IMPROVING THE CURRENT MANAGEMENT OF LIMB MALIGNANT MELANOMA

by

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Declaration

I declare that the preparation of this thesis has been composed by myself. The research described in this thesis was performed by myself except where the help of others has been specifically acknowledged

S M K Lingam

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STATEMENT OF COLLABORATION

Since the unit in Gartnavel General Hospital was set up in 1984 offering isolated limb perfusion treatment for limb malignant melanoma, there have been two research fellows appointed prior to the period of my research from August 1991-July 1993. The two fellows have been Mr R. N. Scott FRCS (1986-1988) and Mr D. S. Byrne FRCS (1988-1990).

The results of ILP presented in chapter 3 includes all the ILP treatments carried out until July 1993 and this includes those carried out during the time of the two previous research fellows. The ILP treatments during my research period had been carried out by myself under the guidance of Mr A. McKay or as first assistant to Mr A. McKay. The analysis of the results presented in chapter 3 has been carried out entirely by myself and this data has not been generated or used by the previous fellows.

All the other treatments carried out in this thesis have been carried out personally by myself and where appropriate under the supervision and guidance of Mr A. McKay. All the animal experiments were carried out with a licence from the Home Office, observing the strict regulations as laid down by the Home Office.

The histological staining of the lymph node described in chapter 6 was carried out by Professor R. M. MacKie and the pathologists from The University Department of Pathology at the Western Infirmary, Glasgow.

SUMMARY

Surgery remains the cornerstone in the treatment of malignant melanoma. The surgical treatment of primary and recurrent limb malignant melanoma ranges from wide local excision of the primary melanoma to elective and therapeutic lymph node dissection. The delivery of a chemotherapeutic agent via an isolated limb perfusion circuit has been available since 1957, yet its exact role remains to be defined.

The theme of this thesis is "how can the current management of limb malignant melanoma be improved?"

The work in this thesis was carried out in the Vascular Unit, at Gartnavel General Hospital, Glasgow and in the Animal and Scientific Laboratories of Glasgow University. In 1984 isolated limb perfusion (ILP) using melphalan was made available in Glasgow for the treatment of primary and recurrent limb malignant melanoma and since then over 250 adjuvant and therapeutic ILPs have been performed.

My analysis of the ILP results in the Glasgow unit showed that in the treatment of primary melanoma, ILP confers an improved disease free interval and possibly a small survival advantage. In the treatment of recurrent limb melanoma, therapeutic ILP is effective in the control of local and regional recurrence. I have analysed the results in specific subgroups of patients, i.e. when patients with subungual malignant melanoma undergoing ILP were matched (for age, site and tumour thickness) with patients treated with amputation only, no survival advantage was demonstrated in the perfused patients. Having analysed these results, I then addressed the issue of how the theoretically attractive concept of ILP could be improved and its role convincingly established.

The components of my study are summarised below:

1) Improving the ILP circuit by:
a) Introducing pulsatility to assess if it may be physiologically superior.
b) Avoiding the compartment syndrome.
c) Using a vasodilator to increase transcutaneous oxygen.
2) Assessing laser ablation as an alternative therapy for recurrent melanoma.
3) Accurate staging of malignant melanoma using lymphatic mapping.
4) Targeting radiotherapy in an animal model.

1) Improving the ILP circuit

Isolated limb perfusion was developed to maximise drug delivery to the target tissue while minimising systemic toxicity. It has been shown that an increase in the skin oxygen tension in the isolated circuit can potentiate the action of the chemotherapeutic agent and may have a selective tumouricidal action.

It was surprising to discover that since the introduction of ILP over 30 years ago, there have been virtually no changes in the basic circuit. Performing clinical studies on ILP patients is a difficult problem, since even in a regional centre, only 30-40 patients each year will be treated by ILP and not all these patients are appropriate for study. Nonetheless, I was determined to attempt to address several basic issues previously ignored:

Pulsatility

1(a) One obvious feature of the ILP circuit that is not physiological is the delivery of the perfusate in a non pulsatile manner via a simple roller pump although pulsatility is an obvious feature of blood flow in arteries. A non randomised study was conducted to investigate if introducing pulsatility into the circuit would confer physiological advantages.

Twenty-two patients were studied. Pulsatility was introduced using a Stockert Frequency Control Module and blood flow was synchronised to the electrocardiogram of the patient. Twenty-two patients matched for sex, age, site and level of perfusion using the standard continuous flow circuit were used as controls. Using pulsatile flow, the time taken to reach physiological skin oxygen saturation was reduced. There was less fluctuation in levels of oxygen tension and there was less fluid shift in the circuit compared to the non pulsatile group. However, there was no significant difference in the maximum oxygen tension achieved between the pulsatile and non pulsatile group. Introduction of pulsatility into the ILP circuit is a simple and safe modification that appears to offer physiological advantages over non pulsatile blood flow.

Compartment pressure

1(b) Treatment by ILP can predispose to the development of a compartment syndrome in the limb and some investigators thus perform routine prophylactic fasciotomy after ILP. A non randomised study was conducted to determine if there was a significant increase in the compartment pressures during ILP and whether the nature of blood flow in the circuit effected these pressures.

Sixteen patients were studied. Eight patients had non pulsatile ILP whilst 8 had pulsatile ILP. The rate of rise of the compartment pressures in the non pulsatile group was significantly greater than in the pulsatile group ($p < 0.001$). The time taken for the tissue oxygen levels to rise to physiological values was significantly lower in the pulsatile group than in the non pulsatile group ($p = 0.002$). These results suggest that the routinely used mode of non pulsatile blood flow in the ILP circuit has the significant disadvantage of greater rate of rise of compartment pressure when compared to pulsatile blood flow. Since the introduction of pulsatility in the operation of the ILP circuit, neither routine prophylactic nor therapeutic fasciotomy has been needed.

Role of Vasodilator

1c) Several reports have shown that vasoactive drugs can significantly affect the nature of blood flow in both experimental tumours in rodents and human tumours. A study was set up to assess the efficacy of vasodilator agents to improve oxygenation in the ILP circuit and potentiate the effects of melphalan. The study was conducted using verapamil hydrochloride in twenty-one patients undergoing ILP. Results of this study showed that this agent produced a significant fall in the peripheral arterial pressure. As the pressures fell, there was a proportional fall in the transcutaneous oxygen tension. Despite the theoretical advantage of the use of vasodilators in chemotherapy, the present study showed that the use of verapamil hydrochloride offers no measurable advantage in the ILP circuit.

2) Assessing laser ablation as an alternative therapy for recurrent melanoma

In Glasgow, ILP is the treatment of choice for the control of recurrent limb malignant melanoma. However not all patients are suitable for this treatment. When cutaneous metastases from malignant melanoma are untreated, they inevitably enlarge in size, ulcerate and become painful. A study was carried out to assess whether a carbon dioxide laser can be used as an effective alternative to ILP in the control of cutaneous metastases. Fourteen patients who were unsuitable for ILP or had failed ILP were recruited into this study. Results showed that in patients with non nodal regional recurrence, when ILP had failed or was unsuitable, carbon dioxide laser therapy is a safe and effective alternative treatment in the palliation of cutaneous recurrence from malignant melanoma.

3) Accurate staging of malignant melanoma using lymphatic mapping

The prognosis of malignant melanoma is ultimately related to the stage of the disease. If staging is incorrect, the patient may not receive appropriate treatment. Controversy surrounds the role of elective of elective lymphadenectomy. If a technique could be developed to identify patients with occult nodal disease, these patients may be selected for elective lymphadenectomy. Intraoperative lymphatic mapping is such a technique which has been developed on the assumption that metastases embolise via lymphatic channels to regional nodes.

Although the technique was reported in 1992, no other workers had been able to replicate the original results. I therefore studied the method in a group of 30 patients. Sentinel nodes were identified in all 30 patients. Eight nodes contained micrometastases. Our evaluation of intraoperative lymphatic mapping shows the method to be promising. The technique is practical and easy to master. If 32% of patients with cutaneous malignant melanoma who are clinically stage 1 have nodal disease, this has great importance in the staging and treatment of disease and for all therapeutic trials in the future. This technique may have potential application in other tumours that metastasise via the lymphatics.

4) Targeting radiotherapy in animal model

The main difficulty for the clinician treating malignant melanoma is that early random metastases occur. If a melanoma specific compound could be labelled with an appropriate radioisotope, this compound could be used to scavenge and destroy melanoma cells beyond the limits of clinical detectability. Targeted radiotherapy is such a treatment that allows deposition of a high radiation dose in targeted tissue with little exposure to the surrounding structures. Methylene blue has been shown to have a high affinity for melanin. A study was designed to assess the efficacy of radioactive iodine labelled to methylene blue to target melanoma cells in nude mice.

Radioisotopes of iodine were injected into 3 groups of nude mice with B16/F10 mouse melanoma. Group A (controls) received tumour cells subcutaneously, Group B received a subcutaneous mixture of tumour cells and radioactive labelled methylene blue and Group C received tumour cells subcutaneously and on macroscopic evidence of tumour growth received intravenous radioactive labelled methylene blue into the tail vein.

Parallel experiments were performed on B16/F10 melanoma cells grown as plate cultures. In both the animal experiments and the cell cultures, the effects of different isotopes were compared. Tumour growth was delayed in the treated groups and the effect was maximal using iodine 125. Targeted radiotherapy using iodine 125 labelled methylene blue may now be clinically possible. The isolated circuit is the obvious vehicle for such a treatment modality.

The overall aim of this thesis was to find ways of improving the efficacy of ILP as a treatment for limb malignant melanoma. My studies have shown that:

1) Pulsatile flow is both logical, easy to produce and confers physiological advantages. The risk of a compartment syndrome in the perfused limb can be considerably reduced.
2) Local laser therapy is a safe and practical alternative for the palliation of local recurrence when ILP is not possible or has failed.
3) Accurate disease staging can be achieved using an intraoperative technique which may have important implications for future studies.
4) An animal model was successfully established showing that targeted radiotherapy is effective and that this mode of treatment may become a clinical possibility for patients with malignant melanoma.

CHAPTER 1

INTRODUCTION

1.1 HISTORY OF MALIGNANT MELANOMA

1.1.1 18TH AND 19TH CENTURY

John Hunter was the first to publish an account of melanoma in 1787 after removing a recurrent lesion from behind the angle of the jaw of a 35 year old man. He did not name the disease as such but in describing the lesion stated, 'part of it was white and part spongy, soft and black'. He called it "cancerous fungous excrescence." Hunter's original specimen in the Royal College of Surgeons of England was examined in 1968 and confirmed to be a melanoma, probably a secondary deposit from an unknown primary tumour[1].

It was Rene Laennec, in 1806 who is accredited with the first description of melanoma as a disease entity. In 1812 he actually used the word melanosis which he said was derived from the Greek word meaning "black"[2].

Fourteen years after Laennec's first description of melanotic growth the first melanoma was described in England by William Norris. It is interesting to note that he was the grandson of John Hunter. Norris, although a country practitioner so loved his profession that he would carry out a complete necropsy in private practice and ride 20 miles for a sight of a case of melanomic sarcoma[3]. His first patient was a 59 year old fair complexioned and light haired man who presented with a tumour midway between the umbilicus and pubis[4]. Norris excised this lesion only to find that it recurred six weeks later in the scar. He noted that the patient remained in good health until just before his death. Norris performed the post-mortem himself and his enthusiasm for pathology can be seen from this account below.

"When the abdomen, chest and cranium were thrown open, it was a most extraordinary phenomenon; thousands upon thousands of coal black spots of circular shape and various sizes were to be seen clearly dotting the shining mucous, serous and fibrous membranes of most vital organs: I should think the most dazzling sight ever beheld by the morbid anatomist. I shall never forget the pleasing thrill which came over me when I first beheld them. It would have puzzled the most powerful descriptive talents to have done full justice to such a novel and striking disease displayed so beautifully in the endless profusion everywhere[3]."

He later found out that his patient's father had also died of melanoma and commented, "he was not acquainted with any case affording so strong probability of the hereditary nature of the disease"[5].

In the 19th century, many other descriptions of primary melanoma were published. In 1834, David William[6] probably gave the first description of the horizontal and vertical growth phases of a superficial spreading melanoma and in 1837 Isaac Parrish[7] reported the first case of melanoma in America.

In 1838, Robert Carswell[8] in his "Illustrations of Elementary Forms of Disease" sub-divided melanoma into two groups; true melanosis and spurious melanosis, and described four modifications - punctiform, tuberiform, stratiform and liquiform. He was the first to coin the term melanoma. In 1851, the first case of surgical excision of a metastatic melanoma was published in the Lancet by Mr Ferguson[9], who removed a groin secondary from a 45 year old woman who two years previously had had a dark tumour excised from the mons veneris.

In 1853, Sir James Paget[10] presented a relatively large series of 45 patients with "melanoma cancer". In his series, there were 27 women and 18 men of whom 20 were between the ages of 20 and 60 years. The primary lesion was removed in 18 patients of whom only 5 were alive at two years. In his famous work "Lectures on Surgical Pathology[10]", he reported that in "spurious melanoma" there was blacking of various structures whose only common character is that they are not tumours. He emphasised that "melanomic cancers [were] ..... medullary cancers modified by the formation of black pigment in their elemental structures".

Oliver Pemberton[11] in 1858 presented a further 60 cases of melanoma, 33 men and 27 women, and described post-mortem appearances in 33 patients. He noted that melanotic cancer occurred frequently in or near a "congenital wart or mole". He also observed that "in colour melanosis has many shades. In its primary form it is almost always brownish. Later the brown shade assumes every intensity of black. Sometimes the first change is of a slate colour". Pemberton[11] also reported the first case of melanoma in a black person. The man was a 29 year old Madagascarian whose lesion was located on the side of his foot but who despite a below knee amputation died from disseminated disease. In 1857, Sir Jonathan Hutchison[12] described the first case of subungual melanoma. He referred to it as a "melanotic whitlow" because it resembled a whitlow[13].

Towards the end of the 19th century more cases of melanoma were being reported including an interesting article by Tennent[14] in the Glasgow Medical Journal. He comments, in a patient with advanced melanoma, that the urine had a greenish black tint and felt this was probably the result of absorption of melanin, as at autopsy no tumour was found in the urinary tract. He concluded, "the mode of origin, development and progress of such a case of melanotic sarcoma strongly indicated the propriety of the more frequent and prompt complete removal of moles or pigmented spots by the hand of the surgeon[14]".

Joseph Coats[15] echoed Tennent's findings at a presentation to the Pathological and Clinical Society of Glasgow where he stated, "outside the obvious boundaries of tumour there are individual cells planted ..... which are capable of multiplying and reproducing tumour tissue ..... when the surgeon removes a sarcoma, therefore he may leave behind many cells ..... capable of forming tumour and so causing recurrence. The operation should be so executed as to remove the tissue some distance outside the apparent limits of the growth".

By the end of the 19th century, wide surgical excision of primary melanoma was well established although controversies on lymph node dissection were being debated. Herbert Snow[16], in his lecture on melanotic cancerous diseases at the Cancer Hospital in 1892, emphasised that "in the surgical treatment of melanoma, the skin tumour is in the majority of cases very small but there is a tendency to rapidly infect the nearest lymph glands. Palpable enlargement of these glands is unfortunately but a late symptom of deposit therein ..... we thus see the utter futility of operative measures which are addressed to the primary lesion only ..... we see the paramount importance of securing whenever possible, the perfect eradication of these lymph glands which will necessarily be first infected and before enlargement takes place radical removal of such organs before they have undergone appreciable increase in bulk, is a safe and easy measure which under the condition indicated should never be neglected".

1.1.2 20TH CENTURY

From the accurate and careful observations made by the 19th century pioneers, it was possible for William Sampson Handley[3], after only studying a single autopsy examination of a patient with melanoma, to present "The Pathology of Melanotic Growth in Relation to Their Operative Treatment", at the Hunterian lectures. He concluded "that the process of dissemination is initiated by the access of malignant cells to the fine lymphatics, followed by the centrifugal spread of permeation along the main lymphatic plexus into which the primary growth pours its lymph and by

secondary permeation of small tributaries of this plexus ..... meanwhile invasion of the bloodstream takes place either by local infiltration of veins from comitant permeated lymphatics or malignant cells carried into the blood along the thoracic duct from invaded lymphatic glands ..... mesoblastic cells of melanotic sarcoma are able to thrive when lodged in a blood vessel. Thus, in later stages of melanotic sarcoma, the slow process of lymphatic permeation usually recedes into insignificance and the patient dies with almost universal deposits resulting from blood embolism". He felt able to advocate wide local excision of the primary lesion, regional lymph node dissection and amputation in selected cases of melanoma. This was to remain the mainstay of treatment for the next fifty years until the question of the effectiveness of lymphadenectomy arose.

Although many advances have been made in various aspects of melanoma, we owe a lot to the early pioneers such as Norris, Paget, Pemberton and Handley who through their dedication, made accurate and detailed observations of the disease and thus laid the foundation upon which advances were possible. Many of their observations have stood the test of time and are still pertinent in the modern day treatment and prevention of melanoma.

1.2 EPIDEMIOLOGICAL FEATURES OF MALIGNANT MELANOMA

1.2.1 INCIDENCE

The world-wide incidence of malignant melanoma is increasing and this upward trend has been sustained over several decades. The first increase was noted in Connecticut, United States in the 1930's and later in Denmark in the 1940's and is now evident world-wide[17]. In the United Kingdom, this increasing incidence is particularly notable in women. Women have twice the incidence of melanoma than men in England, Scotland and Wales and three times the incidence in Ireland[18]. In 1979, there were 2,095 new cases of melanoma in the UK. In 1984 this had increased to 2,624, an increase of 25%. Figure 1 shows changes in the number of patients registered by the Scottish Melanoma Group between the years 1979 and 1986.

These figures depict a rise of 75% over seven years equivalent to 10.5% per year and similar increases are reported in Scandinavia and Australia. Although it has been estimated that approximately 70,000 new cases occur world-wide each year[19], the overall world incidence remains very low due to the small numbers occurring in Asia where over half the world population lives. In the caucasian population, malignant melanoma is now an important cause of death[20].

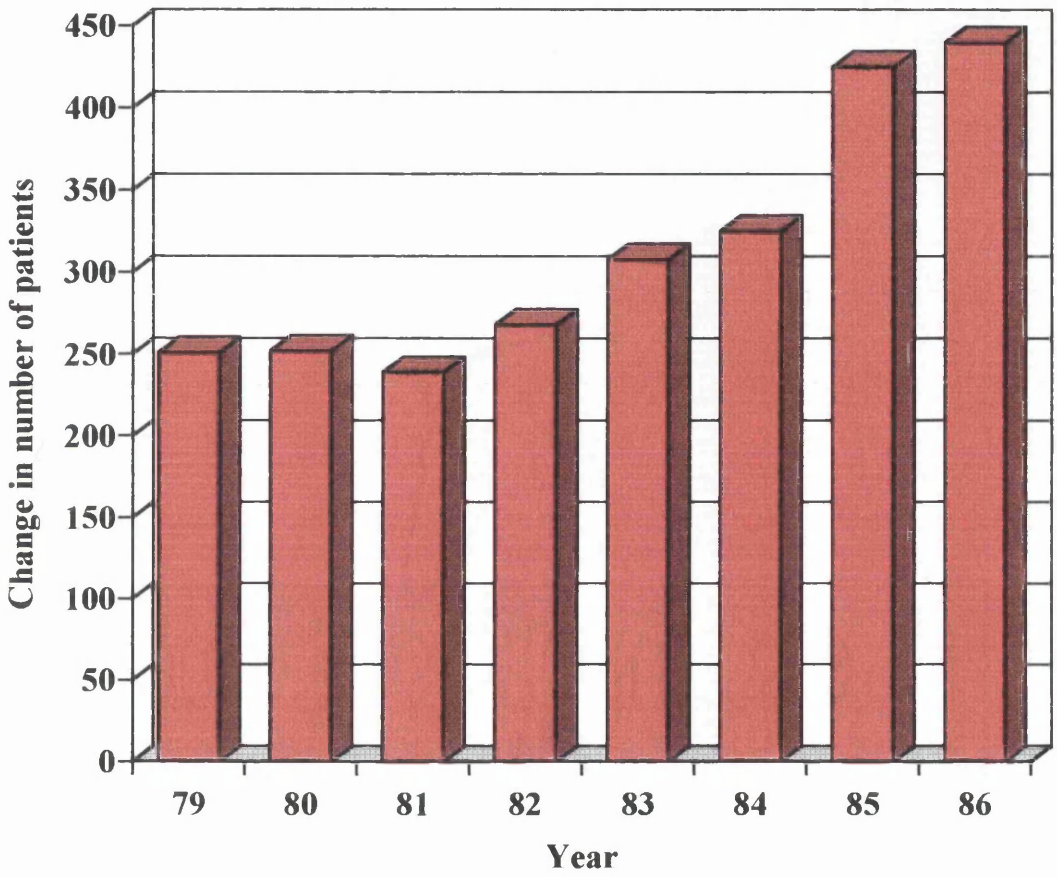


Figure1 The incidence of cutaneous malignant melanoma in Scotland (1979-1986)
(Courtesy of Professor RM MacKie and the Scottish Melanoma Group)

1.2.2 ANATOMICAL DISTRIBUTION

Melanoma in the white population occurs most frequently on the lower limb in females and the trunk in males[21]. However in the black population, melanoma is commonly seen to affect the soles of the feet[22]. The anatomical site of malignant melanoma is reflected in the histology of the tumour[23,24], e.g. superficial spreading melanoma and nodular melanoma commonly occur on the trunk and lower limb whilst lentigo maligna melanoma frequently occurs on the head and neck region and on exposed body surfaces[24].

1.2.3 PLACE OF RESIDENCE AND BIRTH

Place of birth also appears to be an important factor in the incidence of melanoma. This is most clearly seen in people who migrate from a country of low to one of high incidence; initially such migrants tend to have a lower rate of melanoma than the local population[25,26] but with increasing duration of stay and an early age of migration, the risk increases. Conversely this pattern is not, however, observed in white migrants to Hawaii who tend to have a higher incidence of melanoma than Hawaiian born whites[27]. This may be explained by the Southern European or mixed race ancestry of the native whites[28]. A study conducted in Western Australia showed that the age at arrival was a more useful predictor of risk than duration of residence[29]. Those who arrived before ten years of age were exposed to similar risks of developing melanoma as native born Australians whilst arrival after that age conferred a relative risk of approximately 0.5[29].

1.2.4 AGE AND SEX

Melanoma tends to occur in the young and middle aged but is rare before puberty. The incidence rate rises steeply until 50 years of age, after which the rise slows down[30]. Figure 2 shows the age-distribution and age-specific incidence of the rate of melanoma in Scotland from 1979 to 1986. There is often a relationship between the histological type of melanoma and the age at presentation. In the young and middle-aged, superficial spreading and nodular melanoma are more common whereas in the older patient lentigo maligna melanoma predominates. Although world-wide the overall incidence of melanoma does not differ between the sexes, it has been shown that in areas of low incidence, melanoma is commoner in males[30] whereas female predominance is seen in Northern Europe where the ratio of female to male is 2:1. Survival after the diagnosis of melanoma is longer in women than in men[31,32].

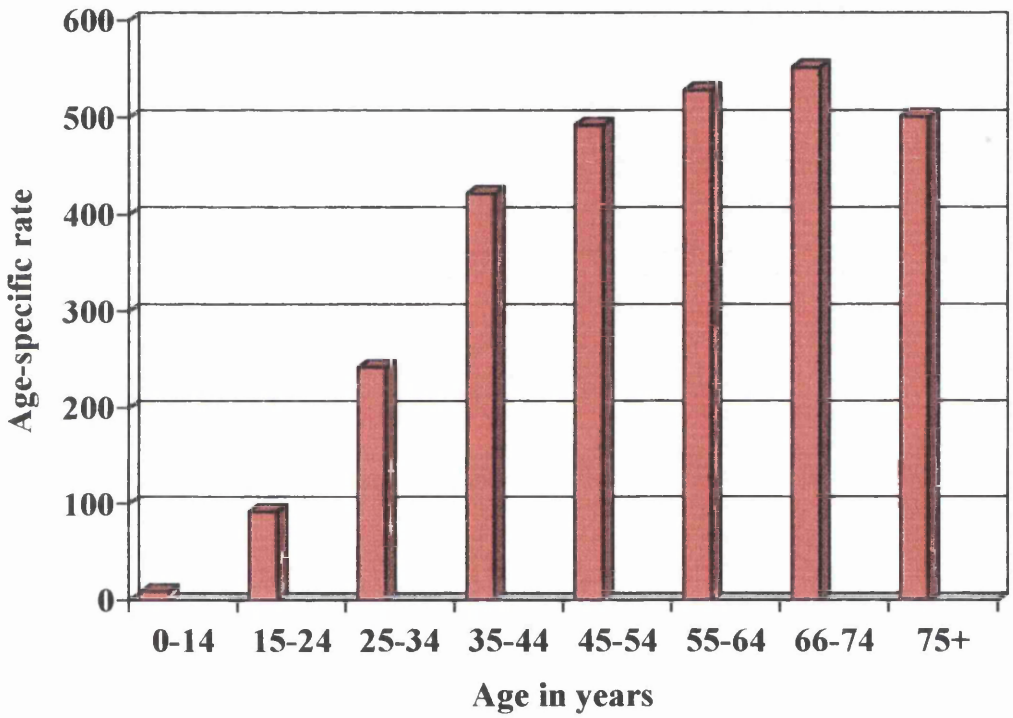


Figure 2 Age-distribution and age-specific incidence rate of melanoma patients in Scotland (1979-1986)
(Courtesy of Professor RM MacKie and the Scottish Melanoma Group)

1.2.5 RACE

Although melanoma is primarily a disease of the white population and is uncommon in the black population, there is a variation in the incidence within the white population, perhaps reflecting the ethnic background. For example, Hispanics have a lower incidence of melanoma than other whites in United States[30]. It has also been shown that people of Southern or Eastern European background have a lower risk of developing melanoma than those who originate in Northern European or the United Kingdom[33,34].

1.2.6 SOCIAL CLASS

Both disease incidence and mortality rates from melanoma are noted as being highest in the higher social classes[35,36]. It has been postulated that this may relate to behavioural pattern. A typical representative of the higher social class tends to work indoors and is more likely to be exposed to sharp short bursts of sunlight whilst a person from a lower social class is often engaged in outdoor work and exposed to constant sunlight and therefore at less risk of developing melanoma[37].

1.2.7 FAMILY HISTORY AND PIGMENTARY TRAITS

Melanoma has long been known to run in families[38]. Holman and Armstrong[39] showed that a patient with one affected relative had a increase risk of 2.3 of developing melanoma and with two affected relatives this more than doubled to 5.0 when compared to the patient with no affected relatives. Blue eyes, fair or red hair and a pale complexion are risk factors for melanoma. Eye colour is a weak risk factor with a relative risk of less than 2. Hair colour is the strongest predictor of all the physical characteristics with a relative risk of 3 in persons with blonde or red hair. Those with pale white skin who never tan and often burn and those who burn initially and tan with difficulty have a relative risk of 3. A history of severe sunburn also gives a relative risk of 3[39]. The most practical method currently available for screening a large population at risk for skin cancer is by determining the skin phenotype. This technique of classifying the sun sensitivity of the skin is based on the ability of the skin to tan.

1.2.8 BENIGN MELANOCYTIC NAEVI

The presence of benign melanocytic naevi increases the risk of melanoma and the relative risk increases with increasing number of naevi. Bain et al[40] initially showed a strong correlation between naevi in the lower limb and the development of subsequent melanoma. Weinstock et al[41], in a later study, found that the number of naevi on any site was not predictive of melanoma at that site. The presence of dysplastic naevi is associated with a significant risk of melanoma. The Scottish study[42] showed that naevi with irregular edges had a seventy fold increase in risk and those with colour variation had a thirty fold increase in the risk of developing melanoma. Holly et al[43] showed that the total number of naevi and atypical naevi were both independent predictors of risk. However MacKie et al[44] found that the total number of naevi was a stronger predictor of risk than the number of atypical naevi.

1.2.9 EXPOSURE TO SUNLIGHT

Several aetiologic agents have been proposed for melanoma of which exposure to sunlight, especially ultraviolet B radiation, has received most attention. Electromagnetic radiation from the sun can be divided into 3 bands: ultraviolet, visible and infra-red. The ultraviolet portion is further divided into ultraviolet-C (200 to 290nm), ultraviolet-B (290 to 320nm) and ultraviolet-A (320 to 400nm). Only ultraviolet B and A reach the earth's surface. The solar energy in the ultraviolet-C and ultraviolet-B bands are absorbed by the earth's ozone layer. However as our ozone layer becomes depleted, more ultraviolet-B rays penetrate the earth's atmosphere and this may be one of the reasons for the increased incidence of melanoma.

In animal studies, ultraviolet radiation has been shown to induce neoplasia in epidermal cells including melanocytes. In human skin it is absorbed by melanosome in the melanocytes and increases melanin activity. The association between melanoma and sun exposure is not a simple one. Studies relating risk of melanoma to occupational exposure to sunlight have been inconclusive. Gallagher et al[45] and Holman et al[46] showed that with increasing occupational exposure there is a decreasing risk of melanoma, whereas several other studies including those by Dubin et al[47] showed the opposite. Cook et al[35] in New Zealand showed that outdoor workers had a higher incidence of melanoma affecting the trunk. Holman et al[46] showed that although outdoor workers had a lower incidence of melanoma than indoor workers, the incidence of melanoma was higher on sites that were exposed to

the sun. It is therefore difficult to be dogmatic on the effect of occupational exposure to sunlight.

In contrast, in several studies, recreational or intermittent sun exposure has been shown to to increase the risk of melanoma. People who sunbathe once or more a week in summer have a relative risk of 2.6 of developing a superficial spreading melanoma on the back. In women between the ages of 15 and 24, the risk of melanoma has been related to the type of bathing suit. Those who wore a one piece suit had a relative risk of 4.0 whilst for those who wore a two piece bathing suit or none at all, the risk of developing melanoma of the trunk rose to 13[46]. It is unclear whether the use of sunlamps and sunbeds increases the risk of the development of malignant melanoma. Different studies have shown the risk to range from 0 to 2.9[48,49,50]. It is widely accepted that inappropriate use of such equipment is harmful to the skin and almost certainly carcinogenic. MacKie et al[51] showed a relative risk of less than unity. However, none of these studies took into account the pattern of exposure to the site at which the melanoma arose.

In summary, the main risk factors for melanoma seem to be high socio-economic class, latitude of residence, white race, migration into an area of high incidence and intermittent sun exposure. If the destruction of the earth's ozone layer continues then ultraviolet-B radiation will increase at a rate of 1% to 2% for every 1% decrease in ozone[52,53]. It has been estimated, provided there are no behavioural changes in the population, that the incidence of melanoma will increase between 1% and 2% for every 1% decrease in ozone layer[53].

1.3 HISTOLOGY OF SKIN

1.3.1 STRUCTURE AND FUNCTION OF SKIN

The skin is the largest organ of the body. It is the frontier with the external environment and its structure and functions all reflect this fact. Functions of the skin include mechanical protection, melanin production that protects against ultraviolet damage, vitamin D synthesis, thermal insulation, fat deposit reserve, thermoregulation and sensory function. The skin is essentially divided into two layers; the outer layer of ectodermal origin, the epidermis, and the inner layer of mesodermal origin, the dermis. Figure 3 is a diagrammatic illustration of the histology of skin.

The epidermis consists of 4 layers - stratum basale, stratum spinosum, stratum granulosum and stratum corneum. A fifth layer, stratum lucidum, is only seen in the thick skin of the palm and sole. The surface cells of the epidermis are continuously being shed and replaced by mitotic division in the basal layer (stratum basale). Keratinocytes form 85% of all cells in the epidermis. As the keratinocytes migrate from stratum basale to stratum corneum, they mature from nucleated cells to flat anucleated squames. Their main function is to provide an impermeable barrier. Other cells of the epidermis include melanocytes, Langerhans cells and Merkel cells. Melanocyte cells produce melanin, which has a photoprotective action.

The epidermis is firmly attached to, supported and nourished by the dermis, a thick layer of fibrous connective tissue. Although epidermis and dermis constitute the skin in the strict sense, beneath the dermis is a layer of loose connective tissue that forms the subcutaneous adipose tissue.

1.3.2 MELANOCYTE, MELANOGENESIS AND SKIN PIGMENTATION

Melanocytes synthesise the pigment called melanin. They are found deep in the basal layer of the epidermis. They are dendritic in form. Melanin appears in their cytoplasm in melanosomes which are oval or spherical bodies. These melanosomes are transferred to keratinocytes through the end feet of the dendritic processes. Each melanocyte is associated with a number of keratinocytes and together they constitute an epidermal-melanin unit. Figure 4 shows a diagrammatic illustration of an epidermal-melanin unit.

There are about 1000 melanocytes per square millimetre of epidermis. Racial difference is not due to increased number of melanocytes but to increased melanogenesis with slightly larger melanosomes. Melanosomes transferred to keratinocytes are broken down more slowly in Negroid than in Caucasian skin by lysosomal enzymes.

Melanocytes contain the copper dependent enzyme tryrosinase (DOPA Oxidase). This enzyme stimulates a sequence of reactions that convert the substance tyrosine (derived from essential amino acid phenylalanine) to stable insoluble melanin. Dihydroxyphenylalanine (DOPA) is formed by oxidation of tyrosine and further reaction results in formation of dopaquinone. Eumelanin, a brown or black pigment, is then formed by random polymerisation of dopaquinone. Figure 5 illustrates the synthetic pathway of melanogenesis.

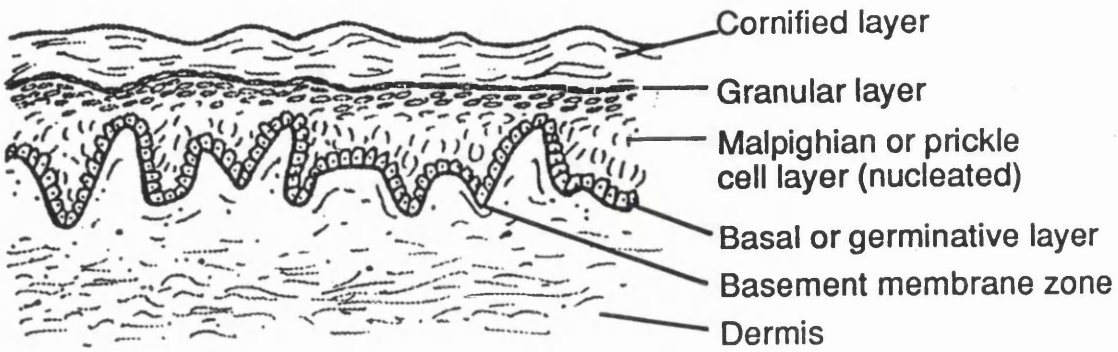


Figure 3 Diagrammatic illustration of the histology of skin (courtesy of Professor R M MacKie)

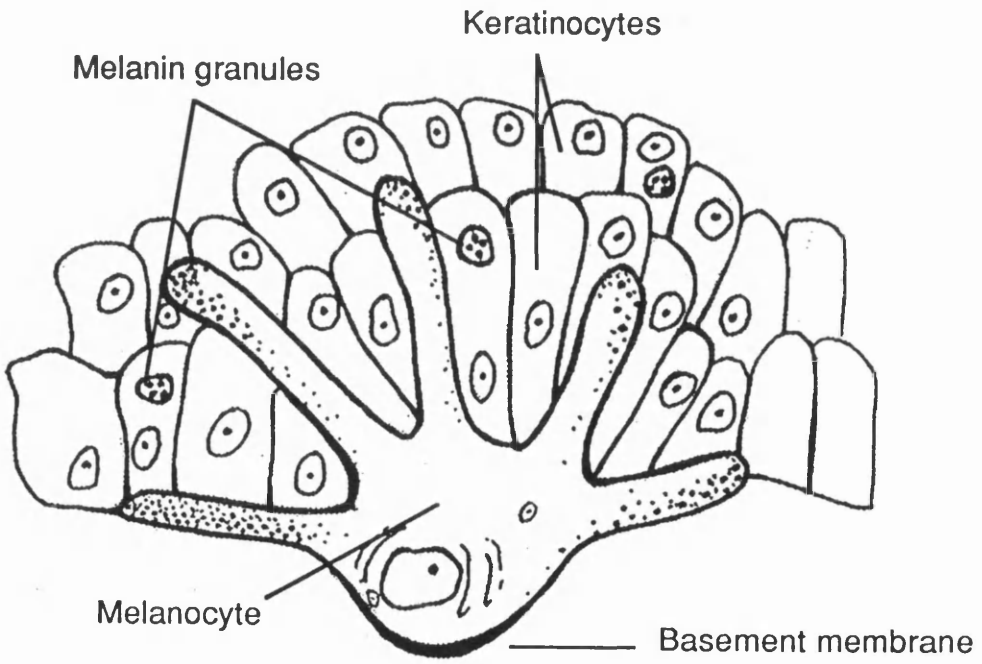


Figure 4 Diagrammatic illustration of an epidermal-melanin unit (courtesy of Professor R M MacKie)

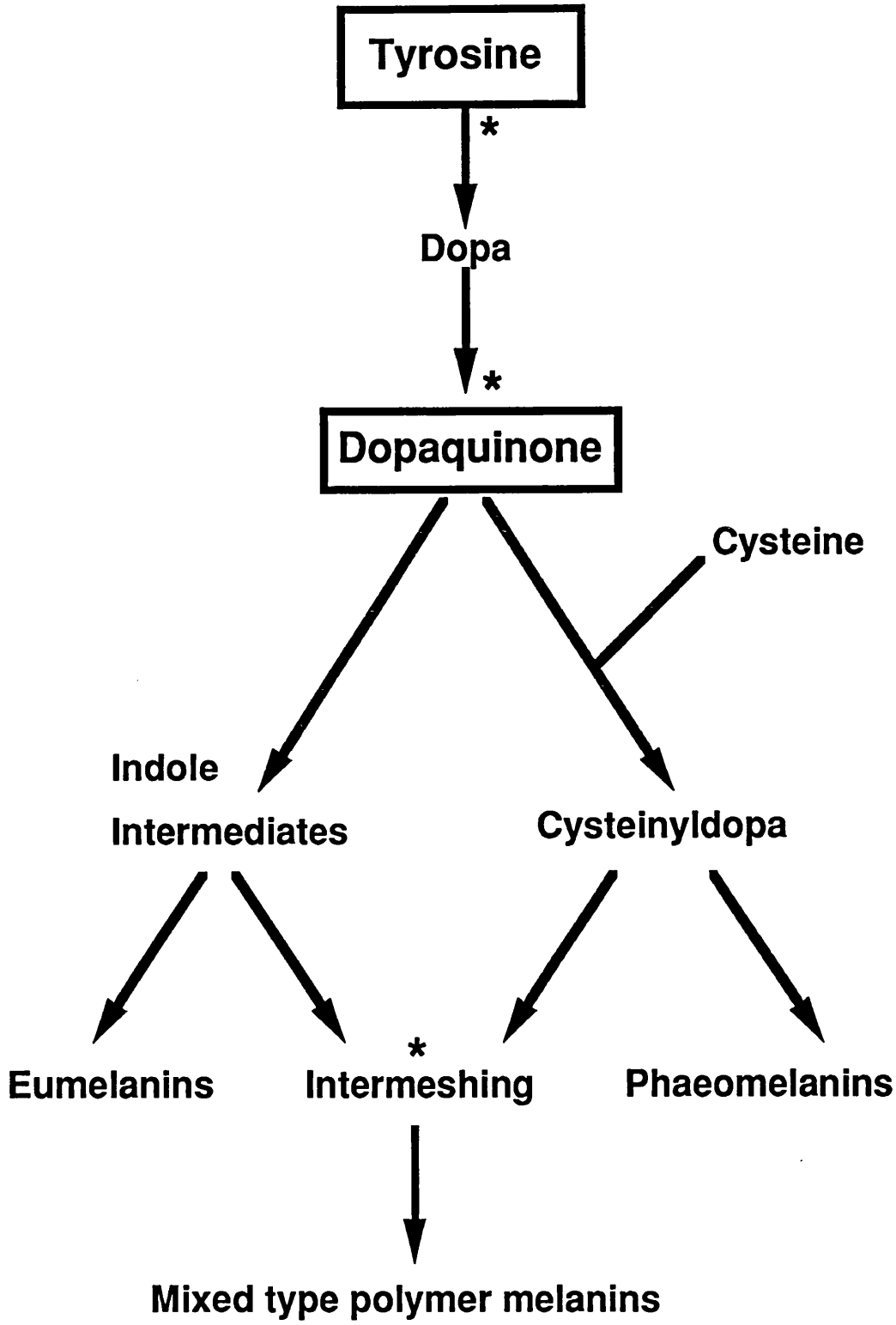


Figure 5 Synthetic pathway of melanogenesis.

