving thoracic radiotherapy for lung (Anscher *et al*, 1994; Groen *et al*, 1997; Anscher *et al*, 1998) and other malignancies (Anscher *et al*, 1997). A number of animal studies (Barcellos-Hoff, 1993; Langberg *et al*, 1994; Richter *et al*, 1996) have shown increased TGFβ1 deposition in tissues in association with the development of late radiation fibrosis. In humans increased TGFβ1 deposition persists in irradiated areas for at least 40 weeks (Canney and Dean, 1990). Elevated plasma TGFβ1 levels predict for the development of late fibrotic complications in patients undergoing radiotherapy for breast cancer (Li *et al*, 1999) and also autologous bone marrow transplantation for locally advanced breast cancer (Anscher *et al*, 1993). This prospective study was designed to investigate the relationship between changes in plasma TGFβ1 levels and radiation morbidity in a cohort of patients treated with curative intent for malignancies of the head

and neck region. Due to the timescale of this thesis, data is only available for acute effects. Samples were obtained from a group of healthy volunteers to establish the range of plasma TGFβ1 levels in a non-cancer population.

4.2 Volunteer samples

Samples were obtained from sixty-six volunteers with no history of malignancy (data summarised in Table 4.1) and processed according to the EDTA method (see Section 2.3). The median age of the volunteers was 62 years (range 20 - 84). There were 44 females and 22 males. One volunteer smoked; 52 did not smoke and for 13 the information was unavailable.

Plasma TGFβ1 levels ranged from 0.71 – 3.21 ng/ml (Figure 4.1). The mean with one standard deviation and median values were 1.47 ± 0.49 ng/ml and 1.41 ng/ml, respectively. The normal range was defined as the mean plus two standard deviations, giving a normal upper limit of 2.45 ng/ml. Significant differences between the volunteer samples were sought using a univariate analysis of variance (uni-ANOVA). This allowed nested comparison of individual samples, as well as comparison of samples taken from the same individual on more than one occasion (n = 4) and the same sample analysed on two or more different occasions (n = 23). The inter-sample CV was 77% and the intra-sample CV was 43%. The assay CV was 4%. Although the intra-sample CV was high, significant differences between the patient and volunteer samples were detectable using ANOVA ($p < 0.001$). Using Spearman's regression test, there was a significant relationship between plasma TGFβ1 levels and volunteer age ($r = 0.41$, $p = 0.001$) (Figure 4.2). When the data

were examined, there were three outliers with plasma TGFβ1 levels greater than 2.45 ng/ml. Although care had been taken not to recruit any volunteers with a history of malignancy, it was possible that these elderly volunteers (ages 68 – 73 years) had a latent malignancy present, which might cause an increased plasma TGFβ1 level. The data were therefore reanalysed following removal of the outliers, but the significant underlying relationship between donor age and plasma TGFβ1 levels remained ($r = 0.39$, $p = 0.001$) (Figure 4.3). Using the Mann-Whitney u test there was no significant relationship between plasma TGFβ1 levels and patient gender ($p = 0.14$). Due to the small number of smokers sampled, no meaningful comparison could be made regarding the effect of smoking.

Table 4.1: Demographic data for volunteer samples processed by the EDTA method

Lab. number	Age	Sex	Current Smoking Habit	TGFβ1 level (ng/ml)
T35	29	F	N	1.76
T36	20	F	N	1.10
T38	38	M	N	1.02
T39	32	F	N	0.93
T40	36	F	N	0.86
T41	30	M	N	0.94
T49	24	M	N	0.77
T52	25	M	Y	0.95
T53	73	F	U[1]	1.56
T54	74	F	U	1.70
T55	76	F	U	1.31
T56	72	F	U	1.57
T57	69	M	U	1.34
T58	71	M	U	1.18
T59	84	F	U	1.68
T60	72	F	U	1.49
T61	65	F	U	1.06
T62	75	M	U	1.38
T63	73	M	U	2.87
T64	77	F	U	1.98
T65	79	M	U	1.79
T66	45	F	N	1.60
T67	21	M	N	1.49
T68	21	M	N	0.71
T69	41	F	N	1.24
T70	31	M	N	1.34
T71	30	M	N	1.30
T72	54	M	N	1.18
T74	42	F	N	1.18
T75	54	M	N	1.17
T76	34	F	N	1.11
T77	46	F	N	1.60
T78	24	F	N	1.78

Lab. number	Age	Sex	Current smoking habit	TGFβ1 level (ng/ml)
T79	55	M	N	1.04
T80	30	F	N	1.70
T81	29	M	N	1.45
T82	34	M	N	1.41
T83	65	F	N	0.99
T84	53	F	N	2.04
T85	70	F	N	2.04
T86	77	M	N	2.17
T87	72	F	N	2.70
T88	68	F	N	3.21
T89	68	F	N	1.43
T90	80	M	N	2.06
T91	80	F	N	1.31
T92	74	F	N	1.52
T93	79	F	N	1.22
T94	82	M	N	1.47
T95	77	F	N	1.41
T96	67	F	N	1.35
T97	78	F	N	2.29
T98	25	F	N	0.95
T99	70	F	N	0.95
T100	74	F	N	1.04
T101	69	F	N	1.09
T102	53	F	N	1.91
T103	45	M	N	1.13
T104	47	F	N	0.97
T105	63	F	N	2.05
T106	74	F	N	1.73
T107	68	F	N	1.62
T108	61	F	N	1.08
T109	52	F	N	1.53
T110	51	F	N	1.45
T111	51	F	N	1.80

[1] – U indicates unknown

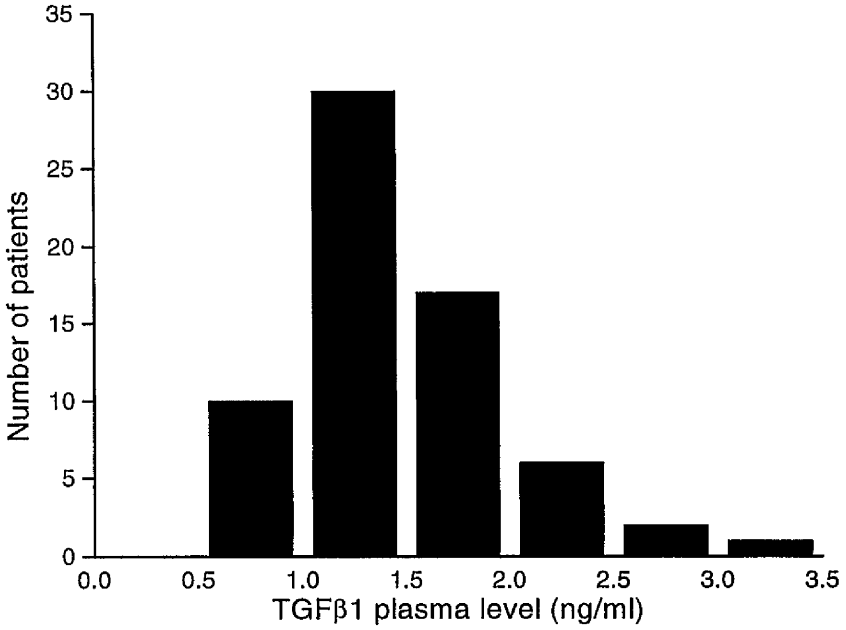


Figure 4.1: Distribution of plasma TGFβ1 levels in volunteer samples processed using the EDTA method (n = 66).

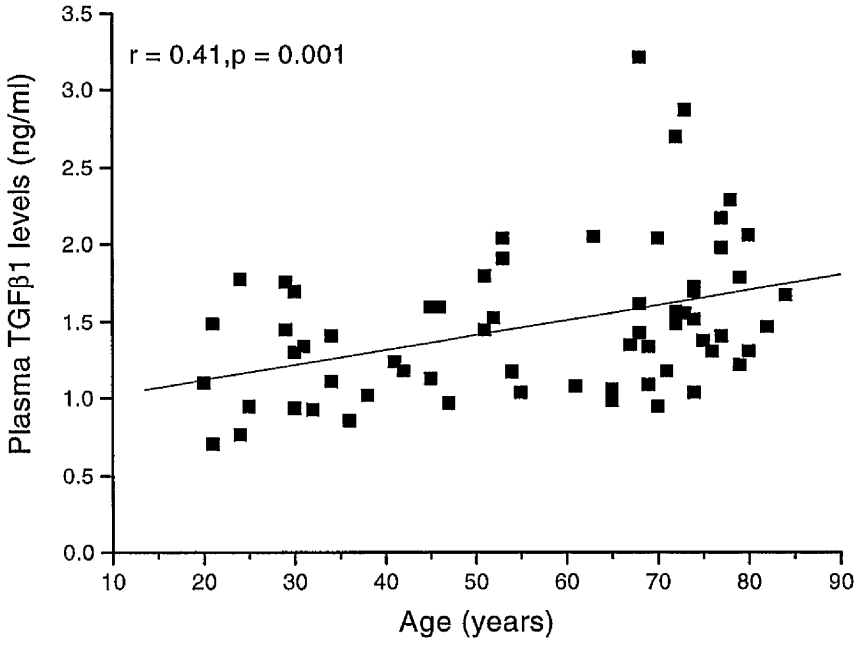


Figure 4.2: Relationship between plasma TGFβ1 levels and donor age in normal volunteers (n = 66)

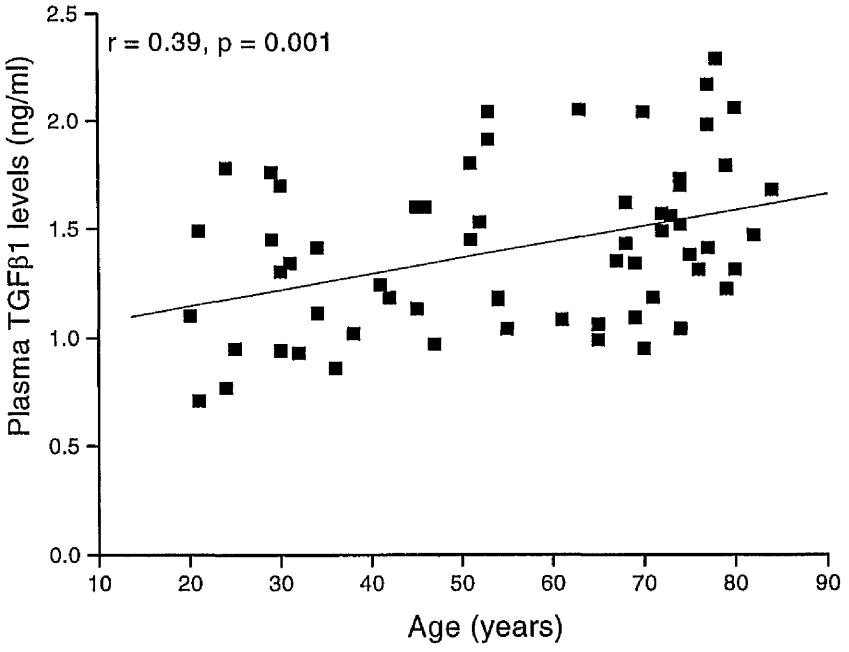


Figure 4.3: Relationship between plasma TGFβ1 levels and donor age in normal volunteers (n = 63)

4.3 Patient samples

Patient characteristics

Seventy-five patient pre-treatment samples were available and 74 from the final week of treatment (Table 4.2). The TGFβ1 data are summarised in Table 4.3. Using the upper limit of the range of TGFβ1 levels obtained from normal donors (see Section 4.2), elevated values were seen in twenty pre-treatment and 16 end of treatment samples.

The median patient age was 60 years (range 38 – 80 years). There were 63 men and 12 women in this series. Thirty-one patients were smoking at the time of recruitment. Thirty-nine patients were ex-smokers and three had never smoked. Data on smoking habit were unavailable for two patients. Using a Kruskall Wallis test, no relationship was found between smoking habit and pre-treatment TGFβ1 level ($p = 0.63$). Table 4.2 summarises the distribution of patients according to stage, histology and treatment. The primary tumour was included in the treatment volume in 70 patients, with two receiving nodal irradiation only and data unavailable for three patients. In forty-two patients, the cervical nodes were not treated. Unilateral cervical nodal irradiation occurred in seven patients and bilateral nodal irradiation in 23 patients. Data were absent for three patients.

Table 4.2: Demographic data for patients with carcinoma of the head and neck

Pat. I.D. no.	Age In Years	Gender	Current smoking habit[1]	Site[2]	Stage[3]	Hist.[4]	Surg.[5]	Chemo.[6]	Total dose given (Gy)[7]	Fraction number
TM39	63	M	E	1	T3N0	1	Y	N	50	20
TM40	59	M	Y	1	T2N0	1	N	N	52.5	16
TM41	67	M	E	3	T2NX	1	Y	N	50	16
TM43	60	M	N	3	T3N1	1	Y	N	50	20
TM44	57	F	Y	3	T2N1	1	N	N	50	16
TM45	65	M	E	3	T2N3	1	Y	N	55	20
TM46	57	M	E	3	T4N0	1	Y	N	50	20
TM47	43	M	Y	2	T4N1	1	Y	N	50	20
TM48	70	F	E	6	T1N0	5	Y	N	50	20
TM49	76	M	E	1	T2N0	1	N	N	52.5	16
TM50	55	M	Y	3	T4N3	1	N	N	50	20
TM51	72	M	E	1	T1N0	1	N	N	52.5	16
TM52	80	M	Y	1	T4N0	1	N	N	50	16
TM53	68	M	U	1	T1N0	1	N	N	52.5	16
TM54	70	M	U	4	TXN0	6	Y	N	47.5	15
TM55	64	M	Y	1	T2N0	1	N	N	52.5	16
TM56	52	M	E	3	T4NX	1	Y	N	52.5	20
TM58	76	M	E	2	T2N2	1	N	N	52.5	16
TM60	53	M	Y	3	T4N0	1	N	N	50	16
TM61	50	M	E	1	T2N0	1	N	N	50	16
TM62	57	M	Y	1	T1N0	1	N	N	52.5	16
TM64	57	M	E	3	T4N0	1	Y	N	50	20
TM65	47	M	N	3	T2N1	1	Y	N	50	16
TM66	42	F	Y	3	T4N0	1	N	N	52.5	20
TM67	38	M	E	1	T1N0	1	N	N	52.5	16
TM68	56	M	Y	1	T1N0	1	N	N	52.5	16
TM69	54	M	Y	2	TXNX	1	Y	N	50	15
TM70	39	M	Y	3	T4N1	1	N	Y(1)	70	35
TM71	70	F	E	6	T3N0	4	Y	N	50	16
TM72	60	M	E	1	T1N0	1	N	N	52.5	16
TM73	56	M	Y	3	T3N2	1	N	Y(2)	50	16
TM74	67	M	Y	3	T1N0	1	N	N	50	16
TM75	61	M	E	2	T2N0	1	Y	N	50	20
TM76	70	M	E	3	T3N1	1	N	N	55	20
TM77	49	M	Y	1	T1N0	1	N	N	50	16
TM78	64	M	Y	1	T3N0	1	N	N	52.5	16
TM79	72	M	E	1	T2N0	1	N	N	52.5	16
TM80	64	M	E	1	T3N0	1	N	N	52.5	16
TM81	69	F	E	6	T1N0	7	Y	N	50	20
TM82	55	F	Y	2	T4N0	1	N	N	50	16
TM83	56	M	E	3	T3N1	1	Y	N	50	20
TM84	65	M	Y	3	T2N0	1	N	N	50	16
TM85	53	M	E	1	T1N0	1	N	N	52.5	16
TM86	74	F	E	1	T4N0	1	Y	N	50	20
TM87	73	M	E	1	T2N0	1	N	N	52.5	16

Pat. I.D. no.	Age In years	Gender	Current smoking habit[1]	Site[2]	Stage[3]	Hist.[4]	Surg.[5]	Chemo.[6]	Total dose given (Gy)[7]	Fraction number
TM88	68	M	Y	1	T3N0	1	N	N	50	16
TM89	68	M	E	1	T2N0	1	N	N	52.5	16
TM90	75	M	Y	2	T2N0	1	Y	N	50	20
TM91	60	M	Y	2	T2N0	1	Y	N	50	20
TM93	67	F	N	3	TXN1	1	Y	N	50	20
TM95	54	M	E	1	T2N0	1	N	N	52.5	16
TM96	58	M	E	1	TXN0	1	N	N	50	16
TM97	70	M	Y	1	T3N0	1	N	N	52.5	16
TM98	66	M	Y	2	T1N1	1	Y	N	50	15
TM99	59	M	Y	1	T2N0	1	N	N	52.5	16
TM100	58	M	Y	1	T1N0	1	N	N	50	16
TM101	51	M	E	1	T1N0	1	N	N	50	16
TM102	54	F	E	2	T1N0	1	Y	N	50	20
TM104	56	M	E	1	T1N0	1	N	N	50	16
TM106	49	F	Y	3	T2N0	1	N	N	52.5	16
TM107	46	M	Y	3	T4N1	1	N	Y(2)	70	35
TM108	50	M	E	3	T2N0	1	N	Y(2)	50	16
TM109	72	M	Y	1	T2N0	1	N	N	52.5	16
TM113	74	M	Y	1	T3N0	1	N	N	50	16
TM114	70	M	E	3	T3N0	1	N	Y(2)	50	16
TM116	74	M	Y	2	T4N0	1	Y	N	47.5	16
TM117	59	M	E	1	T2N2	1	Y	N	50	20
TM118	69	M	E	1	T2N0	1	N	N	52.5	16
TM119	65	F	E	1	T1N0	1	N	N	50	16
TM120	50	M	E	3	T2N1	1	Y	N	52.5	20
TM121	70	M	E	1	T2N0	1	N	N	52.5	16
TM122	61	M	Y	3	T3N0	1	N	N	U	U
TM123	50	M	E	1	T2N0	1	N	N	U	U
TM124	48	F	E	2	T1N0	1	Y	N	50	16
TM125	69	M	E	1	T2N0	1	N	N	U	U

[1] – E = ex-smoker, Y = current smoker, N = never smoked, U – unknown

[2] – For site: 1 = larynx, 2 = oral cavity, 3 = pharynx, 4 = nasal cavity and sinuses, 5 = ear, 6 = salivary glands

[3] – X indicates unknown

[4] – Histology: 1 = squamous carcinoma, 2 = melanoma, 3 = pleomorphic salivary adenoma, 4 = adenoid cystic carcinoma, 5 = myoepithelial carcinoma, 6 = basal cell carcinoma, 7 = small cell neuroendocrine carcinoma, 8 = adenocarcinoma, 9 = acinic cell carcinoma

[5] – Surgery - Yes does not include diagnostic biopsy

[6] – Chemotherapy - numbers in parenthesis indicate number of cycles given

[7] - U indicates unknown

Table 4.3: Summary of patient plasma TGFβ1 levels

TGFβ1 level	N	Mean ± S.D. (ng/ml)	Median (ng/ml)	Range (ng/ml)
Pre-treatment	75	2.31 ± 1.26	1.86	0.88 – 6.72
End of treatment	74	2.32 ± 1.76	1.78	0.69 – 10.87

Plasma TGFβ1 levels

Using Spearman's regression analysis, there was no significant relationship between TGFβ1 levels and patient age (Figure 4.4). Pre-treatment TGFβ1 levels correlated weakly, but significantly with increasing T stage ($r = 0.31$, $p = 0.011$) (Figure 4.5). Using a Mann-Whitney u test the relationships between individual plasma TGFβ1 levels and smoking, chemotherapy, pre-treatment surgery and patient gender were investigated. There were no significant associations found between any of these variables and either pre-treatment or end of treatment TGFβ1 levels. A Kruskall Wallis test showed no significant relationship between either pre-treatment or end of treatment TGFβ1 level and whether the cervical nodes were treated.

An ANOVA showed no significant differences between the age distribution in the volunteer and patient groups ($p = 0.60$). Figure 4.6 is a superimposed plot of volunteer and patient ages, illustrating that age is not a confounding factor in this analysis. There was a positive correlation between the two TGFβ1 levels obtained from each patient (Figure 4.7).

In nine patients, TGFβ1 levels were measured prior to and every week during treatment. Seven patients had a three week course of radiotherapy and hence four sequential TGFβ1 levels were available. One patient had a four week course of radiotherapy and hence 5 sequential TGFβ1 levels were available. In one patient, the aliquot from the second week of a three week course of radiotherapy was unavailable, meaning there were only three values available for this patient. These sequential values are illustrated in Figure 4.8.