Genetic factors act at various stages in the process of melanogenesis but also affect migration from neural crest, melanocyte morphology, melanosomal structure, tyrosinase activity, types of melanin synthesis, melanin transfer to keratinocyte and melanosomal degeneration.

Exposure to sunlight leads to some darkening of skin within five minutes. This is due to migration and redistribution of already existing melanosomes from deep to more superficial keratinocytes and to oxidation reaction. This immediate darkening, which lasts fifteen minutes after cessation of exposure, is responsible for the well known 'false tan'. New pigment formation occurs 24 hours after exposure. More melanosomes are produced and transferred to surrounding keratinocytes.

Ultraviolet light may act by blocking the action of reduced glutathione, a local inhibitor of melanogenesis. It interferes with the function of tyrosinase, combining with the copper in the enzyme and forming complexes with intermediates produced during the conversion of tyrosine to melanin. Although melanin synthesis is stimulated by certain pituitary melanotrophic hormones in animals, this is not so in man. Oestrogens and progestogens also stimulate melanocyte function.

1.4 CLINICAL AND PATHOLOGICAL FEATURES OF MALIGNANT MELANOMA

Malignant melanoma is a tumour of epidermal melanocytes. The tumour is characterised by a biphasic growth pattern, with radial and vertical phases. During the radial growth phase, the primary melanoma undergoes centrifugal enlargement. The lesions tend to be relatively flat with irregular outlines but their overall shape is circular or oval. This phase may persist for years and has little tendency to metastasise. Surgery at this stage is likely to be curative.

The vertical growth phase which subsequently appears as a focal nodularity within the flat lesion represents a new and distinctive clone of tumour cells. Growth is more rapid than the radial phase, with invasion of the underlying connective tissue including blood and lymph vessels. Chromosomal studies have shown differences between the melanocytes in these two phases. Melanoma cells in the radial phase show abnormalities at chromosome 6; melanoma cells of the vertical growth phase show deletions, translocations and duplication at chromosomes 1, 6 and 7, as do cells of metastatic melanoma[54].

There are four major types of malignant melanoma:

1) Superficial spreading melanoma
2) Nodular melanoma
3) Lentigo maligna melanoma
4) Acral melanoma

1.4.1 SUPERFICIAL SPREADING MELANOMA (SSM)

This accounts for about 70% of all melanoma and commonly occurs in the lower limbs in women and on the back in men. SSM develops both on sun exposed and covered surfaces. The lesion tends to be uniform in contour with an irregular indefinite border. It is often multi-coloured, displaying a mosaic of black, brown, tan, blue, red and white. Figure 6 shows a typical SSM lesion. During the radial phase of growth, atypical melanocytes also spread along the dermal-epidermal junction. It is the dermal component that produces the epidermal thickening characteristic of SSM, hence the elevated surface and palpable margins.

1.4.2 NODULAR MELANOMA (NM)

Nodular melanoma has the most aggressive clinical course and accounts for 15-30% of melanoma and is more common in men than in women. These lesions grow very rapidly and are often difficult to diagnose, commonly being mistaken for vascular lesions. It occurs most frequently on the trunk, head and neck. Nodular melanoma exhibits a monophasic growth pattern with vertical growth phase from the outset. NM, as the name suggests, presents as a dome shaped spherical lesion which is smooth or ulcerating. The lesion is generally darker and more uniform in colour than SSM and may resemble a haemangioma. Bleeding is a relatively early sign. The lesions in most cases at the time of clinical detection have already invaded the underlying connective tissue. Polypoidal nodular melanoma behaves in a particularly aggressive manner. About 5% of nodular melanoma lack pigment altogether. Figure 7 shows a typical NM lesion.

1.4.3 LENTIGO MALIGNA MELANOMA (LMM)

This form of melanoma is characterised by a prolonged radial growth phase. In this radial growth phase, the in situ melanoma is known as lentigo maligna. These lesions

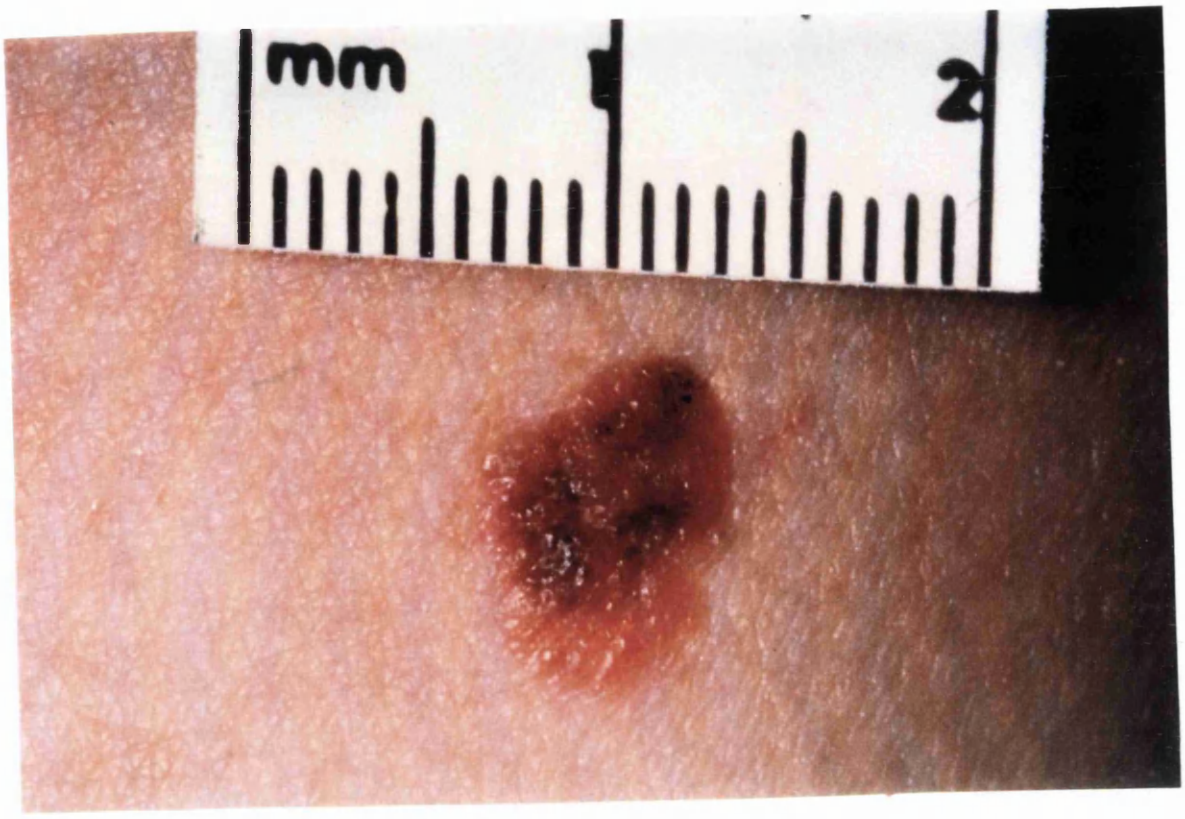


Figure 6 Typical superficial spreading malignant melanoma (courtesy of Professor R M MacKie)

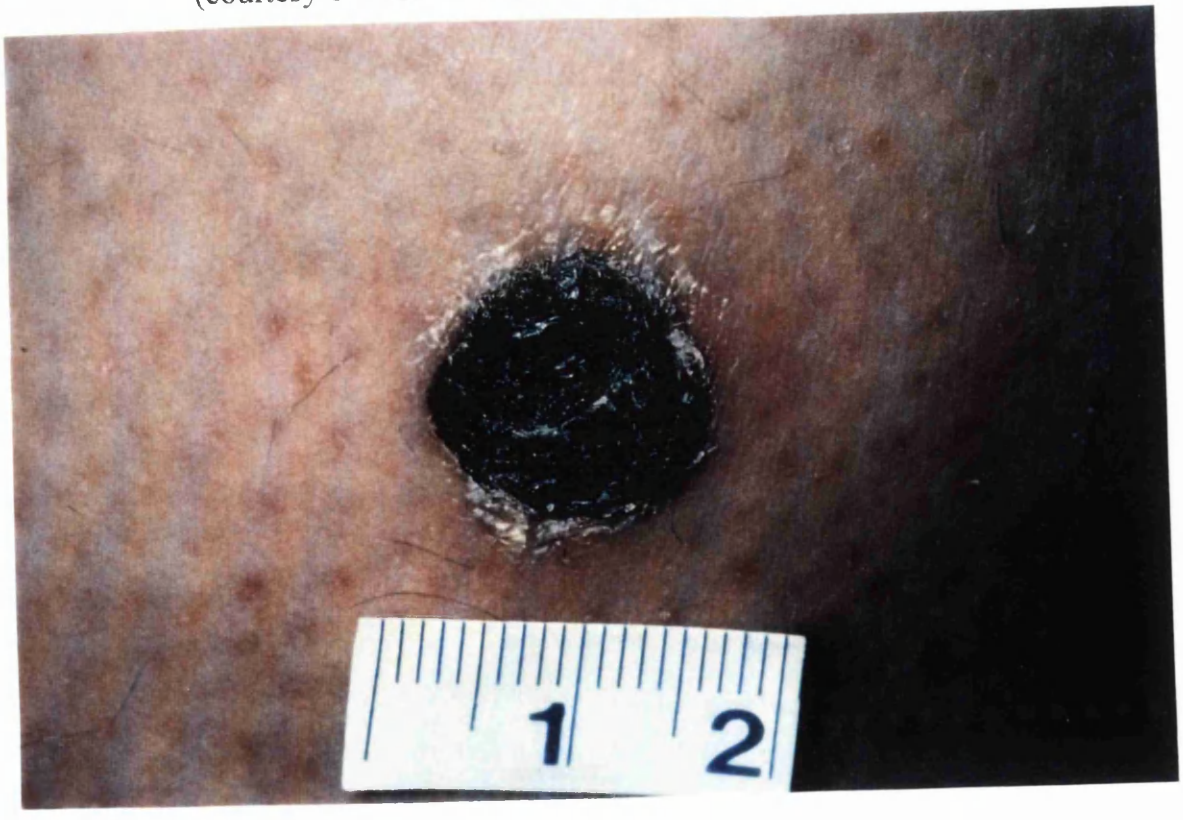


Figure 7 Typical nodular malignant melanoma (courtesy of Professor R M MacKie)

account for 10% of all melanoma and are typically found in the elderly on sun exposed surfaces, especially the head, neck and face.

It is usually a tan to brown patch speckled with minute brown to black flecks. It is characteristically impalpable and palpability indicates dermal invasion. The proliferation of atypical melanocytes is essentially confined to the epidermis. Progression into an invasive lesion clinically presents as one or more blue to black nodules or an area of induration. Figure 8 shows a typical LMM lesion.

1.4.4 ACRAL MELANOMA (AM)

Acral melanoma characteristically occurs on the palm or sole. In the United Kingdom, eight times as many are found on the side of the sole of the foot as on the palm. Acral melanoma has mixed histological features, the common type being acral lentigo melanoma. This represents 2% to 8% of melanoma in whites but substantially more occur in dark skinned patients (35%-90%). The lesion is typically flat with a mosaic pattern of tan, dark brown and black with irregular and ill defined borders. Figure 9 shows a typical acral melanoma. As the lesion advances, the borders become highly irregular and notched. An important variant of acral melanoma is the subungual melanoma. The great toe and thumb are most often affected. Subungual melanoma represents 2% to 3% of melanoma in whites but a higher percentage in black people. It may present as a split nail, ulceration with bloody crusts or a longitudinal black or brown streak in the nail bed. An irregular tan brown colour that diffuses proximally to the cuticle to involve the posterior nail fold is an ominous finding associated with advanced disease.

In summary, malignant pigmented lesions exhibit disorganisation, irregular shape and chaotic pigmentation whereas benign lesions tend to show order in colour, symmetry, regular border and uniformity.

1.5 STAGING AND PROGNOSTIC FACTORS OF MALIGNANT MELANOMA

1.5.1 STAGING

The staging of melanoma is of critical importance. It determines appropriate treatment, prognostic assessment and makes it possible to compare treatment world-wide. Unfortunately there is no universally agreed staging system. The first widely used staging system recognised three stages[55].

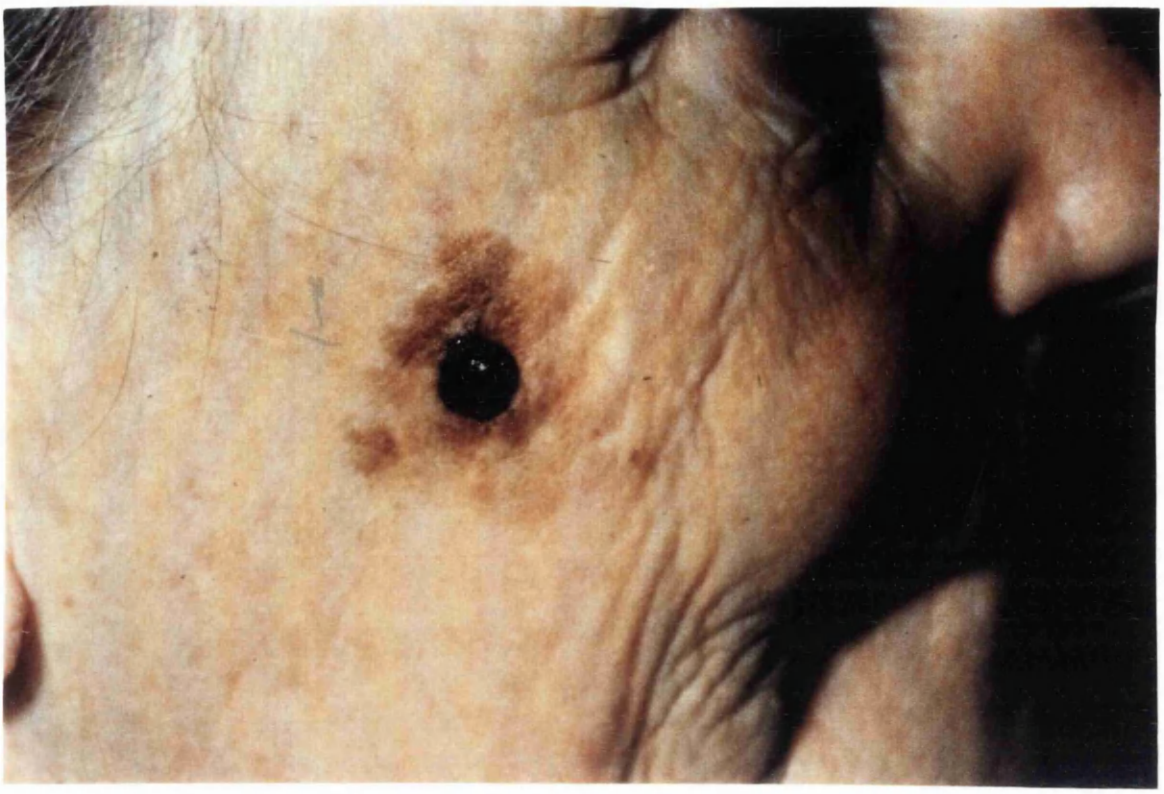


Figure 8 Typical lentigo maligna melanoma
(courtesy of Professor R M MacKie)

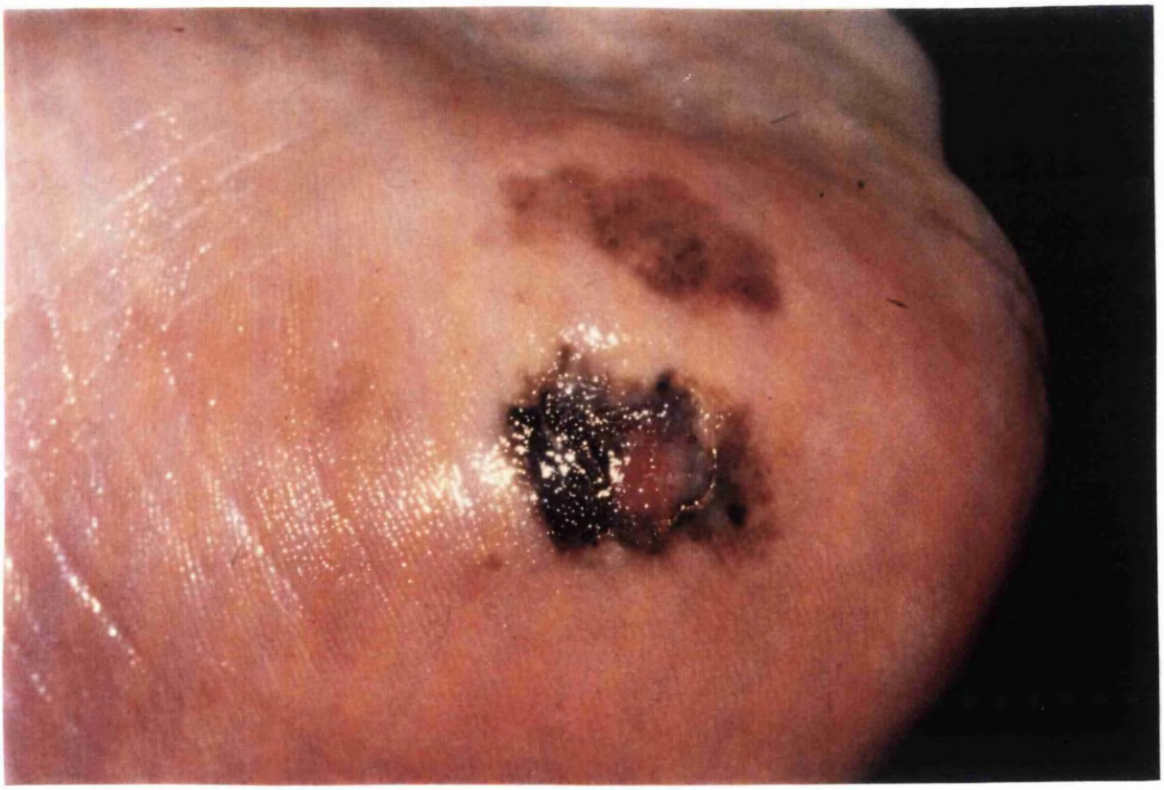


Figure 9 Typical acral malignant melanoma
(courtesy of Professor R M.MacKie)

The greatest asset of the 3-stage system was its simplicity, but it failed to reflect prognostic groups. At the M. D. Anderson Cancer Centre[56] in Houston, Texas, a 4-stage system was devised which addressed a sub stage of melanoma patients with local recurrence, in transit metastases or satellite disease who were suitable for hyperthermic limb perfusion. This staging was introduced before the importance of tumour thickness and level of invasion were appreciated.

In 1978, the Union Internationale Contra le Cancer (UICC)[57] incorporated these prognostic factors in their staging system. This staging system was in part based on the concept that malignant cells tended to spread first to the regional lymph nodes and from there via the thoracic duct lymph to the bloodstream and viscera.

In 1983, the Melanoma Subcommittee of the American Joint Committee on Cancer (AJCC) derived a 4-stage system based on the TNM System[58,59]. The AJCC System addressed the deficiencies in other systems and was able to produce a system better at discriminating risk in patients with early melanoma. Unfortunately, unlike the other TNM Staging, the AJCC System was neither accepted nor used universally. The failings of all these systems were addressed when the AJCC and the UICC produced a mutually acceptable TNM/pTNM Staging for all tumours[60,61].

When clinical diagnostic staging is being carried out, a careful examination is necessary and should include details of primary tumour size, nodularity, ulceration, satellite lesions and palpation for in transit and regional node metastases. To aid clinical staging a chest X-ray, CT scan of chest and abdomen and radioisotope studies of lymph nodes, gallium scan and lymphangiography may be helpful. The staging used in this thesis is the M. D. Anderson Cancer Centre Staging which is listed below:

Stage I	Primary Melanoma IA: Intact primary melanoma IB: Primary melanoma locally excised IC: Multiple primaries
Stage II	Local recurrence or local metastases within 3 cm of the primary site
Stage III	Regional metastases IIIA: Tissues excluding nodes IIIB: Node(s)

IIIAB: Skin etc, plus node(s)

Stage IV — Distant metastases
IVA: Cutaneous metastases only
IVB: Any visceral metastases

1.5.2 PROGNOSTIC FACTORS: PATHOLOGICAL FACTORS

The first prognostic parameter for melanoma was identified by Allen and Spitz[62] in 1953 when they observed that superficial tumours had a better prognosis than deeply invasive ones. Mehnert and Heard[63] introduced a system of four levels of micro-invasion which later Clark[64] modified by introducing a special Level III and his nomenclature became internationally accepted. A year later, Alexander Breslow[65] showed that tumour thickness provided a simpler and more objective method of predicting prognosis.

He defined thickness as the distance in millimetres (mm) from the granular layer in the epidermis to the deepest invasive tumour cell in the underlying dermis. This has been shown to be the single most important factor in the prediction of survival[65,66]. Importantly, this measurement is both objective and reproducible. Several investigators have attempted to identify other parameters that have predictive value for survival and metastasis. These factors include mitotic rate, ulceration, phase of tumour growth, host response, vascular invasion and microscopic satellites[67]. None is as important as Breslow thickness.

TUMOUR THICKNESS - Breslow thickness is the single most important factor for prognosis. The determination of tumour thickness is easy and reproducible. Thickness may have less predictive value on prognosis in ulcerated melanoma, in melanoma with marked hyperplastic epidermis which leads to over-estimation and in desmoplastic neurotropic melanoma when over-estimation leads to poorer prognosis. It is difficult to measure thickness accurately in subungual melanoma.

LEVEL OF INVASION - The Clark[64] system is divided into 5 levels: I - intraepidermal, II - papillary dermis, III - interface between papillary and reticular dermis, IV - reticular dermis and V - subcutaneous fat. This method has disadvantages in that there are difficulties in identifying landmarks at some sites thus making it difficult to reproduce. There is also a greater interobserver variation than with Breslow thickness. Clark[68,69] level of invasion can be used for microstaging in cases when two tumours are of the same thickness, thus giving a different prognosis.

MITOTIC RATE - many studies have shown a significant relationship between mitotic activity and prognosis, especially in the vertical growth phase[70,71,72]. The prognostic effect of mitotic rate varies in different thickness and location subsets, and appears to be most important in tumours that are of 'intermediate' thickness (tumours 1.51 - 3.99mm thick). Elder et al[71] showed that the overall five year survival in patients with intermediate thickness tumour was 70%. However, patients with a mitotic rate of $<6/mm^2$ had an 80% survival whilst the remainder had a 40% survival[72]. Using this argument Schmoeckel and Brown Falco[73] suggested an alternative prognostic index than thickness alone but this formula has not been widely accepted.

PHASE OF TUMOUR GROWTH - The presence of radial and vertical growth has some correlation with thickness and level of invasion. The excellent prognosis of tumours less than 1.0mm thick and anatomical Level I is primarily related to their being in radial growth phase and thus lacking competence for metastasis[69]. Elder et al[69] found that in 69 patients with pure radial phase, all survived at 5-12 year follow up. Thus in their hands, "phase" was more sensitive than level or thickness in identifying low or zero risk melanoma patients.

ULCERATION - This is usually indicative of poor prognosis. One may speculate that ulceration might correlate with 'invasiveness' of tumour into the epidermis or with rapid size of growth. Balch et al[74] and Day et al[75,76] found this to be an independent variable and also found ulcer width of greater than 3mm to be of significance[76]. Although ulceration has some crude correlation to thickness, it may also correspond to tumour volume, rate of proliferation, tumour aggressiveness and architectural configuration.

VASCULAR INVASION - Although seemingly a sine qua non for metastasis, this property is infrequently recognised with certainty in melanoma. It indicates a high risk of later metastasis development[77,78]. While Schmoeckel et al[79] found "unequivocal" vascular invasion to be associated with impaired survival, he found this feature in only about 10% of his cases. It is therefore important to record vascular invasion only when it is seen unequivocally, as false positive results can occur[80]. Recent multivariate analyses have demonstrated no significant influence of vascular invasion on survival[68,80].

LYMPHOCYTE RESPONSE - Severe cellular atypia and small lymphocyte like cell types at the tumour base are signs of poor prognosis[81]. A cellular inflammatory reaction, particularly at the base of the tumour, indicates a favourable prognosis, while its absence correlates with a higher recurrence rate and impaired survival[79,82]. In the vertical growth phase the presence of an infiltrative lymphocytic response is associated with improved survival[81]. Thus patients with the most intense degree of lymphocyte infiltration had better survival compared to those who had mild infiltration[81].

HISTOGENETIC TYPE - Nodular melanoma growth pattern is associated with a much poorer prognosis than SSM and LMM, probably due to the lesion being thicker at presentation[83]. There is no significant difference in survival between NM and SSM when matched for tumour thickness[83,84]. The better prognosis of LMM may be confounded by it usually being less thick[85].

MICROSCOPIC SATELLITES - Satellites are defined as discrete tumour nests > 0.05mm in diameter separate from the main body of the tumour. Day et al[86] studied 95 patients with stage I melanoma and showed that the five year survival was 36% for patients with microscopic satellites compared to 89% for patients without satellites. Satellites are best regarded as local lymphatic and/or haematogenous metastases. Their presence is a clear indication that the tumour has achieved competence for metastasis. This parameter, however failed to demonstrate separate prognostic value in a recent study[68].

TUMOUR CELL TYPE - Many studies have shown no correlation between cell type and prognosis[80]. It has been suggested that since vertical growth is likely to be the determinant of biological behaviour, the cell types in this component are most likely to be of prognostic relevance but this however, has not been specifically studied. In a few studies, well-differentiated spindle cells are associated with better prognosis[87,88]. Drzewiecki and Anderson[89] found a worse prognosis for epitheloid cell melanoma.

1 5 3 PROGNOSTIC FACTORS: CLINICAL FACTORS

AGE - Blois et al[90] found that men aged less than 48 years had a better prognosis than older men. Elder et al[71] found a favourable prognosis to be associated with intermediate age (40-60 years, relative risk 0.49). Advancing age correlated significantly with a shortened survival times [89] and melanoma thickness, with older patients presenting with thicker melanoma.

SEX - Numerous studies have shown that female patients with melanoma have a better survival rate than males[91,92,93]. Most of the differences that persist in the survival rates between males and females when Clark level is matched are probably related to the fact that melanomas in women occur more commonly on the extremities (a more favourable prognostic site) and are found to be thinner and less frequently ulcerated[91].

ANATOMICAL LOCATION OF PRIMARY LESION - The site of the primary tumour appears to be one of the most important survival associated variables[70,82,93]. Patients with melanoma of the extremities have a better survival rate than those with trunk, head and neck melanoma. Analysis of anatomical lesions of the subsites can also reveal a different prognosis, e.g. in head and neck melanoma, the scalp has a worse prognosis than those on the ear, face or neck. Although no overall survival difference was found for women or men when comparing back versus chest lesions, female patients with melanoma on the back had better survival than their male counterparts. Patients with melanoma located on hands and feet had a significantly worse prognosis than did those with a lesion on the arm or leg[72,93].

1.5.4 PROGNOSTIC FACTORS: CONCLUSION

In summary, three dominant factors predict survival in stage I and II melanoma patients.
1 Thickness of melanoma.
2 Melanoma ulceration (present or absent).
3 Anatomical location.
Four other variables also correlate with survival in certain groups of patients: type of initial surgical management, pathological stage, level of invasion and sex.

In patients with nodal metastases (stage IIIB and stage IIIAB) the three dominant prognostic variables are the number of nodal metastases, the presence or absence of ulceration in the primary lesion and the patient's age.

In patients with distant metastases (stage IV) there are no parameters of the primary melanoma that predict the patient's clinical course. The dominant prognostic variations are anatomical site of metastases (visceral versus non visceral), the number of sites of metastases and the remission duration. The sex and age of the patient do not significantly influence survival.

CHAPTER 2

TREATMENT OF MALIGNANT MELANOMA

2.1 TREATMENT OF PRIMARY MALIGNANT MELANOMA

2.1.1 SURGICAL TREATMENT

The surgical treatment of any primary malignant melanoma consists of wide local excision of the tumour with a margin of normal skin. The rationale for wider excision margins is the removal of occult foci of melanoma cells that may lead to metastasis or local recurrence. The current practice is to excise the primary malignant melanoma with surgical margins that are based on tumour thickness and ulceration, as these factors correlate with the risk of local recurrence[94,95]. Breslow and Macht[96] observed that the incidence of local recurrence or metastasis from melanoma less than 0.76mm thick is not affected by the width of the excision margin.

In the World Health Organisation (WHO)[97] randomised study of 612 patients in whom excision was randomised to 1 or 3cm, it was demonstrated that a 1cm margin was adequate for primary melanoma less than 1mm thick. Optimal excision margin for melanoma thicker than 1mm is still controversial. Aitken[98] and associates showed that for melanoma thicker than 2mm, survival was poor if the excision margin was less than 2cm but increasing the margin beyond 3cm did not confer any additional survival benefit. Ackerman et al[99] suggested metastases or microsatellitosis more than 2cm from the primary tumour may reflect disseminated disease and wider local resection may not influence survival outcome.

The current recommendations for surgical margins are as follows:

Type	**Margin**
In situ	0.5 - 1.0cm
<1mm thick	1cm
>1mm thick	2 - 3cm
Lentigo maligna melanoma	1cm

The depth of incision has received less attention than the width. Olsen[100] showed that melanomas without clinical evidence of metastases had more lymph node metastases when the primary excision extended through the underlying fascia than when this was left intact. He suggested that resection of fascia allowed dissemination of melanoma cell from subdermal lymphatics to subfascial lymphatics.

However, in a review carried out by Kenady et al[101] no significant difference in the incidence and site of subsequent recurrence or survival in patients in whom the fascia was excised compared to those in whom the fascia was left intact. In theory, excision of fascia should not affect the incidence of local recurrence or in transit disease since lymphatic drainage to the regional nodes occurs in the subcutaneous tissue superficial to the deep fascia. Resection of fascia is not routinely indicated except when the tumour is deeply invasive in order to ensure that the deep margin is histologically negative for melanoma.

In summary, a 1cm margin is adequate for tumours less than 1mm in thickness whilst a 2-3cm excision margin is required for tumours of more than 1mm in thickness. It is important that the width of excision is tailored to the individual patient based on the assessment of tumour thickness and other prognostic factors.

2.1.2 ADJUVANT THERAPY: PRINCIPLES

The concept and efficacy of multimodality therapy have been proven in animal models of human tumours, where the best results were achieved by combining local treatment with systemic treatment. Experimental models of animal tumours have clearly established the principles of adjuvant therapy as reviewed by Balch and Maddox[102]. When animals were inoculated with a known tumour dose and then randomly allocated to single treatment modalities or combination modalities, the latter produced superior results[103,104].

The four principles that are pertinent to designing treatment plans for any human tumour are listed below[102,103].

1) Effectiveness of any treatment is inversely proportional to tumour burden, i.e. the smaller the tumour the better the response.
2) Occult micrometastases are generally more susceptible to drug treatment than the larger primary tumour.
3) Agents with activity against moderately advanced disease are more active against residual metastatic disease after debulking of tumour by surgery.

4) Curative treatment of cancer involves destroying all cancer cells as lethal metastases may arise even from a single residual malignant cell.

In the clinical situation, patients who are likely to benefit from adjuvant therapy are those who are at high risk of harbouring occult metastases, for example those patients with thick tumours and lymph node involvement.

2.1.3 ADJUVANT TREATMENT: CHEMOTHERAPY

Dacarbazine [5 - (3, 3 - dimethyl 1 - triazeno) - imidazole - 4 - carboxamide (DTIC)] is generally considered to be the most active single agent in advanced disease. It has been used extensively on its own as an adjuvant chemotherapeutic agent or in combination with other agents or immunotherapy. The two largest studies were conducted by the Central Oncology Group (COG)[105] and the WHO[106]. The COG study (174 patients) showed no proven benefit for DTIC whilst the WHO study (761 patients) concluded that adjuvant treatments did not significantly improve either the disease free interval or overall survival. An overall review of 21 randomised trials in 2,850 patients between 1978 and 1986 showed no positive benefit in terms of improving the disease free or the survival interval after adjuvant chemotherapy[107].

2.1.4 ADJUVANT TREATMENT: ENDOCRINE THERAPY

It has been shown that melanoma tissues and cell lines bind to oestrogen and other steroids[108]. Since the report by Fisher et al[109] showing the presence of oestrogen receptors in metastases, several trials have been conducted to examine the effect of tamoxifen but none have shown definite benefit[110,111,112].

2.1.5 ADJUVANT TREATMENT: IMMUNOTHERAPY

Immunotherapy using non specific immune stimulants such as Bacillus Calmette-Guerin (BCG) and Corynebacterium parvum (C parvum) is based on the observation that tumour antigens on melanoma cells induce relatively weak immune responses. To become effective in controlling tumour growth these responses need only to be augmented by immunotherapy. Melanoma patients may have weakened immune defences that can be restored by immune stimulants. Morton et al[113] in a non randomised study comparing surgery, surgery + BCG and surgery + BCG + allogenic irradiated cells, demonstrated a longer disease free interval and overall survival in patients submitted to either adjuvant treatment regimen. The recurrence rate in

surgically treated patients was 50%, 41% and 34% respectively in patients who received immunotherapy.

A positive result by combining BCG and DTIC was also reported by Wood[114]. However, a few reports have suggested that BCG seems to induce earlier recurrence or enhanced tumour growth. In a trial conducted by McIllmurray et al[115] 4 out of 8 patients treated with BCG and autologous irradiated cells, died within one year of treatment whilst none of the surgically treated patients had a recurrence or died. He concluded that adjuvant immunotherapy might be dangerous and discontinued his trial.

Overall the results of several large trials have shown no statistical benefit from BCG vaccinations as an adjuvant to surgery in patients with stage II and III disease. Similar findings apply to the use of C parvum as adjuvant therapy.

Interferon has also been evaluated as adjuvant treatment. The Southwest Oncology Group[116] found that adjuvant treatment with interferon daily for 12 months after surgery for stage II and III disease did not show a significant benefit on disease free or survival interval.

In recent years, workers have tried to prevent tertiary melanoma using different melanoma cell vaccines and purified antigen. Cassel et al[117] reported that prophylactic treatment with Newcastle disease virus lysates of melanoma cells significantly improved survival in 32 patients with stage II and III melanoma when compared to controls. A further study also reported survival rates of approximately 80% at 2 years. Hersey et al[118,119] showed treatment with vaccinia viral lysates improved survival of 80 patients with stage II and stage III melanoma compared to controls. The results of these studies have prompted a randomised controlled study which is now in progress.

2.1.6 ADJUVANT THERAPY: RADIOTHERAPY

Radiotherapy as a primary treatment for cutaneous melanoma is rarely indicated except for extensive facial LMM, especially in the elderly. Adjuvant radiotherapy can be used to eliminate the need for wide surgical excision at sites where this would cause deformity. This approach was first reported by Dickson[120] who treated 234 patients. Seventy-one patients had local excision only, 42 patients had radical surgery and 121 patients had adjuvant radiotherapy following surgery. He found the overall survival of patients receiving radiotherapy was at least as good as that for patients receiving initial radical surgery.

Studies on the role of adjuvant radiotherapy in the management of regional metastases are few. Creagan et al[121] reported that on 56 patients with nodal metastases who were randomly assigned to receive postoperative radiotherapy or no adjuvant treatment following therapeutic dissection, the disease free interval of patients receiving adjuvant radiotherapy was longer.

However, he noted these results were attributed to imbalances in patients' ages and distribution of nodal metastases between the groups. Two other studies have shown encouraging results. Ang et al[122] treated patients with stage I and II melanoma of head and neck. They reported a local control and survival rate for patients treated electively of 95% and 80% respectively compared to those treated before or after therapeutic lymph node dissection of 87% and 70% respectively. Although the study was not randomised, the results seem encouraging.

Radiotherapy does have a role to play in the adjuvant setting of the treatment of melanoma but further prospective randomised studies are needed to evaluate the efficacy of radiotherapy.

Another treatment that has been used is endolymphatic isotope therapy using lead (P^{32}) or iodine (I^{131}). This method delivers radiation directly to regional lymph nodes. The local dose can be high because of the small volumes irradiated. A study undertaken by the Medical Research Council (MRC)[123] to assess the efficacy of this treatment showed that there was no statistical difference between the groups randomised to endolymphatic isotope therapy or to standard surgery. The impression of this study of 146 cases was that there may have been a reduction in the subsequent nodal metastases in the group that had successful endolymphatic therapy but this was not reflected in improved survival. Ariel[124] produced similar results from a series of 120 patients.

2.1.7 ELECTIVE LYMPH NODE DISSECTION: PROS

Although lymphadenectomy had been suggested in the 19th century and later emphasised by Handley[3] in 1907, there are still controversies surrounding lymph node dissection. There is general agreement that all melanoma patients do not need elective lymph node dissection. Some surgeons prefer to excise only clinically obvious nodal metastases. This is termed therapeutic or delayed lymph node dissection. Excision of nodes even when they appear normal is termed elective lymph node dissection (ELND), immediate lymph node dissection or prophylactic lymph node dissection.

It is generally agreed that there is no case for ELND for every patient with melanoma. The questions that arise are:
1) Is it possible to identify accurately a subgroup of patients with a high risk of microscopic regional node metastases?
2) What is the optimal timing of the operation?

The benefits of ELND are based on the hypothesis that microscopic metastases may disseminate sequentially from the primary melanoma to the regional lymph nodes and then to distant sites. A clear case in favour of ELND exists for patients who have occult metastases, i.e. those with primary melanoma with clinically impalpable nodes but in whom tumour is found in the lymph node following dissection. The prognosis deteriorates if a 'wait and see' policy is practised and the patient returns with clinically involved lymph nodes at a later time.

Elective dissection of clinically negative but pathologically positive nodes confers up to 40% 5 year survival advantage in comparison to patients with clinically and pathologically positive nodes[125,126,127]. It has also been shown that the degree of lymph node involvement is important, i.e. patients with microscopic metastases in only a small portion of one lymph node have a better prognosis than those in whom the whole lymph node contains tumour[127,128]. This is perhaps not surprising in relation to reduced tumour burden.

Since there is no method available to detect occult metastases in lymph nodes prior to surgery, the logical approach is to dissect nodes with a high likelihood of occult nodal metastases, e.g. those with primary tumour thicker than 1.5mm. The major advantage of using tumour thickness for choosing patients for ELND is that it can provide a quantitative estimate of the risk for occult metastatic melanoma in both regional and distant sites[91].

Thin melanomas (<1mm) are usually associated with localised disease and a 95% or greater cure rate is achieved after local excision alone. Patients with intermediate thickness melanoma (1.00mm to 4.00mm) have an increasing risk (up to 60%) of occult regional metastases. This group may benefit from ELND[74,91,129,130]. Patients with thick melanoma (>4.00mm) have a high risk of regional nodal micrometastases and occult distant disease[74,91,129] and prognosis is poor. Therefore ELND is not beneficial and is only palliative.

A large randomised study conducted by the WHO[131] further supports the role of lymph node dissection. The ten year survival rate of patients having therapeutic node dissection was 38%; a corresponding group of patients who had ELND as part of their initial treatment had a ten year survival rate of 60%.

The arguments for the 'wait and see' policy is based on two randomised studies conducted by the WHO Melanoma Group[131] and the Mayo Clinic[132]. Opponents of this policy maintain both these studies are flawed. They argue that the WHO melanoma study[131] was restricted to patients with limb melanoma. Most of the patients were women who have been shown to have the lowest rate for metastases[126,133].

On retrospective analysis of the WHO data, when patients were grouped according to tumour thickness and ulceration, the subgroup of patients with intermediate thickness melanoma undergoing ELND had a 27% higher ten year survival rate than those patients without ELND (56% v 39.3%). Patients who had a delayed lymph node dissection had only a 15-20% ten year survival. This meant that almost 75% of these patients had distant microscopic metastases at the time of the clinically evident regional disease and this supports the earlier contention that delayed lymph node dissection is inferior to immediate node dissection for those patients with micrometastases in the lymph node at the time of presentation.

The Mayo Clinic Study[132] also had several flaws. In this study of 173 patients, only 27 patients fell into the 1.5 - 2.99mm tumour thickness of which 16 had ELND, 4 had delayed lymph node dissection and 7 had wide excision only. Thus from such a small study it is not possible to provide evidence for or against the value of ELND.

In conclusion, the protagonists argue that it is reasonable to undertake ELND for patients with intermediate thickness tumours. In this group, at least 56% will require lymph node dissection later and for those in whom lymph node disease is positively proven histologically, survival is better than those patients who have delayed lymph node dissection for clinically positive nodes. Protagonists argue that a 25% five year survival advantage can be expected for intermediate thickness melanoma patients who undergo ELND.

2.1.8 ELECTIVE LYMPH NODE DISSECTION: CONS

The arguments against ELND are based on the following facts: (1) at least 70% of clinical stage I patients subjected to node dissection show no evidence of occult disease after histological examination; (2) the onset of lymph node metastases after treatment of the primary melanoma does not preclude a radical node dissection; (3) there is some evidence that removal of the lymph node group and of the lymphatic vessels may favour local recurrence or of in transit metastases; (4) removal of the lymph node group may favour the haematogenous spread of any tumour cells that may have been left behind in the operative field; (5) not performing ELND obviates the need for extensive surgery which may be unnecessary and may be complicated by postoperative lymphoedema, which may favour growth of residual tumour; (6) it has never been proven that haematogenous spread arises from occult regional lymph nodes; (7) it has never been established beyond doubt that the survival rate in patients who have ELND is improved.

None of the studies that showed benefit from ELND were prospective or randomised trials and may be subject to selection bias[129,134,135].

The first prospective randomised trial was conducted by the WHO Melanoma Group[131]. In this trial patients with $T_{1\text{-}2\text{-}3}N_0M_0$tumour were randomised to receive either: (a) wide excision and ELND, or (b) wide excision and delayed lymph node dissection at the time of appearance of regional node metastases. There were 553 patients in this study and patients with primary lesions located on the extremities were chosen because lymph node drainage from these sites is consistent. The study concluded that ELND does not improve prognosis[131,136,137]. The high female:male ratio in the study is justified by the fact that both this anatomical site and clinical stage of the disease are more common in female patients. The female:male ratios were similar to those in other series and were not due to selection bias. When efficacy of ELND is evaluated within sexes, no difference in survival is observed[137].

In a further study the routes of metastatic spread were studied in 1,164 patients with stage I melanoma[133]. Within this study group, 43% developed local recurrence of the disease, 22.7% had regional lymph node metastases as the first recurrence, 7.8% developed disease in distant sites and 13.8% had both regional lymph node and distant metastases. The pattern of dissemination was evaluated according to sex, tumour thickness, ulceration, Clark levels and anatomical site. The disease was more aggressive in men than in women, but the pattern of dissemination was quite similar.

With reference to Breslow thickness, the thicker the primary melanoma, the more frequent were the metastases. The ratio of regional:distant metastases as a first site of recurrence was not different in the 3 groups of thickness (0.1-1.5mm, 1.51-4.0mm, >4.0mm). The same pattern was detected when ulceration was considered. As regards Clark's level, the risk of distant metastases only increased with increasing levels, but when the ratio of regional node:distant metastases was evaluated, no significant difference was found. The highest incidence of metastases was found in patients with melanoma of the trunk but the patterns of dissemination were similar irrespective of the anatomical site of the primary lesion.

It is thus reasonable to conclude that prognostic criteria evaluate the patient's chance of a cure but does not make it possible to foresee if the first recurrence of the disease will be at regional lymph nodes or distant sites since the ratio of regional node:distant metastases of 1.05 was not modified at a statistically significant level in any group.

To prove or disprove that dissection of microscopically positive nodes gives better results than dissecting clinically palpable nodes, WHO[131] compared the survival of patients who received ELND and were found to have occult metastases with the survival of patients who developed clinically detectable node metastases after wide excision of primary melanoma. They showed that survival was similar in both groups.

2.1.9 ELECTIVE LYMPH NODE DISSECTION: CONCLUSION

Two major prospective randomised trials have shown that ELND is not beneficial. However, proponents of ELND have argued that these trials are flawed.

Currently two surgical trials addressing the efficacy of ELND in selected melanoma patients are under way. The first is being conducted by the American National Cancer Institute in Washington DC. This trial is confined to patients with intermediate thickness melanoma (1.00-4.00mm) who have no evidence of metastases in the regional lymph nodes or distant sites by physical examination and laboratory tests. Patients are grouped according to melanoma thickness, location of primary melanoma (proximal extremity versus trunk or distal extremity versus head and neck) and ulceration of epidermis overlying the melanoma on microscopic sections. The second trial is being conducted by the WHO Melanoma Group and is confined to patients with melanoma of the trunk.

2.2 TREATMENT OF LOCAL RECURRENCE

A local recurrence is defined as any tumour that occurs within 3cm of the scar of a previously excised melanoma. Local recurrences are not only considered as extensions of primary melanoma but also as the first sign of distant metastases. They must be distinguished from satellites and in transit metastases that are intralymphatic in origin and which occur between the primary tumour and the regional lymph nodes. There are four different treatment options: surgical excision, regional chemotherapy, radiotherapy and systemic chemotherapy.

In patients with a solitary local recurrence, surgical excision with a 1-3cm margin is probably adequate. Patients who have multiple recurrences should be considered for regional chemotherapy because the risk of additional recurrence and in transit metastases is substantially increased. In these patients, regional chemotherapy by intra-arterial infusion or intra-arterial infusion using a tourniquet outflow occlusion technique may be used.

Transcutaneous intra-arterial infusion achieves high local drug concentrations comparable to those obtained by isolated perfusion and this technique does not require general anaesthesia. Response rates from cisplatin infusion by this method are comparable to those obtained in cisplatin perfusion but this method of treatment is associated with major complications including compartment syndrome and arterial occlusion[138].

In patients in whom surgical excision is not possible, systemic chemotherapy[139,140] or radiotherapy[122,141,142] are therapeutic alternatives. There is evidence to suggest that isolated limb perfusion is the optimal treatment when local recurrence develops on the extremities; 25% to 50% of patients treated with regional perfusion are alive five years later[143,144,145].

Overall, local recurrence implies a poor prognosis and is usually the first sign of metastatic disease[146,147,148,149]. In a series from University of Alabama (UAB)[150] in America and Sydney Melanoma Unit (SMU)[150], among 95 patients who had local recurrences, the median survival time was only 3 years with a ten year survival rate of only 20%. In a study carried out at Duke University Medical Centre[149], it was found that the location of the primary tumour and length of the initial disease free interval influenced survival.

Patients with local recurrence from lesions of the extremities had a median survival of 6.1 years in contrast to patients with lesions of the trunk and head and neck, who had median survival times of 2.1 years and 3.0 years respectively.

2.3 TREATMENT OF RECURRENT REGIONAL METASTASES

2.3.1 IN TRANSIT METASTASES

Recurrent regional disease is defined either as metastases arising from lymphatics between the site of the primary lesion and the regional nodal basin (in transit metastases) or as tumour recurrence within the area of a previous lymph node dissection.

The choice of treatment of recurrent regional metastases is influenced by the number, anatomical location, size and distribution of in transit metastases. Aggressive local treatment is the most effective means of achieving regional disease control[151]. The treatment options are surgery, isolated limb perfusion, regional chemotherapy infusion, radiotherapy, cryotherapy, immunotherapy and systemic chemotherapy.

Surgery is reserved for patients with one or two in transit metastases. Isolated limb perfusion may be the treatment of choice in this group of patients as it has been shown to have a high likelihood of achieving regional control[143,152,153]. However, this treatment option is limited to patients with recurrent extremity disease distal to the upper third of either the arm or thigh.

Intra-arterial infusion of cisplatin has been used as a salvage procedure in patients with advanced disease in whom surgery, isolated limb perfusion and systemic chemotherapy have failed. Workers at the M. D. Anderson Cancer Centre[154], achieved an overall partial response rate of 37%. Increased response may be achieved using a higher drug concentration, which can also be obtained by using an occlusive arterial-infusion (inflow technique) or an intra-arterial infusion with a tourniquet (outflow technique).

Radiotherapy using high dose radiation may provide significant palliative benefit and occasionally prolongs regional control[155,156]. Recent studies indicate hyperthermia of lesions before or during radiotherapy may enhance response rate[157,158]. Blake et al[159] using fast-neutron therapy reported a 71% complete response rate with a recurrence rate of only 9% in 68 patients. However, in 22 patients complications occurred.

Cryotherapy using liquid nitrogen can be used in patients whose tumours have not responded to other forms of treatment or who are unable to undergo other therapy[160]. It is most effective for intradermal metastases.

BCG has been shown to induce regression when injected into intradermal metastases[161,162]. Bauer et al[163] reported a complete response in approximately 67% of injected nodules and a significant decrease in size in 29%. Systemic chemotherapy using dacarbazine alone or in combination has limited success, usually with a short duration of response[140].

2.3.2 RECURRENT NODAL METASTASES

Clinically detectable metastases within or adjacent to the area of a previous lymph node dissection usually manifests in two clinical situations. The first is in the patient with recurrent disease who is known to have undergone a complete lymph node dissection initially. Recurrent nodal metastases are often seen at the boundaries of the previous surgical site. In this situation, local excision of the recurrence and surrounding soft tissue is sufficient. Surgical margins must be histologically clear of tumour.

In the second situation, the patient presents with recurrent regional metastases but the extent of previous lymph node dissection was incomplete. In this case, a complete lymph node dissection is carried out.

The use of pre-operative chemotherapy is being evaluated at the M.D. Anderson Centre[164]. An initial pilot study of 48 patients receiving two cycles of pre-operative cisplatin, vinblastine and dacarbazine for advanced nodal disease demonstrated an overall response rate of 50%. After nodal surgery, those patients who have at least a partial response are continued on chemotherapy. The rationale is to identify patients with 'resistant' tumours who are not likely to benefit from further chemotherapy. It is not known if patients responding to neoadjuvant chemotherapy have a higher survival rate.

In patients with multiple regional metastases the use of adjuvant radiotherapy can improve local control although it may not increase survival[142].

2.4 TREATMENT OF ADVANCED MELANOMA

The aim of treatment is to relieve symptoms and prolong life. There are several modalities of treatment. The option of not providing treatment is an important one to consider, especially in asymptomatic patients, in those who are terminally ill and in the elderly.

2.4.1 SURGERY

Surgery is an effective palliative treatment for isolated metastases. It offers effective and quick palliation, and in some cases long-lasting survival[165,166]. It is effective only against disease at that local site. Favourable outcomes from surgical resection of distant metastases in selected patients have been shown[167,168,169]. Isolated visceral metastases, especially in the brain and lung, may be amenable for surgery in exceptional circumstances. The decision to use palliative surgery depends on the site of the disease and duration of anticipated survival but each case should be considered on its own merit.

2.4.2 RADIOTHERAPY

Radiotherapy has been shown to be beneficial in treatment of symptomatic patients. The main indications are treatment of dermal, subcutaneous, lymph node, brain and bone metastases. Other indications include spinal cord compression and symptomatic isolated visceral metastases not amenable to surgery. High energy radiation can relieve the pain of bone metastases within one week. Irradiation from a high energy proton beam can effectively treat superficially located metastases in skin or soft tissue. In the majority of cases, high dose, low fraction irradiation induces significant regression of metastases located on skin, subcutaneous tissue or distant lymph node[170,171].

2.4.3 CHEMOTHERAPY

DTIC remains the most widely used agent for treatment of systemic melanoma. The response rate is in the range of 15% to 25%[172,173]. DTIC is well tolerated with the only major side effects being nausea and vomiting. Patients with metastases in skin, subcutaneous tissue or lymph node most frequently respond. The median duration of response is 5 to 6 months. Complete response is observed in only 5% of the patients[174].

The nitrosoureas also have activity against melanoma with response rates between 10% and 18%[175-178]. Carmustine (BCNU), lomustine (CCNU), semustin (methyl CCNU) and fotemustine are the best studied of this group. Combination therapies have been reported to produce higher response rates when compared to single agents, although these studies have not directly compared treatments in prospective randomised trials nor have they demonstrated a survival advantage.

Drug combinations of DTIC and cisplatin[179], DTIC and vindesine[180], and vindesine and cisplatin[181] produced response rates between 20% and 30%. Two other combination regimens were also reported to induce responses in 30% to 40% of treated patients[181,182]. These were BHD (BCNU, hydroxyurea and DTIC) and BOLD (bleomycin, vincristine, CCNU and DTIC). The Southwest Oncology Group[183] carried out a randomised study of BHD regimen versus DTIC plus BCG and showed a response rate of 31% versus 18% but the overall survival was not improved in patients treated with BHD regimen.

More recently a trial was conducted using a three drug regimen containing DTIC and cisplatin in combination with vindesine, vinblastine or BCNU. The combination of DTIC, cisplatin and vindesine showed a 30% to 40%[184,185] response rate and similar results were achieved using vinblastine instead of vindesine with DTIC and cisplatin[186]. Most responses were partial with a median duration of 6 to 9 months.

The Dartmouth four drug regimen developed at the Dartmouth Medical Centre in America using DTIC, cisplatin, BCNU and tamoxifen has shown a 50% response rate[186,187]. It was also seen that tamoxifen was crucial as only a 10% response rate was achieved when it was deleted from the regimen[187].

2.4.4 IMMUNOTHERAPY

Immunotherapy can be considered as an active form of treatment for metastatic melanoma. In patients with localised superficial skin metastases without bulk disease or visceral metastases, BCG will induce regression of most lesions into which it is injected[188,189,190].

Interferon-alpha and interleukin-2 have been shown to be active against melanoma cells and response rates of 10% to 20%[191-194] have been documented, although these are not significantly different from single agent DTIC.

Monoclonal antibodies have also been shown to produce major tumour regression in patients with metastatic melanoma[195-199]. Currently, combinations of interferon-alpha, interleukin-2 and chemotherapy are being evaluated.

Tumour vaccine containing irradiated tumour cell, partially or completely purified melanoma antigen or tumour cell membrane from melanoma cells infected with virus (viral oncolysates) are also being evaluated. The three year survival rate of patients with stage II melanoma who received irradiated allogeneic melanoma cell vaccine (MCV) was 60%, much higher than that of historical controls[200].

In a further study[201] of 95 patients with stage III melanoma who were vaccinated with melanoma antigen vaccine (MAV) it was found that 39 patients who developed a strong delayed type hypersensitivity to the vaccine had longer disease free intervals than the 42 patients who did not respond. The median disease free survival of patients who developed an intermediate response was 24 months. Melanoma vaccines are still experimental and their clinical effectiveness is yet to be established.

CHAPTER 3

GLASGOW EXPERIENCE OF ISOLATED LIMB PERFUSION (ILP)

3.1 INTRODUCTION

Surgery is still the treatment of choice for primary cutaneous malignant melanoma but the optimal extent of surgery remains controversial. Local excision of the primary lesion alone does not always control disease and treatment failure is reflected in the development of local, regional or systemic recurrences. Patients with primary melanoma greater than 1.5 mm thick carry a poor prognosis and as thickness increases, the likelihood of satellite lesions and in transit metastatic disease also significantly increases[202]. Regional chemotherapy is an attractive theoretical option as this treatment can eliminate microscopic metastatic cells and deposits not removed by primary surgery.

Despite the introduction of isolated regional limb perfusion 30 years ago, there still appears to be no consensus for the support of routine use of limb perfusion as prophylactic treatment in the management of stage I melanoma patients. This problem arises largely as the results quoted in support of adjuvant therapy come from non randomised retrospective studies using historical controls that lead to lack of credibility. These studies have often failed to withstand statistical scrutiny.

After reviewing results worldwide it was decided to establish a unit, the first and only unit of its kind in Scotland and the North of England at Gartnavel General Hospital in Glasgow in 1984 to offer ILP to high risk stage I melanoma patients and patients with recurrent melanoma of the limb. In many centres ILP has now become established as the treatment of choice in the management of patients with recurrent malignant melanoma of the extremities. It has been shown to have a high likelihood of achieving regional control with an overall objective response of 80%, with similar proportions of complete and partial remissions and improved limb salvage rates[143,152,153]. When this unit was established several questions needed to be answered.

1) **Could ILP be performed safely?**
2) **Would it have deleterious effects on patient survival or disease free interval?**
3) **Would ILP improve the long term survival rate and disease free interval in patients with high risk primary melanoma of the limb?**

3.2 HISTORY OF ISOLATED LIMB PERFUSION

The unpredictable natural history of malignant melanoma renders it unusual among human tumours. The tendency to arise from a benign lesion, its capacity to spread without regard for anatomical boundaries, its ability to lie dormant for many years only to reappear and bring about the death of its host and finally its tendency for spontaneous regression contribute to our lack of understanding of melanoma and our inability to deal with it more effectively. Until the 1950's, treatment of malignant melanoma was almost exclusively surgical, consisting of wide excision of primary tumour and regional lymphadenectomy.

In 1940, Gilman and Philips[203] first reported the use of nitrogen mustard in the treatment of cancer and within five years, Kloops et al[204] and Bierman et al[205] independently administered intra-arterial nitrogen mustard through a small indwelling polyethylene catheter placed in the artery supplying the tumour bearing area. Responses were seen but the technique was fraught with complications. The best results were obtained when venous return from the area involved was blocked. In 1956, at Tulane University, Ryan et al[206] successfully isolated the limbs, mid gut and livers of dogs and perfused them.

In 1957, Creech et al[207] combined these procedures and formed an isolated circuit with an extra corporeal circulation using a pump-oxygenator which made it possible to administer large drug doses. The first patient was treated at the Charity Hospital of Louisiana, New Orleans[207,208]. Luck et al[209] discovered that melphalan was the most active agent to inhibit the growth of malignant melanoma in man and since then it has become the agent of choice. In 1967, Cavaliere et al[210] laid the foundation for perfusion with hyperthermia when they described the susceptibility of cancer cells to high temperatures.

Since 1961, many investigators have treated patients with a variety of solid tumours with isolated perfusion. The best response rates were seen in malignant melanoma and sarcoma and encouraging results were achieved without undue toxicity. Only in melanoma was complete remission seen. During the development of the isolation perfusion technique, oxygenation and heparinization were reported to have therapeutic potential. Hyperoxygenation of perfused tissue was shown to potentiate the radiomimetic effect of the alkylating agent[211,212].

Heparin was shown to inhibit deposition of metastatic tumour cells and could limit a potential source of in transit metastasis[213]. Cavaliere[210] studied hyperthermic

isolated regional perfusion in 18 patients using no chemotherapy and no tourniquet and noted tumour regression in 14 of 18 patients with 10 complete remissions following perfusion with hyperthermia of 39°C to 41°C for 4 hours. Stehlin[214] also reported an increased response rate using hyperthermic perfusion with the same drug. The application of heat not only has selective tumouricidal action but also enhances the action of chemotherapeutic agents. Thus lower drug doses can be used without loss of therapeutic potential, reducing the risk of local and systemic toxicity.

The most commonly used drug in treating melanoma of the limb is L-phenylalanine mustard (melphalan), an alkylating agent. It is related to nitrogen mustard but melphalan has a longer duration of action and is less of a vesicant. Melphalan was originally selected because phenylalanine, a metabolite of melanin, was thought to carry the attached cytotoxic alkylating radical into the melanin producing neoplastic cell. Dosage is determined on the basis of body weight, limb volume or haematocrit.

3.3 ADVANTAGES OF ISOLATED LIMB PERFUSION

Isolated perfusion was originally developed to increase drug dosage in the treated area over that which could be achieved by systemic administration while avoiding major systemic toxicity. Dose was limited by toxicity to the nerves, blood vessels and muscles in the perfused area.

The advantages of hyperthermic isolated regional perfusion are:-

1) A 6 to 10-fold increase in drug concentration in the isolated limb over that obtained by systemic administration.
2) Improved perfusion of the tumour and surrounding area including the regional lymph nodes
3) An increase in the PO_2 in the isolated tissue which may potentiate the action of alkylating agents and have selective tumouricidal action.
4) Hyperthermia may increase the chemical activity of a drug, increase the metabolism of tumour cells, produce vasodilatation for better perfusion and have a selective tumouricidal action.
5) Heparin used in the extracorporeal circulation may have both antimetastatic effect and selective tumouricidal action.
6) Isolation of the treated area decreases the systemic toxicity and protects "host resistance".
7) There is some evidence that destruction of tumour cells in situ initiates an autoimmunization process.

3.4 RESULTS OF APPLICATION OF MILD HYPERTHERMIA IN ISOLATED LIMB PERFUSION FOR STAGE I MELANOMA OF THE EXTREMITIES

3.4.1 PATIENTS

Between August 1985 and June 1993, 99 patients with clinical stage I malignant melanoma of the upper and lower limb were treated with hyperthermic isolated regional limb perfusion using melphalan in a prospective non-randomised case control study. All the patients in the study had been referred following surgical biopsy or excision from hospitals throughout Northern Britain for ILP. After discussion with statisticians and relevant clinicians, it was decided that a randomised trial could not be completed within a reasonable time scale.

The criteria for inclusion into the study included patients with stage I melanoma affecting the upper or lower limb excluding high posterolateral and deltoid region which are outside the effective perfusion zone, absence of in transit, regional or distant metastatic disease, vascular suitability for catheterisation and adequate medical status for general anaesthesia.

Of the 99 patients, 31 (31.3%) were males and 68 (68.7%) were females with a mean age of 56 (range 21-84) years. The lower limb was involved in 72 (72.7%) and the upper limb in 27 (27.3%). Histology of the tumour included 30 superficial spreading melanoma, 30 nodular melanoma, 3 acral lentiginous melanoma and 5 lentiginous maligna melanoma. On 31 patients no information on the type of tumour was available. The mean Breslow tumour thickness was 3.4 mm (range 0.9-10.0mm). Clark level was available on 71 patients. Table 1 summarises details of the 99 patients.

3.4.2 PROCEDURE OF ILP

ILP is carried out under general anaesthesia. The patient is positioned so that a tourniquet can be placed around the base of the limb. Since this technique uses hyperthemia, the operating room is kept at 21ºC and the patient is placed on a heated water blanket (Hawksley Ripple Heat System with custom blanket) at 40ºC. Temperature is monitored by placing four to six thermistor probes (Yellow Springs) on the skin surface and the temperature readings are displayed continuously on a monitor screen (Siemann Sirecust).

AGE - DISTRIBUTION										
RANGE	0 - 9	10 - 19	20 - 29	30 - 39	40 - 49	50 - 59	60 - 69	70 - 79	80 - 89	TOTAL
NUMBER	0	0	7	11	15	28	14	20	4	99

SEX - EXTREMITY DISTRIBUTION			
	UPPER LIMB	LOWER LIMB	TOTAL
MALE	7	24	31
FEMALE	20	48	68

SIZE OF PRIMARY						
CASES	UNKNOWN	0 - 1.5 mm	1.6 - 3.0 mm	3 - 4.00 cm	> 4.00 cm	TOTAL
NUMBER	3	3	48	18	27	99

ANATOMICAL SITE OF PRIMARY							
	PLANTAR	LEG	THIGH	UPPER ARM	LOWER ARM	PALMAR	TOTAL
NUMBER	15	47	10	11	16	1	99

Table 1 Details of patients undergoing adjuvant isolated limb perfusion

An arterial line is placed in the radial or dorsalis pedis artery of the affected limb to monitor arterial pressure. The transcutaneous oxygen tension in the skin is measured using the Radiometer TCM2 System (Figure 10) at several points in the treated and untreated opposite limb. The leg is placed in a cotton stockingette and a gamgee is placed around the foot to protect the skin from the direct heat and then the heated water blanket is wrapped around the limb (Figure 11).

To carry out perfusion on the lower limb, the external iliac vessels are approached retroperitoneally via an oblique incision in the iliac fossa parallel to the inguinal ligament. Iliac lymph nodes along the vessels are removed. Figure 12 shows the iliac vessels exposed and isolated prior to the perfusion. All the minor branches of the external iliac artery and all tributaries of the external iliac vein are ligated and divided. The inferior epigastric vessels are ligated but the obturator vein is dissected and temporarily occluded to decrease leakage from the perfused limb. The internal iliac vein is clamped throughout the perfusion.

Prior to cannulating the vessels, systemic heparin (150 iu/kg) is given intravenously. A longitudinal arteriotomy and venotomy are carried out and a 16 or 18 French polyvinyl chloride cannula (Bard) is placed in the artery and an 18 or 20 French cannula in the vein. It is important that the tip of the cannula lies in the femoral triangle inferior to the inguinal ligament and distal to where the lower edge of the tourniquet will lie. The cannulae are secured in place using two cotton snares per cannula. A tourniquet is applied using Esmarch's bandage along the gluteal crease, drawing it up through the crotch and over the upper lateral hip (Figure 13). A Steinmann pin is driven into the iliac crest to hold the tourniquet in position.

Perfusion of the upper limb is carried out through the distal part of the axillary vein and artery. The vessels are exposed through an incision in the skin of the axilla. Lymph nodes on the first portion of the axillary vein are removed. A 12 to 16 French cannula is used in the artery and a 16 to 18 French cannula in the vein. A tourniquet is placed around the root of the limb and a Steinmann pin is placed in the head of the humerus to prevent dislodgement of the tourniquet.

The perfusion apparatus traditionally consists of a simple roller pump (Stockerte instrument) attached in series to a disposable hybrid oxygenator (Bard) which incorporates an integral heat exchanger. Figure 14 is a diagrammatic illustration of the isolated limb perfusion circuit. The pump oxygenator is primed with 500 ml lactated Ringer solution and 400 ml of matched packed cell to which is added 3,000 units of heparin prior to connecting the arterial and venous cannula to the circuit.

Figure 10 Radiometer TCM2 System for measuring transcutaneous oxygen

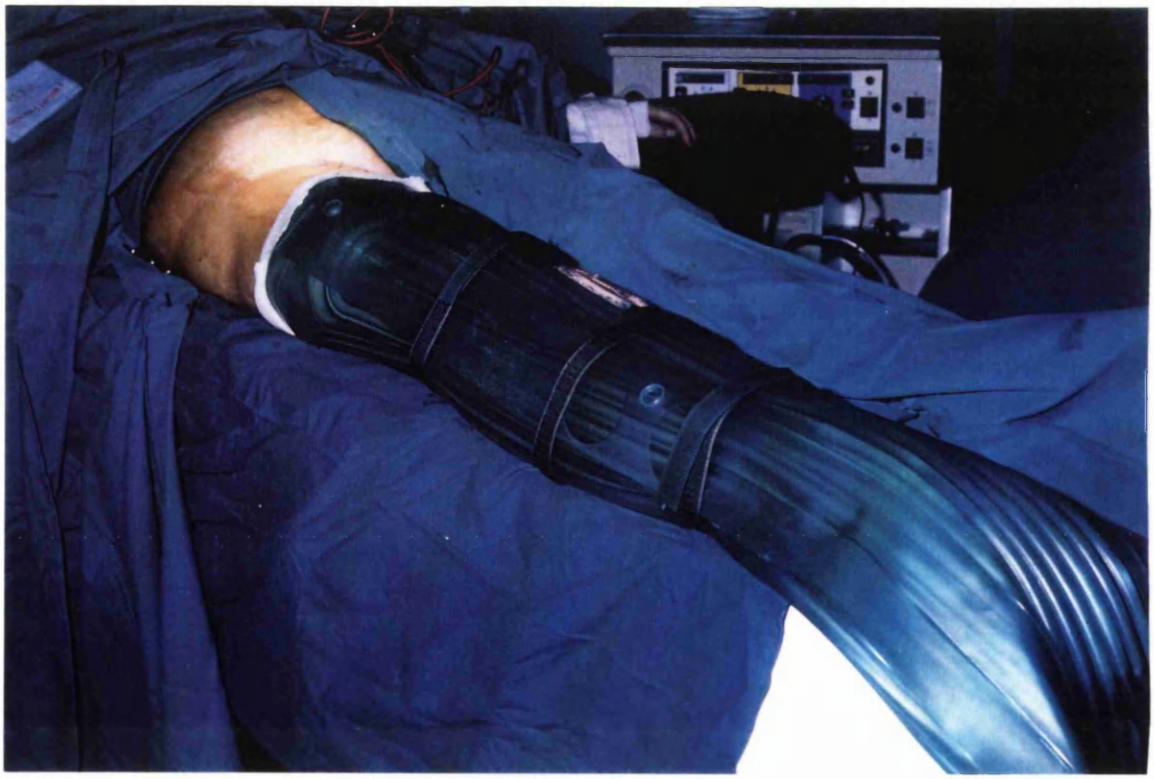


Figure 11 Leg wrapped in a cotton stockingette and gamgee with water blanket wrapped around the limb