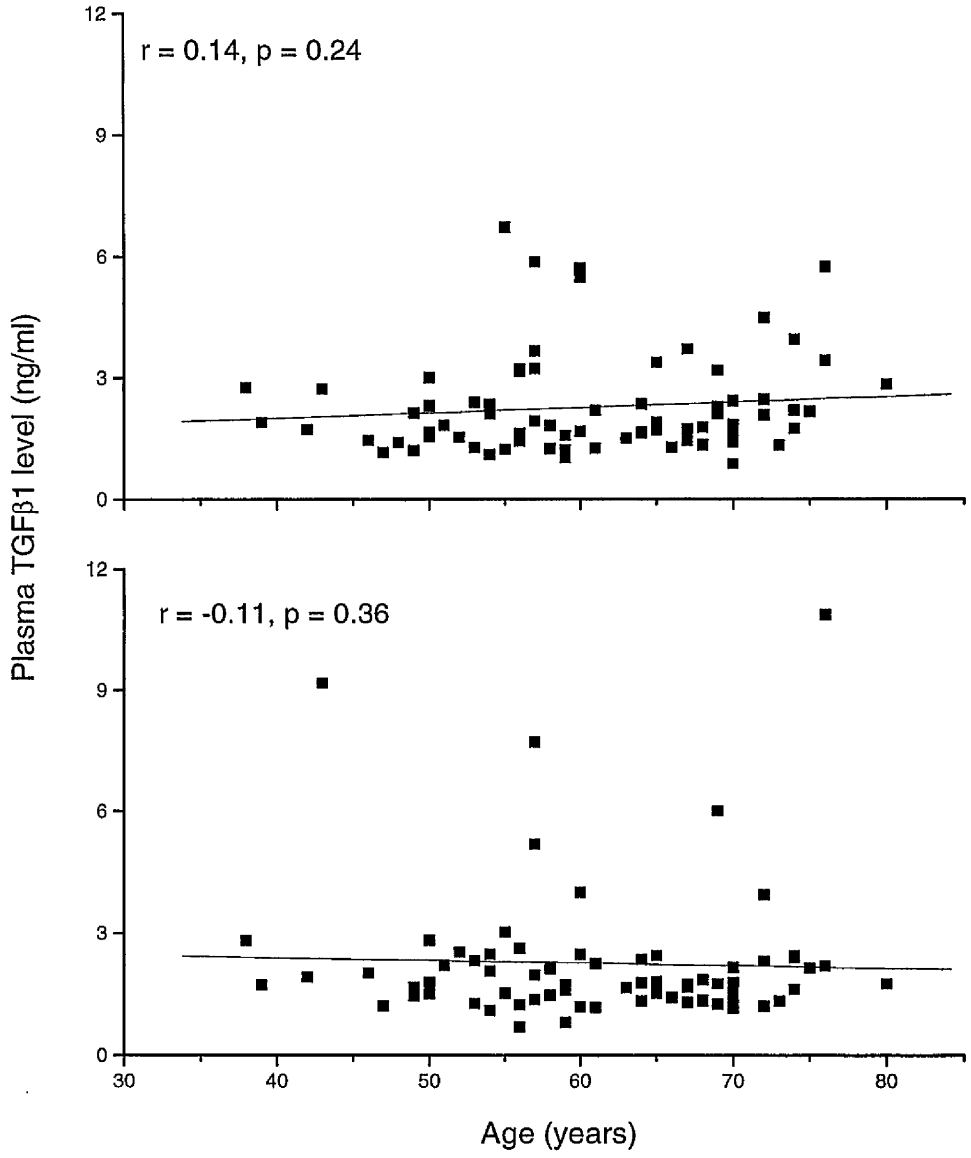


Figure 4.4: Relationship of patient age with pre-treatment plasma TGFβ1 levels (upper graph, n = 75) and end of treatment plasma TGFβ1 levels (lower graph, n = 74)

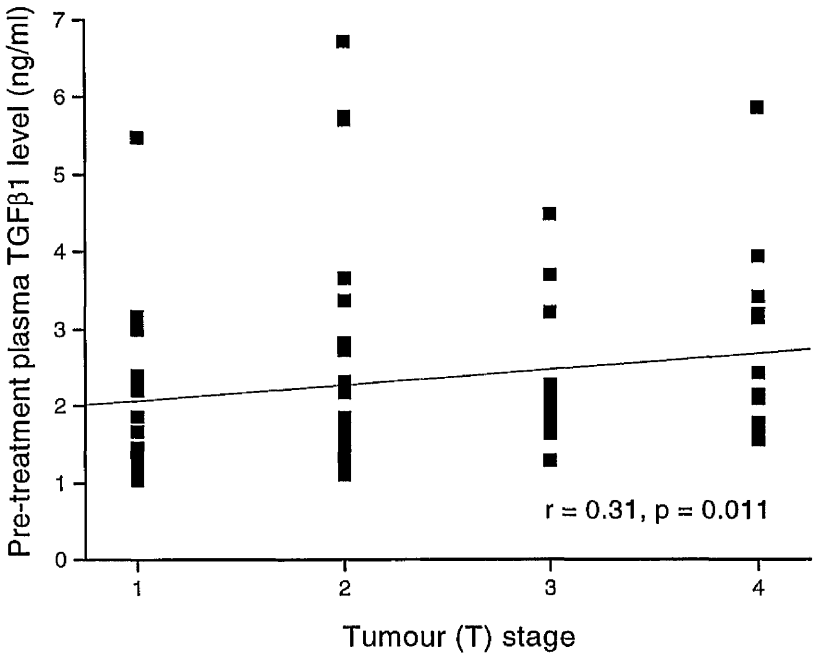


Figure 4.5: Relationship between tumour (T) stage and pre-treatment plasma TGFβ1 levels (n =68)

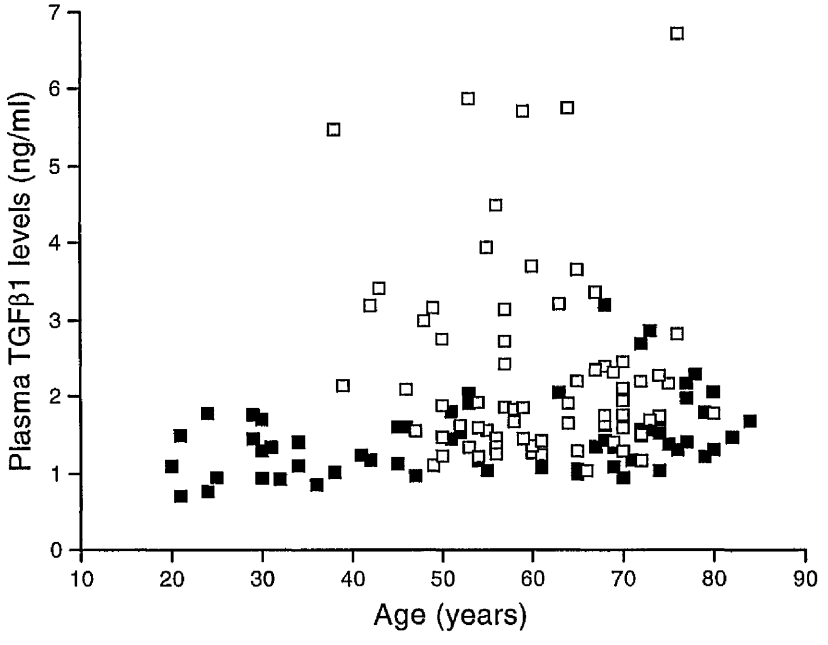


Figure 4.6: Superimposed plot of comparison of TGFβ1 levels and age in volunteers (closed squares, n = 66) and patients (open squares, n = 75)

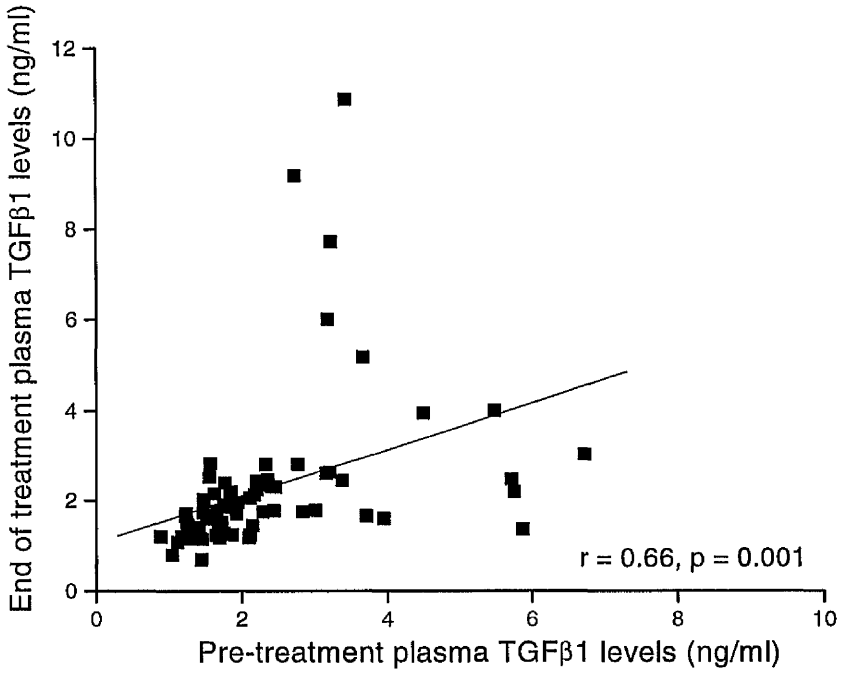


Figure 4.7: Relationship between pre-treatment and end of treatment plasma TGFβ1 levels (n = 74).

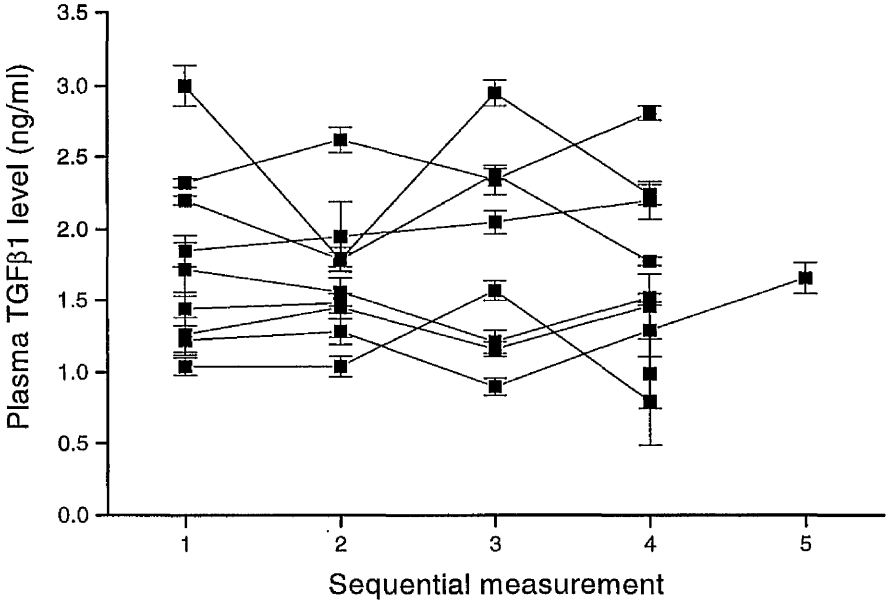


Figure 4.8: Weekly sequential measurements on patients undergoing radiotherapy (n = 9)

LENT SOMA score

Data on the LENT SOMA scores are summarised in Table 4.4. Using the Mann-Whitney u test, the relationship between the individual LENT SOMA scores and pre-treatment surgery, chemotherapy and patient gender were investigated. Women had significantly higher LENT SOMA scores recorded than men, both at the end of treatment and at first follow-up ($p = 0.036$ and 0.008, respectively) (Figure 4.9). Those patients who received radiotherapy as the primary treatment modality had significantly lower LENT SOMA scores recorded at first follow-up than those who had undergone surgery prior to radiotherapy ($p = 0.004$) (Figure 4.10). The LENT SOMA scores for those patients who had received chemotherapy were significantly higher than those who had not, both at the end of treatment and at first follow-up ($p = 0.011$ and 0.050, respectively) (Figure 4.11). In both the pre-treatment and end of treatment groups 42 patients had irradiation of the primary tumour only, seven patients had unilateral cervical nodal irradiation (CNI) and twenty-three patients bilateral CNI. At first follow-up 35 patients had received radiotherapy to the primary tumour only, three had received unilateral CNI and 16 patients had received bilateral CNI. Using a Kruskall Wallis test, the measured LENT SOMA score was significantly higher both at the start and at the end of treatment in those patients who received CNI ($p = 0.005$ and 0.027, respectively). The LENT SOMA score at first follow-up approached significance for this group ($p = 0.068$) (Figure 4.12).

Table 4.4: Summary of LENT SOMA scores

LENT SOMA score	n	Mean ± S.D.	Median	Range
Pre-treatment	75	11 ± 9	9	0 – 36
End of treatment	73	26 ± 14	23	4 – 39
At first follow-up	54	13 ± 12	10	0 - 47

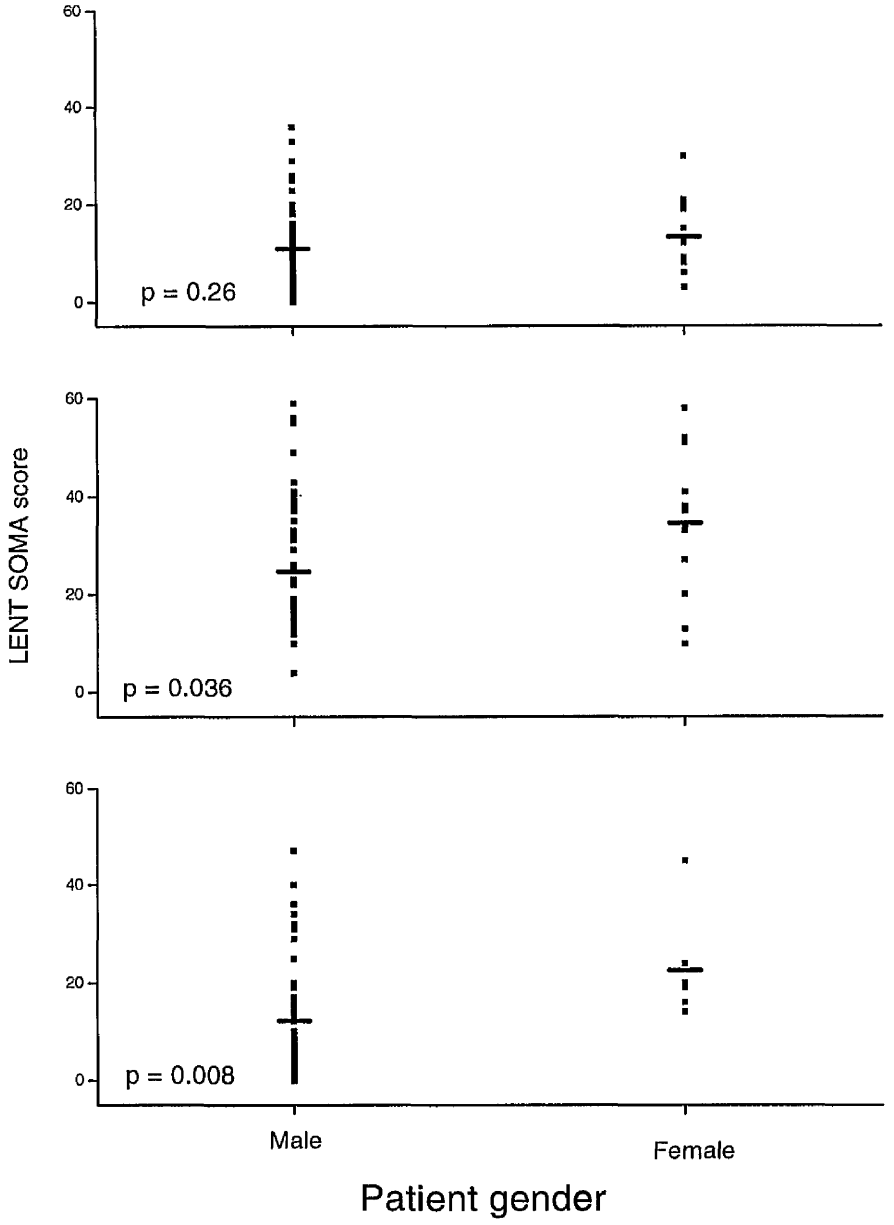


Figure 4.9: Relationship between LENT SOMA scores and patient gender (upper graph – pre-treatment, male = 63, female = 12; middle graph – end of treatment male = 61, female = 12; lower graph – at first follow-up, male = 47, female = 7). Solid bar indicates mean LENT SOMA score.

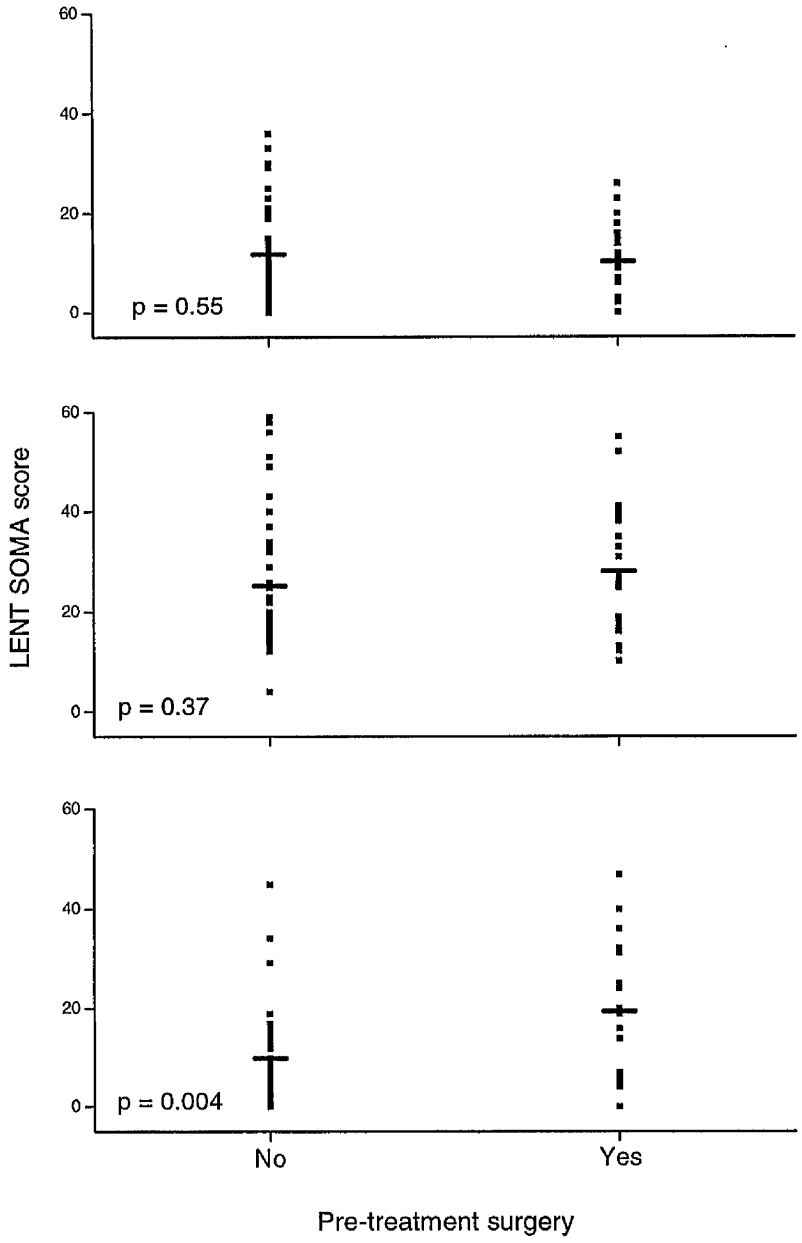


Figure 4.10: Relationship between LENT SOMA scores and pre-treatment surgery (upper graph – pre-treatment, no = 46, yes = 26; middle graph – end of treatment, no = 46, yes = 26; lower graph – at first follow-up, no = 34, yes = 20). Solid bar indicates mean LENT SOMA score.