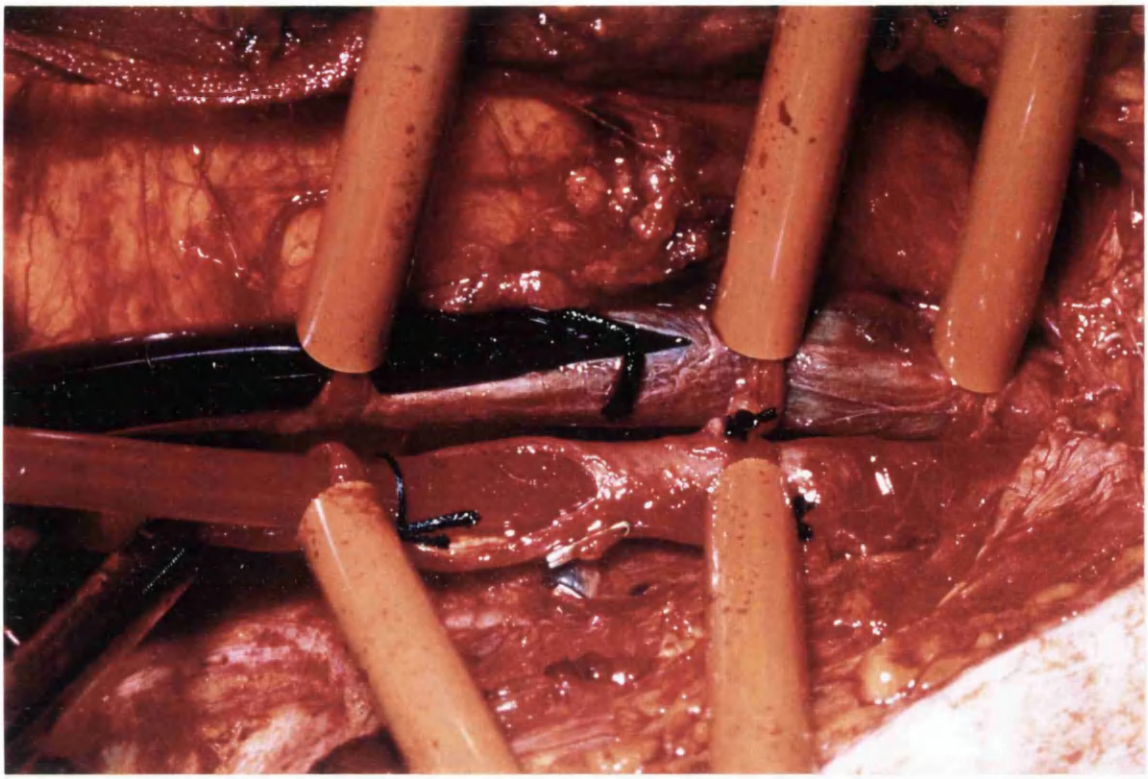


Figure 12 Iliac vessels exposed and isolated prior to the perfusion

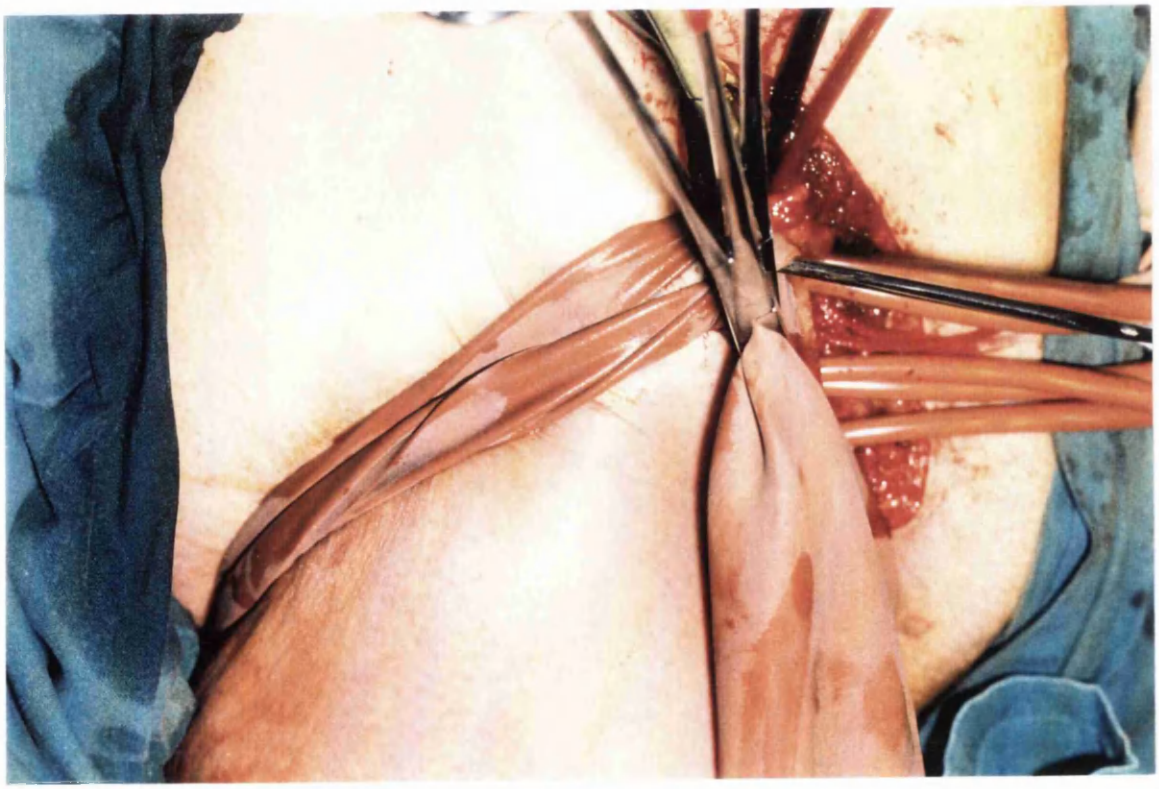


Figure 13 Esmarch's bandage along the gluteal crease

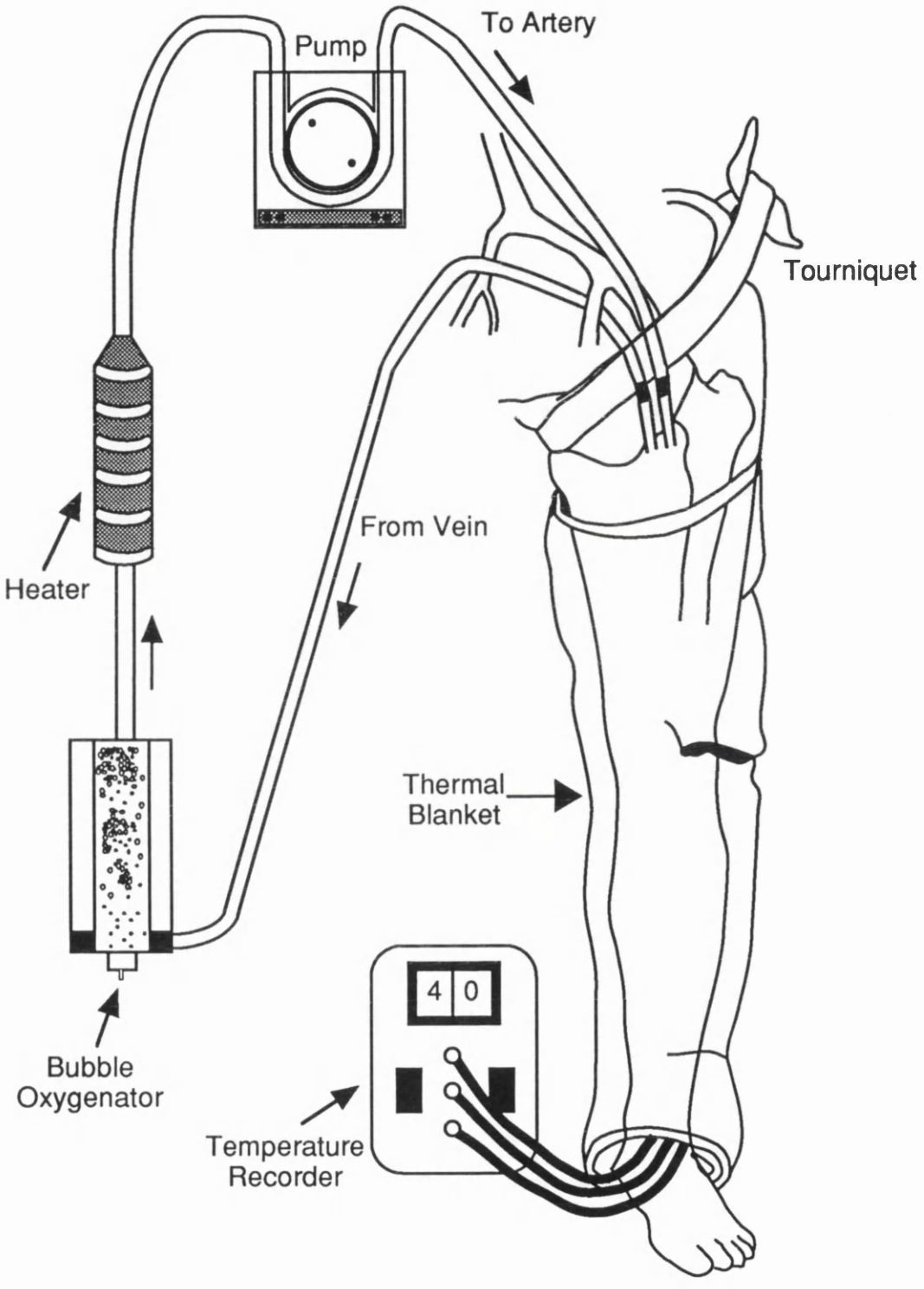


Figure 14 Diagrammatic illustration of the ILP circuit

Once the circuit has been established, 5 ml of 20% fluorescein is injected into the arterial line and with the room darkened, a portable ultraviolet lamp is used to inspect the skin above and below the tourniquet. Any significant leak from the limb to the systemic circulation can be noted and corrected. The perfusate inflow temperature is maintained at 40-41°C resulting in a limb skin temperature of up to 40°C (mild hyperthermia). The limb pressure is ideally maintained just below the systemic arterial pressure and flow rates range from 250 to 900 ml per minute.

Once the limb temperature reaches 38ºC, melphalan is administered into the arterial port of the bubble oxygenator. The dosage used is 1.75 mg per kilogram body weight for the lower limb and 0.75mg per kilogram body weight for the upper limb and is given as a bolus. Perfusion is carried out for one hour during which continuous monitoring of flow rate, oxygen saturation, perfusion pressure and temperature is undertaken. The perfusate is oxygenated using 100% oxygen.

At the completion of perfusion, the circuit is washed out with 2 litres of Ringer lactate, after which the tourniquet is released, the cannulae withdrawn and the vessels repaired with 5'0' non absorbable sutures. The effect of heparin is reversed with the appropriate dose of protamine sulphate. The approximate length of the operation is between 120 and 180 minutes. At the end of the operation, where appropriate, a wide local excision of the primary tumour is carried out.

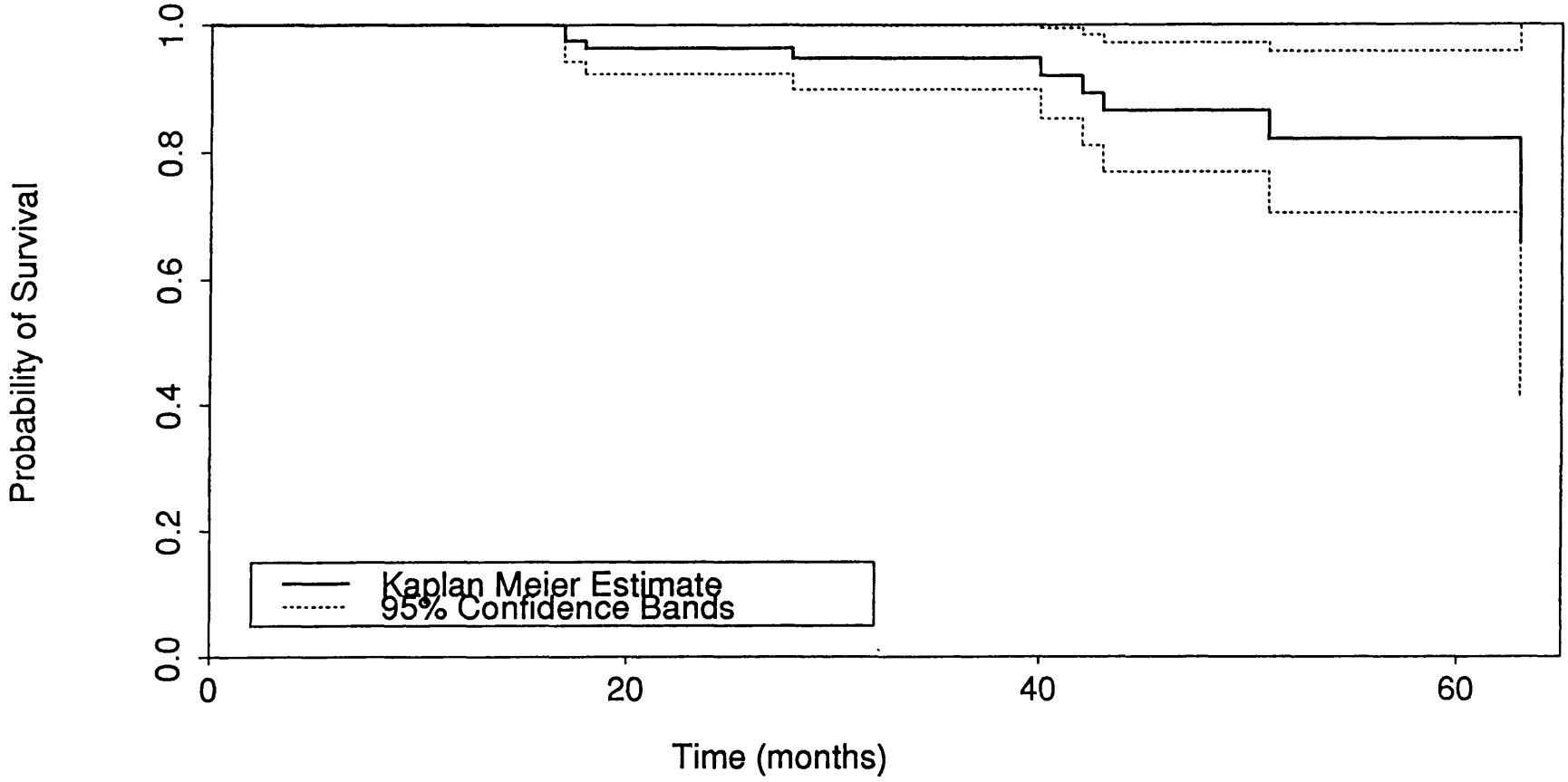
Postoperatively, the patient is nursed with the leg elevated in the high dependency area of the ward. On day 1 patients are encouraged to mobilise. Daily measurements of full blood count and electrolytes are carried out. Patients with haemoglobin less than 9 g/dl are transfused. The average length of stay is 9 days (range 6-12). Patients are followed up at a perfusion clinic 6 weeks postoperatively and at three monthly intervals thereafter for 5 years.

3.4.3 RESULTS

Survival and disease free survival was estimated using the Kaplan-Meier method on all the data. Significance tests of differences among subgroups such as those defined by sex and site were carried out by means of Log-Rank Tests or Proportional Hazards Models as appropriate.

Survival

Nine patients have died from the disease at the time of writing. The 2 and 5 year survival was 96% and 82% (Figure 15) respectively and the disease free survival 83%



Time (t)	0	12	24	36	48	60
Number at risk	99	89	67	43	23	8
Deaths in previous year		0	3	1	3	1

Figure 15 Probability of survival for stage I melanoma patients following ILP

and 61% (Figure 16) respectively. The mean follow up to recurrence was 37 months (range 9-95) and to death was 35 months (range 17-63). There were 20 patients who developed local regional recurrences; 14 lymph node recurrences and 6 patients developed local skin recurrences. Seven patients developed distant metastases. The mean time to recurrence was 21 months (range 1-52 months).

Survival by sex

The 5 year survival and disease free survival for females was 84% and 66% respectively and for males 77% and 50% respectively. For both survival and disease free survival, females appear to have better prospects than males, but this is not confirmed by Log-Rank Tests (p value for survival = 0.284, p value for disease free survival = 0.372)

Survival by site

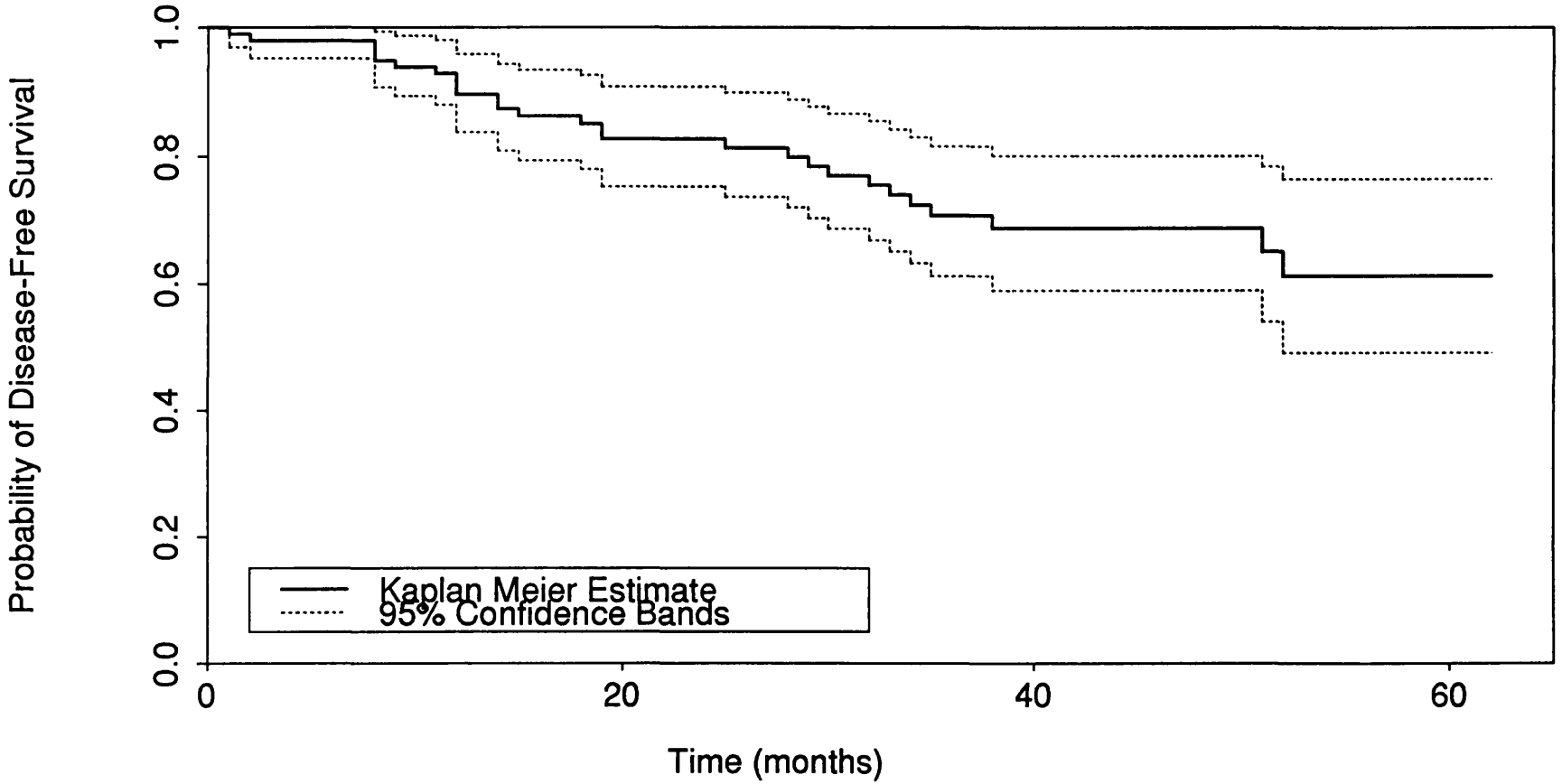
The 5 year survival and disease free survival in patients with lower limb melanoma was 76% and 57% respectively when compared to 95% and 74% respectively for patients with upper limb melanoma. Although the patients with upper limb melanoma seemed to have a better prognosis than patients with lower limb melanoma, the difference was not statistically significant for either 5 year survival (p = 0.582) or disease free survival (p = 0.101).

When all the effects of the six prognostic risk factors, i.e. sex, age, site, Breslow tumour thickness, Clark level and histogenetic type, were considered through a proportional hazards model, none of these prognostic factors proved significant for either survival or disease free survival.

Overall, survival and disease free survival prospects appear reasonable, although there are indications of poorer survival and disease free survival for males and for those with lesions on the legs. These, however fall far short of any significance, possibly due to the small numbers in the different subgroups.

COMPLICATIONS

There were no hospital deaths associated with ILP in the study. No patient required amputation either as a complication of the perfusion or from their disease. Immediate complications included deep vein thrombosis, pulmonary embolus, wound infection, bleeding and haematoma. Systemic complications included leucopenia, thrombocytopenia and pancytopenia, all of which resolved spontaneously (Table 2).



Time (t)	0	12	24	36	48	60
Number at risk	99	82	61	38	22	7
Recurrences in previous year		10	6	8	1	2

Figure 16 Probability of disease free survival for stage I melanoma patients following ILP

REGIONAL	NO. OF PATIENTS
Severe Cellulitis	9
Nerve Symptoms	6
Muscle Symptoms	4
SYSTEMIC	
Leucopenia	18
Thrombocytopenia	6
Pancytopenia	12
GENERAL	
DVT	3
PTE	4
Wound Infection	4
Haemorrage	1
Haematoma	1

Table 2 Postoperative complications following ILP for primary melanoma

Toxicity after perfusion was graded according to the Wierberdink[215] Scale as shown in Table 3. Fifteen (14%) patients were graded I, 42 (40%) graded II, 39 (43%) graded III and 3 (3%) patients were graded IV.

3.4.4 DISCUSSION

Following the publication by Krementz et al[216] in 1962 of the clinical improvement in patients having received isolated limb perfusion, the idea of a prospective randomised study was shelved. In retrospect this was unfortunate in view of the changing nature of the disease and the improvement in survival rates being reported with other therapeutic modalities.

A literature review of adjuvant ILP for stage I malignant melanoma of the extremities is presented in Table 4. None of these studies were randomised and most authors compared their results with literature reports; in only three studies were there any controls. It is, however, important to interpret historical controls with caution.

The only prospective randomised study of adjuvant ILP was reported by Ghusen et al[152] in West Germany. The study consisted of 107 patients with melanoma of the extremities who were randomised to having wide local excision with regional lymph node dissection (RLND) or wide local excision, RLND and hyperthermic isolated perfusion with melphalan. The disease free survival time was chosen as the criterion for evaluation.

Intermediate analysis revealed a highly significant difference between the two groups, with 21 recurrences in the control group and 4 in the perfused group, i.e. recurrence rate in the control group was 27.8% in stage I, 31.6% in stage II and 58.5% in stage III compared to 5.6% in stage I, 5.5% in stage II and 12.5% in stage III in the perfused group.

This study was discontinued prematurely as the authors concluded that adjuvant regional hyperthermic perfusion had been shown to be superior to surgery alone. Five years later, Ghusen et al[227] reported the disease free survival of patients who had perfusion to be still superior. The retrospective breakdown into different risk groups according to tumour thickness also demonstrated a significant difference. They concluded that the overall 5 year survival rate of 90% in the perfused group compared to 60% in the control group and interpreted these results to demonstrate the benefits of adjuvant hyperthermic regional isolated limb perfusion.

GRADE	DESCRIPTION OF REACTION
I	No subjective or objective evidence of reaction.
II	Slight erythema and/or oedema.
III	Considerable erythema and/or oedema with some blistering; slightly distributed motility possible.
IV	Extensive epidermolysis and/or obvious damage to the deep tissues, causing definite functional disturbances, threatening or manifest compartment syndromes.
V	Reaction which may necessitate amputation.

Table 3 Wierberdink grading system for the reaction of normal tissue to ILP

FIRST AUTHOR	YEAR	NO. OF PATIENTS	LEVEL/ DEPTH	TYPE OF EXCISION	RLND	SURVIVAL RATE (%)	
						5 YEAR	10 YEAR
SUGARBAKER[217]	1976	144	II - V	WE	14%	88	84
WAGNER[218]	1976	144	>2 mm	WE	NO	93	
BULMAN[219]	1980	21	>0.76 mm	WE	100%	76	
REGE[220]	1982	39	II - V	WE	100%	91	86
JANOFF[221]	1982	122	II - V	WE	100%	90	76
KREMENTZ[222]	1985	381	III - V	WE	107 Without 274 With	87 84	80 75
SCHRAFFORDT-KOOPS[223]	1987	225	III - V	WE	NO	82	73
KRIGE[224]	1988	95	III - V	WE	NO	83	
LEJEUNE[225]	1989	129	II - V	WE	100%	92	
KETTELHACK[226]	1990	47	III - V	WE	30%	89	

WE = WIDE EXCISION

RLND= RADICAL LYMPH NODE DISSECTION

Table 4 The literature review on adjuvant ILP for stage I malignant melanoma

Sadly, this study has several flaws. Firstly it was prematurely closed, which resulted in a small number of patients being recruited. Thus the data are suggestive but do not show statistically significant evidence for the efficacy of adjuvant ILP. The p value for the difference between the perfused and non perfused groups was at a significance level of P = 0.09 (single factor) and a multivariate analysis had not been done. It was also not clear from the study whether randomisation was according to tumour thickness or not. Furthermore no reference was made to survival or disease free interval with respect to the number of positive lymph nodes. Although this is the only prospective randomised study, the results should be cautiously interpreted.

There are only three studies that have matched patients by prognostic factor. The first study[228] consisted of 120 patients, all women with stage I disease who had ILP and they were matched by sex and tumour location with 116 patients undergoing local excision only in the Sydney Melanoma Unit in Australia.

The second study[229] compared 227 patients with stage I disease having ILP with 238 matched controls (age and site) who had wide local excision only from the same geographical area in the Netherlands. The last study[230] compared 151 patients with stage I disease treated with wide local excision and ILP with melphalan and also DTIC with 151 patients matched for prognostic factors undergoing wide local excision alone from University of Alabama, USA and the Sydney Melanoma Unit, Australia.

The first study[228] showed that women who had ILP and wide local excision had a significantly higher ten year disease free interval, higher ten year survival rate and significantly fewer local regional recurrences than there were in the group treated with wide local excision only. These results should be interpreted with caution as all the patients were female and in stage I disease, females have a better prognosis. Furthermore, all tumours were confined to the lower limb, a site associated with improved survival.

In the second study[229], it was not possible to demonstrate a statistically significant effect of ILP in terms of time to limb recurrence, time to regional lymph node metastases, time to distant metastases, disease free interval and survival. There was no significant difference in prognostic factors of age, sex, ulceration or tumour thickness.

In the third study[230] a subset of patients with lesions > 2.00 mm in thickness showed a significant improvement in disease free and overall survival rates with melphalan but not with DTIC.

Interestingly, in the first study, when the perfused patients in The Netherlands were matched using controls from Australia, there was a significant improvement in survival rates but this was not demonstrated in the second study when the control group came from the same geographic area. This phenomenon, however, was not observed in the third study when controls were taken from another area.

It was also noted that the 5 year disease free survival rate for the non-perfused patients in America and Australia was very low compared to that of the non-perfused patients in The Netherlands. This finding is interesting as the questions that arise are: are there different forms of the disease in different latitudes of the world, or is the difference due to the genetic make-up of individual races?

From these studies, no definite advantage of ILP over wide local excision could be established.

The result from the Glasgow unit is unique in that this is a single centre, single surgeon unit. Thus the treatment of ILP is uniform with no interoperator variation to account for differences in results. It was an interesting finding of this study that there were no significant prognostic factors with relation to either survival or disease free survival. This included the Breslow thickness that has been quoted as the single most important prognostic factor with relation to survival (section 1.5.2).

A possible explanation is that, since in this unit routine lymphadenectomy is not performed, there may be a significant group of patients who may have nodal metastases. The presence of nodal involvement would immediately alter the staging of the patient and subsequently effect the prognosis of the disease. The importance of the proper staging of patients especially those with stage I disease will be shown and fully discussed in chapter 6.

The results of the present study show that ILP can be performed safely with acceptable morbidity. There were no deleterious effects on survival following ILP. The intermediate analysis of the results show that ILP patients appear to have acceptable survival and disease free survival rates. Although there may be a survival advantage, it does not reach statistical significance. Full evaluation must await longer term follow up.

3.5 RESULTS OF ISOLATED LIMB PERFUSION FOR RECURRENT LIMB MELANOMA

3.5.1 PATIENTS AND METHODS

ILP was made available in Scotland in August 1984. Between then and June 1993, 103 patients with stage II-III malignant melanoma underwent therapeutic limb perfusion for recurrent melanoma of upper and lower limb. All patients in the study were referred from hospitals throughout Northern Britain and Ireland. The criteria for inclusion were patients with recurrent or advanced melanoma confined to a single limb, vascular suitability for catheterisation and adequate medical status for general anaesthesia. Disseminated disease was generally considered to be a contraindication, unless the local disease was so gross that ILP was considered justifiable to achieve local disease control.

There were 25 males and 78 females with a mean age of 62 (range 21-92) years. The lower limb was involved in 95 patients and the upper limb in 8 patients. The clinical details of the patients are summarised in Table 5. Prior to the treatment with ILP, 75 patients had primary tumour and recurrences treated with excision, 24 patients had had excision and groin dissection at the time of presentation with the primary tumour, 2 patients presented with satellite lesions with the primary intact, 1 patient had had excision, radiotherapy, followed by groin dissection and further chemotherapy and 1 patient was diagnosed following a groin dissection and later admitted that he had a "warty" mole on his thigh that had fallen off one year prior to the groin dissection.

The number of recurrences prior to ILP was 1 in 45 patients, 2 in 33 patients, 3 in 12 patients, 4 in 5 patients and 5 in 4 patients and more than 5 recurrences in 4 patients. The mean recurrence interval prior to ILP was 20 months (range 1-129). Six patients had other associated malignancies. These included breast cancers in 2, chronic lymphatic lymphoma in 1 prior to developing the primary melanoma, and 3 patients developed breast, uterine and ovarian tumours following ILP. One patient developed a second primary melanoma.

The technique of regional isolated limb perfusion was performed as described on pages 58-60. For upper limb recurrence, axillary perfusion and reperfusion was performed if necessary. In the lower limb, the perfusion was carried out at the iliac level and if reperfusion was needed, it was done at iliac, femoral or popliteal level. The mean time to ILP was 48 months (range 1-290) from the time of surgery for their primary melanoma.

SEX - SITE DISTRIBUTION

	UPPER LIMB	LOWER LIMB	TOTAL
MALE	3	22	25
FEMALE	5	73	78
TOTAL	8	95	103

LEVEL OF PERFUSION

LEVEL	FIRST PERFUSION	REPEAT PERFUSION
Iliac	95	11
Femoral		5
Popliteal		3
Axillary	5	
Subclavian	2	

Table 5 Details of patients undergoing ILP for recurrent limb malignant melanoma

3.5.2 RESULTS

One hundred and twenty-two therapeutic perfusions were performed on 103 patients. The response rates were classified according to the Union Internationale Contra le Cancer (UICC) Criteria[231] (Table 6). Eighty-seven patients had complete response, 34 had partial response and 1 had no response from the perfusion (Table 7). Figures 17-20 show the sequence of changes in a single recurrent malignant melanoma nodule following ILP. Sixty-six patients have died at the time of writing. The mean time to death was 22.5 months and the average follow up time of the 37 survivors was 34.5 months. The overall 2 and 5 year survival was 50% and 26% respectively (Figure 21). Eight patients died from other causes including myocardial infarction, uterine carcinoma, ovarian carcinoma and pneumonia.

Survival according to limb: The overall 2 and 5 year survival for the lesions in the upper limb was 38% and 18% respectively whilst in the lower limb the survival was 52% and 27% respectively.

Survival according to sex: The overall 2 and 5 year survival for the females was 60% and 35% respectively and 18% and 4% respectively for the males.

Survival according to sex and limb involved: There were no survivors in the males with upper limb lesions at 2 years whilst in the females with upper limb lesions the 2 year survival was 75%. In the group with lower limb lesions, the 2 and 5 year survival was 18% and 6% respectively in the males compared to 61% and 34% respectively in females (Figure 22).

Survival according to local recurrence

Of the 42 patients who developed a local recurrence, 36 patients were dead within the first 24 months of the perfusion; of the 11 who developed both systemic and local recurrences, none were alive after twelve months.

Rate of local recurrence

Local recurrence in this study is defined as having recurrence either in or around the scar of the primary lesion, regional lymph node recurrence or a combination of these findings.

Of the 103 patients, 42 developed local recurrences after perfusion, of these 29 were regional skin recurrence and 13 were regional lymph node recurrence. Thirty-six patients developed non local recurrence or systemic metastases, of whom 9 also

Complete Response (CR)	Disappearance of all known disease
Partial Response (PR)	50% decrease in measurable lesions and objective improvement in evaluable but non measurable lesions. No new lesions. It is not necessary for every lesion to have regressed to qualify for partial response, but no lesion should have progressed

Table 6 Union Internationale Contra le Cancer (UICC) grading response following therapeutic ILP

Response Rate To Therapeutic Isolated Limb Perfusion				
	CR	PR	NR	TOTAL
First Perfusion	78	24	1	103
Repeat Perfusion	9	10	0	19
	87	34	1	122