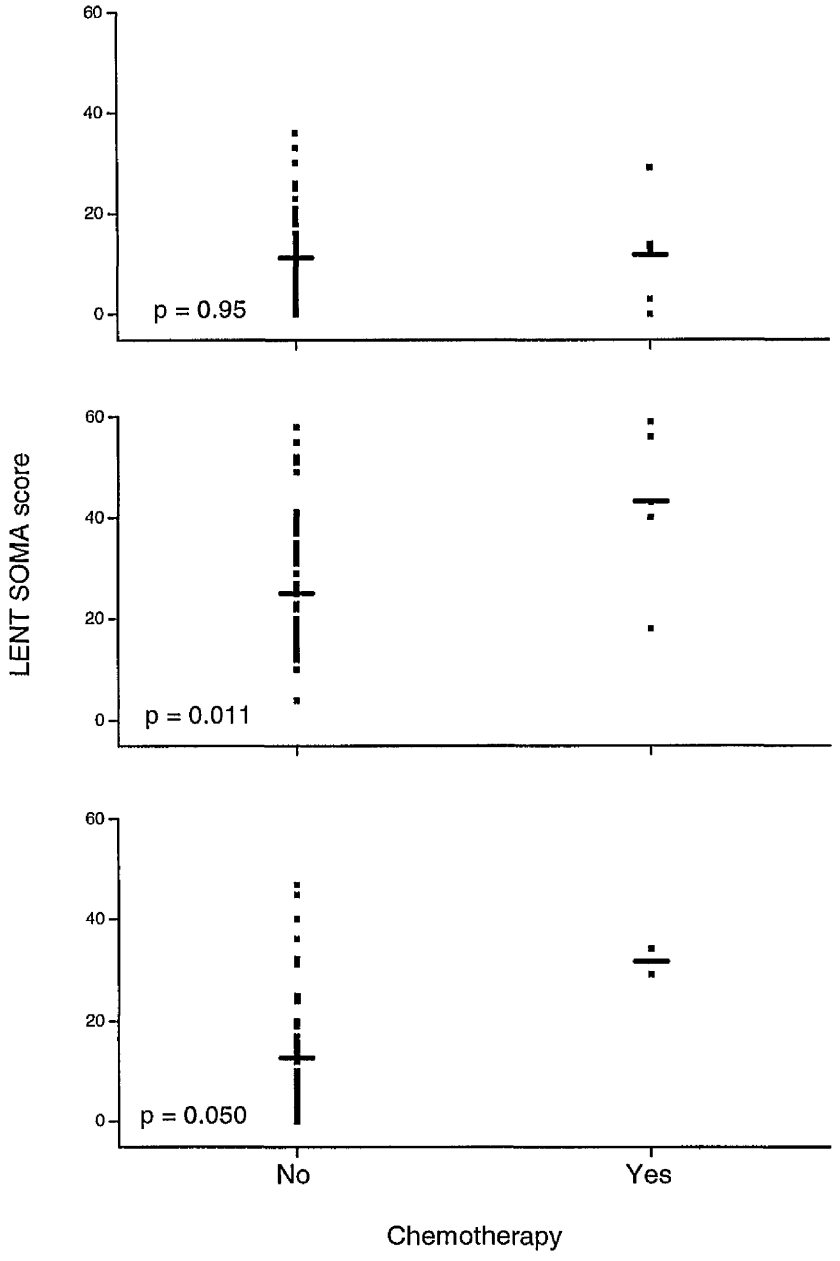


Figure 4.11: Relationship between LENT SOMA score and chemotherapy administration (upper graph – pre-treatment, no = 67, yes = 5; middle graph – end of treatment, no = 67, yes = 5; lower graph – at first follow-up, no = 52, yes = 2). Solid bar indicates mean LENT SOMA score.

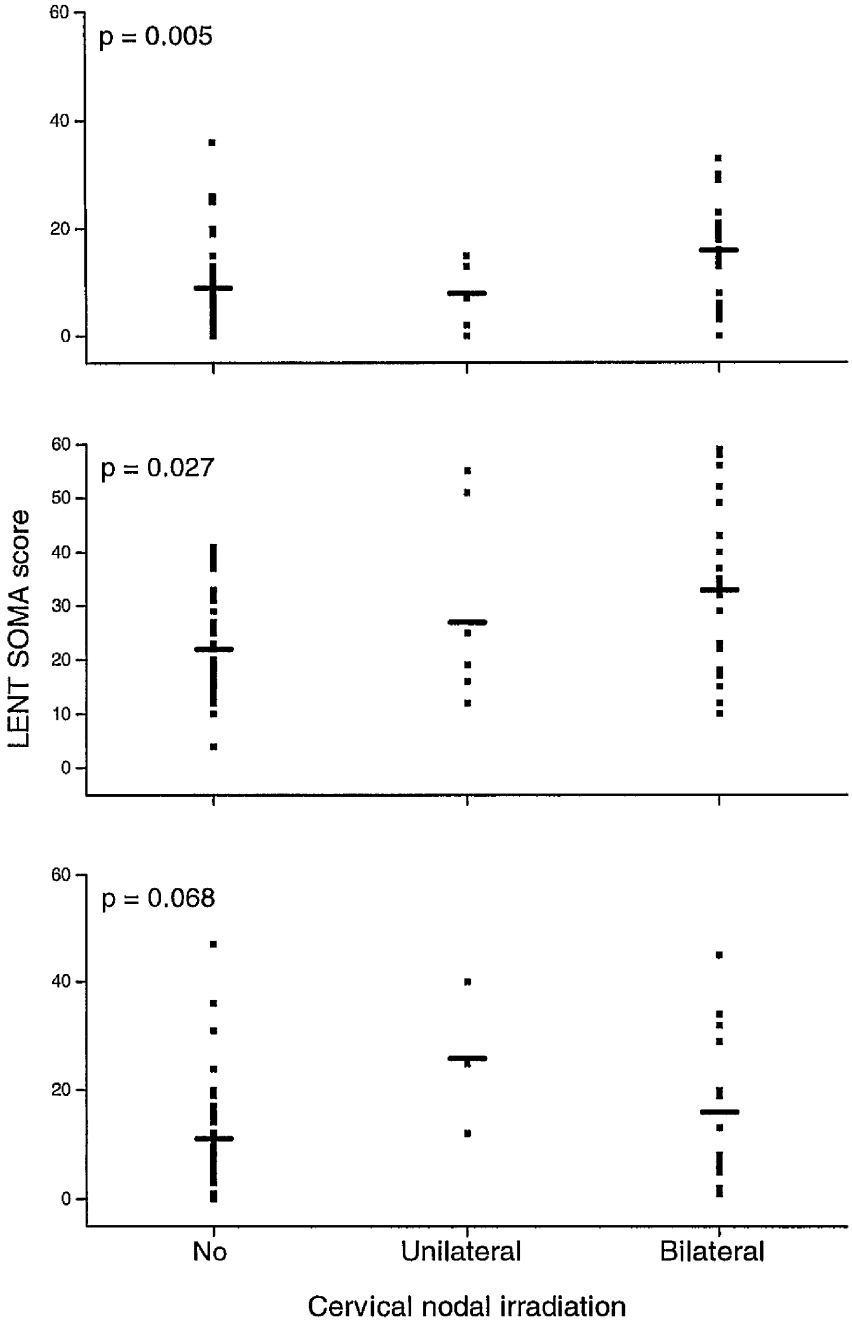


Figure 4.12: Relationship between LENT SOMA scores and irradiation of the cervical nodes (upper graph – pre-treatment, middle graph – end of treatment, lower graph – at first follow-up). Solid bar indicates mean LENT SOMA score.

4.4 Changes in plasma TGFβ1 levels

In radiation oncology, most interest has focussed on the clinical utility of changes in plasma TGFβ1 levels being predictive of symptomatic acute radiation toxicity. The TGFβ ratio (defined as the end of treatment TGFβ1 value divided by the pre-treatment value (see Section 1.12)) was calculated for all patient samples. The ratio was available on 74 samples (data summarised in Table 4.5). The ratio ranged from 0.23 - 3.36 (Figure 4.13). Using criteria defined elsewhere (Anscher *et al*, 1995; Anscher *et al*, 1997) patients were grouped into three categories. Category one patients had a TGFβ ratio of less than one and a normal end of treatment TGFβ1 level. Category 2 patients had a TGFβ1 ratio of less than one and an elevated end of treatment TGFβ1 level. Category three patients had a TGFβ ratio of greater than one. In this series there were 37 category one patients, 4 category two patients and 33 patients in category three. According to the published literature (Anscher *et al*, 1995; Anscher *et al*, 1997; Groen *et al*, 1997), it is patients in category two that are most at risk of developing symptomatic acute radiation toxicity.

Table 4.5: LENT SOMA and TGFβ1 data for patient samples processed by the EDTA method

Patient I.D. number	LENT SOMA score (pre-treatment)	LENT SOMA score (post-treatment)	LENT SOMA score (at first follow-up)	TGFβ1 level (pre-treatment) (ng/ml)	TGFβ1 level (post-treatment) (ng/ml)	TGFβ ratio[1]	Category of TGFβ ratio[2]
TM39	7	10	6	3.22	7.72	2.4	3
TM40	13	4	9	5.71	2.47	0.43	1
TM41	15	55	40	3.37	2.45	0.73	1
TM43	14	10	5	3.7	1.66	0.45	1
TM44	21	37	U	2.73	9.17	3.36	3
TM45	18	40	7	3.66	5.17	1.41	3
TM46	16	33	20	2.43	1.77	0.73	1
TM47	20	38	47	3.42	10.87	3.18	3
TM48	15	27	24	1.67	1.31	0.78	1
TM49	4	18	4	2.83	1.74	0.61	1
TM50	6	12	8	1.56	2.83	1.81	3
TM51	8	12	0	1.17	1.2	1.03	3
TM52	36	40	14	1.78	1.85	1.04	3
TM53	6	37	4	2.4	2.31	0.96	1
TM54	0	17	0	2.46	2.3	0.93	1
TM55	3	26	0	5.75	2.19	0.38	1
TM56	26	41	36	1.62	2.15	1.32	3
TM58	11	26	31	6.72	3.03	0.45	2
TM60	19	49	U[3]	5.87	1.36	0.23	1
TM61	2	22	0	2.76	2.81	1.02	3
TM62	7	13	6	1.86	1.24	0.67	1
TM64	20	18	32	3.15	2.61	0.83	2
TM65	2	41	16	1.55	2.53	1.63	3
TM66	30	58	45	3.2	2.63	0.82	2
TM67	20	23	16	5.48	4	0.73	1
TM68	7	29	1	1.46	1.73	1.18	3
TM69	0	16	U	1.92	1.71	0.89	1
TM70	29	59	U	2.14	1.44	0.67	1
TM71	3	41	16	2.11	2.06	0.97	1
TM72	4	12	1	1.27	1.16	0.91	1
TM73	3	18	29	4.49	3.95	0.88	2
TM74	2	32	U	2.36	2.35	0.99	1
TM75	7	19	6	1.24	1.51	1.22	3
TM76	33	33	19	1.94	1.96	1.01	3
TM77	12	16	6	3.17	6	1.89	3
TM78	15	22	13	1.65	1.77	1.08	3
TM79	25	22	17	1.51	1.65	1.09	3
TM80	15	15	2	1.91	1.8	0.94	1
TM81	9	38	19	1.41	1.41	1	3
TM82	8	34	U	3.94	1.6	0.41	1
TM83	0	13	4	1.35	1.31	0.97	1
TM84	5	23	8	1.29	1.26	0.97	1
TM85	8	14	12	1.34	1.31	0.98	1
TM86	6	10	14	1.74	1.91	1.1	3

Patient I.D. number	LENT SOMA score (pre-treatment)	LENT SOMA score (post-treatment)	LENT SOMA score (at first follow-up)	TGFβ1 level (pre-treatment) (ng/ml)	TGFβ1 level (post-treatment) (ng/ml)	TGFβ ratio[1]	Category of TGFβ ratio[2]
TM87	4	17	1	1.69	1.17	0.69	1
TM88	23	32	6	1.64	1.23	0.75	1
TM89	20	29	13	1.75	1.28	0.73	1
TM90	8	12	8	2.17	2.13	0.98	1
TM91	10	39	U	1.36	1.35	0.99	1
TM93	20	52	20	2.35	2.47	1.05	3
TM95	9	15	6	1.59	1.61	1.01	3
TM96	5	4	5	1.83	2.12	1.16	3
TM97	7	12	12	1.29	1.4	1.09	3
TM98	7	12	25	1.04	0.8	0.77	1
TM99	12	14	10	1.85	2.2	1.19	3
TM100	1	17	3	1.67	1.5	0.89	1
TM101	10	26	15	1.44	.99	0.69	1
TM102	12	33	20	1.22	1.66	1.36	3
TM104	3	13	7	1.26	1.46	1.15	3
TM106	13	51	U	1.11	1.08	0.97	1
TM107	14	43	U	2.09	1.18	0.56	1
TM108	0	56	34	1.47	2.02	1.38	3
TM109	7	19	3	2.2	2.44	1.11	3
TM113	15	19	U	2.28	1.74	0.76	1
TM114	13	40	U	1.76	2.39	1.36	3
TM116	2	31	U	1.72	1.52	.88	1
TM117	23	35	U	1.45	1.15	0.79	1
TM118	11	19	U	2.32	2.81	1.21	3
TM119	19	20	U	2.2	2.24	1.02	3
TM120	2	25	U	1.23	1.71	1.38	3
TM121	9	25	U	1.59	1.59	1.01	3
TM122	25	U	U	1.42	U	U	U
TM123	3	15	U	0.88	1.2	1.36	3
TM124	3	13	U	3	1.78	0.59	1
TM125	2	U	U	2.11	1.24	0.59	1

[1] – see text for definition of terms

[2] – see text for definition of terms

[3] – U indicates unknown

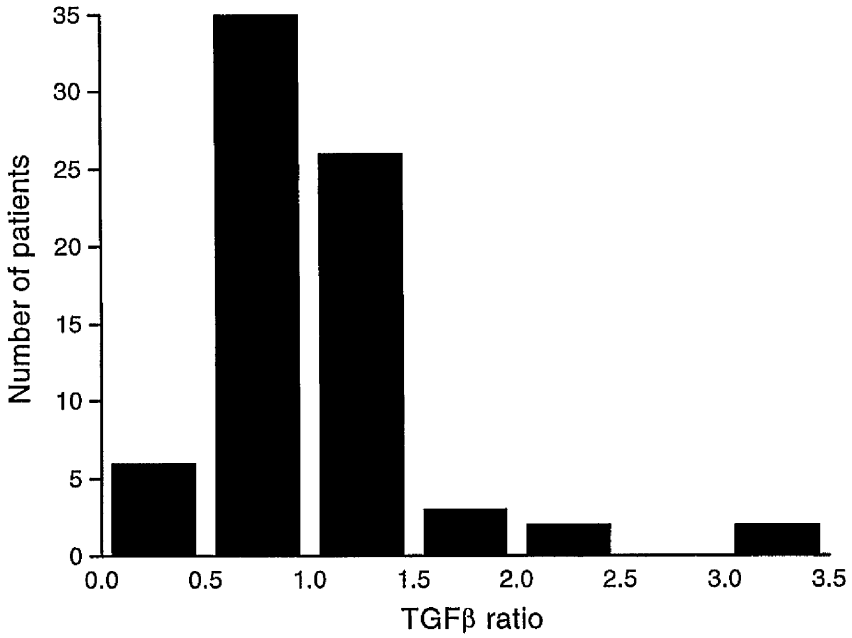


Figure 4.13: Distribution of TGFβ ratios

Relationship between TGFβ results and LENT SOMA score

The relationship between the individual LENT SOMA scores and plasma TGFβ1 values were investigated using Spearman's non-parametric regression test. There was no significant relationship between TGFβ1 levels prior to (Figure 4.14) or at the end of treatment (Figure 4.15) and any LENT SOMA score. Using the Mann-Whitney u test there was no significant difference between the LENT SOMA scores pre-treatment, at the end of treatment and at first follow-up for a TGFβ ratio of less than or greater than one ($p = 0.64$, 0.80 and 0.77, respectively).

Using a Kruskall Wallis test, the distribution of LENT SOMA scores between the three TGFβ categories was examined (Figure 4.16). The LENT SOMA score at first follow-up

was significantly higher in category two patients, compared to categories one or three ($p = 0.008$). The mean LENT SOMA scores in categories 1, 2 and 3 were 10, 34 and 13, respectively. This finding was confirmed by a t-test, which showed that the LENT SOMA score at first follow-up was significantly different in category two patients compared with those in categories one or three ($p = 0.002$ and 0.003, respectively).

The LENT SOMA scores at the end of treatment correspond to the height of the acute radiation reaction. After a three week course of radical radiotherapy, the acute reaction can intensify beyond the end of treatment for 10 - 14 days. After this, a steady improvement occurs over the following weeks. The bulk of the acute reaction should have settled by six weeks, which is the date of first follow-up. In this series, a severe acute reaction was one that had persisted until first follow-up. This was defined as a LENT SOMA score at first follow-up in the highest quartile (greater than 19). This level was chosen for analysis as it approximated the level of the mean LENT SOMA score at the end of treatment, when the acute reaction is intense. The sensitivity and specificity of category 2, in predicting a severe acute radiation reaction were 31% and 100%, respectively. The positive predictive value of a category 2 score was 100%.

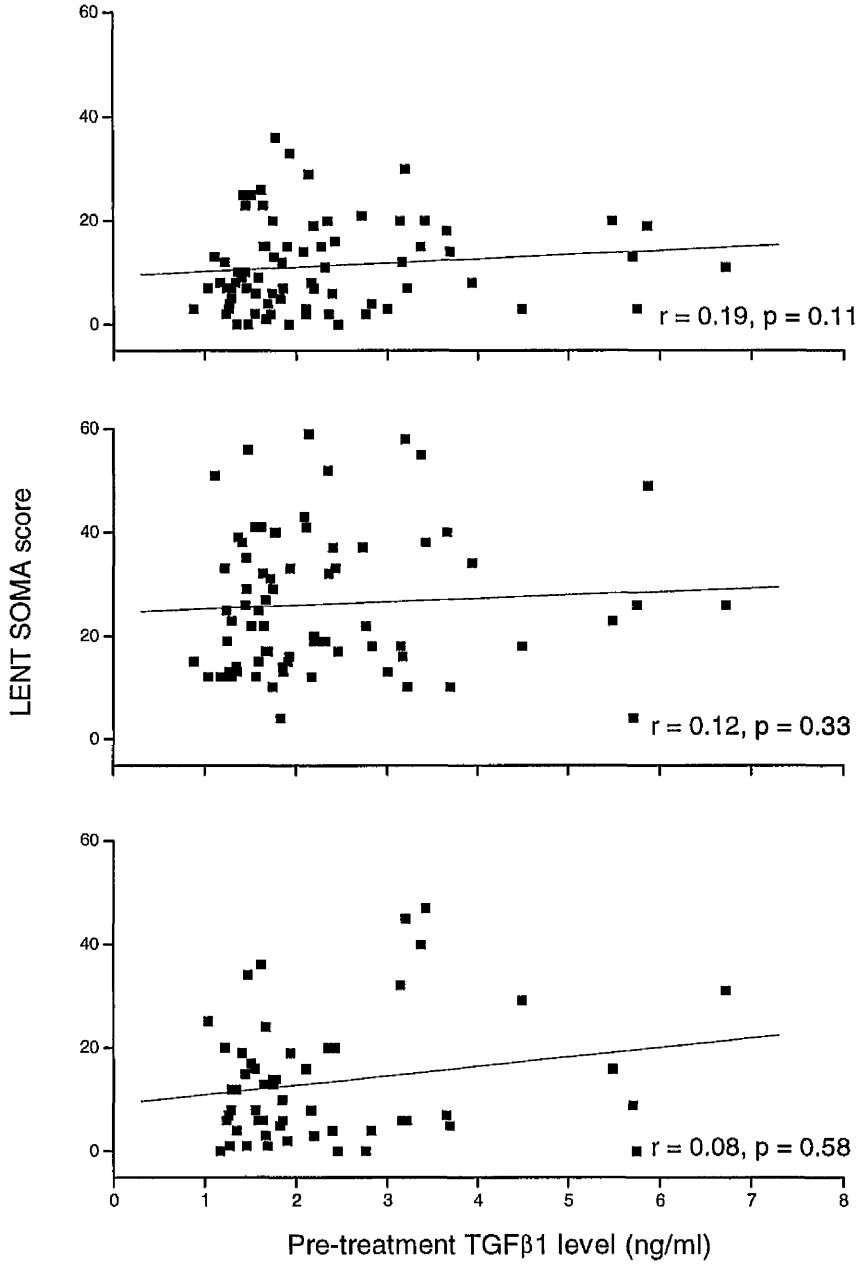


Figure 4.14: Relationship between pre-treatment TGFβ1 levels and LENT SOMA score (upper graph – pre-treatment, middle graph – end of treatment, lower graph - at first follow-up)

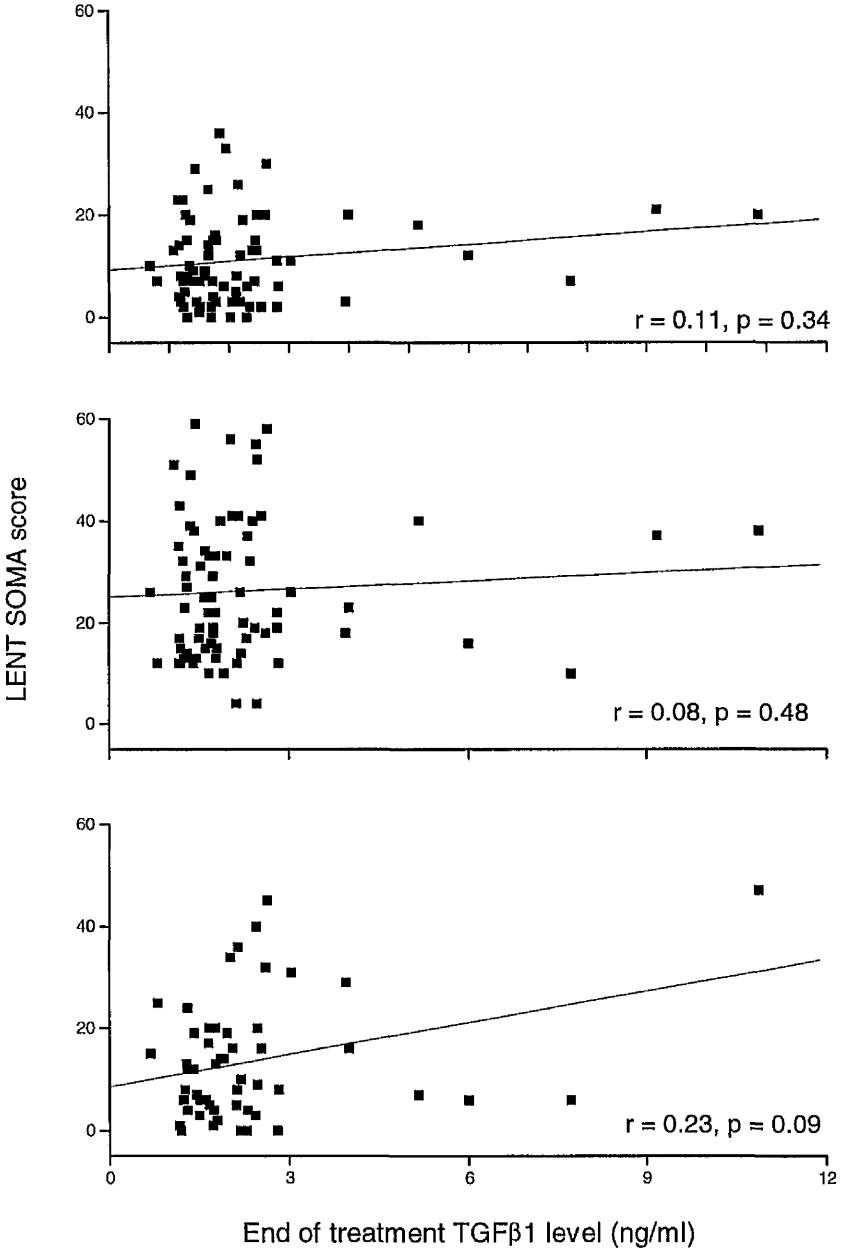


Figure 4.15: Relationship between end of treatment TGFβ1 levels and LENT SOMA scores (upper graph – pre-treatment, middle graph – end of treatment, - lower graph – at first follow-up)

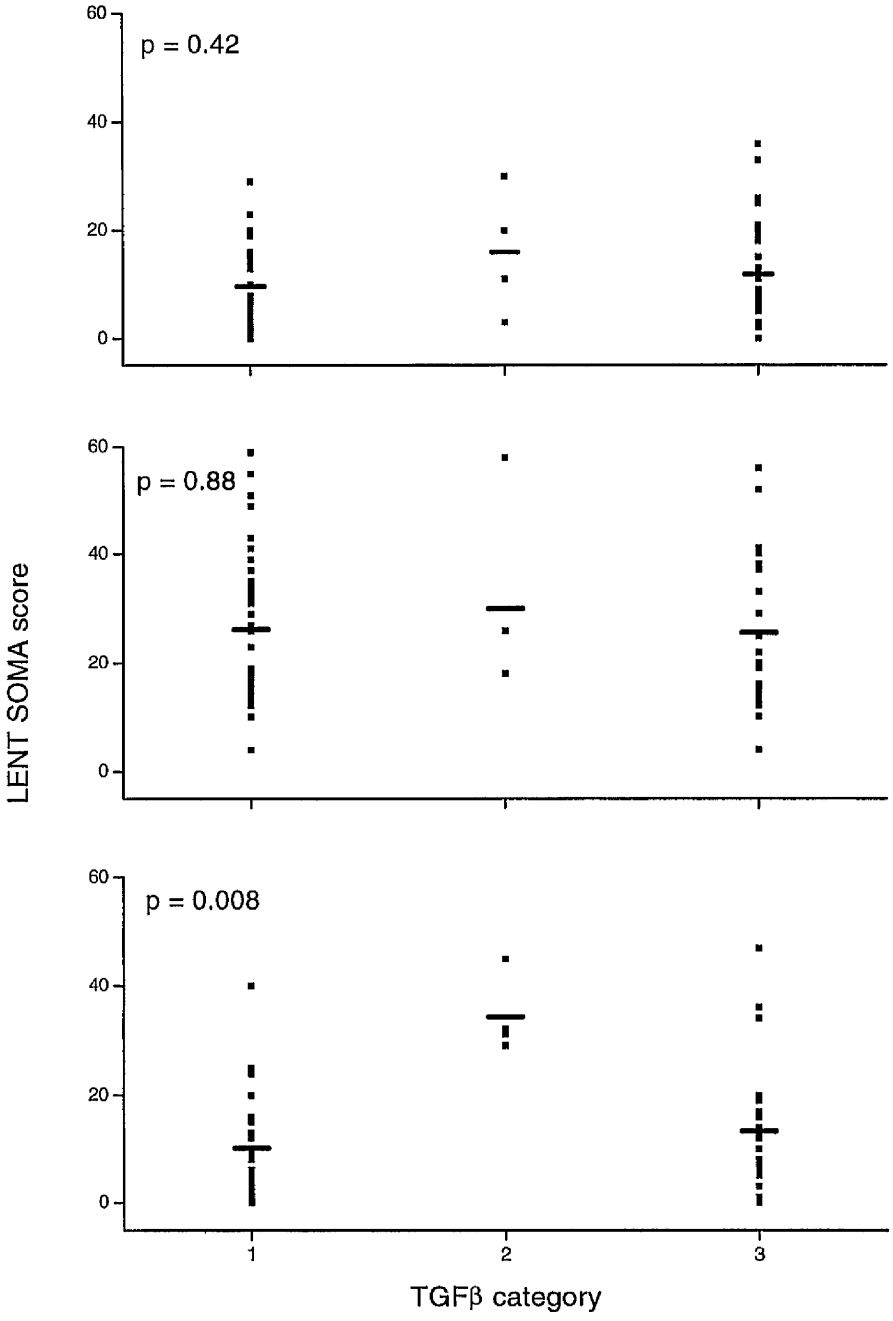


Figure 4.16: Relationship between LENT SOMA score and category of TGFβ ratio (upper graph – pre-treatment, middle graph – end of treatment, lower graph – at first follow-up, solid bar shows mean LENT SOMA score)

4.5 Discussion

The volunteer samples processed according to the EDTA protocol were in keeping with the published literature (Anscher *et al*, 1994; Reinhold *et al*, 1997). There was a significant age-related increase in plasma TGFβ1 levels, which is not borne out by the literature (Wakefield *et al*, 1995). This may have occurred for a number of reasons. First, the volunteer group in this study comprised 66 individuals. This is larger than most control groups quoted in the literature. Second, the ages of normal controls are not specified in the literature and probably do not span the wide range sampled in this study. Third, although every attempt was made to sample only volunteers with no history of malignancy, because of the ages of some of the volunteers a latent malignancy may have been present. However, removal of these donors with elevated TGFβ1 levels did not remove the significance of the age-related rise in TGFβ1 levels. This implies that this is a real phenomenon that merits further study. There were no differences between plasma TGFβ1 levels in men and women, which is in keeping with the literature (Wakefield *et al*, 1995). The number of smokers (1) in the volunteer group meant that no meaningful conclusion could be drawn about the effects of smoking on normal plasma TGFβ1 levels. Patient smoking habit did not appear to influence measured TGFβ1 levels.

Pre-treatment TGFβ1 levels were positively correlated with increasing T stage, which lends support to their role as a marker of tumour burden (Sminia *et al*, 1998, Gridley *et al*, 1998) and agrees with the data from patients with cervical cancer (see Section 3.3). Patients who had received multi-modality therapy, in the form of either chemotherapy or primary surgery, had significantly increased LENT SOMA scores at first follow-up. Those who had

chemotherapy only also had increased LENT SOMA scores at the end of treatment. There are a number of possible reasons for this. First, most patients receiving chemotherapy had longer fractionation patterns and therefore would have been at the height of their reaction at the end of treatment. Second, patients receiving combined modality therapy experience greater side effects (Tseng *et al*, 1997). LENT SOMA scores from this group of patients contained mainly subjective categories i.e. those dealing with the patients' perception of their illness. Objective data were only available in the form of amount of analgesia used and this was self-reported by the patients. The LENT SOMA scores recorded at the end of treatment and at first follow-up were significantly higher in women. This relationship is unexpected. It is difficult to explain as only men received chemotherapy in this cohort of patients. It may reflect a better articulation of subjective feelings in women, or male inability to articulate feelings to the female research nurses that administered the questionnaire. Acute side effects are dependent on the volume irradiated. The increased LENT SOMA score in patients receiving nodal irradiation is probably due to the larger volume irradiated.

The mean LENT SOMA score at first follow-up was significantly elevated in those patients whose TGFβ1 levels remained elevated above normal. Changes in plasma TGFβ1 levels have been shown to identify those patients with an increased risk of developing symptomatic acute radiation toxicity (Anscher *et al*, 1994; Anscher *et al*, 1998; Groen *et al*, 1997) in lung cancer and other thoracic malignancies. The specificity of a category 2 TGFβ ratio in identifying those at risk of a severe acute reaction in this study was 100%. The positive predictive value of a category 2 TGFβ ratio was also 100%. This study has demonstrated the clinical utility of measuring TGFβ in order to predict acute toxicity in a

group of predominantly squamous malignancies. Thus, those patients who will go on to suffer a prolonged severe acute reaction can be accurately identified at the end of treatment and targeted with increased support from a clinical nurse specialist and other input e.g. enteral feeding.

If the TGFβ ratio is obtained in the final week of treatment, the option exists to omit the final fraction of radiotherapy. This would be an unlikely option for a number of reasons. First, most acute reactions are self-limiting and have completely resolved by six months following the end of treatment. Second, time to development and rate of progression of telangiectasia are the only late toxicities for which there is a proven link with the severity of the acute reaction. Unless convincing evidence of a link between the severity of acute and all late reactions became available; most clinicians would be highly averse to compromising a potentially curative treatment modality for the sake of symptoms that, despite their severity, will settle spontaneously.

If analysis of this data at a future date demonstrates a relationship between TGFβ1 levels and late radiation toxicity, then a different emphasis would be placed on the available treatment options. If pre-treatment TGFβ1 levels determined late radiation toxicity, then patients could be more accurately assigned to primary surgical management. If the TGFβ ratio were to determine the incidence of late radiation toxicity, then the option to omit the final fraction of radiotherapy still exists. It may be that in this situation clinicians would be more willing to potentially compromise curative treatment as there is some evidence of a link between normal and tumour cellular radiosensitivity (West *et al*, 1995).

Molecular changes are present in irradiated tissues very soon following the delivery of radiotherapy. Late toxicities can take years to become manifest. This implies that there is a time interval, following radiotherapy, where the potential to influence the molecular environment exists. Changes in the tissues during this time would have the potential to either ameliorate or eliminate the late toxicity. This is known as post radiation modification. If the TGFβ ratio were to accurately predict the development of late radiation toxicity, then trials of post-radiation response modifiers could be accurately targeted at those patients for whom they would offer the most benefit.

CHAPTER 5: BREAST CARCINOMA

5.1: Introduction

Using PFGE, a significant correlation was demonstrated between residual DNA double strand breaks and the development of late radiation fibrosis in a group of thirty-nine patients treated for early breast cancer between 1985 and 1989 (Kiltie *et al*, 1999). These patients were all treated at the Christie Hospital, Manchester using an identical treatment protocol. PFGE thus showed promise as a predictive assay of intrinsic cellular radiosensitivity in breast cancer patients. This group formed the training cohort, in which the hypothesis was generated. However, any new hypothesis must be retested and validated on a different group of patients (validation cohort) (Miranda *et al*, 1992; Hoskins *et al*, 1998). This study had two main aims. First, to investigate the usefulness of PFGE as a predictive assay in a validation cohort of patients. Second, investigate the relationship between late radiation sequelae and TGFβ1 levels taken prior to treatment in a group of breast cancer patients.

5.2 Patient characteristics

Fifty patients were randomly recruited from a cohort of 190 patients who were treated at the Christie Hospital, Manchester for early breast cancer in 1993 and 1994. The demographic details are displayed in Table 5.1. These patients had taken part in a study investigating normal cell radiosensitivity in relation to intensity of the acute radiation reaction. Therefore, a clinical photograph taken prior to the start of treatment was available for all patients. All patients also had a blood sample taken prior to the start of treatment

and processed according to the heparin method (see Section 2.3). All patients were women. The mean age of the patients at recruitment was 61 years (range 45 – 75 years). Ten patients were smokers at the time of recruitment into the study. Seventeen patients had a first degree relative with breast cancer. Twenty- two patients had a right-sided tumour and 28 a left-sided tumour. Tumour (T) stage was available for 49 patients. Forty-three patients had a T1 and 6 patients a T2 tumour. Nodal status was available for 38 patients. Twenty-eight patients had N0 disease and ten patients had N1 disease. Thirty-seven patients reported having an acute skin reaction at the time of radiotherapy, while thirteen patients remembered no acute reaction.

Table 5.1: Demographic data for breast carcinoma patients

Patient number	Lab number	Age at diagnosis (years)	Age at biopsy (years)	Affected side	Disease stage[1]	Smoking history	Family history	Acute reaction
1	SP55	58	62	L	T1N0	N	Y	Y
2	SP56	65	69	L	T1N1	Y	Y	Y
3	SP57	58	62	L	T1N0	N	Y	Y
4	SP58	62	66	L	T1N1	N	Y	Y
5	SP59	58	62	R	T1N0	N	N	N
6	SP60	60	65	R	T1NX	N	Y	Y
7	SP61	67	72	L	T1NX	Y	Y	Y
8	SP62	57	62	R	T1N0	N	N	Y
9	SP63	44	48	L	T1N0	N	Y	Y
10	SP64	55	60	R	T1N0	N	N	Y
11	SP65	64	69	L	T1N1	N	Y	Y
12	SP66	44	48	L	T1N0	Y	N	Y
13	SP67	58	63	L	T2N1	N	N	Y
14	SP68	40	45	L	T1N0	N	N	Y
15	SP69	67	71	L	T1N0	N	Y	N
16	SP70	60	65	L	T1N0	N	N	Y
17	SP71	51	57	R	TXNX	N	N	Y
18	SP72	48	53	R	T1N0	N	Y	Y
19	SP73	42	47	R	T1N0	Y	N	Y
20	SP74	58	63	R	T1N0	Y	N	Y
21	SP75	56	61	R	T2N1	N	N	Y
22	SP76	60	66	R	T1N0	N	N	Y
23	SP77	65	70	L	T1N0	N	N	Y
24	SP78	48	53	R	T1N0	Y	Y	Y
25	SP79	55	60	L	T1N0	Y	N	Y
26	SP80	63	68	L	T2N0	N	Y	N
27	SP81	63	68	R	T1NX	Y	N	Y
28	SP82	63	68	L	T1N0	N	N	N
29	SP83	52	57	L	T1NX	N	N	Y
30	SP84	62	67	L	T1N0	N	N	N
31	SP85	52	58	R	T1N1	N	N	N
32	SP86	60	65	R	T2N0	N	N	Y
33	SP87	49	55	R	T1NX	N	Y	Y
34	SP88	59	65	R	T1N0	N	N	Y
35	SP89	49	54	R	T2NX	N	N	Y
36	SP90	58	63	L	T1N0	N	Y	Y
37	SP91	65	60	L	T1NX	N	Y	Y
38	SP92	53	58	R	T1NX	N	N	Y
39	SP93	53	58	L	T1N1	Y	N	N
40	SP94	54	59	L	T1NX	N	N	N
41	SP95	52	57	L	T2N0	N	N	Y
42	SP96	52	58	L	T1N1	N	Y	N
43	SP97	65	70	R	T1N0	Y	N	Y
44	SP98	56	61	R	T1N1	N	N	Y
45	SP99	57	62	L	T1N0	N	N	N
46	SP100	61	66	R	T1N0	N	N	N
47	SP101	51	56	L	T1NX	N	N	Y
48	SP102	56	61	R	T1NX	N	N	Y
49	SP103	70	75	L	T1N1	N	Y	N
50	SP104	60	65	L	T1N0	N	N	N

[1] – X indicates unknown

5.3 PFGE results

The results of the PFGE assay are displayed in Table 5.2. Although viable fibroblast cultures were obtained from all fifty primary biopsies, FDR (fraction of damage released) results were only obtained from 49 patients. In the remaining patient, the background FDR results were too high to produce meaningful results. The mean with one standard deviation and the median FDR results were $8 \pm 2\%$ and 8%, respectively. Using ANOVA significant differences were detected between FDR results from individual patients ($p < 0.001$). The distribution of FDR results is shown in Figure 5.1. Using Spearman's regression test, there was no relationship between age and FDR (Figure 5.2).

Table 5.2: Experimental data for breast carcinoma patients

Patient number	Lab. Number	TGFβ1 level (pre-treatment) (ng/ml)[1]	Days elapsed[2]	Number of experiments[3]	FDR (%)
1	SP55	8.85	33	4	10.7
2	SP56	25.54	17	4	5.5
3	SP57	U	28	3	7.6
4	SP58	26.99	19	4	6.4
5	SP59	5.95	14	4	7.9
6	SP60	U	12	3	10.3
7	SP61	U	12	4	8.3
8	SP62	U	12	2	4.8
9	SP63	11.78	12	4	8.1
10	SP64	U	12	4	9.9
11	SP65	10.56	22	5	6.9
12	SP66	14.18	13	3	11.1
13	SP67	10.64	13	3	9.1
14	SP68	U	12	7	7.3
15	SP69	14.61	13	6	8.1
16	SP70	11.16	14	2	8.3
17	SP71	U	14	3	3.8
18	SP72	9.66	14	4	6.1
19	SP73	13.75	14	4	8.2
20	SP74	9.78	13	4	13.4
21	SP75	11.08	21	3	9.0
22	SP76	4.97	19	4	6.8
23	SP77	15.63	14	5	12.0
24	SP78	12.49	20	4	12.5
25	SP79	13.69	17	3	8.8
26	SP80	19.04	14	3	10.6
27	SP81	U	14	3	7.7
28	SP82	U	12	4	12.8
29	SP83	9.45	14	4	7.1
30	SP84	7.14	12	4	9.9
31	SP85	11.36	12	3	9.2
32	SP86	11.59	26	4	9.2
33	SP87	6.90	19	4	7.9
34	SP88	U	19	3	5.3
35	SP89	8.38	28	3	8.1
36	SP90	15.75	20	4	7.6
37	SP91	12.63	21	5	8.5
38	SP92	U	14	5	7.5
39	SP93	11.25	20	4	4.2
40	SP94	10.61	13	5	5.9
41	SP95	6.53	21	3	3.8
42	SP96	U	21	4	5.7
43	SP97	12.05	19	2	6.2
44	SP98	7.22	19	U	U
45	SP99	U	19	2	11.1
46	SP100	10.60	12	2	6.8
47	SP101	13.48	15	3	7.7

Patient number	Lab. Number	TGFβ1 level (pre-treatment) (ng/ml)[1]	Days elapsed[2]	Number of experiments[3]	FDR (%)
48	SP102	8.39	15	3	7.4
49	SP103	7.48	15	3	3.3
50	SP104	9.55	15	4	5.2

[1] – U indicates unknown

[2] – number of days between biopsy and first passage of fibroblasts in culture

[3] – number of individual experiments averaged to obtain FDR (%) result

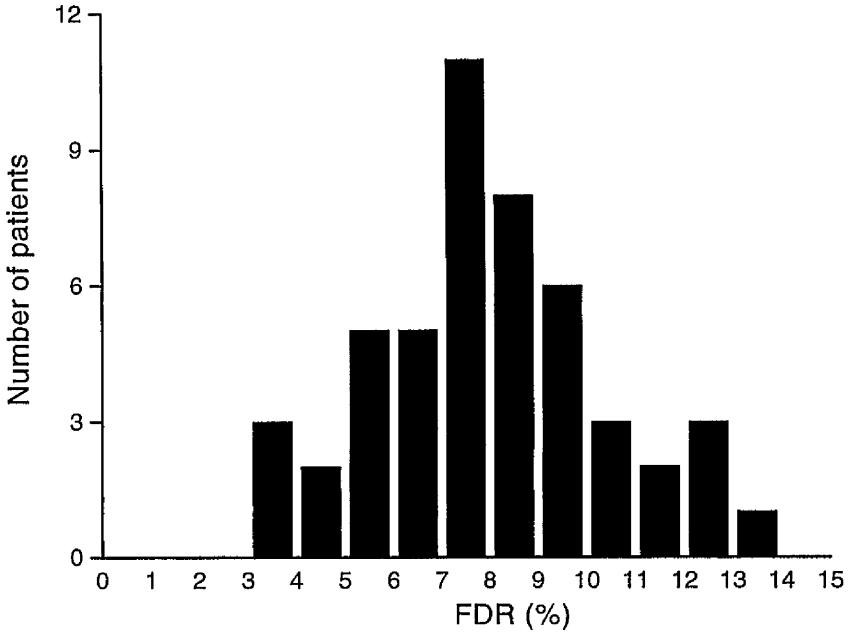


Figure 5.1: Distribution of FDR (%) results (n = 49)

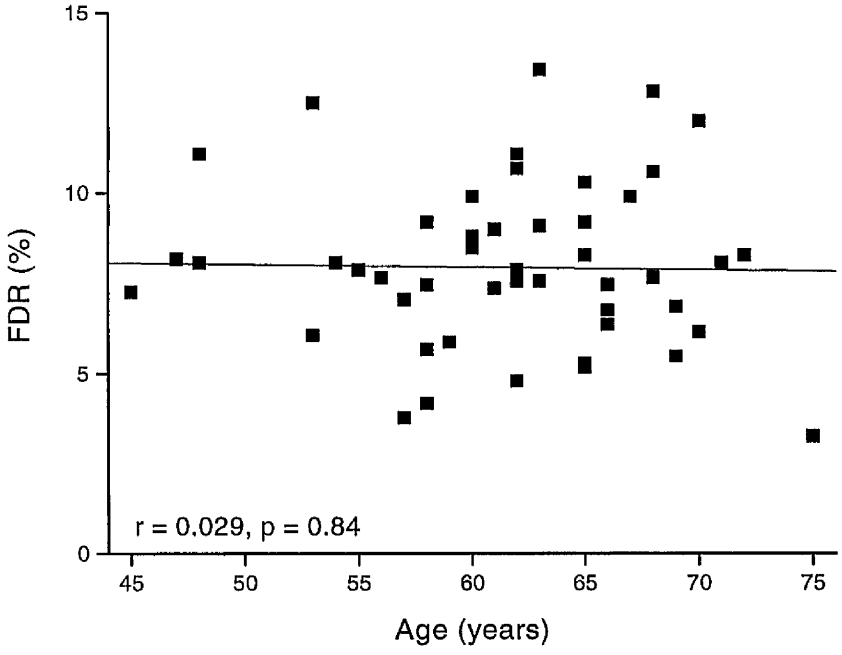


Figure 5.2: Relationship between patient age at biopsy and FDR (%) (n = 49)

5.4 LENT SOMA results

The LENT SOMA scores are displayed in Table 5.3. The maximum LENT SOMA score possible was 31. The mean with one standard deviation and the median values were 4 ± 2 and 3, respectively. The distribution of LENT SOMA scores is shown in Figure 5.3. Using Spearman's regression test there was a weak but significant relationship between LENT SOMA score and patient age (Figure 5.4). No relationship was found, using a Mann-Whitney u test, with the presence of an acute reaction (Figure 5.5), but there was a significant relationship between LENT SOMA score and a positive family history of breast cancer (Figure 5.6). This was confirmed by a t-test comparing the mean LENT SOMA score in those patients with and without an acute reaction ($p < 0.001$).