Table 7 Measurable response according to UICC grading following ILP

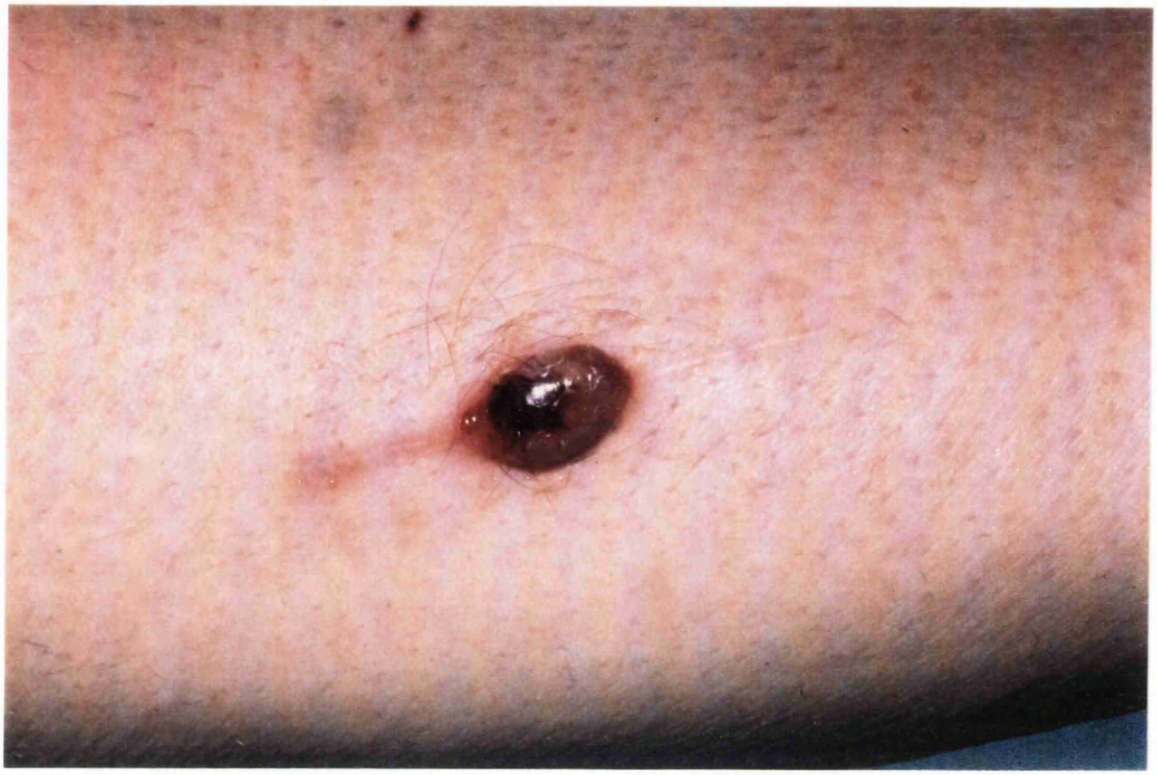


Figure 17 A single recurrent malignant melanoma nodule

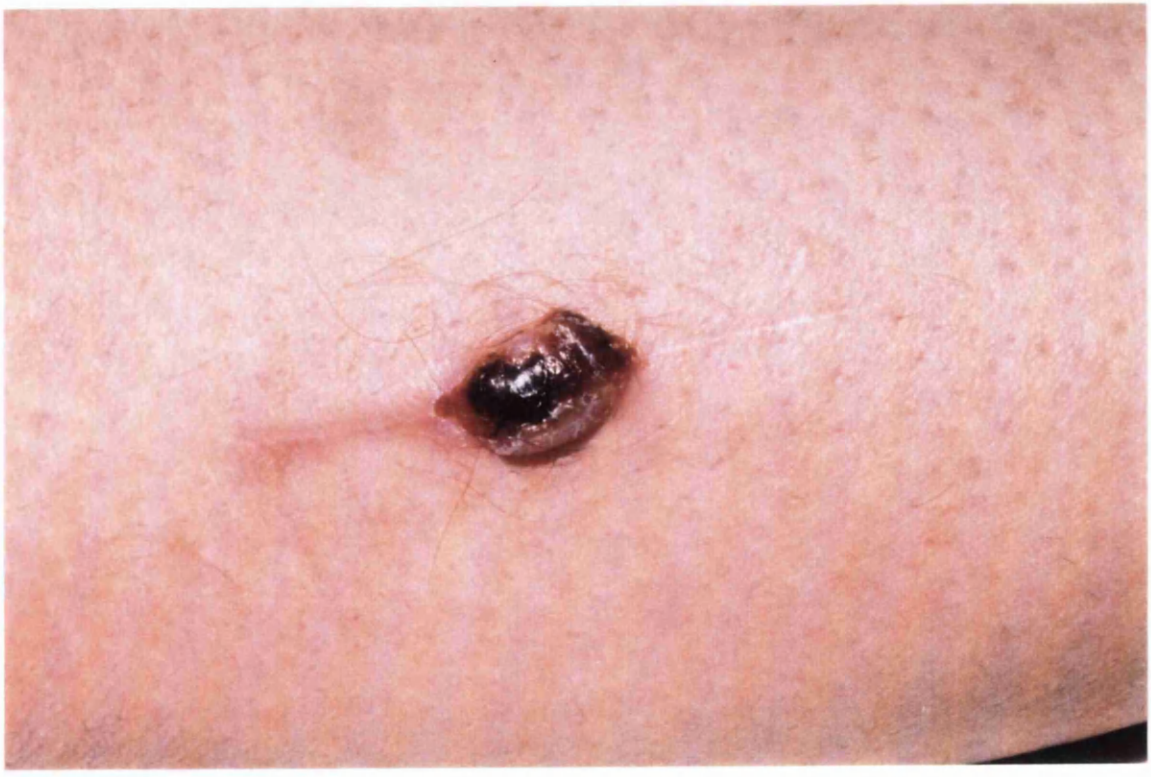


Figure 18 The same nodule three days after ILP treatment

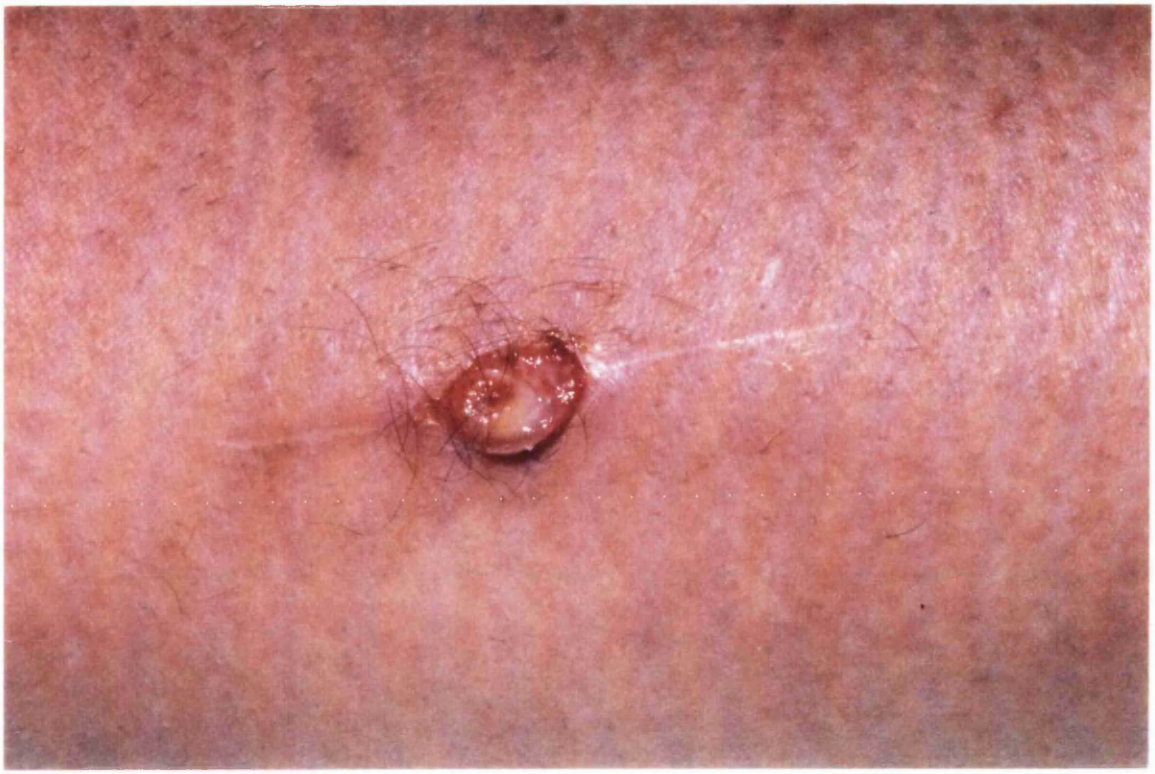


Figure 19 The same nodule one week after ILP treatment

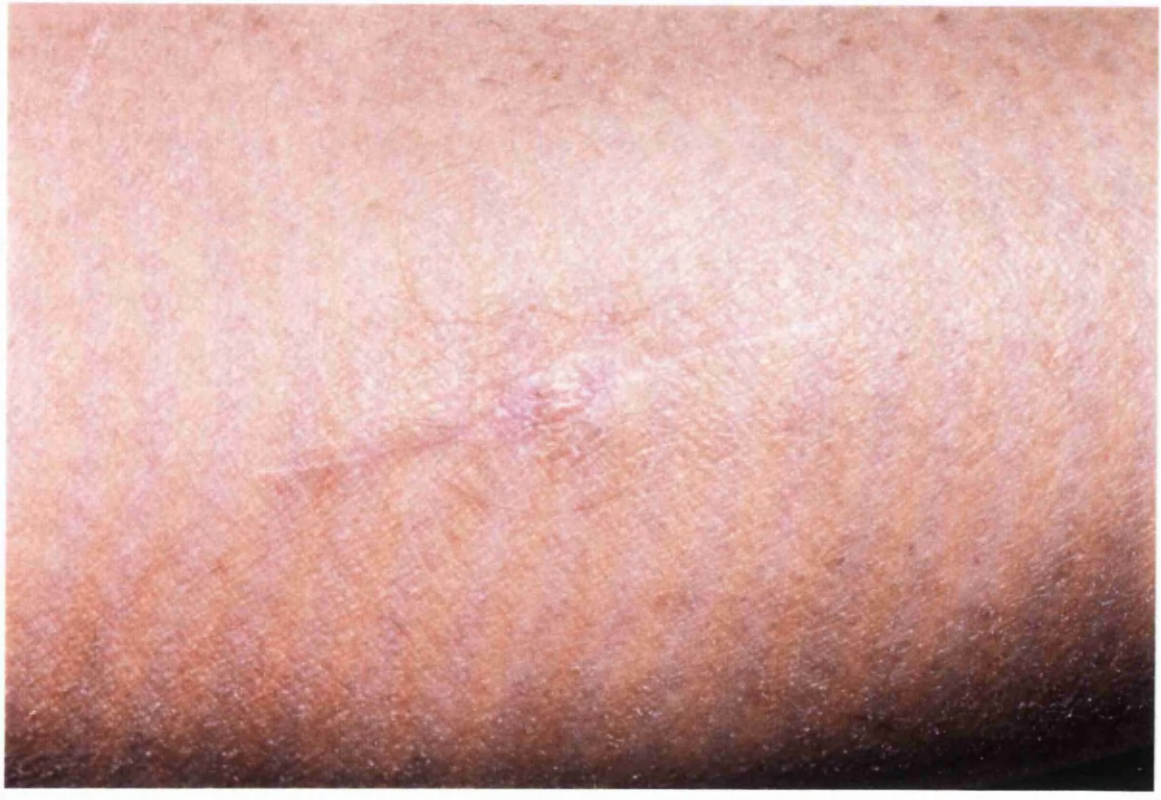
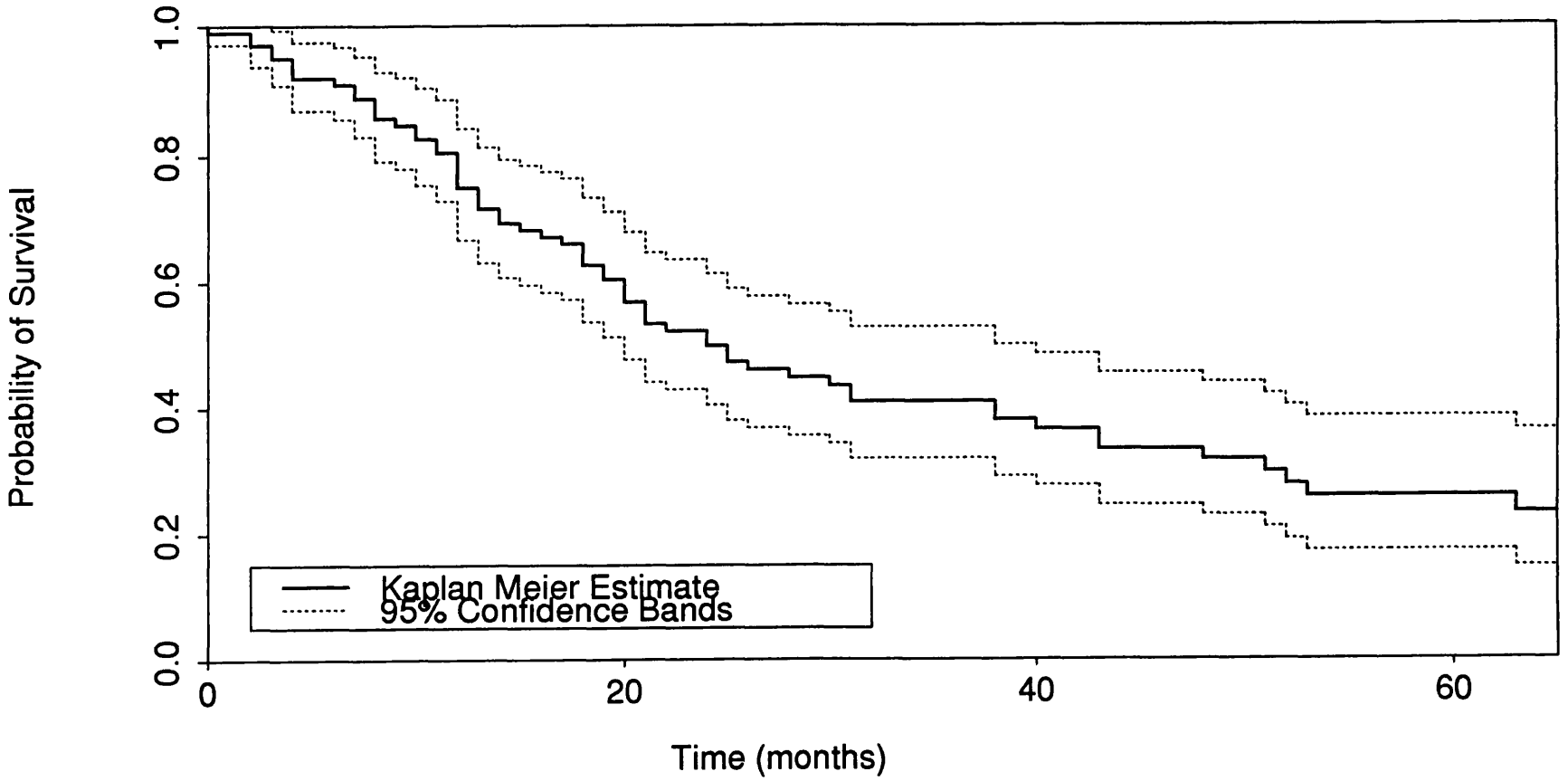


Figure 20 The same nodule one month after ILP treatment



Time (t)	0	12	24	36	48	60
Number at risk	103	68	40	28	17	10
Deaths in previous year		24	22	7	6	3

Figure 21 Probability of survival for stage II-III patients following ILP

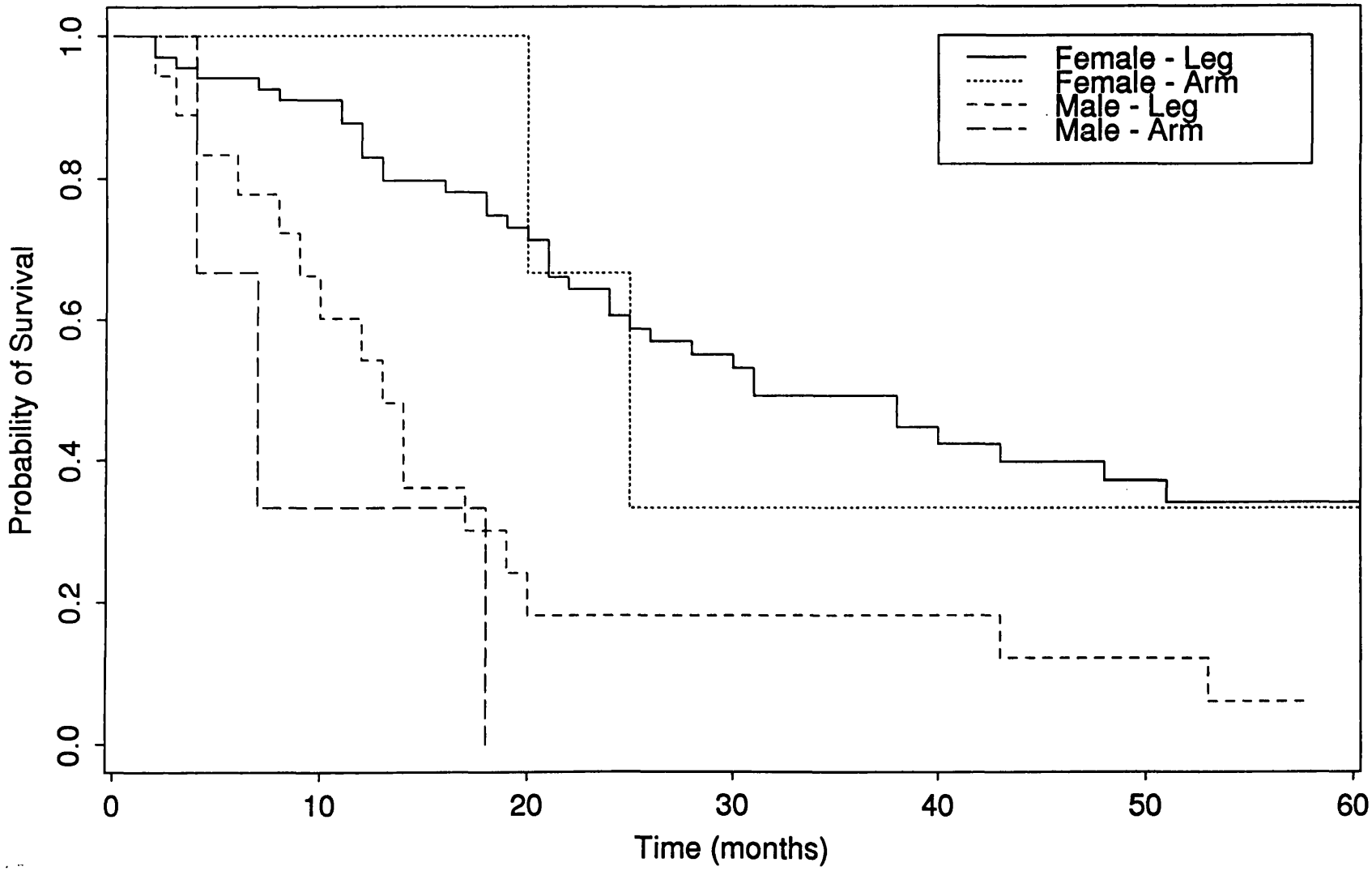


Figure 22 Probability of survival according sex and site for stage II-III patients following ILP

developed skin recurrence and 2 developed regional lymph node recurrence simultaneously. The 2 and 5 year disease free survival was 23% and 12% respectively (Figure 23).

Interval between perfusion and local recurrence: The mean time to local recurrence was 12 months with a mean time of 10 months to skin recurrence and 16 months to regional lymph node recurrence. The mean time to non local recurrence was 18 months. The mean follow up for the patients with no recurrence was 37 months.

Local recurrence according to sex: The sex distribution of the 42 local recurrence was 34 females and 8 males. The time to local recurrence in the female was 9 months and in the male was 12 months. The 2 and 5 year disease free survival for the female group was 26% and 16% respectively and in the male group of 16% and 0% respectively.

Local recurrence according to limb involved: 3 recurrences occurred in the upper limbs and 39 in the lower limbs. There was no difference in the rate of recurrence between the limbs.

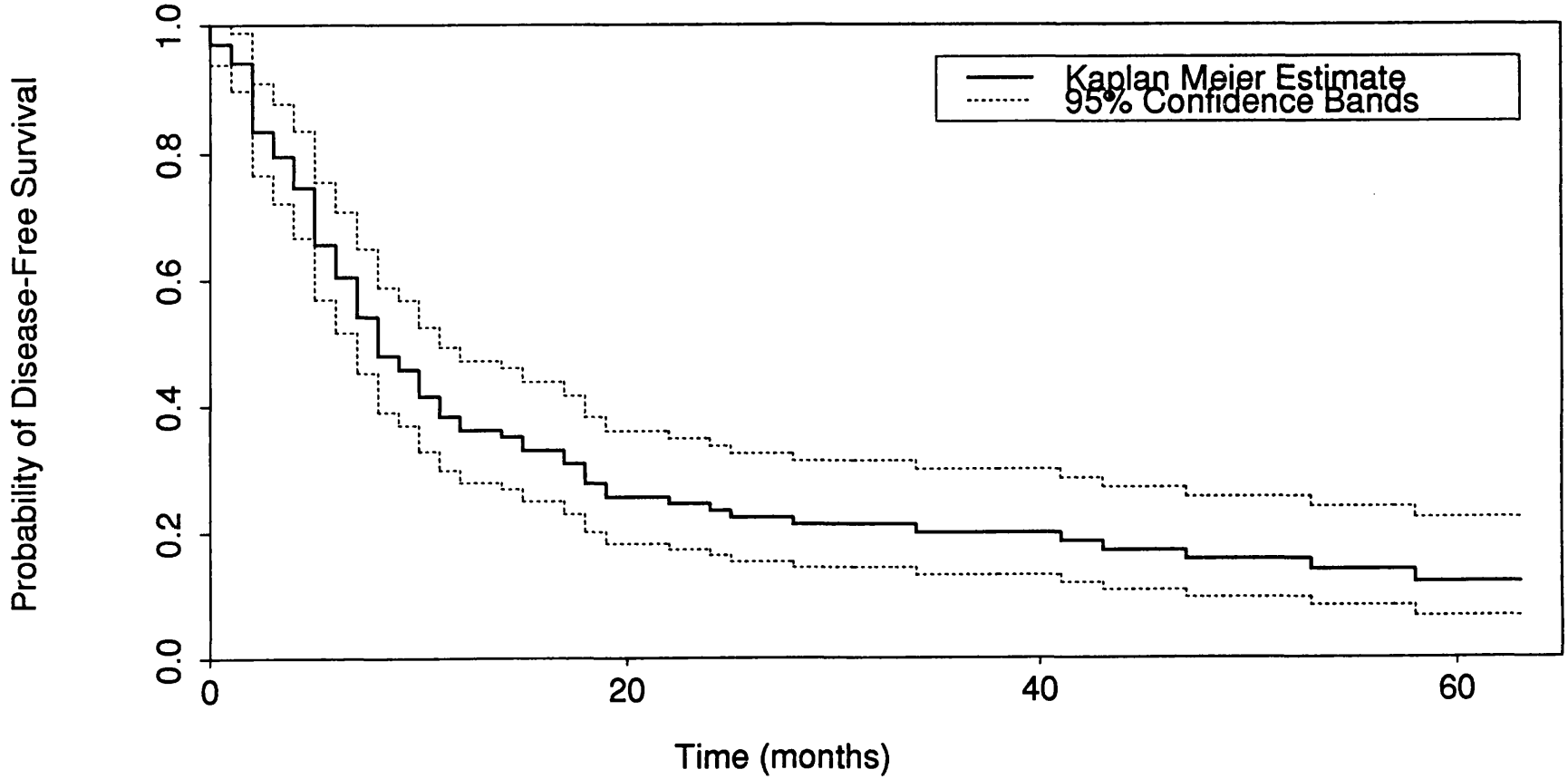
Local recurrence according to sex and site: The 2 and 5 year disease free survival for females with a lesion in the lower limb was 28% and 16% compared to 14% and 0% respectively in males with a lower limb lesion (Figure 24).

For both survival and disease free survival, females appear to have a better prognosis than males and this is confirmed by Log Rank Tests (p value for survival < 0.0001, p value for disease free survival <0.01). With respect to site, the lower limb seems to show a slightly better prognosis but this is not significant for either survival ($p = 0.49$) or disease free survival ($p = 0.52$).

When the four subgroups of sex and site were compared, there was a significant difference for both survival ($p < 0.0001$) and disease free survival ($p < 0.001$) intervals. These differences, however were between the sexes rather than between sites. A Stepwise Proportional Hazards Analysis was also carried out and this confirmed the effect on sex, but not of site.

COMPLICATIONS

There were no intraoperative complications during the procedure. There was one death within a month of surgery from an upper gastrointestinal bleed.



Time (t)	0	12	24	36	48	60
Number at risk	103	34	21	15	10	6
Recurrences in previous year		63	12	3	3	2

Figure 23 Probability of disease free survival for stage II-III patients following ILP

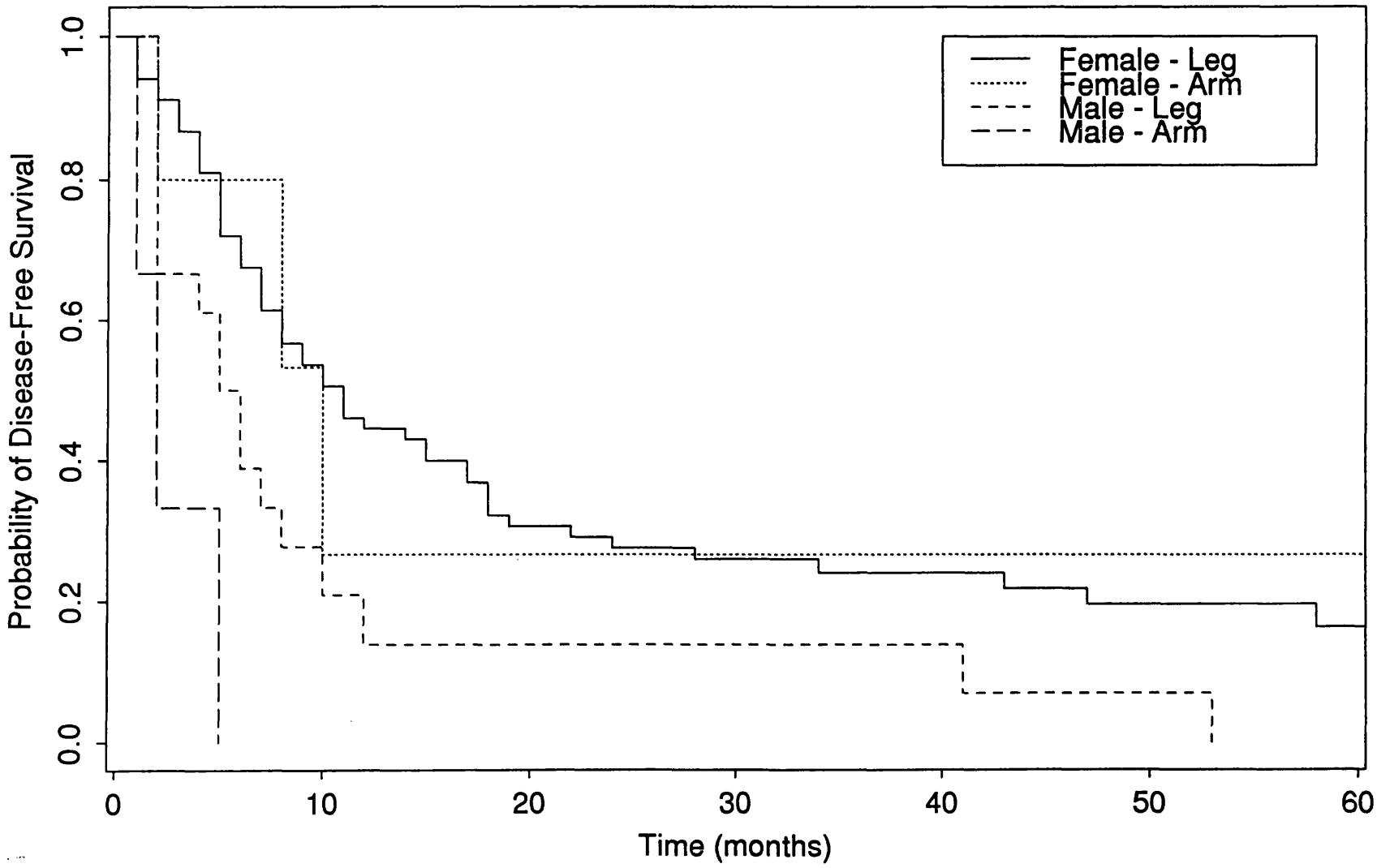


Figure 24 Probability of disease free survival according to sex and site for stage II-III patients following ILP

No amputations were necessary either as a result of complication from the perfusion or from the disease. Immediate complications included deep vein thrombosis in 3, wound infections in 4, leucopenia in 16, thrombocytopenia in 3 and pancytopenia in 18 patients, all of which resolved spontaneously.

Skin reaction to isolated perfusion was recorded according to Wieberdink[215] grading system. The majority of patients showed Grade II reactions. Figure 25 shows the typical appearance of a grade III skin reaction according to the Wieberdink scale.

3.5.3 DISCUSSION

The usual treatment offered to patients with recurrent malignant melanoma located on an extremity has been surgical excision of varying extent. Amputation has been applied less often despite the fact that this is about the only method for the definite cure of all patients without distant metastases. Since the introduction of ILP, it is being increasingly used on patients with recurrent melanoma on an extremity. Thus when analysing ILP, the results should be compared with those results obtained by excisional surgery alone or by amputation.

All the reports on regional perfusion treatment of melanoma are afflicted with the weakness that they lack adequate control groups. The literature consists of numerous reports of the results of treatment with ILP of recurrent malignant melanoma of the extremities (Table 8). The survival rates range from 29% to 71%. With regard to survival rates for recurrent malignant melanoma following ILP, the 5 year survival is 26% in this unit.

The low 5 year survival rate in this study needs explanation. Fifty-eight patients (i.e. over 50%), received two or more surgical excisions as the treatment for their recurrences prior to being referred for ILP. When McNeer and Cantin[148] reviewed local failure following surgery in the treatment for melanoma, they stated "Once local failure develops after definite treatment for melanoma, the prognosis, while not hopeless, is grave". They reported a 20% five year survival rate for 64 patients following surgical resection alone in the treatment for recurrences clinically confined to an extremity.

Fortner et al[147] reported a 40% five year survival after resection of local recurrence and 8.6% five year survival after surgical treatment of intransit disease. Amputation for stages II-IIIA has failed to produce better results than more conservative surgery.

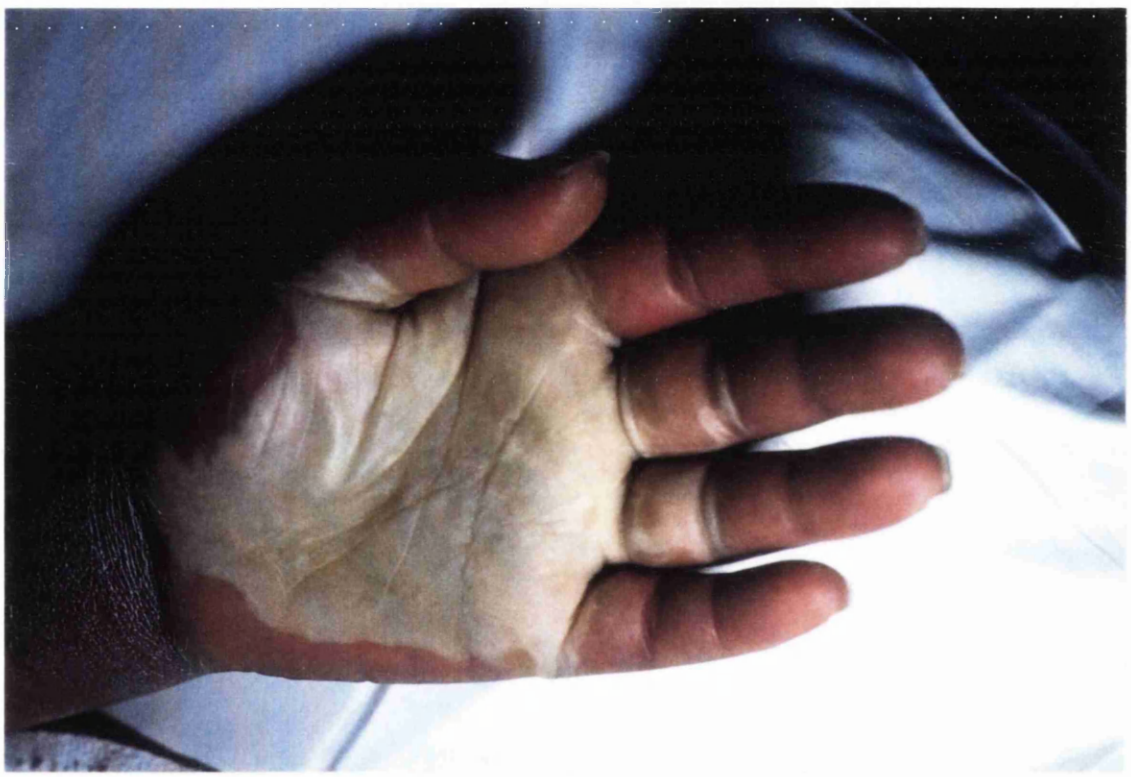


Figure 25 A grade III reaction according to the Wieberdink scale

AUTHOR	YEAR	STAGE	NUMBER OF PATIENTS	5 YEAR SURVIVAL
WAGNER[218]	1976	II IIIB	41 57	2-10 Year 68% 2-10 Year 41%
KREMENTZ[232]	1979	II III IV	20 253 115	64% 50% 10%
STEHLIN[233]	1965	II III	18 61	50.7% 46.5%
MARTIJN[234]	1981	II III	12 48	42% 46%
STEHLIN[235]	1988	II,III	73	52.5%
KETTELHACK[226]	1990	II,III	46	40%
LEJEUNE[225]	1991	II	29	53.5%

Table 8 Results of major therapeutic ILP trials in stage II-IV malignant melanoma

Bowers[236], McPeak et al[237] and Cox[238] reported 5 year survivals of 18%, 33% and 15% respectively following amputation.

Even when the survival rate from amputation, which is the most radical of form of treatment, are compared, the 5 year survival rate from ILP is superior.

When the disease free survival following ILP is compared with surgical excision alone, in this study it was 12% when compared with 11% and 27% in the series published by McNeer and Cantin[148] and Fortner et al[147]. The mean time to local recurrence was 20 months prior to ILP and was 12 months following ILP. This result may be explained by the fact that over 50% of the patients were being treated after more than two recurrences and received ILP at a mean interval of 48 months from the primary excision.

The result of this study reinforces the fact that patients with recurrences have a poor prognosis. Since they did not receive any treatment to modify the progress of the disease at an earlier stage, ILP in these patients was used to control local disease rather than to improve survival or disease free survival. Avoiding amputation is a clear benefit.

In many of the studies published on the treatment of recurrent melanoma of the limb with ILP, the treatment of ILP was performed at the time of first recurrence. In this study over 50% of the patients had at least two or more surgical excisions prior to treatment of their recurrence with ILP. Thus results from this study cannot be directly compared with other published series as ILP in most of these studies were carried out when the patient developed the first recurrence.

In the early years when the Glasgow unit was being established, the patients that were referred were those with intolerable limb disease following failed surgical treatment and various other therapies. The only other option for many of these patients was amputation. With time, and as the referring surgeons and physicians gained more insight and confidence in the treatment of ILP, patients were being referred earlier in the course of their disease. The unit now treats more patients at first recurrence and also patients with primary disease for adjuvant ILP.

Hansson et al[239], showed that perfusion plus excisional surgery prolongs the recurrence free interval in comparison to surgery alone. Stehlin et al[233] also was able to demonstrate that perfusion and surgery was superior to surgery alone.

The objective response rates from this study are encouraging. A complete response of 75% and 48% and a partial response of 23% and 52% in the first and second perfusions respectively is comparable to other studies[239,240]. It is interesting to note that repeat perfusion can also achieve good control of recurrence. ILP offers effective palliative control of the disease.

The objective response achieved using ILP is high and the use of ILP is easily justified for recurrent melanoma. As Au and Goldman[241] stated, "the disease has already demonstrated aggressive behaviour in these cases, and will progress unless it can be modified". The original clinical report by Creech et al[242] described the effects of perfusion without excision in 6 patients with recurrent cutaneous metastases involving the extremity. They described a characteristic post perfusion sequence of changes in these lesions, including darkening, flattening, crusting, and slough of the crust, with persistence of a freckle at the tumour site.

Several investigators undertook similar studies and have achieved reasonable long term survivals in selected patients with recurrent limb melanoma treated by surgical excision and adjuvant perfusion.

Female patients had a significantly better survival than males. Although there is a slightly better prognosis for lesions on the lower limb, this is not statistically significant in terms of either survival or disease free survival.

To summarise, results from this unit show that ILP is effective in the control of recurrent melanoma confined to a limb with high objective response rates. The achievement of ILP has been that amputation, a procedure that was used widely in the treatment of this disease 20 years ago is seldom, if ever needed to control limb melanoma. In this centre, to date, there has been no amputation performed to control recurrent limb melanoma.

3.6 RESULTS OF ISOLATED LIMB PERFUSION FOR SUBUNGUAL MELANOMA

3.6.1 INTRODUCTION

Hutchison[11] has been attributed with the first description of a subungual melanoma although it was reported earlier by Bayer[243] in 1854 and Dermargnay and Manod[244] in 1855. It was Hutchison who realised the malignant nature of the disease and recommended radical surgery in the form of amputation of the digit involved. However, it was much later, in 1976, that the description and definition of most volar and subungual melanoma as a fourth clinical variety of cutaneous malignant melanoma was reported by Reed[245].

Although the incidence of malignant melanoma is rising, subungual melanoma is very rare and represents only approximately 1% to 3% of all diagnosed melanoma in the Western World[246,247,248]. It is, however, commoner in dark skinned and oriental populations, representing 8% to 31% of all cutaneous melanoma[249,250,251]. Because of its rarity, subungual melanoma is often mistaken for benign conditions such as subungual haematoma, pyogenic granuloma, chronic paronchyia and onchymysosis[252]. This delay in diagnosis is quoted as a reason for the poor prognosis when compared with melanoma at other sites.

Metacarpophalangeal or metatarsophalangeal amputation has long been considered the treatment of choice for subungual melanoma[247,253]. Although adjuvant isolated limb perfusion has not been proven to improve survival in the treatment of stage I melanoma[229,254], perfusion has been advocated in deeply infiltrating subungual lesions following amputation[50,255,256]. The results of regional isolated limb perfusion in the treatment of subungual melanoma carried out in this unit are presented in this section.

3.6.2 PATIENTS AND METHODS

Between August 1987 and April 1993, 24 patients with subungual malignant melanoma were treated with ILP following amputation. Of the 24 patients, 11 were men and 13 were women, with a mean age of 66 years (range 33-84). The lesions were situated on a finger in 11 patients and on a toe in 13 patients. The great toe was involved in 8 of the 13 lesions occurring in the foot whilst the thumb was involved in 5 of the 11 lesions occurring in the hand. Two patients gave a history of trauma prior to the onset of symptoms.

All patients were treated with either axillary or iliac perfusion and sampling of lymph nodes carried out if indicated. The histological specimens were microstaged according to Breslow thickness and Clark levels. One patient could not be staged. The median Breslow thickness was 4.0 mm (range 1.5- 8.0 mm).

The disease was classified according to the M. D. Anderson classification: stage I (primary tumour)17 patients; stage IIIA (satellitosis and/or in transit metastases) 1 patient; stage IIIB (regional node metastases) 2 patients and stage IIIAB (satellitosis and/or in transit metastases with regional lymph node metastases) 4 patients.

Acral lentiginous melanoma was the most common histological type (15/23), followed by superficial spreading melanoma (5/23), nodular melanoma (2/23), and lentiginous maligna melanoma (1/23). In one patient the histology of the tumour was not available. Tables 9 and 10 summarises the details of the patients. For statistical analysis the Kaplan-Meier method was used.

The technique of regional isolated perfusion has been described previously on pages 58-60. The patients were perfused using melphalan, 1.75 mg/kg body weight for the lower limb and 0.75 mg/kg body weight for the upper limb. The patients were all treated with mild hyperthermia (39-40°C). Eleven perfusions were carried out using the axillary vessels and thirteen using the iliac vessels. At the end of the perfusion, amputation of the involved digit was carried out whenever indicated. Metatarsophalangeal joint amputation was carried out for lesions of the toes and proximal interphalangeal joint amputation for lesions on the fingers. The mean overall follow up of the entire group was 30 months (range 6- 109). The median follow up for the surviving patients was 32 (range 6 - 104) months.

3.6.3 RESULTS

There were no intraoperative complications and no deaths as a result of the perfusion. Two to three days after the perfusion, using the Wieberdink[215] grading system, 3 patients showed Grade I reaction, 17 showed Grade II reaction and 4 showed Grade III reaction. Post operative complications included wound infection in 1 patient, seroma in 1 patient and diminished sensation in the index finger in one patient, probably as a result of tourniquet trauma to the brachial plexus.

In patients with stage II and III disease, 5 (5/7) patients had a complete response and 2 (2/7) patients had a partial response classified according to the UICC Criteria[231].

AGE	SEX	SITE	TYPE	CLARK LEVEL	BRESLOW THICKNESS	DATE OF DIAGNOSIS	DATE OF ILP
75	F	(L) 2ND TOE	ALM	IV	3.5	06/02/85	24/04/85
49	M	(R) GREAT TOE	ALM	IV	2.5	25/01/87	20/08/87
83	M	(R) THUMB	ALM	V	5.0	01/05/89	17/08/89
76	M	(R) FINGER	ALM	V	3.5	14/08/89	01/11/89
71	M	(R) 5TH FINGER	LMN	V	4.3	04/01/90	02/02/90
56	M	(L) GREAT TOE	ALM	IV	1.5	18/05/90	06/07/90
70	M	(L) 4TH FINGER	SSM	V	5.0	13/06/90	17/08/90
77	F	(L) INDEX FINGER	SSM	IV	1.9	22/06/90	24/08/90
60	F	(L) THUMB	SSM	IV	6.6	11/09/90	12/12/90
44	F	(L) 3RD TOE	ALM	IV	3.0	15/12/90	01/02/90
62	F	(L) THUMB	ALM	V	5.5	05/11/90	04/02/91
57	M	GREAT TOE	ALM	V	5.1	06/01/91	26/03/91
80	F	(L) 3RD FINGER	NM	V	2.7	15/01/91	01/05/91
68	M	(L) THUMB	ALM	IV	1.9	07/02/92	29/03/92
75	F	(R) THUMB	ALM	V	8.0	08/11/91	01/04/92
75	F	(L) GREAT TOE	ALM	V	5.0	18/05/93	16/06/93
64	F	(R) 4TH TOE	ALM	IV	2.8	24/05/93	07/07/93

Table 9 Details of patients with stage I subungual malignant melanoma

AGE	SEX	SITE	TYPE	CLARK LEVEL	BRESLOW THICKNESS	STAGE	DATE OF DIAGNOSIS	DATE OF ILP
84	F	(R) GREAT TOE				IIIAB	25/05/73	16/11/84 02/05/86
60	F	2ND TOE	SSM	IV	4.0	IIIAB	12/11/86	03/06/88 17/02/89
33	M	(L) GREAT TOE	NM	V	3.8	IIIAB	01/04/89	18/04/90 10/07/90
81	F	(L) INDEX FINGER	ALM	V	3.4	IIIAB	01/08/90	31/05/91
60	M	(R) GREAT TOE	SSM	V	4.6	IIIB	06/06/91	28/08/91
67	F	(L) 4TH TOE	ALM		3.0	IIIA	31/12/92	25/03/93
62	M	(R) GREAT TOE	ALM	V	5.0	IIIB	11/11/92	02/12/92

Table 10 Details of patients with stage III subungual malignant melanoma

These two patients underwent further ILP with partial response only. The mean hospital stay after perfusion was 9 (range 6-15) days.

There were 7 local and regional recurrences, 5 in the perfused limb and 2 in the regional lymph nodes. No skin recurrence was seen in the stage I patients with two regional node recurrences only. Seven patients developed distant metastases including 4 patients with stage I and 3 patients with stage III disease. Two patients developed a bowel carcinoma (Duke B) and one bronchial carcinoma a year following diagnosis and another patient had a successful pregnancy eighteen months following perfusion.