Table 5.3: LENT SOMA data for breast carcinoma patients

Patient number	Lab. number	Patient Build[1]	Breast Size[2]	Fibrosis Score[3]	LENT SOMA Score[4]	Modified LENT SOMA score[5]
1	SP55	2	3	2	6	5
2	SP56	2	2	0	8	7
3	SP57	3	2	1	3	3
4	SP58	2	2	2	5	5
5	SP59	2	2	0	0	0
6	SP60	2	2	1	3	2
7	SP61	2	1	2	4	4
8	SP62	3	2	1	3	1
9	SP63	2	2	2	7	4
10	SP64	2	3	0	1	1
11	SP65	3	2	2	5	5
12	SP66	1	1	1	2	2
13	SP67	3	2	2	5	4
14	SP68	2	2	2	4	4
15	SP69	3	2	2	4	4
16	SP70	2	3	2	4	3
17	SP71	2	2	2	2	2
18	SP72	3	2	3	5	5
19	SP73	2	2	1	3	3
20	SP74	3	2	1	2	1
21	SP75	2	3	1	4	3
22	SP76	2	2	2	3	3
23	SP77	2	2	2	6	5
24	SP78	2	2	3	6	6
25	SP79	2	2	1	3	3
26	SP80	2	3	2	5	5
27	SP81	2	2	2	3	3
28	SP82	3	2	1	2	2
29	SP83	2	2	1	6	6
30	SP84	2	2	3	5	5
31	SP85	3	2	1	3	3
32	SP86	2	3	1	3	3
33	SP87	2	2	1	3	3
34	SP88	2	3	1	5	5
35	SP89	1	2	2	2	2
36	SP90	3	1	1	4	3
37	SP91	2	2	2	3	3
38	SP92	2	2	1	3	3
39	SP93	2	2	0	0	0
40	SP94	2	2	0	0	0
41	SP95	2	1	1	2	2
42	SP96	3	3	3	8	6
43	SP97	3	2	2	5	5
44	SP98	2	2	0	3	3
45	SP99	2	2	1	1	1
46	SP100	3	2	2	5	5
47	SP101	2	3	0	2	2
48	SP102	1	1	2	2	2

Patient number	Lab. number	Patient Build[1]	Breast Size[2]	Fibrosis Score[3]	LENT SOMA Score[4]	Modified LENT SOMA score[5]
49	SP103	2	2	2	5	4
50	SP104	3	2	3	6	5

[1] – scored by clinical photograph: 1 = small, 2 = medium, 3 = large

[2] – scored by clinical photograph: 1 = small, 2 = medium, 3 = large

[3] – scored by clinical examination: 0 = none palpable, 1 = barely palpable, 2 = definitely palpable, 3 = marked density present

[4] – derived from LENT SOMA questionnaire, a combination of: breast pain intensity score (max. 4), analgesia requirement score (max. 4), retraction score (max 4), retraction management score (max.1), breast oedema score (max. 2), breast oedema management score (max 2), skin ulceration score (max. 4), skin ulceration management score (max. 4), telangiectasia score (max. 3) and fibrosis score (max. 3) i.e. maximum score possible = 31

[5] – total LENT SOMA score minus telangiectasia score i.e. maximum score possible = 28

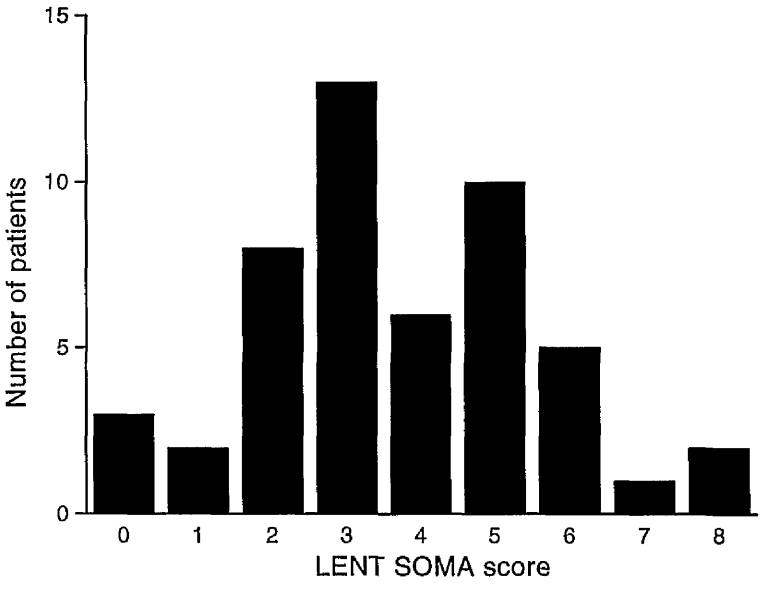
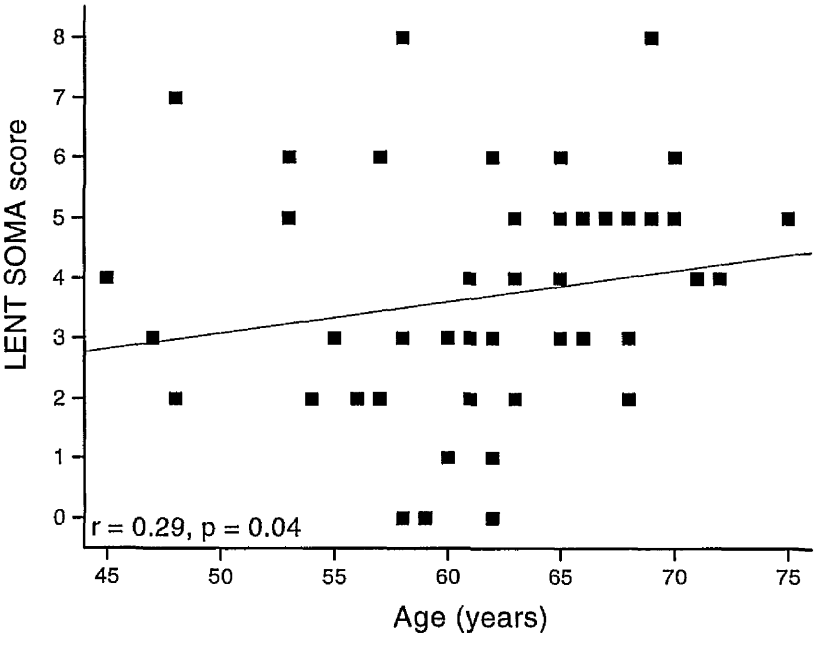


Figure 5.3: Distribution of LENT SOMA scores (n = 50)



5.4: Relationship between patient age and LENT SOMA score

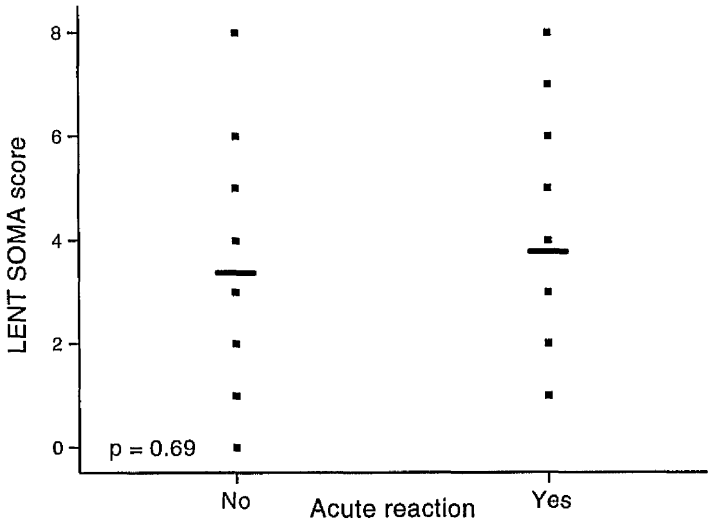


Figure 5.5: Relationship between LENT SOMA score and presence of an acute reaction. Numbers: no = 13, yes = 37. Solid bar indicates mean LENT SOMA score. Data points may represent more than one patient, refer to text for details.

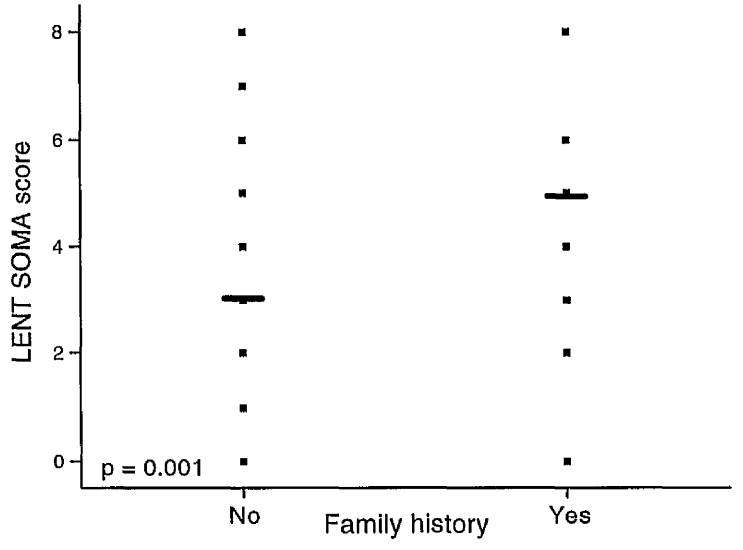


Figure 5.6: Relationship between LENT SOMA score and family history of breast cancer. Numbers: no = 33, yes = 17. Solid bar indicates mean LENT SOMA score. Data points may represent more than one patient, refer to text for details.

5.5 Comparison of PFGE results and LENT SOMA score

Using Spearman's regression analysis there was no correlation between FDR and fibrosis score or overall LENT SOMA score (Figures 5.7 and 5.8, respectively). Again using Spearman's regression test, the relationship between the combined FDR and fibrosis scores of the training and validation cohorts was investigated and no significant relationship was found ($r = 0.20$, $p = 0.063$) (Figure 5.9).

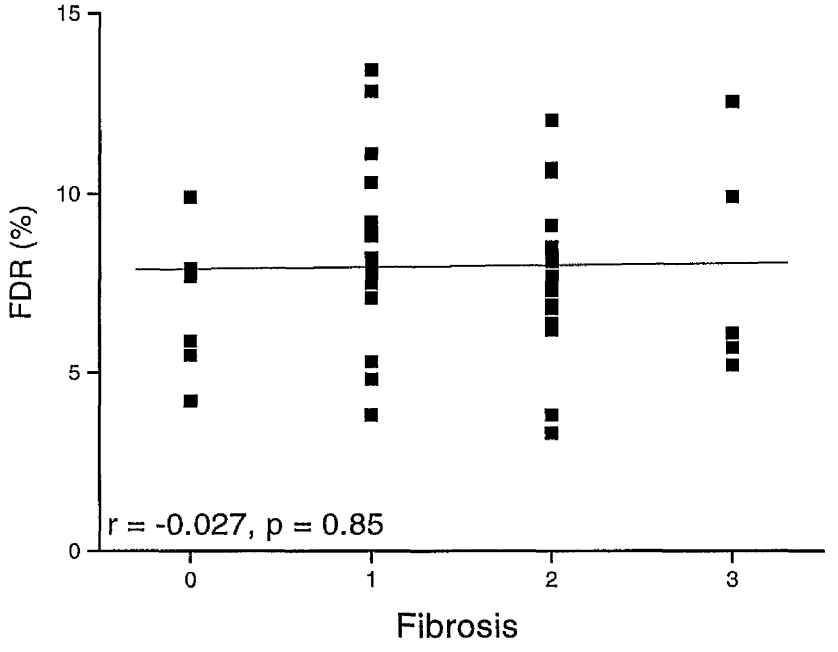


Figure 5.7: Relationship between FDR and fibrosis score (n = 49).

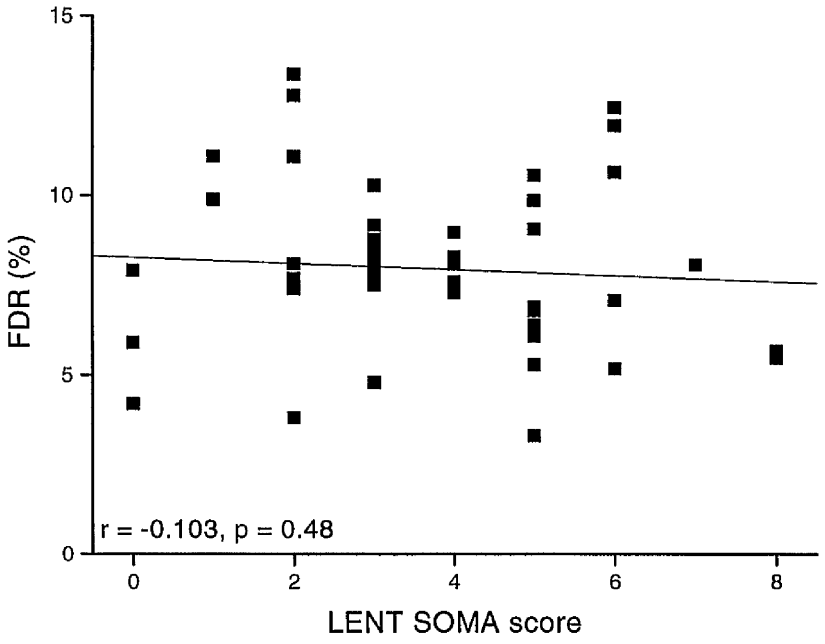


Figure 5.8: Relationship between FDR and total LENT SOMA score (n = 49).

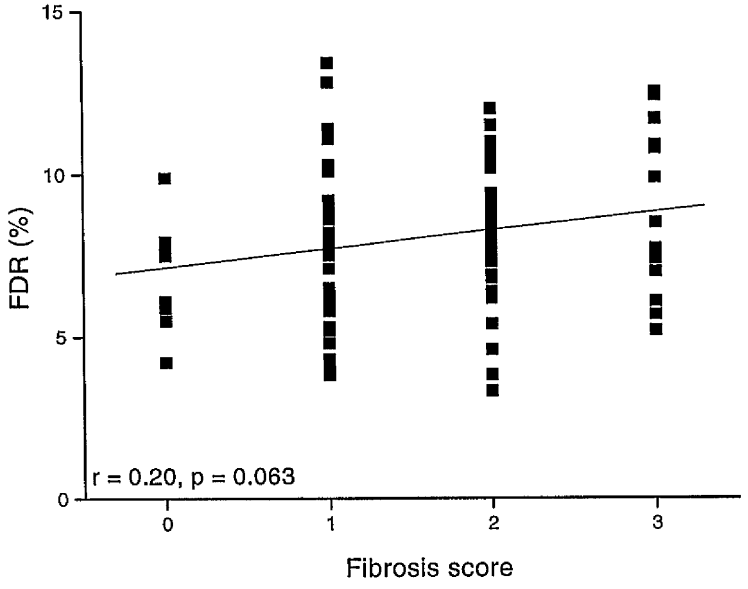


Figure 5.9: Relationship between FDR and fibrosis in the combined cohort (training and validation), n = 88

5.6 TGFβ1 results

The individual TGFβ1 levels are given in Table 5.2. There were 37 pre-treatment TGFβ1 levels available. The mean with one standard deviation and the median values were 11.64 ng/ml ± 4.65 ng/ml and 11.08 ng/ml, respectively. The distribution of pre-treatment TGFβ1 levels is displayed in Figure 5.10. Using Spearman's regression test, there was no correlation between TGFβ1 levels and either patient age (Figure 5.11) or T stage (Figure 5.12). Using a Mann-Whitney u test, there was no relationship between TGFβ1 levels and either a family history of breast cancer (Figure 5.13) or presence of an acute reaction to radiotherapy (Figure 5.14). However, there was a relationship between pre-treatment TGFβ1 levels and smoking habit (Figure 5.15).

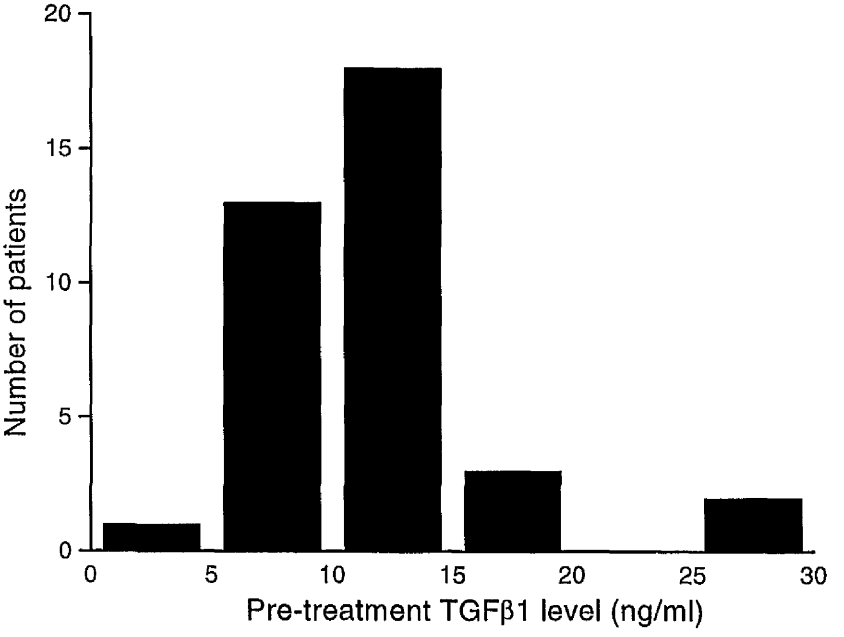


Figure 5.10: Distribution of TGFβ1 levels taken prior to treatment (n = 37)

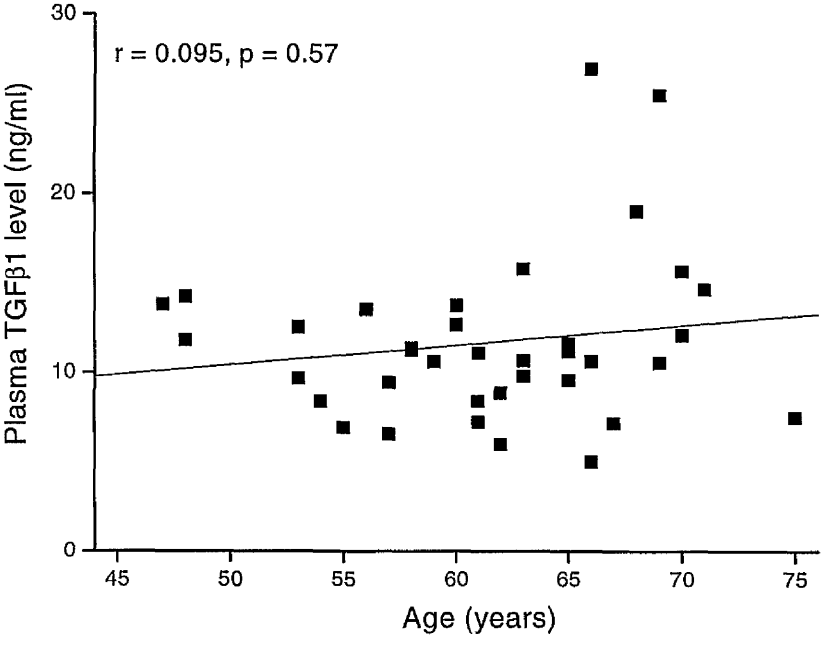


Figure 5.11: Relationship between pre-treatment TGFβ1 levels and patient age (n = 37)

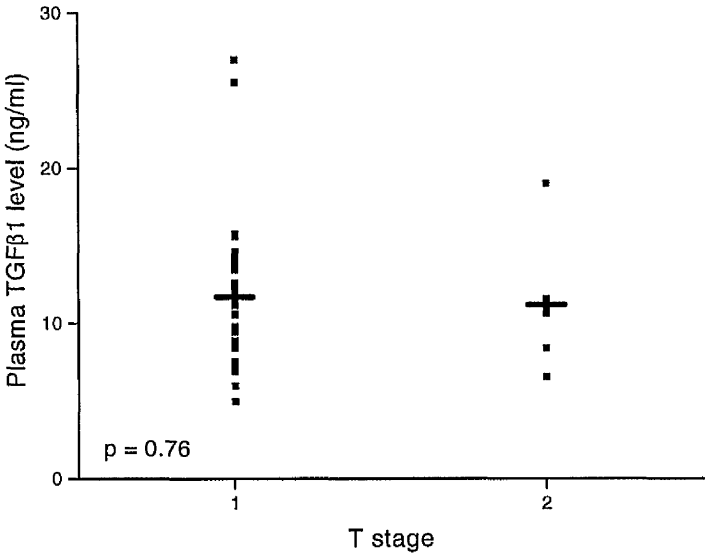


Figure 5.12: Relationship between pre-treatment plasma TGFβ1 levels and tumour (T) stage (n = 37)

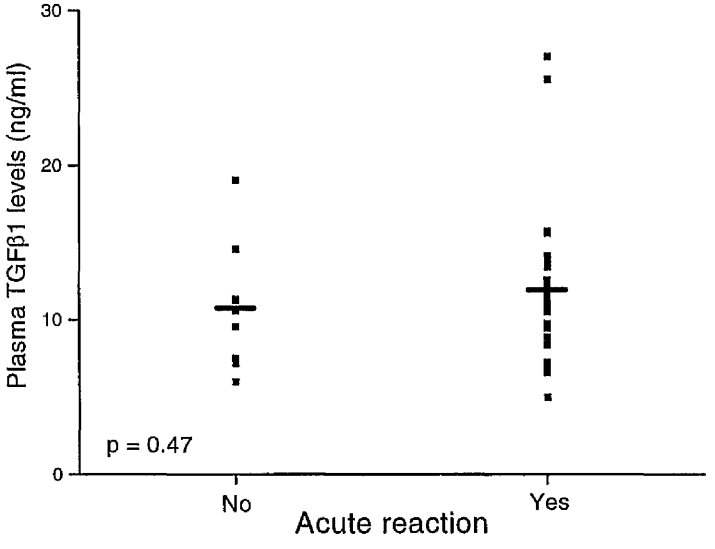


Figure 5.13 Relationship between pre-treatment plasma TGFβ1 levels and presence of an acute reaction

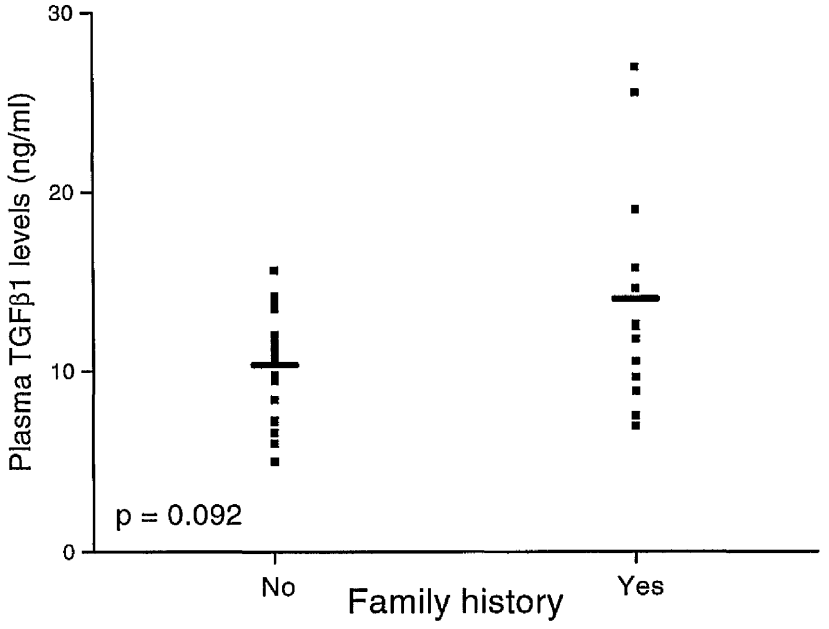


Figure 5.14: Relation ship between TGFβ1 levels and family history of breast cancer (no = 24, yes = 13). Solid bars indicate mean plasma TGFβ1 levels.

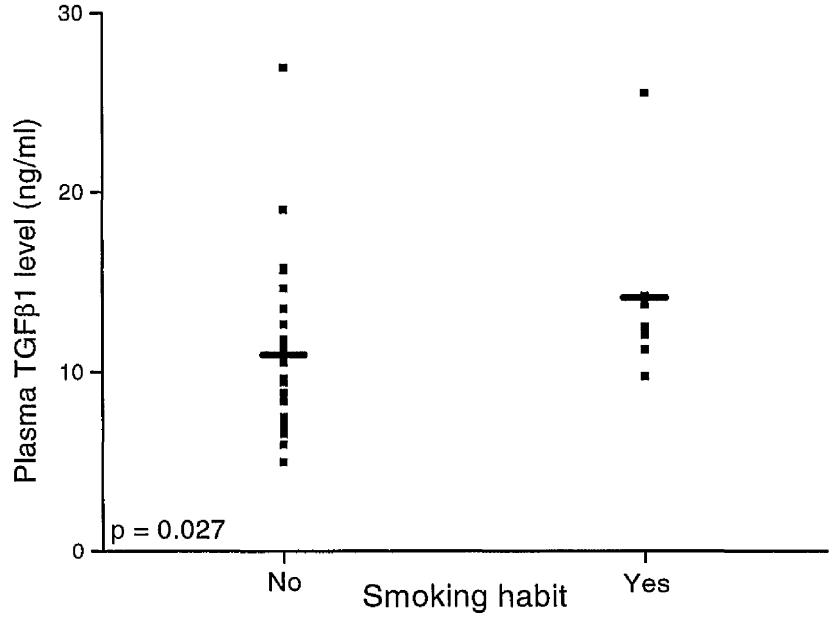


Figure 5.15: Relationship between TGFβ1 levels and smoking habit (no = 29, yes = 8). Solid bars indicate mean plasma TGFβ1 levels.

5.7 Comparison of TGFβ1 levels with LENT SOMA score and PFGE results

In this analysis, fibrosis score (one component of the LENT SOMA score) as well as total LENT SOMA score were correlated, using Spearman's regression test, with pre-treatment TGFβ1 and FDR. No relationship between either total LENT SOMA score or fibrosis score and either TGFβ1 levels or FDR was found (Figures 5.16 - 5.18, respectively).

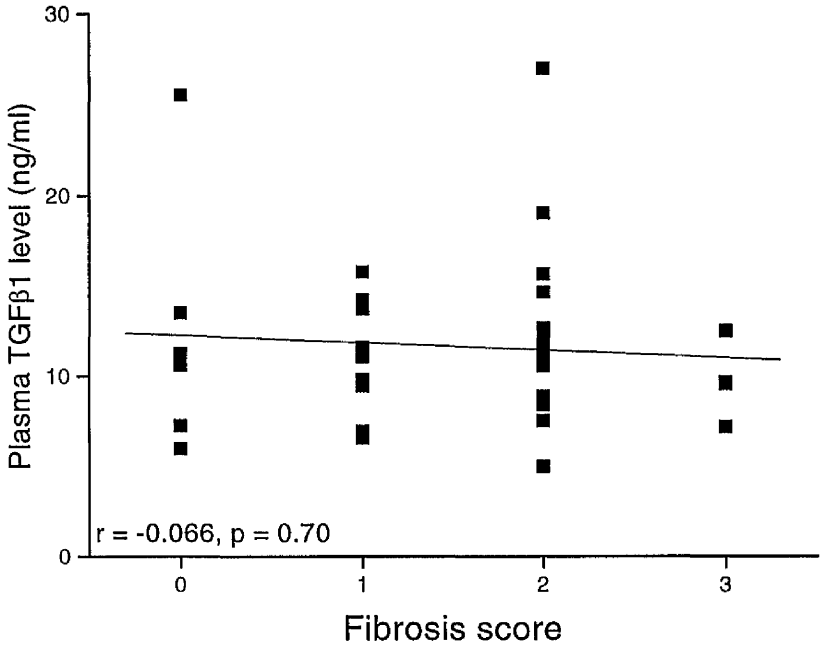


Figure 5.16: Relationship between pre-treatment TGFβ1 levels and fibrosis score (n = 37).

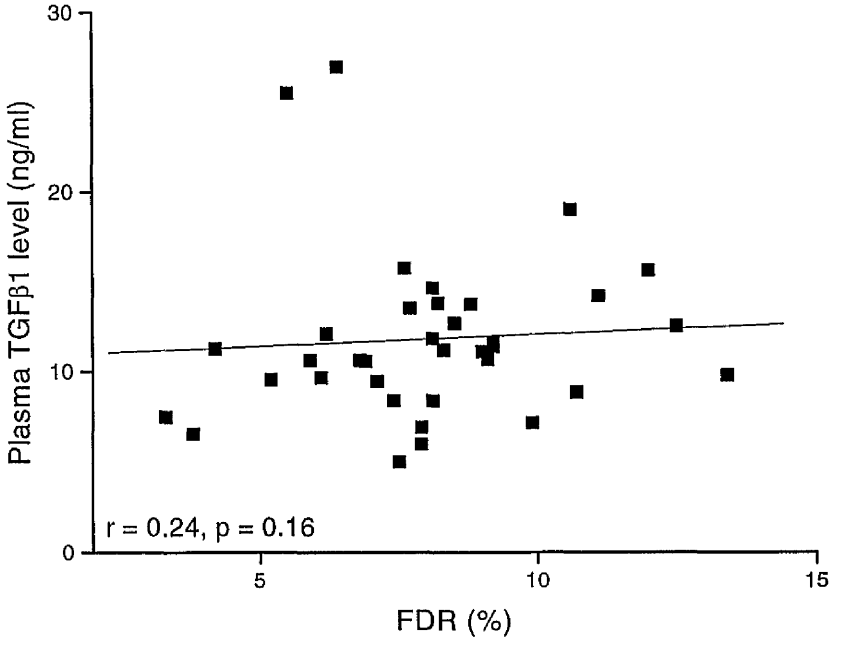


Figure 5.17: Relationship between pre-treatment TGFβ1 levels and FDR (n = 37)

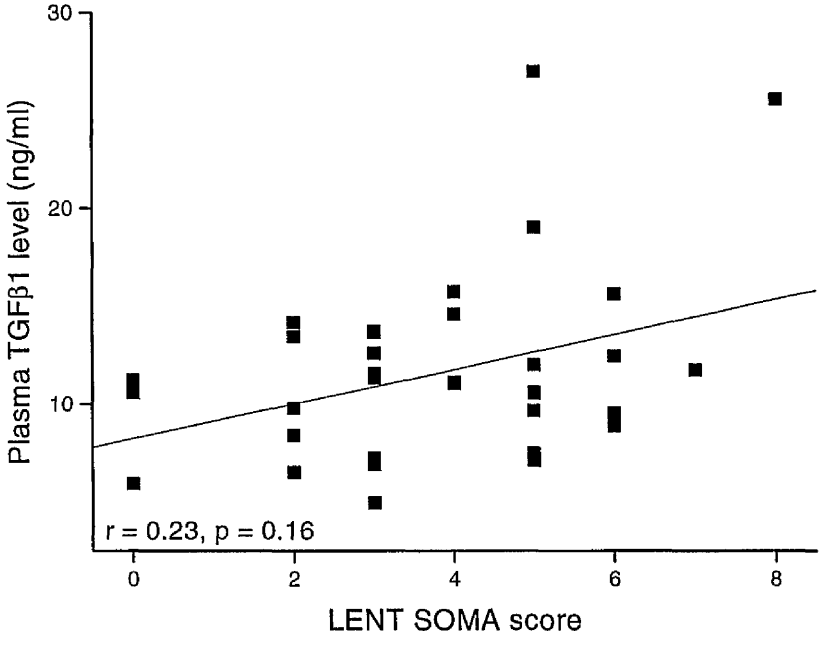


Figure 5.18: Relationship between pre-treatment TGFβ1 levels and LENT SOMA score (n = 37).

5.8 Discussion

Whenever a new hypothesis is generated it is necessary to test it on a new, distinct cohort of patients (Miranda *et al*, 1992; Hoskins *et al*, 1998). Thus only hypotheses with a firm evidence base are admitted into routine practice. The primary aim of the work in this chapter was the validation of a new hypothesis that residual DNA double strand breaks at 24 hours following irradiation, as measured by PFGE, were a predictive assay for the incidence of late radiation fibrosis in patients with breast carcinoma. This hypothesis was not validated. There may be a number of reasons for this. First, although the FDR results were obtained from both patient cohorts in the same laboratory, using the same equipment, the fact that results from the two cohorts were obtained by two independent researchers may have made a difference. However, Figure 5.19 illustrates the similar distribution of FDR results in both cohorts. Using an ANOVA no significant difference was seen between the two sets of results ($p = 0.72$). Second, the patients in each cohort were examined over a different time range following their radiotherapy (9 – 14 years in the training cohort and 4 – 5 years in the validation cohort). Figure 5.20 shows a comparison of the distribution of fibrosis scores in each treatment cohort. Allowing for varying numbers in each cohort, they are broadly similar. This was confirmed by an ANOVA, which showed no significant differences between them ($p = 0.13$). It has been demonstrated that severity of telangiectasia progresses over time (Tucker *et al*, 1992; Turesson *et al*, 1996) and there is some evidence that post-radiotherapy fibrosis may also progress over time (Bentzen *et al*, 1990). Thus, it may be that if the training cohort were to be reevaluated in a further five years time, the distribution of fibrosis scores may have changed sufficiently for a positive correlation to become evident. Third, although treatment within the cohorts was standardised, the elapse of five years meant that a number of changes in treatment

technique had occurred. Patients in the training cohort were treated with an unwedged, two-field treatment where the breast was enclosed in a perspex box. The box acted as a bolus material, significantly increasing the dose received by the skin. The patients in the validation cohort received a wedged, isocentrically planned treatment, with no perspex box. The comparison of telangiectasia scores between the cohorts (Figure 5.21) demonstrates a reduction in both the incidence and severity of this late radiation toxicity in the validation cohort. A comparison of the total LENT SOMA score, corrected for telangiectasia score, in each cohort (Figure 5.22) demonstrates that the validation cohort had less late radiotherapy reactions than the training cohort. This may have reduced the chance of detecting a correlation between LENT SOMA score and fibrosis. With a smaller range of LENT SOMA scores and a reduced prevalence of late radiation reactions, the correlation between LENT SOMA score and fibrosis may need to be stronger to be discernible in the validation cohort.

The secondary aim of the work in this chapter was to examine the relationship between plasma TGFβ1 levels taken prior to treatment and clinical endpoints. There was no relationship between age and TGFβ1 levels. This is in agreement with the published literature (Wakefield *et al*, 1995). It also agrees with the findings in other patient groups, but not for the cancer-free volunteers studied in this thesis. This may be due to the smaller age range of the patient group compared with the volunteer group. Pre-treatment TGFβ1 levels were not significantly related to T stage or N stage, meaning they were of no use as measures of tumour burden. There may be a number of reasons for this. First, in this study, pre-treatment plasma TGFβ1 levels were measured at least six weeks following surgery. TGFβ1 levels can decrease following surgery due to a reduction in tumour burden (Kong *et al*, 1995; Tsushima *et al*, 1996). Therefore, the lack of correlation with disease stage

may be as a consequence of surgery. Second, only patients with stage T1 or T2 disease were available in this study. If patients with stage T3 or T4 disease or with heavy nodal involvement were available, a correlation with disease stage might emerge. Due to the small numbers of smokers recruited in our volunteer group (see Section 4.2), no conclusion could be drawn about the impact of smoking on plasma TGFβ1 levels. In this series, pre-treatment TGFβ1 levels were significantly higher in those patients who admitted smoking. This does not agree with our findings in patients with carcinoma of the head and neck (Chapter 4). Patients were asked about current smoking habit, rather than smoking habit at the time of surgery and radiotherapy. Thus the observed relationship between plasma TGFβ1 levels and smoking may be spurious. However, on balance, patients are more likely to have stopped than started smoking in the intervening period.

Pre-treatment plasma TGFβ1 levels did not predict for an acute reaction to radiotherapy. There may be a number of reasons for this. First, acute reaction was scored retrospectively from the patients' memory and may therefore be biased. Second, changes in plasma TGFβ1 levels between those measured prior to treatment and at the end of radiotherapy may be required for this correlation (see Sections 1.12 and 4.3). There was no relationship found between pre-treatment TGFβ1 levels and fibrosis-related clinical endpoints. This does not support the conclusions of Li *et al* (1999). There may be a number of possible explanations for this. First, we used a different assay from that used by Li *et al.* As described in Chapter 2, we used a commercially available kit, with mass-produced antibodies. Li's group used their own antibody in an ELISA system that reported by enhanced chemoluminescence. However, given that we have successfully demonstrated a correlation with acute reactions in patients with carcinoma of the head and neck region (see Chapter 4), this is likely to be unimportant. Second and of greater significance, there were

a larger number of patients in Li *et al*'s series (91 compared with 37 in this series). A prospective study is now required measuring plasma TGFβ1 levels at the start and end of treatment to reach a firm conclusion about the clinical utility of predicting severe acute and late radiation sequelae in patients with carcinoma of the breast.