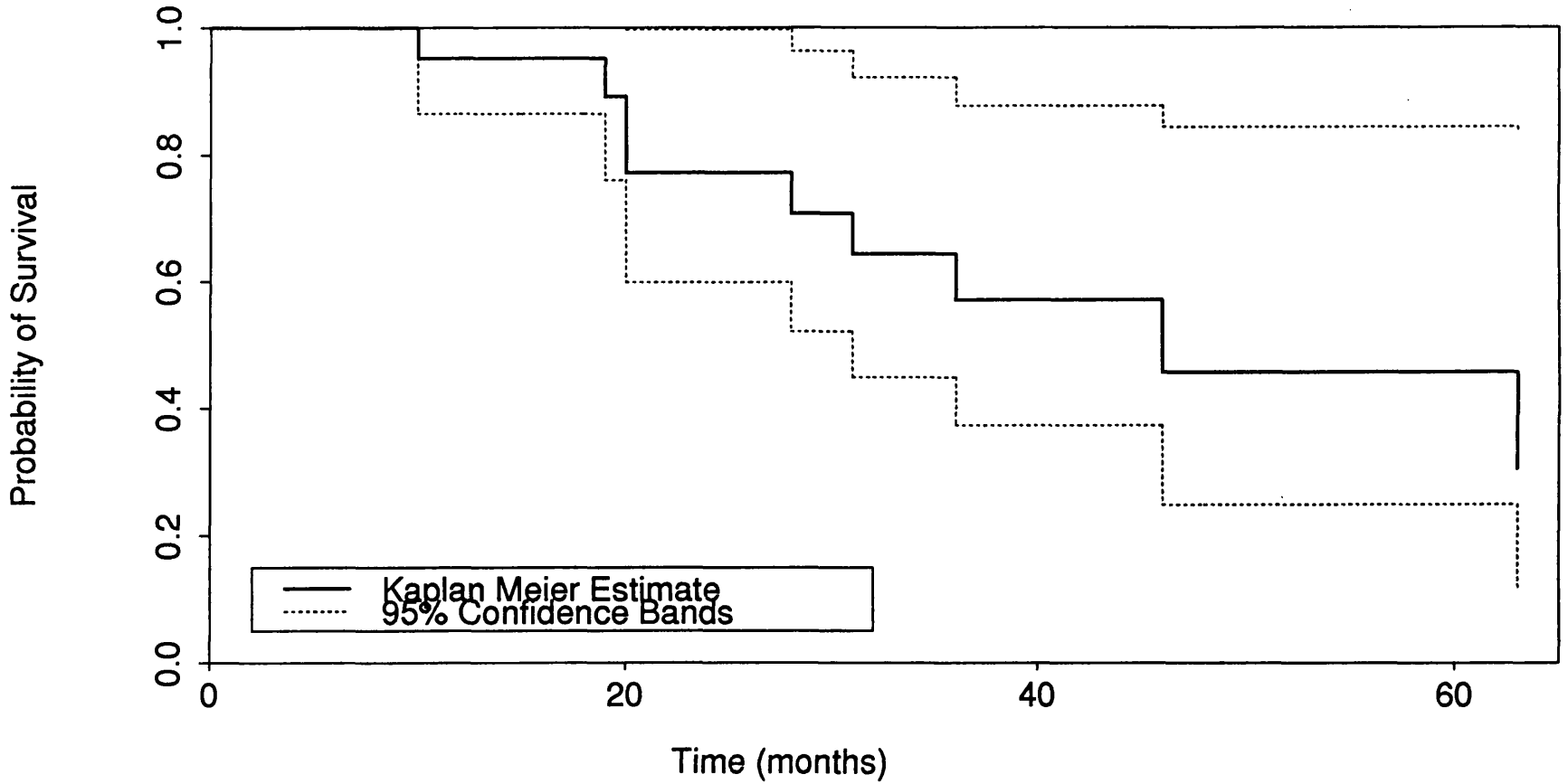
Survival and disease free survival were estimated using the Kaplan-Meier method. Significance tests of differences among the subgroups such as those defined by sex and/or site were carried out by means of Log-Rank Tests or by Proportional Hazards Models as appropriate.

The overall 2 year and 5 year survival was 77% and 46% (Figure 26) respectively with a 2 and 5 year survival for stage I of 92% and 54% and for stage III of 42% and 21% respectively (Figure 27). The overall 2 and 5 year disease free survival was 58% and 51% respectively with a 2 and 5 year disease free survival for stage I of 78% and 70% respectively. Figure 28 shows the survival by sex and site.

Log Rank Tests were carried out to assess any prognostic significance for sex, site or stage. Only the clinical stage was found to be significant for both survival and disease free survival (p value = 0.01 for survival, and p-value <0.01 for disease free survival). A stepwise Proportional Hazards Analysis confirmed that only the clinical stage significantly affected survival and neither sex nor site influenced survival outcome. Fourteen patients are currently alive and disease free, 1 patient is alive with metastatic disease and 9 patients have died of metastatic disease and 1 from bronchial carcinoma.

The outcome of 24 patients in this study was then compared with 111 patients with subungual melanomas treated with amputation only. These 111 patients formed part of the Scottish Melanoma Group data base which registers all malignant melanoma in Scotland.

First, the survival prospects for the perfused and non perfused patients were analysed without correcting for other prognostic variables. This was done by obtaining a Kaplan-Meier survival probability of each groups separately and then carrying out a Log Rank Test to see if there was any difference in survival between them. This



Time (t)	0	12	24	36	48	60
Number at risk	24	19	13	6	3	3
Deaths in previous year		1	3	3	1	0

Figure 26 Probability of survival of patients with subungual melanoma following ILP

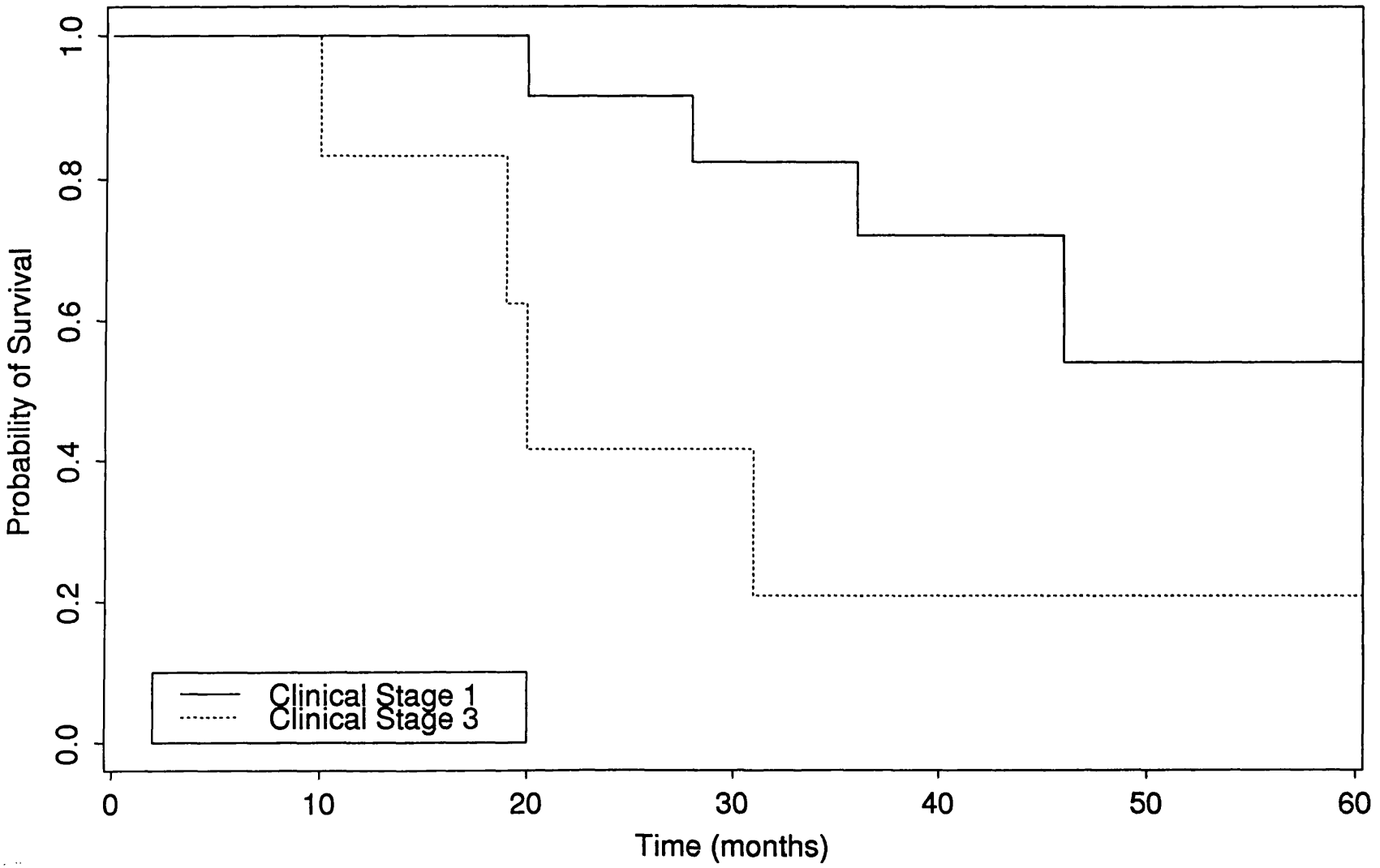


Figure 27 Probability of survival according to the stage in patients with subungual melanoma following ILP

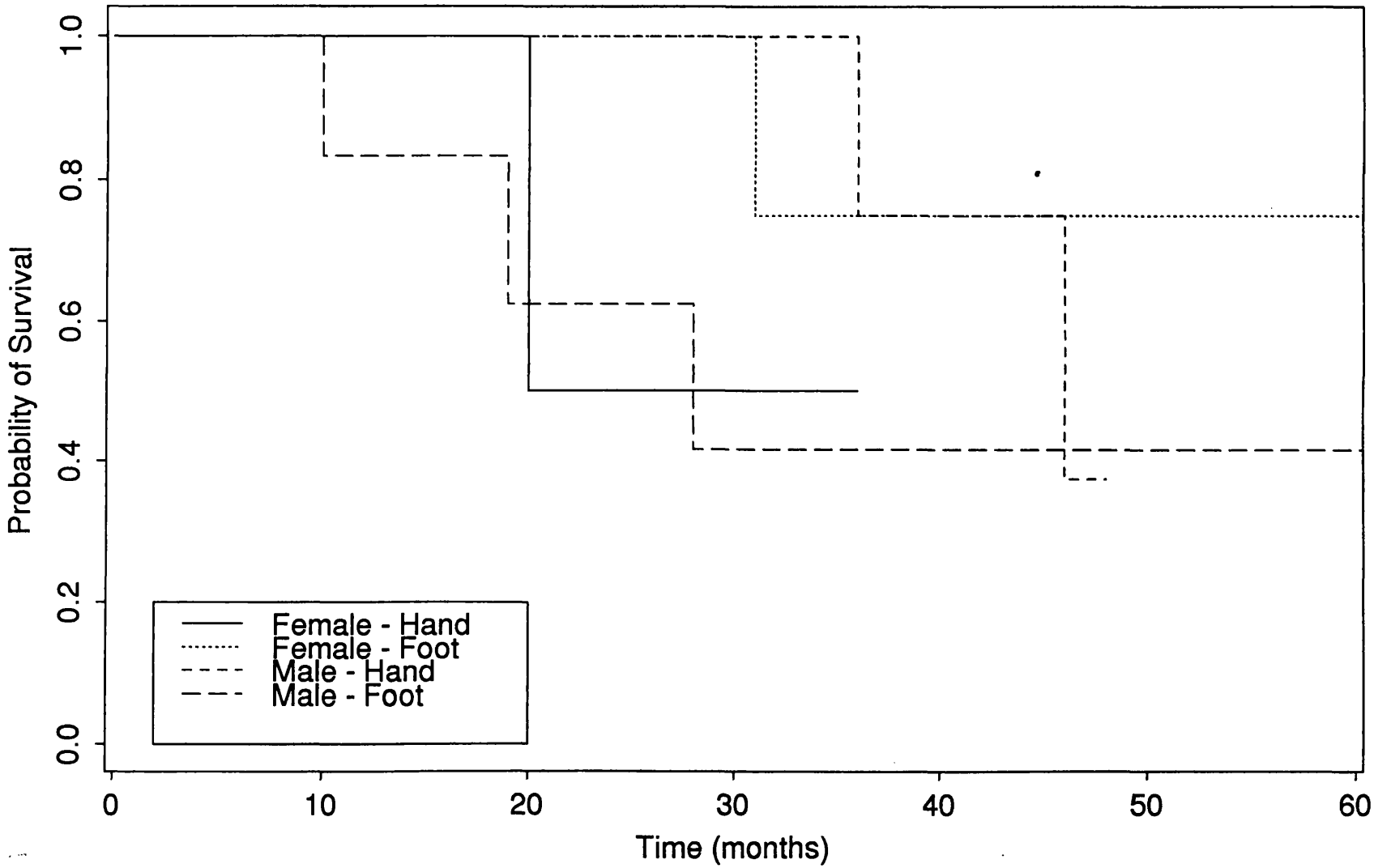


Figure 28 Probability of survival according to sex and site for patients with subungual melanoma following ILP

analysis gave a p value of 0.90 which was non significant, i.e. there was no evidence of difference in survival between perfused and non perfused patients.

A Stepwise Porportional Hazards Analysis was then performed to investigate if there was a difference in survival between perfused and non perfused patients, this time correcting for any variables found to be significant. Again, perfusion did not make any difference to survival.

3.6.4 DISCUSSION

Since subungual melanoma is rare in the Western World, there are only a few published series in the literature. Table 11 summarises the published series. Subungual melanoma commonly occurs in the sixth and seventh decades and has no sex preponderance. It occurs with equal frequency on the hand and the foot, with the thumb and big toe being most commonly affected. In our series 65% (13/20) were found on the big toe or thumb. The majority of lesions are acral lentiginous melanoma but nodular, superficial spreading and lentiginous maligna melanoma can also occur.

There are only 3 studies of patients with subungual treated with ILP. None of the studies was randomised. Baaset et al[253] reported in 22 patients a 5 year survival of 56% for stage I disease and 27% for stage III disease; there was no loco regional recurrence. Muchmore et al[250] reported a 61% 5 year survival rate for stage I and 17% for stage III patients. Neither, however, were able to demonstrate a marked improvement in survival compared with historic patients not perfused. Although Vrouenraets et al[257] have reported an improved survival in patients treated with ILP their results should be reviewed with caution as the follow up is short and the number of patients small.

In this series, the 5 year survival was 54% for stage I patients and 21% for stage III patients. These results are comparable to other studies. When these results are compared to the 111 patients with subungual though unmatched lesions from the Scottish Melanoma Group, no survival advantage was demonstrated for patients who were perfused.

The control of local regional disease was better in the perfused group than in the non perfused group with only 5 patients in the perfused group developing recurrence in the affected limb. Although there is no survival benefit, ILP prolongs the disease free interval and is effective in controlling local limb recurrence, which in many cases can

AUTHOR	YEAR	TREATMENT	MD STAGE	5 YEAR SURVIVAL
BASS[253]	1989	ILP + Amputation	I III I & III	56 27 40
MUCHMORE[250]	1990	ILP + Amputation	I III I & III	61 17 35
VROUENRAETS[257]	1993	ILP + Amputation	I III I & III	68 19 54
DAS GUPTA[247]	1965	Amputation + RLND	I III	52 15
PACK[246]	1967	Amputation + RLND	I	46
PAPACHRISTOU[258]	1982	Amputation + RLND	I & III	53
TAKOMATSU[259]	1985	Amputation + RLND	I & III	40

Table 11 Published series on the treatment of subungual malignant melanoma

pose a difficulty. None of the patients in this study needed amputation to control local disease.

How then should this tumour be treated? Pack and Adaur[260] advocated amputation at the metocarpophalangeal joint and metatarsophalangeal joint, but this was later found to be inadequate as far as local recurrence is concerned. Most recently Krementz et al[256] suggested that this lesion should be treated with distal phalangeal amputation, whilst in those with extensive involvement a ray amputation is recommended.

The practice in this unit is to amputate at the metatarsophalangeal joint for lesions of the toes and to amputate at the metacarpophalangeal joint for lesions of the thumb and proximal interphalangeal joint for lesions on the fingers. Ray amputation is reserved for cases when the lesion has not been completely excised or for local regional recurrence. At present, ILP for patients with subungual melanoma is not recommended.

The results from this unit show that there is no significant survival advantage in the adjuvant treatment of stage I subungual lesions in comparison to non perfused patients. However with the encouraging reports[261] on the use of Tumour Necrosis Factor (TNF) and melphalan for recurrent melanoma, the use of this combined treatment may be justified for patients with subungual lesions. It will also be the practice in this unit to carry out lymphatic mapping using blue dye (see chapter 6) on all patients to try and select those patients with microscopic nodal disease, so that early nodal clearance can be offered.

3.7 CONCLUSION

At the time of writing, over 300 ILPs have been performed in Glasgow. It has been shown that ILP can be performed safely with minimal morbidity. There has only been one death within 30 days from a gastrointestinal bleed. ILP has not adversely affected the prognosis of patients. In the treatment of adjuvant ILP of stage I melanoma there is a trend towards survival advantage in the perfused group but this does not reach significance. Longer follow up is required to show significant improvement in survival.

The European Organisation for Research and Treatment of Cancer (EORTC) and the WHO are conducting a multicentre prospective randomised trial evaluating adjuvant ILP. The patient randomisation is based on patient and tumour parameters such as sex, age, anatomical location, Clark level, Breslow thickness and ulceration of

tumour. Hopefully this EORTC/WHO ILP trial will add to our knowledge. As yet, it is not possible to exclude the possibility that a small subgroup of patients will benefit from adjuvant ILP.

Isolated limb perfusion is now an established treatment for recurrent malignant melanoma in Glasgow. An objective response is seen in more than 80% of patients with improved limb salvage rate. The five year survival rate is 30-70% for patients with local recurrence, satellitosis and in transit metastases with or without lymph node metastases.

Even in patients with in transit and regional metastases, five year survival is about 35%. More recently patients are being referred for ILP at an earlier stage in their disease and in the next few years, it may be possible to show an improved survival.

In the small group of patients affected by subungual melanoma, treatment with ILP did not demonstrate survival benefits and thus it is not possible to recommend adjuvant ILP. This may be related to the fact that it is difficult to accurately measure tumour thickness resulting in underestimation of the thickness and thus affecting survival. Furthermore, there is often a delay in the diagnosis from the time of appearance of the lesion. A combination of these two factors may contribute to the poor survival seen. However these patients may benefit from a combined treatment with TNF and melphalan.

In conclusion, a small subgroup of patients with stage I melanoma may benefit from adjuvant ILP. In the treatment of recurrent limb melanoma, ILP is the treatment of choice. It is a treatment that can alter the progress of the disease and may thus improve the patient's survival and disease free survival.

CHAPTER 4

OPTIMISING THE ISOLATED LIMB PERFUSION CIRCUIT

4.1 INTRODUCTION

Given my own experience of ILP and having reviewed the published literature on the works of others, it was clear that many questions remained unanswered in relation to the current methods used in the ILP circuit. Optimising the efficacy of the circuit has the potential to improve the results of ILP. I therefore decided to analyse various aspects of the circuit in an attempt to derive objective criteria that could be applied to establish the efficiency of the perfusion.

When an oxygenator was incorporated into the ILP circuit, attention was focused on the necessity for oxygenating the blood perfusate. It was originally presumed that oxygenation was necessary to ensure the viability of the perfused tissues[262,263,264]. Since regional perfusion usually lasts for not more than 60 minutes, the lack of oxygen during this time should not be dangerous. But when hyperthermia was introduced and the time of the perfusion lengthened, viability of the tissues may become compromised.

Furthermore, it has been established that the effects of ionising radiation can be enhanced by increasing the tissue oxygen tension[265,266,267]. It has also been noted that many chemotherapeutic agents are thought to exert effects on the cell similar to that of ionising radiation[268]. High oxygen tension in the tissues may thus potentiate the tumouricidal effects of these agents. Ryan et al[269] showed a response to chemotherapy beyond that expected by intra-arterial administration of the drug by isolated perfusion utilising a bubble oxygenator that raised the oxygen tension of the blood to 400-600 mmHg. On the dog's hind leg they showed that the toxicity of fluorouracil was increased by oxygenation of the perfusate.

Later, Krementz et al[270] carried out a controlled experiment utilising standard transplantable tumours in mice to study the effect of increased tissue oxygen tension on the tumouricidal action of alkylating agents. They found that the tumouricidal effect of nitrogen mustard was enhanced by increased tissue oxygen tension.

Based on this principle that increased oxygen tension was necessary not only for viability of the tissues but also improved the tumouricidal effect of the chemotherapeutic agent, I decided to evaluate several factors in the isolated limb perfusion circuit to see if the oxygen tension achieved in the circuit could be optimised. Clearly, only factors that could be modified or measured, were studied.

The factors examined were the time taken to achieve maximum flow rate, time to maximum oxygen, maximum oxygen and volume change in the reservoir.

There is usually considerable variation in the flow rates in the isolated circuit. There is no preset value and normally the perfusion is commenced with a low flow rate of 50-100ml/min and is increased until a maximum stable flow rate is achieved. This stable level is judged to be adequate when there is no rapid change in the reservoir volume and the level of oxygenation is adequate with minimal fluctuation. It is not clear whether there is any benefit from very high flow rates but it is important that physiological flow rates are achieved to prevent ischaemia of normal tissues. Thus it is beneficial if the maximum flow rate could be achieved quickly with minimal fluctuation.

The reservoir volume is important in the maintenance of flow rate and tissue oxygenation. When there is vasodilatation or fluid loss from the circuit, there is a fall in the reservoir volume. This has the effect of resetting the circuit and maintaining stable conditions. The reservoir thus behaves as an "auto regulator" in the isolated limb perfusion circuit. It is obvious that a constant need to add additional fluid into the reservoir, indicates significant loss of the fluid from the circuit into the extravascular space of the isolated tissues. This can be detrimental to the tissues.

Thus maximising tissue oxygen tension is important as it will not only prevent ischaemia of normal tissue, but more importantly, the antitumour action of melphalan may be enhanced.
The chapter presents the results of the study on:
1) The introduction of pulsatile flow (never before attempted in the ILP circuit).
2) Measurement of compartment pressure (never before attempted in the ILP circuit).
3) Use of vasodilator agents (never before attempted in the ILP circuit).

4.2 PULSATILE VERSUS NON PULSATILE FLOW IN THE ISOLATED CIRCUIT

4.2.1 INTRODUCTION

Pulsatility is the most obvious feature of blood flow in the arteries.

Over one century ago Ludwig and Schmidt[271] initially ascribed physiological significance to the pulsatile nature of blood flow. Since then several studies have shown that pulsatile perfusion is necessary to maintain a proper function of the microcirculation. Recent evidence indicates that normal capillary blood flow is pulsatile[272]. Studies of the brain[273] and omental[274] microcirculation during cardiac bypass suggest that pulsatile perfusion maintains better capillary and venule perfusion than non pulsatile blood flow. Pulsatile perfusion of isolated organs causes less damage and maintains better organ function than non pulsatile perfusion[275].

The concept of an extracorporeal circulation was first described by Le Gallois[276] in 1812 and the first artificial oxygenation of blood is credited to Ludwig[276] in 1869. The first report of an isolated organ perfusion system using a blood film spread on a rotation cylinder was by Frey and Grubber[276] in 1885. However the pioneering work of Gibbon[277] in the 1930's advanced cardiopulmonary bypass (CPB) from the laboratory into clinical reality, when in 1953 he performed the first successful cardiac operation. This was followed by the development of the bubble oxygenator by De Wall[278].

By the time ILP was introduced for use in the treatment of malignant melanoma of the limb, CPB had become a routine procedure in many countries. At that time non pulsatile CPB was the method of choice. Inevitably therefore, the pioneers of ILP based their technique on the non pulsatile circuit. However since the introduction of this technique over 30 years ago, changes were seen in the CPB circuit that now utilises the pulsatile nature of blood flow. Attracted by the theoretical advantages of pulsatile flow in the ILP circuit, the author was determined to investigate whether such a change would confer physiological benefit.

The ILP circuit is very similar to the cardiopulmonary bypass circuit but it differs in certain respects. Pulsatility in the CPB circuit is created with the use of a pulsatile assisted device (PAD) which is inserted in the arterial line during CPB and the PAD is synchronised with the electrocardiogram of the patient in diastole.

Both the CPB and the isolated circuit utilise the bubble oxygenator where oxygen is bubbled through the circulating blood and gas exchange occurs at a direct blood gas surface. In the ILP circuit, a heat exchanger is used to create hyperthermia whereas CPB utilises hypothermia. The pump oxygenator in the ILP circuit utilises a combination of blood and Hartmann's solution as the priming fluid unlike CPB that uses isotonic dextrose-saline solution.

4.2.2 PHYSIOLOGY OF BLOOD FLOW

The arterial system is considered as being in steady state oscillation. A wave can be regarded either as an isolated phenomenon or as a repetitive one. Regularity of the heart beat is one of its most characteristic features and this leads to a condition of steady state oscillation. In its simplest form, circulation consists of a pump (the heart) which forces blood periodically and rhythmically into a branching system of elastic tubes. The pulsations generated travel centrifugally but are partially reflected to travel backwards at points of discontinuity. The pulsations are damped by the time they reach the smallest branches (the capillaries) which are in intimate contact with the cells of the tissues.

Blood returns in a more or less steady stream to the heart with secondary pulsations imposed by muscular activity and by the heart itself. In reality the human circulation is extremely complicated. In the arterial tree allowances must be made for the varied geometric patterns of branching, non uniform elasticity of arteries, non linear wall properties and anomalous viscosity of blood in smaller blood vessels.

As blood travels away from the heart, the mean pressure falls slowly but the pulsatile pressure variation increases until in the femoral artery it may be double that at the root of the aorta. Flow oscillation, on the contrary, diminishes markedly. Such behaviour is due to the presence of a 'closed' type reflection in the small peripheral vessels. In the absence of reflections, damping would cause a parallel fall in pressure and flow oscillations. Ultimately pressure oscillation is damped out in the region of the smaller arteries.

The increase in the ratio of the pulsatile pressure amplitude to that of the flow amplitude is largely determined by the increase in the fluid impedance of the low frequency components: the change in impedance of the various frequency components in terms of their distance from the main reflecting sites is at a minimum at a quarter wavelength distance from these peripheral sites.

The shape of the arterial pressure wave is dependent on a number of factors including duration of systole, mean arterial pressure, vasomotor tone, pulse wave velocity and wave reflections. In the abdominal aorta and the lower limb arteries, the foot of the wave is progressively delayed whereas the peak of the pressure wave is almost synchronous in time. The diastolic pressure fluctuations are reciprocal with those in the proximal aorta. With ageing, the pulse wave velocity increases markedly. This single factor accounts for the change in the arterial pressure wave constant and pressure wave transmission with ageing.

Diastolic flow waves are usually apparent in the thoracic and abdominal aorta and in the iliac and femoral arteries. The diastolic flow wave is associated with backflow in these vessels and is related to the diastolic pressure wave. Backflow is more apparent below the major branches of the abdominal aorta. Such backflow is reduced when these vessels are clamped and reappear when they are unclamped. In small peripheral arteries, there is no backflow and flow fluctuations are very small.

The major factors that determine blood flow are pressure difference and resistance to flow. The pressure difference (perfusion pressure) can be altered by a change in inflow (arterial pressure) or a change in outflow pressure (venous pressure). Resistance can be calculated as analogous to Ohm's Law relating to electrical resistance, potential difference and current. Total vascular resistance (TVR) thus can be calculated from the ratio:

$$TVR = \frac{\text{(arterial pressure - venous pressure)}}{\text{Blood Flow}}$$

4.2.3 PATIENTS AND METHODS

Forty-two patients undergoing isolated limb perfusion for malignant melanoma between January and June 1993 were recruited into the study. Twenty-one patients underwent pulsatile perfusions and 21 patients matched for site, age and type of perfusion received non pulsatile isolated limb perfusions. There were 36 females and 6 males with a mean age of 61 (range 35 to 76) years. There were 32 lower limb perfusions and 10 upper limb perfusions.

All patients underwent isolated limb perfusion by the technique described in pages 58-60. To create pulsatile blood flow in the circuit, a Stockert Frequency Control Module (Figure 29) was used. This pulsatile creating machine was employed externally in series with the isolated limb perfusion circuit as shown in figure 30.

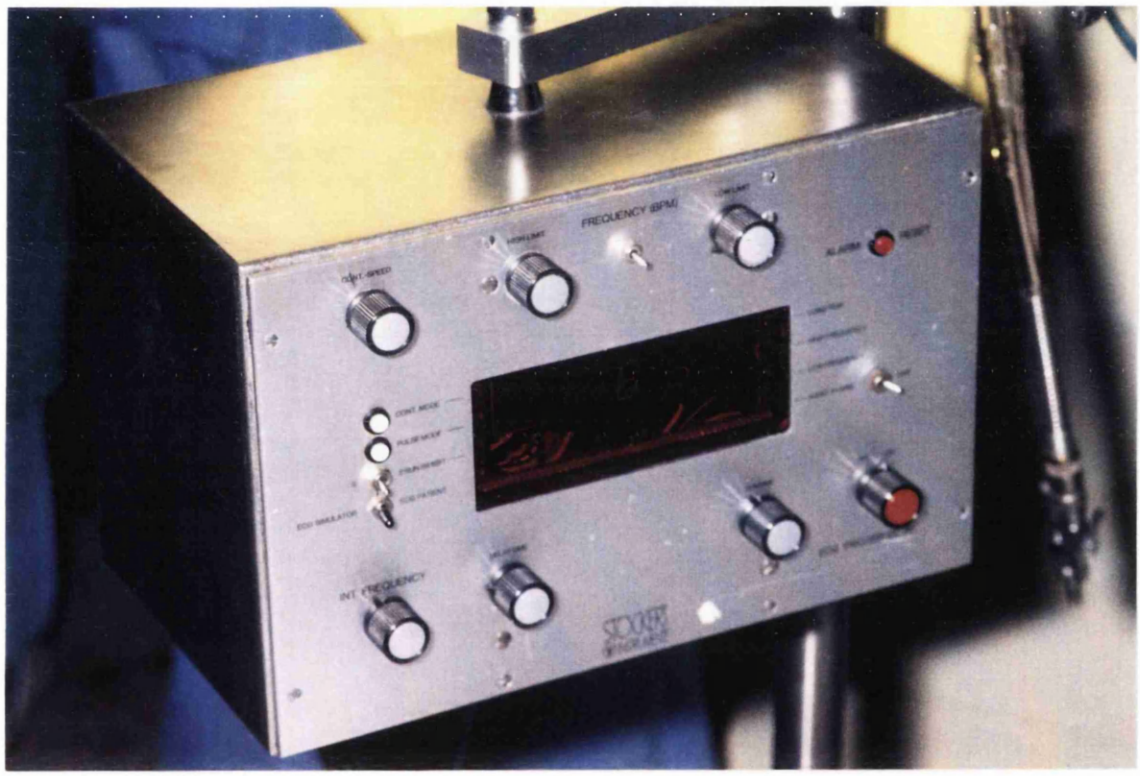


Figure 29 Stockert Frequency Control Module
(courtesy of Cardiothoracic Department, Western Infirmary, Glasgow)

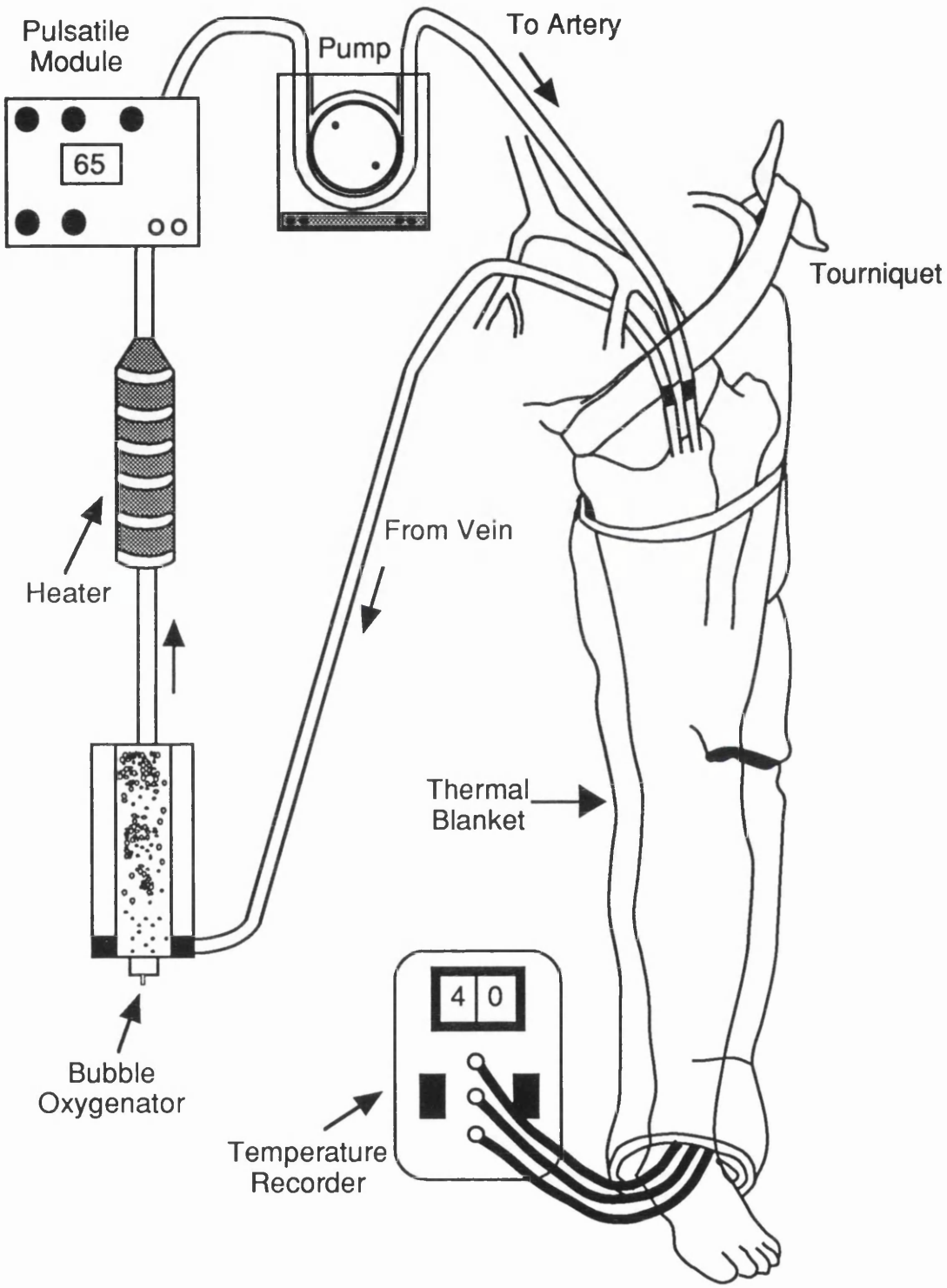


Figure 30 A diagrammatic illustration of the pulsatile ILP circuit

The frequency created by the machine was synchronised to the electrocardiogram of the patient in diastole. Skin perfusion was monitored using the Radiometer TCM2 transcutaneous monitor.

During perfusion, recordings of the time to maximum flow rates, volume shift in the reservoir, time taken to achieve maximum skin oxygen concentration and maximum oxygen achieved were made.

4.2.4 RESULTS

The results of the pulsatile and the non pulsatile data were compared in relation to:
1) Time to maximum flow.
2) Time to maximum oxygen achieved.
3) Volume shift in the reservoir.
4) Maximum oxygen achieved.
5) All the variables were then compared with relation to the effect on the limb.

Time to maximum flow.
The time to maximum flow rate was shorter in the pulsatile group than in the non pulsatile group. There was also less fluctuation in the flow rates in the pulsatile than in the non pulsatile group. There was a strong correlation between pulsatile and non pulsatile matched observation (0.79). This strong correlation was seen even when the proportional differences between the matched pulsatile and non pulsatile values were analysed. On average, the non pulsatile values were higher than their pulsatile equivalents by 8 - 41% (95% confidence intervals).

Time to maximum oxygen achieved
The time to maximum oxygen achieved was shorter in the pulsatile than in the non pulsatile group. There was also less fluctuation in the oxygen tension in the pulsatile than in the non pulsatile group. However when the correlation between the pulsatile and the non pulsatile values were calculated, the matching was poor (0.17). The correlation between the difference (pulsatile minus non pulsatile) and the raw measurements were then calculated and from this analysis it was clear that there was a dependence on the magnitude of the measurement. Accordingly the proportional difference between the matched pulsatile and non pulsatile values were analysed.

Analysis of the results showed that the non pulsatile values were higher than the pulsatile values. These results were formally verified by producing 95% confidence intervals for the mean proportional difference. This showed that the non pulsatile values were higher than their pulsatile equivalents by 16-74%.

Volume shift in the reservoir

In the group undergoing pulsatile perfusion, less additional fluid was required to maintain the reservoir volume than in the non pulsatile group. There was less fluctuation in the pulsatile group than in the non pulsatile group. When the proportional differences were analysed between the matched pulsatile and the non pulsatile values, the non pulsatile values were higher than the matched pulsatile values. The 95% confidence intervals for the mean proportional differences were 3-75% higher in the non pulsatile group than their pulsatile equivalents.

Maximum oxygen achieved

Although the maximum oxygen level achieved in the pulsatile group appeared to be overall higher than in the non pulsatile group, there was no significant difference between the matched pulsatile and the non pulsatile values when the proportional differences were analysed. The 95% confidence interval was between -42 to 26% (not significant as it contains zero in the range, i.e. in some cases the pulsatile values were lower than the non pulsatile values). Table 12 shows the summary of the statistical analysis.

Measurements between the upper and lower limb

With respect to the time to maximum flow, time to maximum oxygen achieved and the volume shift in the reservoir, there was no difference between the values seen in the upper limb from that seen in the lower limb.

In the upper limb, the maximum oxygen level achieved in the non pulsatile group was greater than the pulsatile group but in the lower limb, the maximum oxygen level was greater in the pulsatile group than the non pulsatile group.