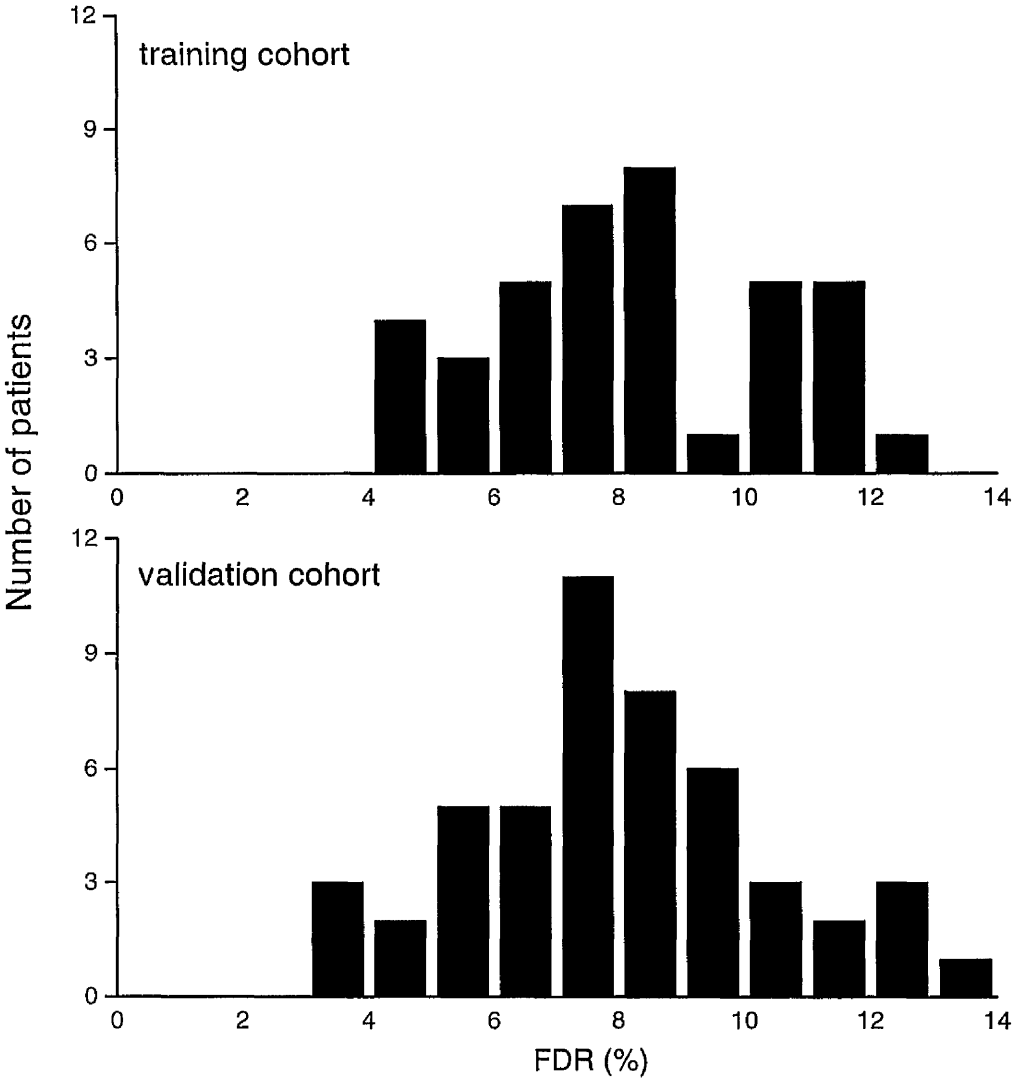


Figure 5.19: Comparison of the distribution of FDR between the training (n = 39) and the validation (n = 49) cohorts

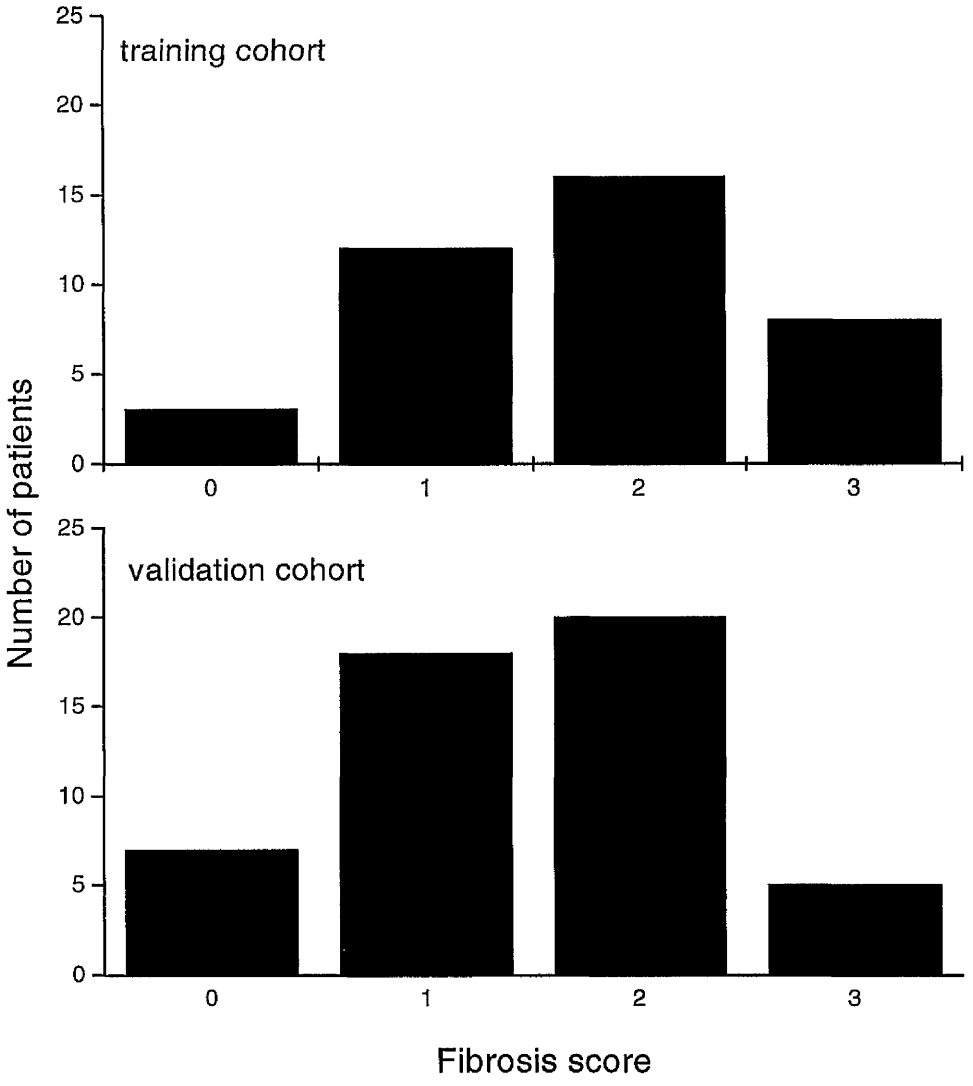


Figure 5.20: Comparison of the distributions of fibrosis scores between the training (n = 39) and the validation (n = 49) cohorts

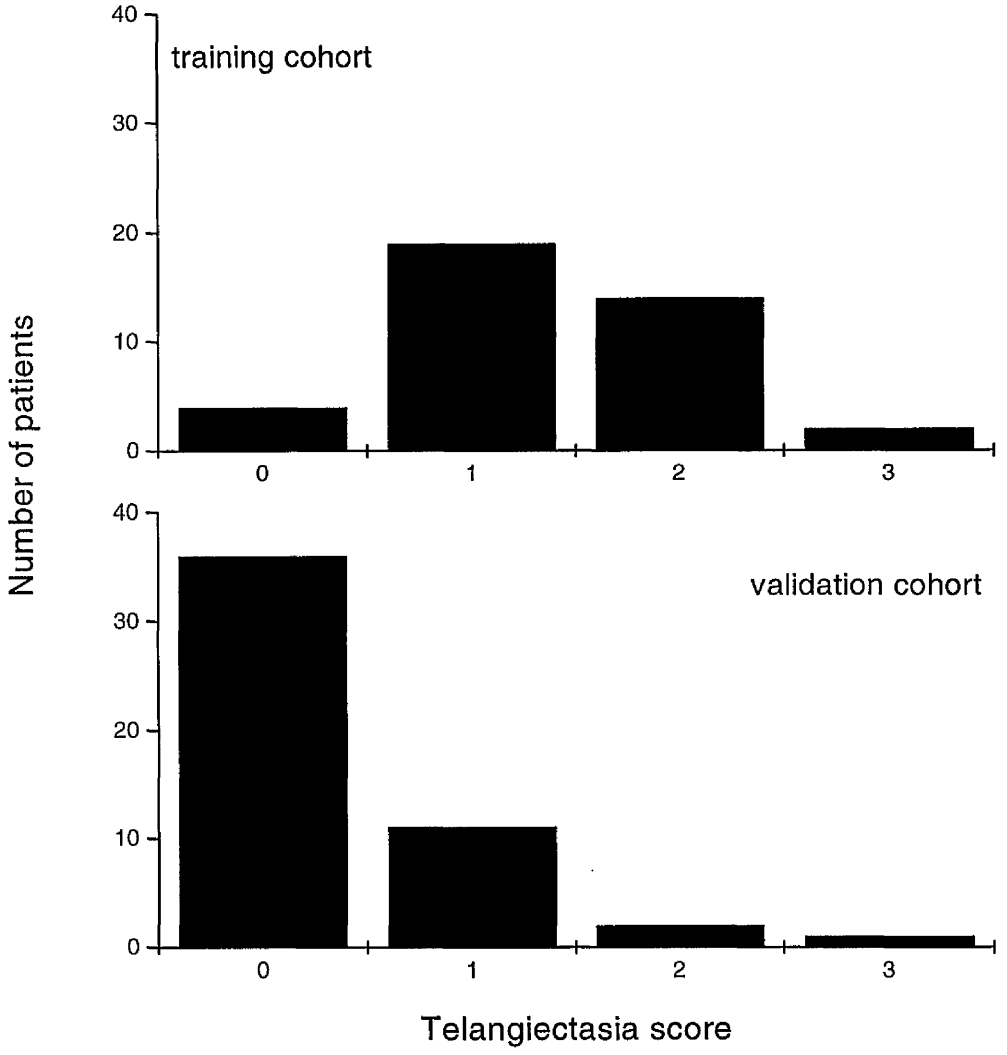


Figure 5.21: Comparison of telangiectasia scores between the training (n = 39) and the validation (n = 49) cohorts

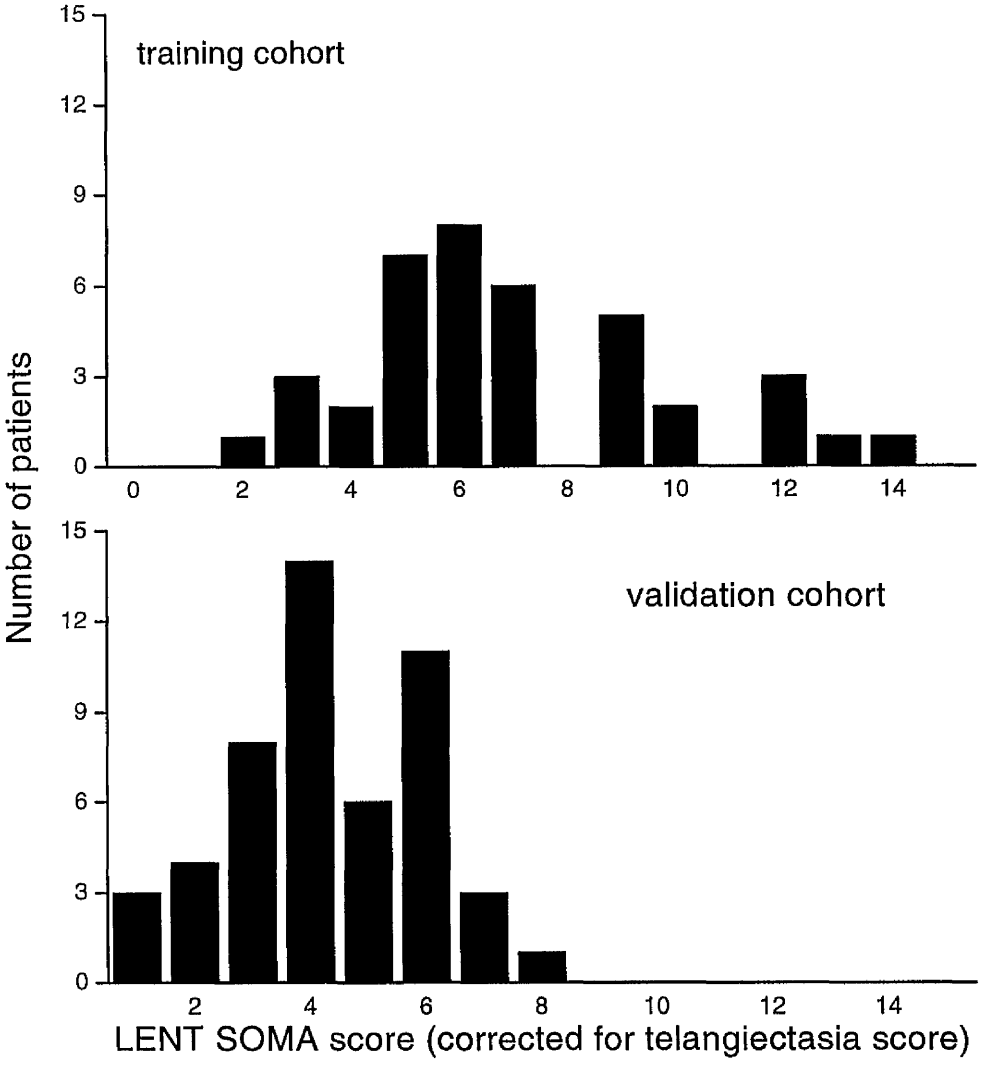


Figure 5.22: Comparison of total LENT SOMA score (minus the telangiectasia score) between the training (n = 39) and the validation (n = 49) cohorts

CHAPTER 6: DISCUSSION

Significant inter-individual variation occurs between patients in the severity of acute and late side effects experienced as a result of radiotherapy. Although a number of factors may play a part (see Section 1.7), genetic factors have been estimated to account for between 80 and 90% of this variability (Geara *et al*, 1993; Turesson *et al*, 1996). This variation in normal cell radiosensitivity limits the amount of radiotherapy that can be prescribed because of the tolerance of the surrounding normal tissues. It also limits the dose that can be safely administered to a tumour and hence ultimately the clinical radiocurability of that tumour. The development of an assay to predict an individual's normal cell radiosensitivity offers the possibility of individualising radiotherapy schedules. In theory this would result in an increase in local tumour control with no corresponding increase in late normal tissue toxicity (McKay *et al*, 1998; Hendry 1998). This is an important issue, as the number of patients with cancer in the population requiring radiotherapy will rise in the 21st century.

This thesis was concerned with the assessment of assays predicting normal cell radiosensitivity. Two different assays were studied. First, plasma TGFβ1 levels were measured using ELISA and a commercially available kit. This methodology is attractive as a predictive assay for normal cell radiosensitivity as it is simple to perform and would not require significant investment on the part of most radiotherapy departments to implement if proven useful. Second, residual DNA double strand breaks were measured using normal fibroblasts and pulsed-field gel electrophoresis (PFGE). This is a technically difficult method requiring tissue biopsy and some financial investment (tissue culture facilities,

PFGE apparatus), making it less attractive to clinical departments. The assays were examined in a number of tumour types.

Cervical carcinoma

Patient samples were obtained from a cohort treated at the Christie Hospital, Manchester between 1990 and 1993. These patients were initially recruited in an unselected fashion and investigated prospectively. Both clonogenic assay parameters and tumour markers were ascertained at the time of initial recruitment. The TGFβ1 levels investigated as part of this thesis were analysed from stored samples. Analysis of TGFβ1 was performed by one researcher on unselected samples, blinded to the available clinical endpoint data. Thus it is unlikely that any selection bias contributed to the results obtained. Only patients who were suitable for tumour biopsy were included in the initial study, but this is unlikely to have introduced any systematic selection bias.

Any patient who required surgery for any late radiation morbidity was classified as having grade 3 morbidity under WHO criteria. Morbidity scoring was performed retrospectively by means of the Franco-Italian Glossary. For most disease sites, late radiation morbidity scoring is anecdotal, or worse still, absent. Few trials comprehensively report late radiation morbidity. Cervical carcinoma is unusual in that there is an internationally recognised and validated late radiation morbidity reporting system - the Franco-Italian Glossary (Chassagne *et al*, 1993). However, the glossary is retrospective in nature and thus prone to the shortcomings this implies. These include incomplete recording or under-reporting of morbidities; reliance on interpretation of patient records by clinicians not directly involved

in the patient's care; and possible inter-clinician variation in the reporting of patient records. A single clinician, highly experienced in the field of gynaecological oncology, scored late radiation morbidities from every patient's record, using the Franco-Italian Glossary. This abolished any inter-clinician variation in late radiation morbidity recording. With these reservations, however, the morbidity data available, while not conforming to the "gold-standard" of prospectively collected data, reflects the experience of morbidity in this cohort.

In patients with cervical cancer, pre-treatment plasma TGFβ1 levels demonstrated a significant positive correlation with tumour stage. This is in keeping with other published data in cervical cancer patients (Ngan *et al*, 1996; Chopra *et al*, 1998). The correlation, although significant was relatively weak. It is widely acknowledged in cervical cancer that tumour stage does not take bulk of disease into account and therefore does not fully describe burden of disease. Excess plasma TGFβ1 circulating prior to treatment is thought to be directly related to tumour burden (Anscher *et al*, 1994). Were accurate volume of disease measurements available on these patients, for example ultrasound or magnetic resonance volume estimates, it is likely that pre-treatment plasma TGFβ1 levels would show a stronger correlation with burden of disease, rather than stage alone.

Circulating plasma TGFβ1 levels measured prior to commencement of treatment are derived from the tumour itself and directly relate to tumour burden. In patients with breast (Kong *et al*, 1995) or colorectal (Tsushima *et al*, 1996) carcinoma, plasma TGFβ1 levels can normalise by six weeks post-operatively in those undergoing curative resection. Most patients treated with radical radiotherapy for cervical cancer do not undergo initial

debulking surgery. Plasma TGFβ1 levels at the end of radiotherapy are therefore derived from both residual tumour burden and production of TGFβ1 by normal tissues in response to radiation. The work of Anscher *et al* (1993, 1998a) and Groen *et al* (1997) emphasises the clinical utility of the TGFβ ratio in predicting the severity of both acute and late radiation morbidities. The relationship between pre-treatment plasma TGFβ1 levels and stage may explain the observed lack of correlation with late radiation morbidity. This implies that were samples available post-treatment, i.e. when the contribution from disease burden is less, any correlation present might be unmasked. Unfortunately, no post-treatment samples were available from this cohort of patients.

This retrospective series confirmed that pre-treatment plasma TGFβ1 levels correlate with disease stage and tumour outcome. As the ELISA technique used would be easy to apply clinically, these findings should be confirmed by a prospective study. In addition, any prospective study should measure TGFβ1 levels at the end of treatment. This would allow correlation between the TGFβ ratio and the development of late radiation toxicity. This study has now been established at the Paterson Institute.

Healthy volunteers

Plasma samples were obtained from sixty-six volunteers in order to investigate the reproducibility of using a commercial kit to measure TGFβ1 and to provide a normal control range. These samples showed an unexpected age-related rise in the level of plasma TGFβ1 in the normal population. This was mirrored in the patient population, taking into

account those samples with grossly elevated levels. The control group in this thesis was larger and had a wider age range than is generally quoted in the literature. However, this finding should be confirmed by other groups. If this age-related rise in TGFβ1 levels is borne out by further studies, it may need to be controlled for in all patient populations studied (i.e. age-matched controls will be required). This is especially important in oncology, where a significant proportion of the patients are over 65 years of age.

Assay parameters

Significant differences were demonstrated between the patient and volunteer samples using ANOVA, despite a considerable overlap in their ranges. The intra-individual assay variability was high (42%) compared with inter-individual variability (77%). This implies that the day to day variation in plasma TGFβ1 levels is high and may limit the potential clinical utility of the assay. As such, it deserves further study. The intra-individual variability of the assay was ascertained by sampling a number of the volunteers on more than one occasion. Those volunteers were at the younger end of the age spectrum sampled and it may be that they have more variability in their plasma TGFβ1 levels. The volunteer group contained a number of people with chronic non-malignant disease (diabetes mellitus, psoriasis with arthropathy, systemic lupus erythematosus and hypothyroidism) and it may be that their TGFβ1 levels fluctuate with changes in their disease state/medication. Plasma TGFβ1 levels may be increased in those with surgical wounds. Although none of the volunteers had recent surgery, it is possible that they had minor skin breaches due to e.g. gardening that affected their plasma TGFβ1 levels. Other possible confounding factors include viral illness (there is some evidence that TGFβ1 can behave like an acute phase

protein) and the menstrual cycle. The former is likely to affect both the volunteer and patient cohorts equally. As for the latter, the majority of patients were male and only four female patients were under the age of 55 years, thus this is a potential confounding factor. In short, there needs to be more investigation into variations of plasma TGFβ1 in the normal population.

Head and neck carcinoma

In a prospective series of patients with carcinoma of the head and neck region, plasma TGFβ1 levels were measured at the start and in the final week of treatment. Initially, only those patients whose follow-up would be at the Christie Hospital itself were recruited. However, when it was realised that accrual was lower than anticipated, recruitment was widened to include those patients whose follow-up would be at other centres. All patients were treated with radical intent at the Christie Hospital by one of two dedicated site-specialised radiotherapists. Thus, initially these patients were subjected to a degree of geographical selection, within the north west of England. It is possible differing socio-economic factors between these two patient groups gave rise to a bias in the results obtained. However, the number of patients subject to geographic selection as a proportion of the total patient population studied is relatively small and unlikely therefore to have a major influence on the overall results obtained.

Pre-treatment plasma TGFβ1 levels were significantly positively correlated with increasing tumour (T) stage. This lends support to their role as a marker of tumour burden, which is in

agreement with the published literature and findings in this thesis related to cervical cancer (see Section 3.3).