The above results were validated using 95% confidence intervals for the difference across limbs between the proportional differences of the non pulsatile versus the pulsatile. There was no difference between limbs for the time to maximum flow, time to maximum oxygen achieved and volume shift in the reservoir. However, there was a significant difference for maximum oxygen. On average, the pulsatile values were greater than the non pulsatile values in the lower limbs but pulsatile values were lower than non pulsatile values in the upper limb.

Correlations between Pulsatile and Non-Pulsatile matched observations.

Variable	Correlation
Time to max Flow	0.79
Volume Shift	-0.22
Time to Max Oxygen	0.17
Maximum Oxygen	-0.11

This suggests strongly that the matching is POOR for all variables except Time to Max Flow.

Correlations between the DIFFERENCE (i.e. Pulsatile minus Non-Pulsatile) and the RAW measurements are

Variable Name	Correlation with Pulsatile	Non-Pulsatile
Time to Max Flow	0.423	0.229
Volume Shift	0.716	0.841
Time to Max Oxygen	0.694	0.594
Maximum Oxygen	0.662	0.757

All further calculations are carried out on the Proportional differences,

i.e. $\frac{\text{Pulsatile - Non-Pulsatile}}{(\text{Pulsatile + Non-Pulsatile})/2}$ expressed as a Percentage

to remove this Dependence on the Magnitude of the measurement.

Summary of Proportional Differences (i.e. Percentage means)

Variable	Lower limb	Upper limb	Total
Time	28.3	11.6	24.3
Volume	42.5	27.4	38.9
Time to max oxygen	62.7	**16.0**	45.2
Max. Oxygen	**28.6**	56.3	8.4

Values with ** around them denote samples where the Pulsatile group are higher than Non-Pulsatiles.

Table 12 Stastistical analysis of the pulsatile study

The overall results showed that the time to maximum flow, volume shift in the reservoir and time to maximum oxygen level were significantly greater in the matched non pulsatile than the matched pulsatile values for both limbs.

4.2.5 DISCUSSION

Many of the studies showing the advantages of pulsatile flow have been based on the CPB circuit. There have been no studies on the effect of pulsatile blood flow in the isolated limb perfusion circuit. However, isolated organ perfusion has been studied in kidney and brain where a significant advantage of pulsatile perfusion over non pulsatile perfusion has been demonstrated.

The advantage of pulsatile over non pulsatile perfusion during isolated organ or whole body perfusion has been the subject of continuing controversy. Wesolowski et al[279] and Selkurt et al[280] found no difference in renal function with pulsatile and non pulsatile perfusion. Randall et al[281] detected no significant difference in blood flow in the hind limb of dogs perfused with non pulsatile and pulsatile blood flow at equal blood pressures. Harken et al[282] showed that oxygen consumption in the isolated perfusion of the hind limb of the dog was independent of the pulsatile nature of the perfusing blood.

On the other hand, the preponderance of data seems to point to the superiority of pulsatile perfusion. Parsons and McMaster[283] demonstrated superior lymph flow and function with pulsatile perfusion. Sanderson et al[284] found signs of ischaemic nerve cells in animals subjected to non pulsatile perfusion. These signs were not present in animals undergoing pulsatile perfusion.

Dalton et al[285] demonstrated moderate to severe hydropic degeneration of kidney tubules after non pulsatile perfusion. Many et al[286] found that sodium excretion and urine volume decreased during non pulsatile perfusion.

In studies on the whole body perfusion by Jong-Bloed[287] and Thomas et al[288] pulsatile flow was found to be superior to continuous flow. Ogata et al[289] and Nonoyama[290] demonstrated increased acidosis, oedema and splanchnic pooling with continuous rather than pulsatile flow in dogs. Recently Dunn et al[291] showed that pulsatile flow during CPB offers significant advantages over continuous flow. Oxygen consumption was higher in dogs that were perfused with pulsatile flow. This meant better cellular oxygenation and is reflected in the more alkalotic blood found in the pulsatile circuit.

A theoretical advantage of pulsatile over non pulsatile flow has been suggested by Shepard[292]. He suggested that it is an energy gradient and not a pressure gradient that produces blood flow. For this purpose he defined an 'energy equivalent pressure' that is the pressure which represents the effect of pulsatile energy.

It has been hypothesised that the increased 'energy equivalent pressure' of pulsatile flow may enhance interstitial diffusion by oscillating cell-fluid boundary layers and thus ensuring the patency of end arterioles which may otherwise collapse during non pulsatile flow. This analysis predicts higher mean flow for pulsatile than for non pulsatile flow at equal mean arterial blood pressure.

Jacobs et al[293] and Trinkle et al[294] found increased oxygen consumption, improved tissue and acid base status and lower peripheral vascular resistance in animals subjected to pulsatile CPB compared to those subjected to non pulsatile perfusion.

The results of this present study confirm that there is a consistent difference between pulsatile and non pulsatile perfusion in the ILP circuit. Firstly, in the pulsatile perfusion, time to maximum flow in the circuit was significantly shorter than the non pulsatile perfusion with less fluctuation in the flow. This is important as there is steady and stable tissue oxygenation once the maximum flow rate is achieved.

Secondly, the volume shift in the reservoir was significantly greater during non pulsatile perfusion than during pulsatile perfusion. The blood in the reservoir functions to maintain a constant flow and oxygenation in the circuit. The greater change in the reservoir volume seen in the non pulsatile perfusion reflects an unstable circuit with significant loss of fluid and fluctuating oxygen levels. Since this is an isolated circuit, it implies that the fluid must be lost into the surrounding tissue. The amount of volume shift from the reservoir is important as will be seen in section 4.3.

Thirdly, the time to maximum oxygen in the circuit was significantly greater during non pulsatile perfusion than during pulsatile perfusion. The time to maximum oxygen is dependent on flow rate. The sooner the optimal flow rate is established, the sooner maximum tissue oxygenation is achieved, thus maximising the oxygen enhancing effect of the chemotherapeutic agent.

However, there was no significant difference in the overall maximum oxygen level eventually achieved between pulsatile and non pulsatile perfusion.

There is no ready explanation for this result. It is possible that high flow rates in this study prevented the detection of a difference in the oxygen concentration between the two groups. The final high flow rate in both the groups may have compensated for any intrinsic disadvantage that occurs with the non pulsatile perfusion.

In conclusion, pulsatile perfusion in the ILP circuit improves tissue oxygenation by reducing the time to maximum oxygen, the time to maximum flow rate and the volume shift in the reservoir thereby producing a more stable circuit with minimal fluctuation. This effect may enhance the antitumour effect of melphalan by providing a constant level of tissue oxygenation. The results of this study support the other studies described above in that pulsatile perfusion, similar to blood flow in normal vessels in nature, is superior to non pulsatile perfusion. Introduction of pulsatile blood flow into the ILP circuit thus confers physiological advantages.

4.3 COMPARTMENT PRESSURES IN THE ISOLATED CIRCUIT

4.3.1 INTRODUCTION

Compartment syndrome is characterised by increased tissue hydrostatic pressure within a closed fascial space and by secondary compromise of skeletal muscle capillary perfusion with resultant ischaemia, tissue necrosis and potential systemic toxicity. The term compartment syndrome has also been referred to as Volkmann's ischaemia, muscle compartment syndrome, anterior tibial syndrome, rhabdomyolysis and many others. The above definition is useful because it can be employed whenever increased tissue pressure produces similar circulatory and functional effects, irrespective of the location of the process and the initiating cause.

The isolated limb perfusion circuit is a closed circuit where oxygenated blood is circulated under pressure. In theory, in such a closed circuit where a large amount of blood is being circulated in a limited space under pressure and at temperatures higher than normal body temperature, the possibility of increased compartment pressure exists. Some investigators perform routine prophylactic fasciotomy to avoid the complication of compartment syndrome. A study was designed to establish if there was a significant increase in the compartment pressure during ILP and whether pulsatile or non pulsatile blood flow in the circuit affected these pressures.

4.3.2 PATHOPHYSIOLOGY OF INCREASED TISSUE PRESSURE

The normal function of tissue is maintained by circulation that is sufficient to meet the tissue's metabolic need. However, when tissue pressure increases in osteofascial compartments, the ability to accommodate changes in the volume is limited. This compromises local circulation to a point where metabolic needs of the tissues are no longer met and functional abnormalities ensue.

The fact that increased tissue pressure compromises local circulation was demonstrated by Ashton[295], Rorabeck and Clarke[296] and other investigators[297]. All these studies indicate that pressure as low as 20 mm Hg applied to a limb can significantly reduce local blood flow. This reduction in blood flow becomes increasingly severe at higher pressures. However, in none of these studies was there evidence for a "critical pressure" above which tissue blood flow suddenly became compromised.

One of the most important functions of the local circulation is to deliver oxygen to the tissue. It has been shown in both animal and human studies that higher tissue pressures result in lower muscle PO_2 values and muscle PO_2 is significantly reduced by an applied pressure of 20 mm Hg[298,299]. Muscle PO_2 values decrease essentially linearly as the applied pressure is increased[300]. No critical pressure was observed but greater compromise of muscle PO_2 was seen at higher tissue pressures. Increased tissue pressure also compromises neuromuscular function[298]. Higher pressures and longer periods of pressure application produce more frequent functional losses.

There are several proposed mechanisms of pressure induced circulatory compromise. These include arterial spasm[301,302,303], critical closure theory[295,304,305] and tidal wave theory[297]. However, the theory currently accepted is the arteriovenous gradient theory[306,307,308,309]. According to this theory, increase in tissue pressure reduces the local arteriovenous gradient and thereby reducing local blood flow. When blood flow is reduced to the point where it no longer meets the metabolic demands of the tissue, functional abnormalities and thus the compartment syndrome results. The relationship between arteriovenous gradient and local blood flow is as below[310]:

$$LBF = P_A - P_V / R$$

LBF	=	local blood flow
P_A	=	local arterial pressure
P_V	=	local venous pressure
R	=	local vascular resistance

Because veins are collapsible, the pressure inside them cannot be less than local tissue pressure. Thus when tissue pressure increases, the pressure in local veins must also rise. This increased venous pressure results in a reduction in the arteriovenous gradient. Some reduction in this gradient can be compensated for by changes in the local vascular resistance. This process of autoregulation maintains local blood flow over a range of arteriovenous gradients[311].

When the arteriovenous gradient is significantly reduced, the process of autoregulation becomes relatively ineffective[312]. At this point, local blood flow is determined primarily by the local arteriovenous gradient. When the tissue pressure increases further, local blood flow is reduced to the point where it no longer meets the metabolic demands of the tissue and functional abnormalities ensue.

This theory predicts that a reduction in local arterial pressure (e.g. by limb elevation) will exaggerate the circulatory effect of any increase in tissue pressure. Lowering local venous pressure by decompressing the tissue is an effective method of restoring circulation if the compartment syndrome ensues. This theory also predicts the preservation of pulses and distal circulation frequently seen in compartment syndrome. What is more important is the amount of pressure the tissue can tolerate before its function becomes abnormal. The pressure tolerance of tissue depends on several factors:

i) The specific effect of increased tissue pressure on local blood flow in the tissue.
ii) The metabolic demand of the tissue.
iii) The duration of increased tissue pressure.

The specific relationship of tissue pressure and tissue blood flow is affected by the presence of hypertension, shock, limb elevation and arterial occlusion. The metabolic demands are related to the presence and severity of local tissue injury. The duration of increased pressure depends on the rate of onset of the compartment syndrome and the promptness with which it is treated. Thus the tolerance of tissue for increased tissue pressure varies considerably from patient to patient.

4.3.3 AETIOLOGY AND CLINICAL FEATURES OF COMPARTMENT SYNDROME

There are two pre-requisites for the development of compartment syndrome:

i) an envelope limiting the available space;
ii) increased pressure within that envelope.

A limiting envelope may be any structure of limited compliance that surrounds tissue, e.g. in the anterior compartment of the leg, the envelope consists of fascia and bone. A limiting envelope may also be produced iatrogenically in the form of a tight external dressing or casts.

The second pre-requisite may be a decrease in the volume of the envelope (e.g. closure of fascial defects), an increase in the content of the envelope (e.g. bleeding, trauma, burns, intra-arterial drug injection), or application of pressure to the outside of the envelope.

The diagnosis of compartment syndrome may be made on clinical symptoms and signs alone. These include pain out of proportion to what is anticipated from the clinical situation, weakness of muscle in the compartment, pain on passive stretching of the muscle of the compartment, hypoaesthesia in the distribution of the nerves coursing through the compartment and tenseness of the compartment envelope. The period of risk for compartment syndrome appears to extend at least to three and possibly to six days after the initial cause of compartment swelling[313].

4.3.4 MEASUREMENT OF TISSUE PRESSURE

Several methods of tissue pressure measurement have been described. These include the capsule method[314,315], the collapsible segment method[316,317], servonull technique[318], injection technique[319,320] and continuous infusion technique[321,322]. The technique that has been used in this study is the wick technique[295,323,324] which will be described in detail.

4.3.5 PATIENTS AND METHODS

Over a period of six months, from January 1993 to July 1993, 16 patients undergoing isolated limb perfusion with melphalan were recruited into the study. There were 12 females and 4 males with mean age of 62 (range 42-80) years. All patients underwent iliac perfusion. Eight patients underwent ILP in the pulsatile mode whilst 8 had ILP in the non pulsatile mode.

To measure compartment pressure a soluble braided vicryl suture of fibre diameter of 8 to 16 micrometer and a vascular access cannula is used (Figure 31). The cannula is of gauge size 12, and 20 cm long. It is made of two components, a needle with a valve at the end to which a cap is attached and a plastic sleeve that is placed over the needle. The long end of the suture is passed through the cannula and this is done by removing the cap and threading the suture through from the back to the tip of the needle with the plastic sleeve in situ. The cannula with the wick in position is filled with heparinised saline (10 units) and calibrated to zero hydrostatic fluid pressure. Then the two ends of the vicryl suture are held taut (the suture is now doubled back) and the skin is pierced with the needle under a sterile technique.

Once the deep fascia is penetrated (approximately 2-3cm in depth), the needle is withdrawn leaving the suture and plastic sleeve in place. The suture is placed in the anterior tibial muscle compartment. The system is then flushed with heparinised saline. The cannula is secured by taping it to the skin (Figure 32) after which it is connected to a pressure transducer and the system is filled with heparinised saline solution. The circuit is tested by raising the limb above the level of the heart and by this manoeuvre the pressure will be seen to rise and on lowering the limb, the pressure falls.

The wick increases the surface area in contact with the tissue. The system is heparinised to minimise clotting around the fibres. If patency is in question, it may be flushed with the flushing syringe. Continuity of the fluid column between the tissue and the transducer is necessary for accurate pressure measurement. The wick prevents tissue blockage of the orifice of the cannula and allows free exchange of fluid between the interstitium and the fluid filled catheter. Figure 33 is a diagrammatic illustration of the wick technique showing the tip of the wick catheter in the muscle and its connection to the pressure transducer and recorder.

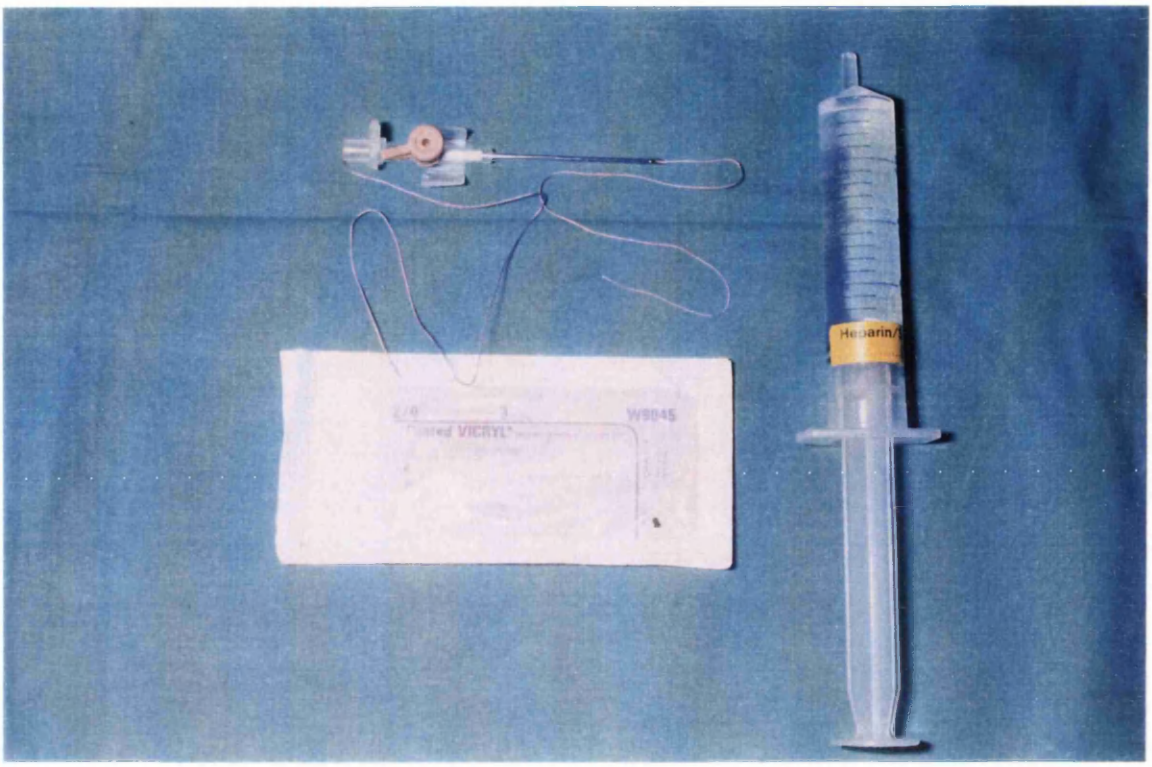


Figure 31 Apparatus used in the wick technique

Figure 32 Wick in position in the lower limb

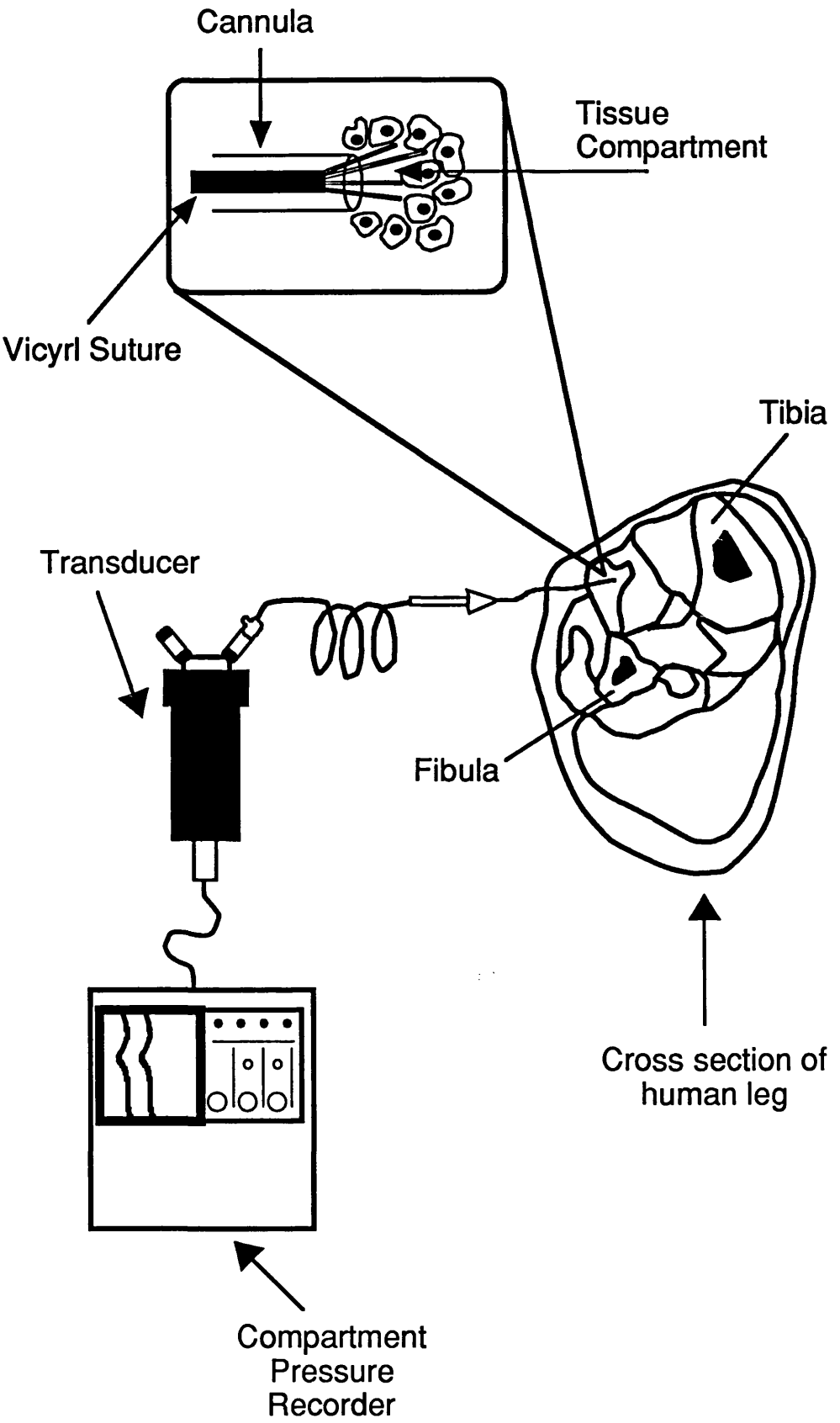


Figure 33 Diagrammatic illustration of the wick technique

Measurement of compartment pressure was carried out as described above. Recordings were made preoperatively and at 5 minute intervals during the hour of the perfusion. Pressures were measured in the anterior compartment of the leg. Continuous transcutaneous oxygen measurements, circuit flow rates and reservoir volumes were recorded. The transcutaneous oxygen tension in the skin was measured using the TCM2 System (radiometer) at several points in the limb.

4.3.6 RESULTS

The compartment pressures in patients undergoing pulsatile ILPs were compared with the compartment pressures in patients undergoing non pulsatile ILPs. The two groups were studied in relation to:

1) compartment pressure and time.
2) compartment pressure and reservoir volume.
3) compartment pressure and flow rate.
4) compartment pressure and maximum oxygen tension.

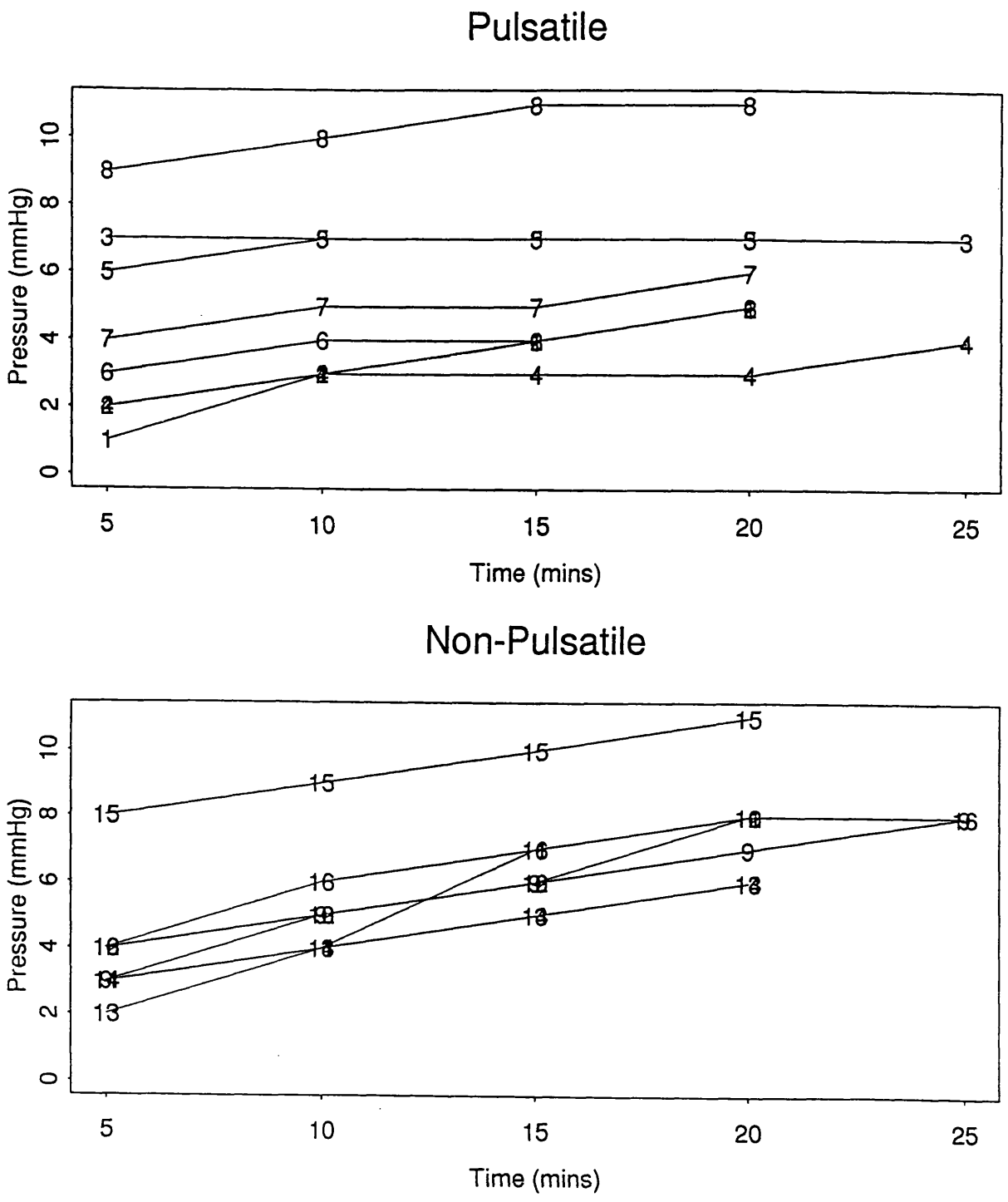
Compartment pressure with time

Analysis showed that there was an increase in the compartment pressure with time and this increase was quicker in the non pulsatile group (Figure 34). The strong positive relationship between pressure and time (pooled correlation of 0.97) for both groups were confirmed in formal analysis using an F-test. Although the two groups appeared to start at the same average value (i.e. there were no significant differences in the intercepts, $p = 0.65$), there was a significant difference in the rate of increase of pressure with time, i.e. significant difference in the slopes between the pulsatile and non pulsatile group $p < 0.001$.

The general rate of increase of pressure with time was 0.242 mmHg per minute in the non pulsatile group whereas it was only 0.106 mmHg per minute in the pulsatile group (i.e. the rate of increase was effectively at least doubled in the non pulsatile group).

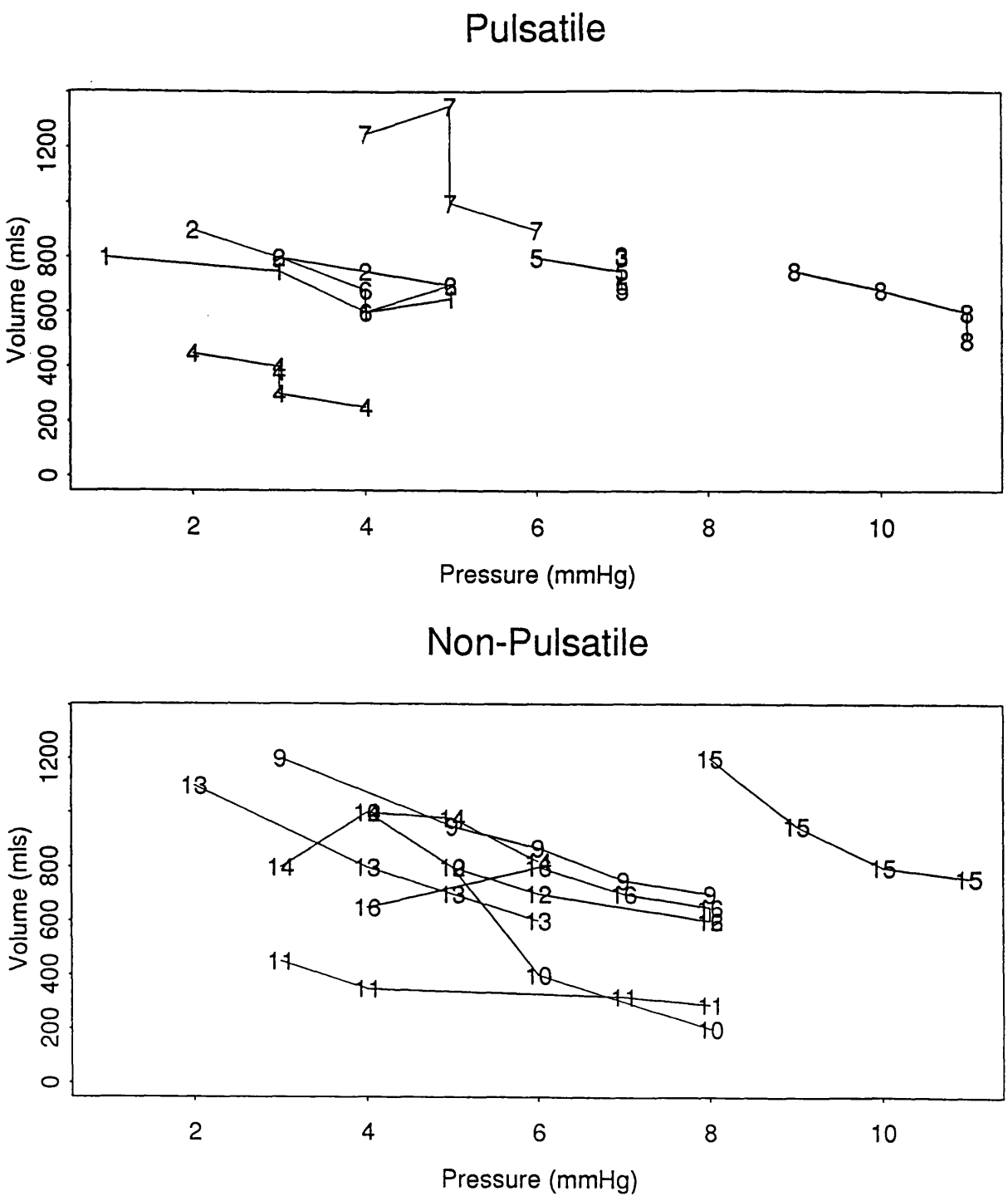
Compartment pressure and reservoir

When the reservoir volume was analysed with respect to compartment pressure (Figure 35), in both the pulsatile and the non pulsatile groups, a negative relationship was seen. A formal analysis based on estimating individual correlation for each subject was carried out and then pooled across subjects' first within their own group and then across both groups together.



Subjects are labelled 1-8 for pulsatile group
Subjects are labelled 9-16 for non pulsatile group

Figure 34 Relationship between compartment pressure and time in the compartment pressure study



Subjects are labelled 1-8 for pulsatile group
Subjects are labelled 9-16 for non pulsatile group

Figure 35 Relationship between compartment pressure and volume shift in the compartment pressure study

The overall estimated correlation was -0.85 with the individual correlation ranging from -0.99 to +0.05. Overall there was evidence of a fairly strong negative relationship between volume and pressure in general across individuals, i.e. for most individuals, as the compartment pressure increased, the volume in the reservoir decreased.

Compartment pressure with flow rate

The relationship between flow rate and compartment pressure was also studied (Figure 36). A positive relationship was seen between flow and pressure in both the pulsatile and the non pulsatile groups. The spread of both variables was greater for the non pulsatile group but the correlation was in general very similar (pooled correlation of 0.94 and 0.95 respectively for the two groups).

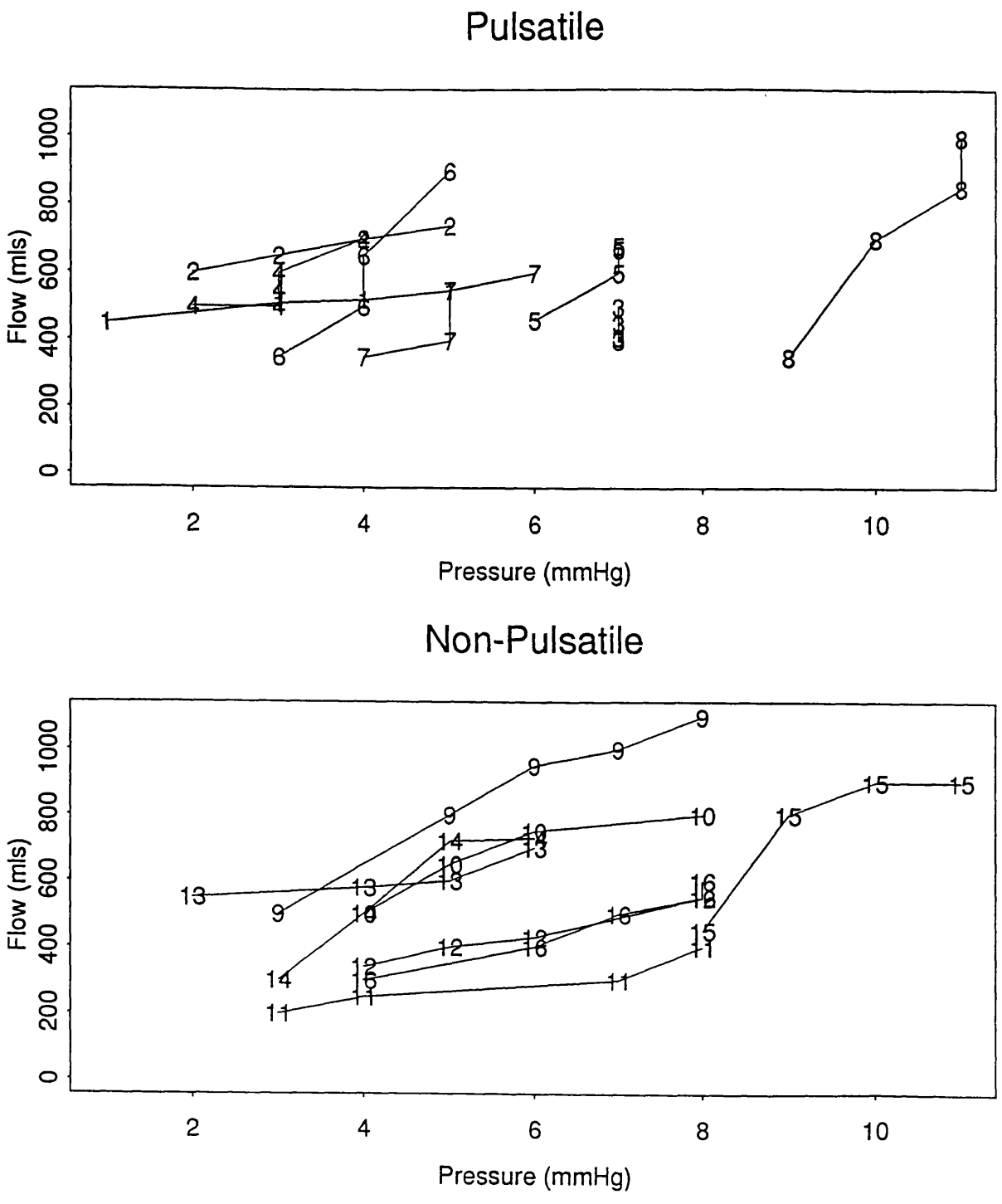
Compartment pressure and oxygen

When the relationship between oxygen and compartment pressure (Figure 37) was analysed, the pooled correlation for the pulsatile group was 0.24 and 0.52 for the non pulsatile group. When the F-Test was applied, there was a significant difference ($p < 0.005$) between the pulsatile and the non pulsatile group suggesting that there was a moderately stronger relationship between oxygen and compartment pressure for the non pulsatile group when compared to the pulsatile group.

In summary, there was a significant rise in the compartment pressure with time and the rate of rise was at least twice that in the non pulsatile group than in the pulsatile group. As the compartment pressure rose, there was a significant fall in the reservoir volume along with a significant increase in the flow rate in both the pulsatile and the non pulsatile group. There was also a significant decrease in the tissue oxygen tension with the decrease being more significant in the non pulsatile group when compared to the pulsatile group.