Radiation morbidity reporting

In published series, reporting of acute and late radiation morbidities is both patchy (Nori *et al*, 1993) and anecdotal (Sakamaki *et al*, 1993). Until recently, most published data were concerned with rates of local control rather than morbidity. With increased clinician and patient awareness and education, many trials now incorporate quality of life measures (Ringash *et al*, 2000). These include the EORTC QoL questionnaire (Bjordal *et al*, 1994) and the Rotterdam symptom checklist (de Haes *et al*, 1990), both of which have been validated in prospective trials. However, reporting of specific acute and late radiation morbidities is still suboptimal. There are a number of reasons for this. Reporting of acute effects is often graded according to WHO criteria and thus tends to be more systematic. However, these criteria were designed specifically with chemotherapy toxicities in mind and are often not flexible enough or are inappropriate for the toxicities experienced by patients undergoing radical radiotherapy. With late radiation morbidities the situation is worse. Late side effects are highly dependent on the tumour site treated. Although they are easy to categorise, they are often difficult to grade either accurately (e.g. degree of fibrosis) or objectively (e.g. dryness of mouth). Usually they are defined in terms of functional impairment (e.g. tooth loss) or medical interventions (e.g. surgical debridement required). The aetiology of late radiation morbidity is complex, poorly understood and often multi-factorial. For example, diarrhoea following pelvic radiotherapy may be due to bile salt malabsorption through a damaged terminal ileum or due to a sub-clinical stricture in the

large intestine. When available, records of late radiation morbidity tend to be descriptive rather than structured with a reproducible grading schema (Liao *et al*, 2000). There is little or no international cooperation and thus promising locally derived schema are often never validated on sufficient patient numbers for them to be adopted with any confidence. Some disease sites e.g. cervix have attempted, with some success, to produce relevant uniform scoring systems. These are often very disease specific and therefore not easily adapted for use in other tumour types.

A formal attempt was made to unify the reporting of late radiation morbidity in 1996 with the publication of the LENT SOMA scales (Rubin *et al*, 1995a; Rubin *et al*, 1995b; Pavy *et al*, 1995). These were published simultaneously in the international journals of the European and American therapeutic radiation oncology societies. LENT SOMA stands for late effects in normal tissues - subjective, objective, management and analytic. They were designed to be relevant to the late toxicities experienced by those undergoing radiotherapy to any site. All possible radiation-induced toxicities in all sites were described. The patient's view of their quality of life was measured in the subjective category, allowing patients to grade their own experience of morbidity. Functional impairment was categorised in a systematic manner allowing objective measure of morbidity. Medical and surgical interventions required by the patient can be documented in the management scales. This allows sequential assessment of patients and thus demonstrates objectively the progressive nature of some late radiation toxicities. In the analytic scales, relevant raw data e.g. body weight is recorded to compare with the subjective and objective data. Results of investigations e.g. haemoglobin levels are also recorded. Each area of the scales stands alone. Therefore centres which are relatively materiel poor e.g. in terms of scanning

equipment and have little on the analytic scales can be compared like for like with better resourced centres on the subjective and objective scales giving the widest available data to those investigating late radiation morbidity.

Adoption of these scales into widespread clinical practice has been slow and erratic. This is partly due to the scales being large and often unwieldy to use alone and the need to develop a scoring system based on the scales. It also reflects the lack of validation available for the scales. Validation depends upon either formal comparison with current established scales or independent validation using a semi-structured questionnaire approach. Given that few disease sites have a validated late radiation morbidity scoring system with which the LENT SOMA can be compared and that independent validation is complex and expensive, this hurdle may take a long time to overcome.

In this thesis, treatment morbidity was recorded using a LENT SOMA questionnaire. This was derived from the head and neck LENT SOMA scales. It has not been validated as there is no universally accepted head and neck morbidity scoring system available for comparison. Every patient answered questions pertaining to possible morbidity at all treatment sites. Thus, it is unlikely that any morbidity was missed entirely. The questionnaires were administered prior to treatment, in the final week of treatment, at the first follow-up visit and six monthly thereafter. As a result, sequential assessments of each patient were available using the same tool. This allowed accurate comparison of patients' symptoms with a pre-treatment baseline. There remains the possibility of a systematic bias in the data collected. Correlation of the data collected with an independently validated scoring system for these tumour sites would go a long way to disprove this, however, as

there is no such scoring system for these sites, then it is unlikely that this will ever be fully resolved. The questionnaires were administered by one of two dedicated research sisters. Their inter-individual variability in administering the questionnaires had been assessed independently and found to be low. This reduces the risk of a systematic bias in these data.

For patients with head and neck carcinoma, only acute radiation toxicities were available for analysis, during the time of this thesis. All but one of the categories was subjective in nature. The only objective category was concerned with patients' use of analgesia in the preceding week. As this was self-reported by patients and not validated independently it may also be inaccurate. Each patient will be examined clinically annually from one year post-treatment by a clinician specialising in treatment of head and neck carcinoma. Thus objective long-term data concerning late radiation morbidity will be available in due course from the cohort of patients studied.

Prediction of acute radiation toxicity

The LENT SOMA questionnaire was administered prior to commencing treatment, in the final week of treatment and at the first follow-up visit, scheduled for six weeks following the completion of treatment. At the end of a three-week schedule of radical radiotherapy, most patients developed some acute radiation toxicity. This may intensify over the following week or so and then gradually resolve, with most patients showing a major improvement by six weeks post-treatment. Some patients suffered an intense and/or prolonged acute reaction. The LENT SOMA scores obtained in this series support these observations. The median LENT SOMA score at the end of treatment (26) was more than

double that obtained prior to treatment (11) or at first follow-up (13). The fall in median LENT SOMA score at first follow-up implies that most patients suffered fewer symptoms at that time than at the end of treatment. The range of LENT SOMA scores obtained at first follow-up (0 - 47) was wider than that obtained either prior to treatment (0 - 36) or at the end of treatment (4 -39). The high pre-treatment scores represent both tumour and patient related symptoms, e.g. breathlessness, which may relate to other pre-morbid conditions. Tumour related symptoms comprise symptoms related to the continuing presence of the tumour itself (for patients treated with primary radiotherapy) and post-surgical morbidity (for patients treated with primary surgery). Post-surgical morbidity can relate to the tumour site itself or the area of donor tissue when the surgery has incorporated reconstruction or skin grafting. Inquiries about analgesia use in the LENT SOMA questionnaire did not specify tumour-related pain. However, the highest *individual* values of LENT SOMA scores were obtained at first follow-up. The low median LENT SOMA score at first follow-up (13) when compared to the range of LENT SOMA scores (4 - 39) implies that although the bulk of the patients have a LENT SOMA score below the median end of treatment score (26), a number have a LENT SOMA score that is elevated above this. This implies that some patients are suffering a prolonged acute reaction. For the purposes of this thesis a severe prolonged acute reaction was defined as a LENT SOMA score at first follow-up in the upper quartile.

TGFβ ratio

For patients with carcinoma of the head and neck region, all those who had an initially elevated TGFβ1 level that was reduced, but not normalised by the end of treatment had a

severe prolonged acute reaction. The total number of patients in this category was four from a possible total of 54. The sensitivity and specificity of this pattern of TGFβ1 levels in predicting a persistent acute reaction was 33% and 100%, respectively. The value for sensitivity is rather lower than would be expected in a diagnostic test. However, as these figures depend on only four samples it is likely that there is a wide confidence interval surrounding the calculated specificity, meaning that the actual specificity of a category two ratio may be much higher. In support of this, the positive predictive value of a category two result was 100%, implying the actual specificity of the test is higher than it appears. However, as the absolute number of patients was small, caution should prevail in applying these results to other patient groups without further confirmatory studies. If the data are accurate, it may be possible to accurately predict those patients who will develop a severe reaction, but only towards the end of their treatment. Options for alteration of the treatment schedule are therefore limited. Indeed, most clinicians would not compromise a potentially curative treatment for the sake of symptoms that, although severe, are self-limiting. However, it would allow extra support, in the form of clinical nurse specialists or enteral feeding to be accurately targeted at those who are most likely to need support. Also, a persistent acute reaction can lead to a prolonged period of mucosal damage which can promote the development of consequential late damage. Targeting clinical nurse specialist support to these patients may potentially decrease the incidence of late morbidity. Late radiation toxicity is more important in terms of functional impairment and quality of life. Data on these patients will continue to be accrued over time and the relationship between late toxicity and TGFβ1 levels will be examined in the future.

The pathophysiological effects of TGFβ1 *in vivo* are varied (see Table 1.2). Activated TGFβ1 promotes an increased production and decreased breakdown of the ECM and acts as an indirect promoter of angiogenesis. TGFβ1 activation in the ECM is increased immediately following irradiation. Increased activated TGFβ1 persists in irradiated tissues for many months at least. Late morbidity, in the form of post-radiation fibrosis, has a latent period of at least six months and often years before it becomes clinically apparent. Thus, while it seems highly likely that excess, activated TGFβ1 promotes the development of post-radiation fibrosis, it does not produce this in isolation. During the latent period, other micro-environmental factors influence the expression of post-radiation damage. The latent period is variable in duration. Thus late events can increase cumulatively year on year and patient cohorts examined at different time points may differ in their incidence of late radiation toxicity. To demonstrate an existing positive correlation between late radiation toxicity and plasma TGFβ1 levels, it may be necessary to ascertain patient morbidities at more than one time point during their follow-up, as the experience of morbidity in the cohort will not be static. This is an important fact to bear in mind when analysing long-term follow-up data.

Breast carcinoma

Between 1993 and 1994, one hundred and ninety unselected patients with early breast cancer were entered into a prospective study of the relationship between biological factors and acute radiation reaction (Scott *et al*, 1998). These patients were uniformly treated with the then current Christie Hospital technique. These patients had a clinical photograph taken post-surgery, but prior to the commencement of radiotherapy. Residual DNA damage and

TGFβ1 levels were examined in a cohort of 50 patients drawn randomly from the initial study population of 190 (the validation cohort). The training cohort had demonstrated a positive correlation between residual DNA damage and late radiation morbidity in 39 patients. Results from the fifty patients in the validation cohort, would have the same power to detect the same strength of correlation. It would also allow for possible failures in cell culture or PFGE techniques. The researchers were blinded as to the acute and late radiation toxicities experienced by all patients prior to recruitment. However, some selection bias was inevitably introduced when entering patients into this study, most obviously in that all patients were alive and free of disease at the time of entry (this would also have applied to the training cohort). Accurate figures on the precise number of patients with relapsed disease are not available for this group of patients. However, from memory, somewhere between ten and thirty of the patients had documented disease relapse, which is in keeping with expected survival figures for this group of patients. Other more subtle factors are also likely to introduce some selection bias. The most obvious example of this was seen in patients refusing to enter the retrospective study. Once patients had been recruited, their previously documented acute reactions were examined. The vast majority of patients who had experienced a severe acute radiation reaction and who had been approached, had declined entry to the retrospective study. It is likely those patients recruited to the training cohort also had a similar selection bias, although no objective data are available to support this theory. This implies consequential late radiation morbidities e.g. necrosis secondary to severe acute reactions are likely to have been under-represented. Thus the distribution of acute toxicities seen in the retrospective series was not the same as in the original series (again this may be true of the training cohort).

Only thirty-seven pre-treatment TGFβ1 samples were available for analysis. No relationship was demonstrable between TGFβ1 levels and any clinical endpoint. This is not in keeping with findings published by Li *et al* (1999). The most likely reason for this is the small number of samples studied. However, there are likely to be other factors influencing this result. All retrospective studies have inherent flaws, including selection bias, and this could contribute to the lack of positive correlation seen. This study was performed using ELISA and a commercially available kit that included mass-produced antibodies. In a positive published study (Li *et al*, 1999), also using ELISA, the antibodies were produced by the investigators themselves and the assay involved enhanced chemoluminescence. The antibodies used by Li *et al* also measured a different fraction of TGFβ1 in the plasma. It is possible, therefore, that the different assays used contributed to the conflicting findings. The samples analysed as part of this thesis were 4 or 5 years old. Although they had been stored at -80°C, the TGFβ1 may have been subject to degradation over time. However, as degradation is likely to have been uniform in all samples, this is unlikely to explain the lack of correlation seen.

Fibrosis was assessed clinically using palpation by one clinician in all patients. Clinical photographs were scored for telangiectasia and retraction/atrophy independently by three experienced clinicians. There was a high level of agreement between the clinicians in all categories scored. Development of telangiectasia is unlikely to be directly influenced by TGFβ1, thus it is not surprising that no correlation was found. Both palpable fibrosis and atrophy/retraction, i.e. visible fibrosis were expected to show some correlation either alone or in combination with TGFβ1 levels. It is possible that, although assessment of these end-points was accurate (according to agreed criteria) and consistent, the end-points do not

properly represent late radiation toxicity mediated by fibrosis and hence TGFβ1. Changes in the breast following treatment are complex and multifactorial in aetiology. Vascular disruption can occur secondary to both surgery and radiotherapy. This can lead to loss of specialised breast tissue and consequent fibrosis. Radiation damage to epithelial cells can lead to involution and atrophy of specialised breast tissue. Breasts consist of predominantly adipose tissue. Fat necrosis can occur leading to further shrinkage of the breast, but without palpable fibrotic changes. Most of the patients in this study were treated with tamoxifen. This drug has a predominantly anti-oestrogenic effect in the breast. This can lead to further atrophy, possibly accompanied by fibrotic changes. It may be that simple clinical observation and palpation are not sensitive enough to accurately measure late radiation fibrosis. This is a recurring theme of the published literature in this area: clinical end-points are poorly defined; they are often subjective, depending on the experience and exposure of the clinicians who assess them; and the aetiology of a given end-point with respect to late radiation toxicity has never been validated. In the series of breast cancer patients studied in this thesis all categories of late radiation reaction were represented in reasonable proportions. Therefore, under-representation of any given late toxicity is unlikely to be responsible for the lack of correlation seen. Nevertheless, in view of the small number of samples and the retrospective nature of the study it may be worthwhile investigating further the relationship between plasma TGFβ1 levels and radiation toxicity in patients with carcinoma of the breast in a prospective study.

As part of the LENT SOMA questionnaire, patients were asked about the presence or absence of an acute reaction at the time of their treatment. The patients were not asked to grade this reaction, merely if it had been present or absent. Patient recall is notoriously poor and unreliable (Hinton, 1996; Bruera *et al*, 1999). Patients were not asked for the definition of what an acute reaction meant to them. It may be that they only reported a reaction occurring during radiotherapy or a reaction that was greater than expected. With a three-week course of radiotherapy, the acute reaction can intensify beyond the end of treatment and it may be that patients did not associate this intensification with the treatment itself, rather with their own actions. If patients are fully consented for radiotherapy and informed of all the potential side effects, they might deny having a reaction if they experience only what they expect. Patients often discuss treatment and toxicities with each other during their radiotherapy. Patients may rate the presence or absence of an acute reaction in relation to other people's experiences. If the patients were hesitant about answering the question, or requested a definition, they were supplied with the options of breast tenderness and/or pinkness of the skin. Not all patients were prompted in this way and it may be that this influenced the responses obtained. A more accurate representation of acute radiation toxicity might be obtained by showing clinical photographs or giving a verbal description of varying acute radiation reactions and asking whether the patient could remember a similar reaction. Extent and intensity of acute radiation reaction can predict the development and progression of telangiectasia. It can also indicate those at increased risk of consequential late morbidities. There was no positive correlation found between telangiectasia score and the presence of an acute reaction. This

highlights the deficiencies of retrospective morbidity scoring. However, as the incidence of telangiectasia in the validation cohort was low, it may be that the low number of events was also partly responsible for the observed lack of correlation.

The total LENT SOMA score for the patients was derived from subjective criteria (breast pain intensity, analgesia requirement); objective criteria (retraction, breast oedema, skin ulceration, telangiectasia, fibrosis); and management criteria (retraction, breast oedema and skin ulceration management). Thus it incorporated most recognised late radiation complications in the breast. A correlation was sought between the LENT SOMA score and the FDR(150). This is a measure of fibroblast intrinsic radiosensitivity. It is likely that fibroblast radiosensitivity is unrelated to a number of the factors studied e.g. breast oedema. Thus a correlation was specifically sought between FDR(150) and fibrosis score.

Correlation between residual DNA damage and late radiation endpoints

The residual DNA damage, in normal fibroblasts, as measured by PFGE did not correlate with clinical endpoints in the form of post-radiotherapy fibrosis. This did not support the findings of Kiltie *et al* (1999) which was performed in the same institution using identical equipment. There are likely to be a number of reasons for this. First, the two patient cohorts were studied at different times following radiotherapy. The training cohort was studied at 10 - 14 years post-treatment and the validation cohort at 3 - 4 years post-treatment. While there is some evidence that fibrosis may continue to progress over time (Bentzen *et al*, 1990), this is poorly documented, but it may account for the results seen. Second, inter-researcher variation may have played a part. Although retraction and telangiectasia were

scored from photographs by the same three clinicians, the fibrosis scores were derived from a different researcher for each cohort. Both researchers were clinicians with a similar degree of experience in clinical oncology and late radiation toxicity. However, no formal assessment of their inter-individual variation was undertaken. Third, changes in treatment technique at the Christie Hospital over this period meant that the spread of late radiation toxicities observed was less in the validation cohort. This narrow range of toxicities may have obscured a weak correlation. More importantly, however, all degrees of late radiation toxicity were represented in the validation cohort, in reasonable proportions, making it less likely that a weak correlation was lost due to the experience of toxicity in the patients' sampled. Finally, an important reason for the lack of a significant correlation is the fact that residual DNA damage in normal fibroblasts is not a robust clinical predictor of late radiation fibrosis.

In contrast, prospectively measured plasma TGFβ1 levels show promise in a number of situations. They are positively correlated with tumour burden and hence stage in cervical carcinoma (retrospectively) and head and neck carcinoma (prospectively). In cervical carcinoma, they are positively correlated with survival. This implies they may be of use as a prognostic factor. Prospective measurements of plasma TGFβ1 levels show promise in predicting acute radiation toxicity in patients with carcinoma of the head and neck. This merits further investigation in other tumour sites, especially where radiation is the main treatment modality. Acute radiation toxicity can lead to significant, albeit mostly self-limiting, morbidity. The ability to accurately predict those most at risk of acute radiation toxicity would allow targeting of limited support resources at those people with the highest risk of consequential late radiation morbidities. If a relationship between plasma TGFβ1

levels and late radiation morbidity is demonstrated, then the ability to target trials of post-radiation modifiers to those most likely to benefit is realised. In conclusion, plasma TGFβ1 levels show promise in the field of predictive testing.

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APPENDICES

Appendix 1: Patient Information Sheet for Breast Carcinoma Study

DNA DOUBLE STRAND BREAKS AS A MEASURE OF INTRINSIC RADIOSENSITIVITY.

We would like to invite you to take part in a study which involves taking a small sample of skin and blood from patients treated by radiotherapy.

We know that when patients are given radiotherapy treatment, some are more likely to experience side effects from the treatment than others. We are developing two tests in the laboratory, one of which is carried out on blood samples from patients who have had radiotherapy. The other test is carried out on small samples of skin taken from the thighs of patients who have had radiotherapy in the past. It is hoped that this may identify patients who are more sensitive to the radiotherapy. Taking the sample will not affect any treatment you may be on. Any side effects you had during or after your treatment will be compared with the results of our tests in order to find out whether they could be used in the future to predict which patients are likely to experience side effects from their treatment.

You will be given local anaesthetic, which will numb the skin, then a tiny sample of skin will be taken from your upper thigh. You will not need any stitches. A swab will be put on, and this should be left for a week before being removed.

You are free to refuse to take part for any reason. This will not affect your relationship with your doctor.

Patient Name..... Hospital Number.....

Contact name: Dr. Jeanette Dickson
Section of Genome Damage and Repair
Paterson Institute for Cancer Research
Christie Hospital NHS Trust
Wilmslow Road
Manchester M20 9BX

Contact telephone number: 0161 446 3528 (direct line)

Appendix 2: Patient Information Sheet for Head and Neck Carcinoma Study

TRANSFORMING GROWTH FACTOR BETA MEASUREMENTS AS A PREDICTOR OF RESPONSE TO RADIOTHERAPY

We would like to invite you to take part in our study.

Some people are more sensitive to radiotherapy treatment than others. At present we cannot tell these people apart. We are hoping to develop a blood test that will allow us to do this.

We want to take two blood samples, one before you start treatment, and one in the final week of treatment. The test will be performed on these samples, and the result compared with any effects which you experience during treatment.

You are completely free to refuse to take part in this study. It will not affect your treatment in any way.

Patient Name:.....Hospital Number.....

Contact Name: Dr Jeanette Dickson
Section of Genome Damage and Repair
Paterson Institute for Cancer Research
Christie Hospital NHS Trust
Wilmslow Road
Manchester M20 9BX

Contact Telephone Number: 0161 446 3528 (direct line)

Appendix 3: Pro forma for scoring clinical photographs

Breast Photograph Assessment Sheet

Investigators initials			
SP number			
Patients initials			

Hospital number							

Retraction/Atrophy: Ring the number that gives the most accurate representation of breast shrinkage

Degree of retraction or atrophy	nil	0
	10 – 25%	1
	25 – 40%	2
	40 – 75%	3
	100%	4

Telangiectasia in the whole breast: Ring the number that describes the average amount of telangiectasia in the whole breast

Telangiectasia in the rest of the breast	none	0
	minimal	1
	moderate	2
	severe	3

Body build and breast size: Ring the numbers which best describe the general body build and breast size of the patient

General body build	slight	1
	average	2
	heavy	3

Breast size	small	1
	medium	2
	large	3