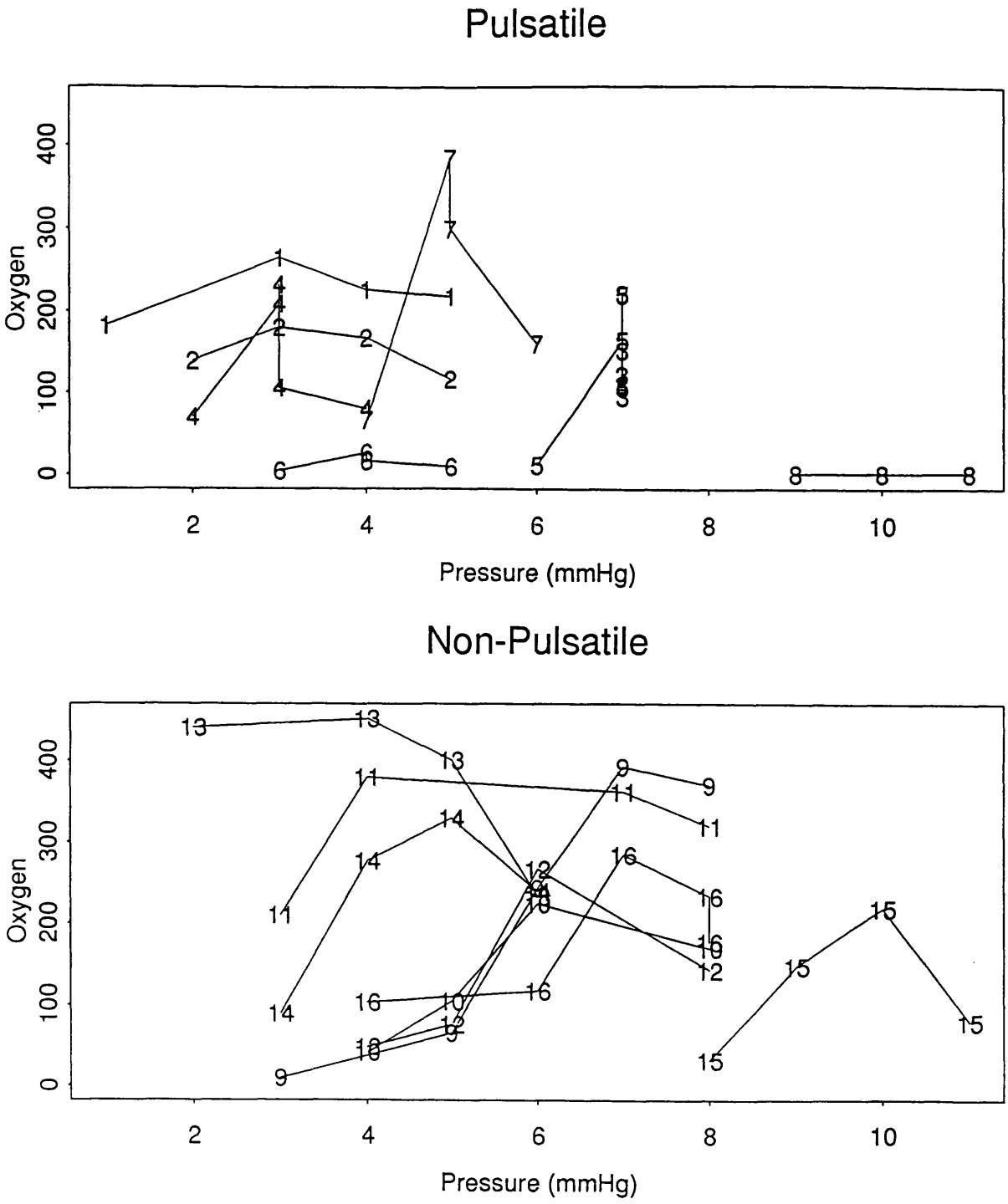
4.3.7 DISCUSSION

The wick technique employed in this study for the measurement of compartment pressure has several advantages. The wick allows a relatively large volume of tissue fluid to permeate and permits transmission of interstitial fluid pressure into the fluid filled catheter. The wick keeps the orifice open and no artefact of injected fluid exists around the end of the catheter. The wick is completely permeable allowing even the passage of red blood cells[324]. The wick cannot act as an osmometer, and is an ideal means of measuring hydrostatic pressure in the muscle. The wick technique is easy to master, inexpensive and its component parts are readily available in most hospitals.



Subjects are labelled 1-8 for pulsatile group
Subjects are labelled 9-16 for non pulsatile group

Figure 36 Relationship between compartment pressure and flow in the compartment pressure study



Subjects are labelled 1-8 for pulsatile group
Subjects are labelled 9-16 for non pulsatile group

Figure 37 Relationship between compartment pressure and oxygen in the compartment pressure study

The results of this study show that there is a significant increase in the compartment pressure in the ILP circuit with time in both the pulsatile and the non pulsatile group. The oxygen levels also drop inversely to the compartment pressure and the fall is significantly greater in the non pulsatile group. This is not suprising as the rate of increase of the compartment pressure is greater in the non pulsatile group and is at least double that of the pulsatile group.

The flow rate rises with the rise in the compartment pressures. But the opposite relationship is seen between the reservoir volume and the compartment pressure i.e. as the compartment pressure increases, the reservoir volume decreases.

These observed results are perhaps not suprising. As flow is increased in the perfusion circuit to achieve high oxygen levels, the compartment pressure also rises because as flow rates increase, an increased fluid volume is being perfused in a closed compartment causing an inevitable increase in the pressure in a fixed volume compartment. This resultant increase in compartment pressure alters the balance between the venous pressure and interstitial pressure, resulting in fluid being pushed into the interstitial space.

This loss of fluid from the circuit is seen as a decrease in the reservoir volume as fluid from the reservoir is pumped into the circuit to maintain the flow rate necessary to prevent fall in tissue oxygenation. Initially the rise in the compartment pressure does not affect the oxygen saturation achieved, but with time, as the flow rate increases and the volume loss into the interstitial space increases, compartment pressure rises significantly compromising local circulation and oxygen delivery.

These observations are in keeping with the arteriovenous gradient theory. As the local tissue pressure rises, the local venous pressure also rises causing a resultant decrease in the arteriovenous gradient. When this happens, the local blood flow is compromised and tissue oxygenation is affected. Thus at higher compartment pressures, it is not surprising to find that the oxygen level decreases.

Furthermore, in this study there was a highly significant difference in the time to maximum oxygen with the non pulsatile group taking on average 2 to 7 minutes longer. This reinforces the finding of the first study of the advantage of pulsatile blood flow over non pulsatile blood flow.

The maximum compartment pressure did not rise above 20 mmHg in both groups of patients. Compartment syndrome is normally seen when pressures exceed 20 mmHg. Only one patient undergoing non pulsatile perfusion developed postoperative compartment syndrome and this was due to arterial occlusion secondary to an artheromatous plaque that was dislodged during the surgery.

Heppenstall et al[325] compared tolerance of skeletal muscle to tourniquet application (ischaemia) with acute compartment syndrome (ischaemia and pressure). They showed that during ischaemia cellular levels of phosphocreatinine decreased at an identical rate in both groups. In contrast, the level of adenosine triphosphate diminished rapidly in animals with the compartment syndrome but remained unchanged in the tourniquet group.

Elevated tissue pressure appears to act synergistically with ischaemia to produce more severe cellular deterioration than ischaemia alone. This study of the ILP circuit reinforces the fact that even though ischaemia is important, increasing tissue pressure in a fixed volume space such as in the ILP circuit can compromise the local circulation.

For many years the ILP circuit operated under normothermia. When Cavaliere et al[210] reported biochemical and clinical studies showing selective sensitivity of cancer cells to heat, hyperthermia then became the "norm" for ILP. Although these changes brought about improved results, a greater number of complications were seen including injuries to nerves. Schraffordt Koops[326] observed these complications and on the basis of his experience, he recommended routine prophylactic fasciotomy to prevent loss of function on the perfused limb.

Fasciotomy is currently the only treatment available for the treatment of compartment syndrome. Fasciotomy facilitates microvascular circulation[327] but in the fasciotomised muscle, mitochondria are unable to resynthesise phosphocreatinine due to either intrinsic metabolic alteration or limited oxygen available[325]. Although fasciotomy may be effective, it is an imperfect treatment with associated morbidity.

This study has shown that there is a significant rise in the compartment pressure with time in the current ILP circuit where blood flow is non pulsatile. The rate of rise of the compartment pressure is at least twice in the ILP circuit operated in the non pulsatile mode as in the pulsatile mode. Introducing pulsatile perfusion into the ILP circuit may be an effective alternative to performing prophylactic fasciotomy and its attendant morbidity. As a result of this study, pulsatile perfusion has been introduced

into the ILP circuit and to date there has been no necessity to perform fasciotomy either prophylactically or therapeutically.

4.4 EFFECT OF VASODILATORS IN THE ISOLATED CIRCUIT

4.4.1 INTRODUCTION

There have been several reports of vasoactive drugs significantly affecting the nature of blood flow in experimental rodent and human tumours[328,329,330]. It is theoretically possible that in the isolated limb perfusion circuit, the use of such drugs may be beneficial. By decreasing the blood pressure and the peripheral arterial resistance, these drugs can be used to enhance blood flow with the resultant effect of increased tissue oxygenation at lower flow rates. It has already been shown in the previous section that high flow rates predispose to higher compartment pressures and ultimately lower oxygen levels.

These vasodilators could also have potential benefits in certain patients in whom a significant transcutaneous oxygen level cannot be achieved by the current techniques employed. Furthermore, as effective concentration of melphalan is dependent on the volume of perfusate, a lower flow rate is more favourable as the concentration of the drug can be increased with higher tissue concentration of melphalan being achieved.

The aim of this study was to investigate if vasodilators could be used to advantage in following situations:
1) To increase the transcutaneous oxygen tension in patients whom a sufficiently high transcutaneous oxygen cannot be achieved.
2) The disadvantages of high flow rates are overcome by the ability to lower flow rate while maintaining a similar level of oxygenation.

4.4.2 PHARMACOLOGY OF VASODILATORS

Verapamil hydrochloride is a calcium channel blocker and is classified as a Class IV anti-arrhythmic agent. A calcium channel blocker acts by inhibiting the inward movement of calcium in the smooth muscle cells of the systemic and coronary arteries and in the cells of cardiac muscle and the intracardiac conduction system. It lowers peripheral vascular resistance with little or no reflex tachycardia. Its efficacy in reducing both raised systolic and diastolic blood pressure is thought to be primarily due to this mode of action.

When verapamil is given intravenously, a dose of 5 to 10 milligrams is injected over a period of 30 seconds and if necessary a further 5 milligrams may be injected 5 to 10 minutes after the first.

4.4.3 PATIENTS AND METHODS

Twenty-one patients who were undergoing isolated limb perfusion for malignant melanoma of the lower and upper limb during the period January 1993 to August 1993 were recruited. There were 13 women and 8 men with a mean age of 60 (range 50-77) years. They underwent isolated limb perfusion as described in chapter 3. Sixteen patients had ILP carried out in the non pulsatile mode whilst 5 patients had ILP in the pulsatile mode. There were 15 iliac perfusions, 4 axillary perfusions and 2 popliteal perfusions.

Once the circuit had been stabilised, 10 milligrams of verapamil was given intra-arterially into the arterial line of the circuit over 30 seconds. Perioperative and intraoperative recordings of dorsalis pedis/radial artery pressures and transcutaneous oxygen tensions were made at five minute intervals. Transcutaneous oxygen was measured using the Radiometer TCM2 System transcutaneous oxygen meter and dorsalis pedis/radial artery pressures were measured by using an intra-arterial cannula that was connected to a transducer and a recorder. The transducer used in this study was a complete pressure monitoring kit directly attached to the intra-arterial line. For safety reasons, the transducer was used only once and disposed.

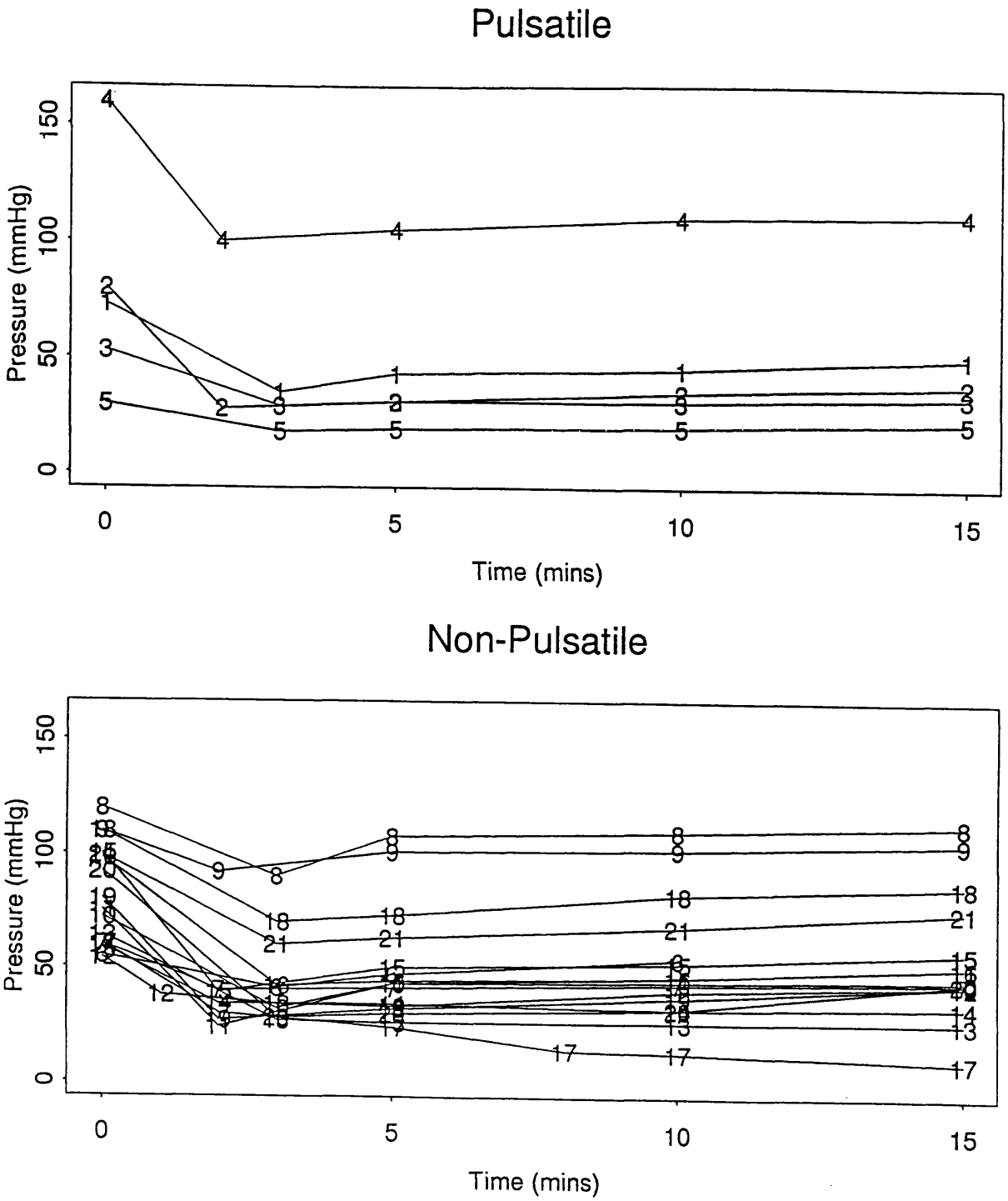
4.4.4 RESULTS

The results were analysed with respect to the following parameters:

1) Peripheral arterial pressure with time.
2) Transcutaneous oxygen with time.
3) Flow rate with time.
4) Flow rate with oxygen.

Peripheral arterial pressure with time

Following injection of the vasodilator, there was a fall in dorsalis pedis and radial artery pressures and a steady state was reached on average after five minutes (Figure 38). When the pressure with time was formally analysed, there was an overall negative relationship between pressure and time with pooled correlations of -0.42 and -0.37 respectively for the pulsatile and non pulsatile groups.



Subjects are labelled 1-5 for pulsatile group
Subjects are labelled 6-21 for non pulsatile group

Figure 38 Relationship between arterial pressure and time in the vasodilator study

Since the fall in pressure with time occurred within the first five minutes, the change in pressure from 0 to 5 minutes was then analysed using the paired sample t-test. This test showed that in both the pulsatile and the non pulsatile group, there was a significant but equal decrease in pressure from 0 to 5 minutes.

Transcutaneous oxygen with time

Following injection of verapamil, (Figure 39), there was a fall in the oxygen level with time with a steady state reached on average between 5 and 10 minutes. When the relationship between transcutaneous oxygen tension and time was formally analysed, there was a negative relationship between oxygen and time with pooled correlations of -0.73 and -0.59 respectively for the pulsatile and non pulsatile groups.

Flow rate with time

When the flow rate was plotted against time (Figure 40), an increase with time was seen. When the relationship between flow rate and time was formally analysed, there was a strong but fairly constant positive relationship between flow and time with pooled correlations of 0.95 for both the pulsatile and non pulsatile groups.

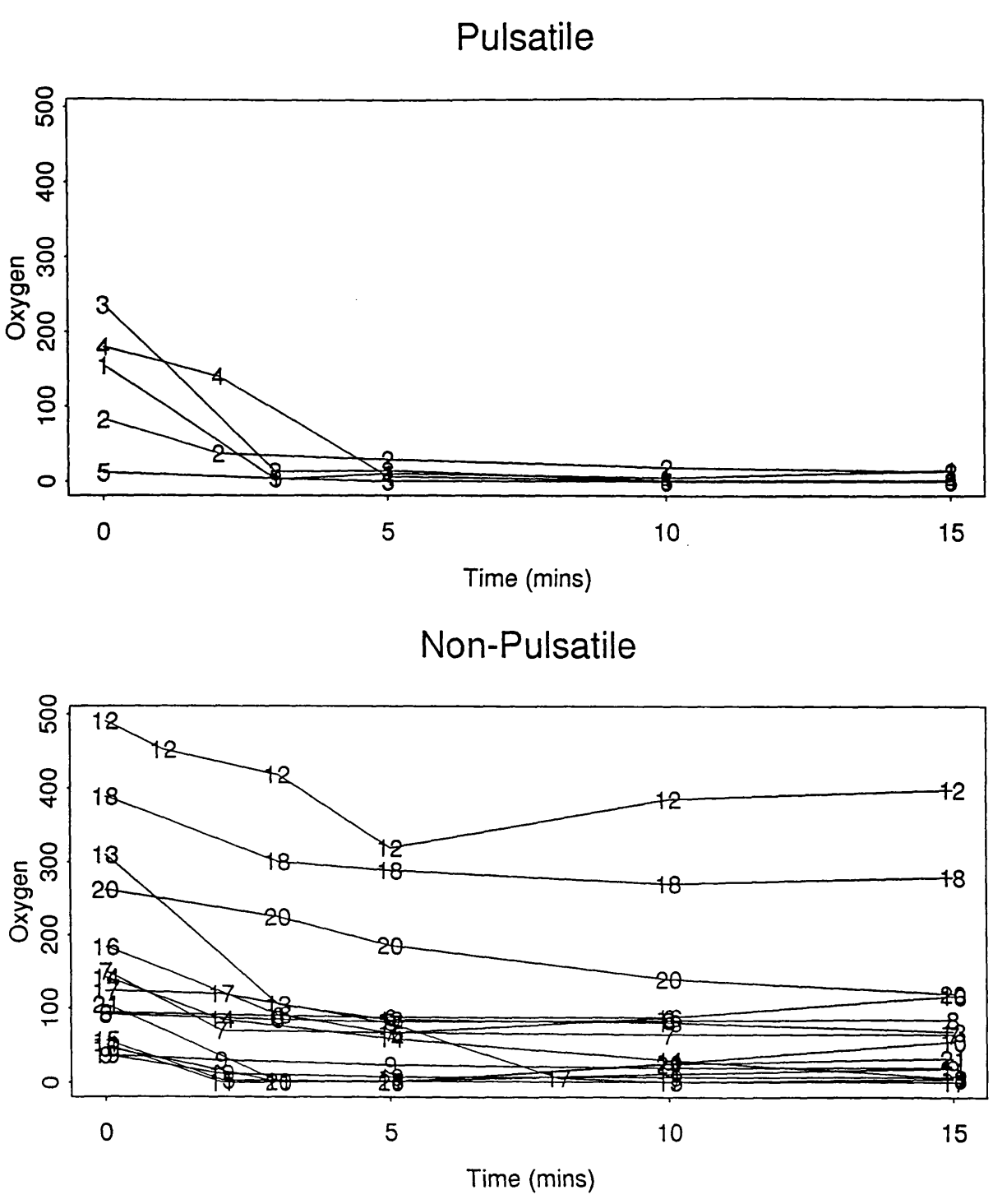
Flow rate with oxygen

When the relationship between flow and oxygen was analysed, there was a moderate negative correlation between flow and oxygen with pooled correlations of -0.68 and -0.67 respectively for the pulsatile and the non pulsatile groups.

4.4.5 DISCUSSION

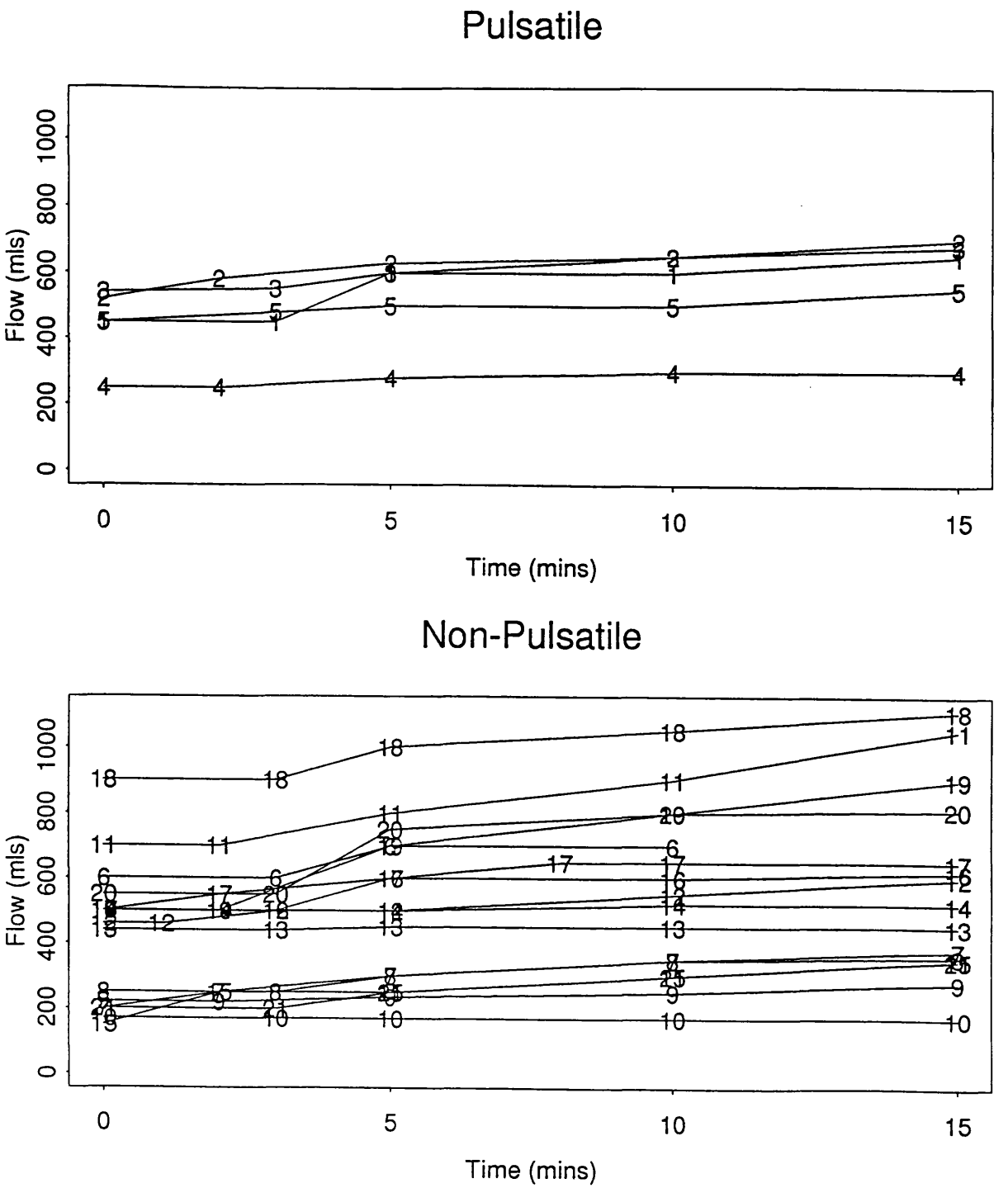
Changes in the blood flow in and around tumour masses can significantly alter the effectiveness of chemotherapy, radiotherapy and thermotherapy. The distribution of anticancer drugs to the tumour is influenced by blood flow to the tumour as compared with blood flow to other tissues[331]. This is because there are selective structural and functional abnormalities of tumour vasculature in relation to the surrounding normal vasculature that could provide a potential target for selective cancer therapy.

The destruction of tumour cells in the ILP circuit is temperature dependent. Heat dissipation during local hyperthermia treatment depends largely on blood circulation and a relatively low perfusion of tumour tissue is essential for selective heating of tumour masses[332]. Vasoactive drugs could be used to increase blood cooling of normal tissues thus allowing greater overall temperature rise in the tumour. This is because tumours are nourished by sinusoidal capillary beds that have poorly developed smooth muscle wall[333] and thus will show little response to vasoactive



Subjects are labelled 1-5 for pulsatile group
Subjects are labelled 6-21 for non pulsatile group

Figure 39 Relationship between oxygen and time in the vasodilator study



Subjects are labelled 1-5 for pulsatile group
Subjects are labelled 6-21 for non pulsatile group

Figure 40 Relationship between flow and time in the vasodilator study

agent acting on smooth muscle whilst the normal capillaries of the surrounding tissues will respond to these agents. Thus vasoactive agents could be used to achieve differential blood flow between the tumour and normal tissue.

The aim of this study was to assess whether a vasoactive agent could be used to reduce the peripheral resistance thus improving local blood flow with resultant increase in transcutaneous oxygen levels. This study demonstrated the expected fall in arterial pressure and increase in the flow rates in the perfused limbs following injection of the vasodilator. However, the expected increase in the transcutaneous oxygen level was not seen. Instead, a significant fall in the transcutaneous oxygen level resulted.

From our results, we found that the use of vasodilators to increase inflow and possibly oxygen in the tumour circulation in situations of high flow rates, high pressure states and low transcutaneous oxygen still remains a theory. Although verapamil did reduce peripheral resistance and arterial pressure, this was not associated with an increase in transcutaneous oxygen levels. This was reflected in the negative correlation seen between flow and oxygen.

Robinson[334] and associates studied the effect of verapamil and alcohol on blood flow, melphalan uptake and cytotoxicity in murine fibrosarcoma and human melanoma xenograft. They showed that in subcutaneous human melanoma xenograft, verapamil had no effect on the fractional distribution of cardiac output, nor on melphalan uptake and did not effect blood melphalan levels, suggesting absence of effects on the cytotoxicity and cellular uptake.

Although in theory there may be several advantages in using a vasodilator, in practice it may be a disadvantage because if the tumour resistance remains constant, the fall in arterial blood pressure, as demonstrated in this study, will cause a drop in the tumour blood flow and this may ultimately effect the uptake of melphalan.

From this study, it can be concluded that the use of verapamil confers no advantage with respect to the transcutaneous oxygen levels necessary for enhancing the antitumour action of melphalan. Verapamil may reduce tumour blood flow and uptake of melphalan in the isolated circuit.

However there may still be a place for vasodilators in hyperthermia treatment. They may help by acting on smooth muscle in the capillaries of the normal tissues thereby increasing blood flow and causing cooling of the normal tissue without affecting the vasculature of the tumour with overall greater input and temperature rise in the

tumour cells, optimising the cytotoxic effect of the treatment and reducing the effect of hyperthermia in normal tissues[335].

4.5 CONCLUSION

The effectiveness of ILP with melphalan is dependent on several factors.

When pulsatile blood flow was introduced into the ILP circuit, the time to maximum flow and the time to maximum oxygen was significantly shorter. There was also significantly less volume shift in the reservoir, indicating less "leak" from the circuit. Since the ILP circuit is a closed system, the fluid that leaked could increase the volume in a fixed compartment producing detrimental effects on the local blood flow and possibly tissue oxygenation. The next study was to see how this effected the compartment pressure and tissue oxygenation.

Compartment pressure in the ILP circuit was found to increase with time. The rate of increase was double in the non pulsatile perfusion. The rate of rise of the compartment pressure was directly related to the volume shift in the reservoir. With the increase in the compartment pressure, there was a significant fall in the transcutaneous oxygen level with the fall being greater in the non pulsatile blood flow mode.

From the first two studies it was clear that pulsatile blood flow in the ILP circuit, had definite advantages over non pulsatile blood flow. The next step was to determine if the high pressures and flow rates in the isolated circuit could be reduced with the use of vasodilators. There were also patients in whom tissue oxygenation continued to be low despite high flow rates. Several published studies had shown that tumour blood flow could be increased with the use of vasoactive agents.

However, in the third study, it was not possible to show that verapamil could be used to increase tissue oxygenation despite a significant decrease in arterial pressure and increase in flow rates.

In conclusion, introduction of pulsatile blood flow into the ILP circuit is a simple and safe modification that has significant physiological advantages over non pulsatile blood flow in the current ILP circuit. It also reduces the risk of developing compartment syndrome and is an effective alternative to carrying out routine prophylactic fasciotomy.

CHAPTER 5

CLINICAL EXPERIENCE OF CARBON DIOXIDE LASER ABLATION AS AN ALTERNATIVE TREATMENT FOR CUTANEOUS METASTASES FROM MALIGNANT MELANOMA

5.1 INTRODUCTION

Metastatic melanoma has a remarkably variable clinical course. There is a diverse pattern of metastatic spread via lymphatic and haematogenous routes. Metastases to skin and subcutaneous tissues are often the first sign of haematogenous spread. Local recurrence implies a poor prognosis and is often the first sign of metastatic disease[146-148]. Although the overall outlook for patients with local recurrence is poor, the probability of long term control exists.

Several studies have shown that isolated limb perfusion is the optimal treatment when local recurrence develops on the extremities. Twenty-five to fifty percent of patients treated with this therapy are alive 5 years later. In Glasgow, for patients presenting with local recurrence of the extremities, the treatment of choice is isolated limb perfusion. This form of treatment can modify the already aggressive nature of the disease and improve the survival and disease free survival in these patients.

Ideally all patients with local recurrence confined to the extremities should be considered for isolated limb perfusion treatment with melphalan. However not all patients respond to isolated limb perfusion or are suitable for this treatment. Even if long term cure in these cases is not possible, the opportunity for palliation is considerable, since subsequent regional recurrence can be disabling.

Skin and subcutaneous metastases are generally 0.5cm to 2.0cm in diameter and are readily detectable on examination. Metastases can be single or multiple and can occur anywhere on the body. The choice of treatment is influenced by the number, anatomical location, size and distribution of the cutaneous metastases and by the presence of disease at other sites. Aggressive local therapy is the most effective means of achieving regional disease control[151]. The treatment options available are surgery, chemotherapy, radiotherapy, immunotherapy and cryotherapy.

Surgery is feasible only when a few cutaneous metastases are present. Regional chemotherapy, radiotherapy and systemic chemotherapy have limited success in controlling local disease[336]. Cryotherapy has been used successfully for treating intradermal metastases but no studies have been carried out to assess the efficacy of the treatment.

In patients with a poor prognosis in whom ILP is not suitable or has failed, the use of carbon dioxide laser treatment might offer control of local recurrence. The present study was designed to assess the efficacy of the carbon dioxide laser treatment as an alternative to ILP in the control of the local recurrence of melanoma on the extremities.

The aim of treatment in this group of patients is not to cure, but to produce relief of symptoms and local control of disease with minimal disruption to the patient's life.

5.2 PHYSICS OF LASER

5.2.1 GENERAL PRINCIPLES

Atoms and molecules can only exist in certain strictly defined energy levels. The transition and resultant photon energies are therefore strictly defined. Einstein showed that the relaxation of an excited atom to release a photon could happen not only spontaneously but also as a result of interaction with another photon of the correct energy. The incident photon remains unchanged and the newly emitted photon is identical to the incident photon with respect to wavelength, phase, polarisation and direction of propagation. This process is called 'stimulated emission' and is the principle upon which the laser action is based.

Nikolai Basov, Alexander Prokhorov and Charles Townes realised that a photon emitted by an excited atom could, by initiating a chain of repeated interactions with other excited atoms of the same species, stimulate the emission of numerous other identical photons. Thus the intensity or flux of the electromagnetic wave associated with the original photon would be amplified.

To achieve this, more atoms in the upper level high energy state than in the lower level low energy state are needed, otherwise photons would tend to be absorbed by the lower level rather than stimulate emission of further photons from the upper level.

This 'population inversion' could not be easily obtained but this difficulty was overcome by Townes, who constructed the first MASER, the acronym for Microwave Amplification by Stimulated Emission of Radiation. The first LASER, acronym for Light Amplification by Stimulated Emission of Radiation, was constructed by Theodore Mainman in 1960.

5.2.2 PROPERTIES OF LASER LIGHT

1) Divergence of a laser beam is very small. The full power of the laser beam can therefore be focused onto a very small spot.

2) Due to the high collimation of a laser beam, the power per unit cross-sectional (irradiance) area of the beam is very high and remains so to great distances. Again, due to low divergence the laser can be focused onto a very small spot to produce still greater irradiance.

3) Laser light is monochromatic. The output of a laser, therefore, is a beam of light the entire output power of which is centred on one wavelength.

5.2.3 INTERACTION OF LASER LIGHT WITH TISSUE

Laser-tissue interactions are thermal. There are three types of laser currently used for their thermal effects. They are the carbon dioxide (CO_2) laser, the neodymium yttrium aluminium garnet (Nd YAG) laser and the argon laser. The absorption characteristics of these lasers vary enormously due to the differing wavelengths. The CO_2 laser beam is strongly absorbed in water whereas the other two lasers are absorbed more in pigmented cells.

Initially, heating of soft tissue causes thermal contraction, higher energies kill the cells and ultimately vaporise cellular material leaving a crater. Since the CO_2 laser is very strongly absorbed by water, it causes explosive vaporisation of intracellular water and this phenomenon forms the basis of the use of CO_2 laser as a tissue cutter or laser 'scalpel'.

The effect of laser on tissue can be maximised if the light energy is delivered using a pulsed laser in which the duration of the pulses is shorter than the time it takes for heat to diffuse out of the capillaries and the surrounding dermis.

5.2.4 ADVANTAGES OF CO_2 LASER

The CO_2 laser generates electromagnetic radiation at a wavelength of 10.6μm. The laser medium of CO_2 laser is a mixture of carbon dioxide, nitrogen and helium gas. Because of the affinity of water for mid-infrared radiation such as generated by the CO_2 laser, this laser displays unique surgical properties[337-341]:-

a) Diseased tissue volumes can be vaporised under precise visual control.
b) There need be little or no mechanical contact with the intended target.
c) Heat propagation to adjacent tissue can be minimal.
d) Micro-organisms at the site of treatment are automatically destroyed.
e) Vessels smaller than 0.5mm will be thermally sealed.

5.2.5 PRINCIPLES OF USING CO_2 LASER

1) Choice of Appropriate Wavelength.
This parameter defines both type of interaction and volume of tissues that will be denatured. CO_2 laser is an ideal choice for vaporising sharply localised tissue volumes to a shallow depth.

2) Rapid Delivery of the Required Energy Dose.
It is important to realise that a given amount of energy will denature the same mass of tissue, regardless of the rate of energy delivery. But different rates of energy delivery produce different surgical effects. Since lateral heat propagation to adjacent tissues depends on exposure, slow rates will produce conduction burns. Thus when using CO_2 laser, the surgeon must learn to use high power outputs[342].

3) Choice of the Best Temporal Mode.
Thermal spread can be decreased by selecting a superpulse mode at the expense of reduced average power to about one third of the output attainable in the continuous mode. A compromise between the high precision of rapid superpulse and the higher power of the continuous mode is obtained from the chopped mode which preserves most of the power output at the expense of allowing more lateral heat conduction.

4) Selection of an Appropriate Power Density.
Power density is defined as the number of watts used for a given spot size. This is expressed as watts/cm^2

The number of watts used is controlled by the power output of the laser to the desired level. The area of the spot size is equal to pi times the radius of the spot size squared. Laser energy can be focused through lenses of different focal lengths. The greater the focal length the larger the spot size. Larger spot sizes are produced by defocusing the laser beam or increasing the distance from the focal point to the tissue.

5) Adjusting Beam Geometry to Produce a Crater of an Appropriate Shape.
The crater shape mirrors the intensity profile of the incident energy and thus the geometry of the beam is important. Sharply focused beams have narrow spot diameters and high amplitudes, producing impact craters like drill holes. Enlarging spot diameter by partially defocusing the beam will reduce the central amplitude, thereby "flattening" the geometric shape of the intensity profile[343]. Incisions are best made with a tightly focused beam geometry whilst surface ablation should be done at a point of partial defocus. Fully defocused beams cause extensive char with negligible penetration and therefore have no surgical applications.

5.2.6 SURGICAL PRINCIPLES OF CONTROL OF CO_2 LASER

a) Choice of an Appropriate Beam Delivery System.
- See above.

b) Minimising Thermal Damage by Tissue Cooling.
Chilling the tissues with iced saline has been shown to diminish postoperative pain and swelling and contributes to a two thirds reduction in healing time[344]. Tissue should be chilled prior to initial laser treatment and at frequent intervals during the operation with reapplication of iced saline immediately following laser irradiation.

c) Control of Intraoperative Bleeding.
This problem can be overcome by using vasoconstrictor injections. Most bleeding arises when the laser punches a hole in the side of a small vessel. Resealing the bleeding point with a high powered beam for 2 to 3 seconds will secure haemostasis by transecting the perforated arteriole and sealing the cut ends[345].

d) Good exposure and a perpendicular beam impact.

e) Accurate delineation of the treatment margins.

The laser used in this study was the Sharplan 1030 CO_2 laser with 80 watts maximum output (Figure 41). It generates a wavelength of 10.6μm and 98% of the incident energy is absorbed within 0.01mm of tissue[338]. The CO_2 laser causes heating of cellular water and subsequent vaporisation. Thermal transmission to adjacent tissue is minimal and extends to no more than 0.3-0.5mm from the laser impact site[340,345]. Since the CO_2 laser beam wavelength is in the invisible range of the spectrum, a visible helium-neon beam is incorporated.

The CO_2 laser is focused through a manoeuvrable handpiece containing the lens which is used for freehand laser surgery. This handpiece has a focal point guide tip and when the handpiece is drawn away from the tissue in the defocus mode, the spot size will increase. The surgeon controls the power density by controlling the watts and spot size

5.3 PATIENTS AND METHODS

Between September 1992 and March 1994, fourteen patients (ten females and four males) with median age of 69 (range 45-94) years with cutaneous metastases from malignant melanoma which were too numerous or too large for local surgical excision, were recruited for laser ablation. All patients except two had had isolated limb perfusion in the past. Eight patients had previously had one ILP, three patients had two ILP and one patient had three ILP.

There was no clinical or radiological evidence of liver or lung metastases at the time of treatment. The number of lesions per patient ranged from three to forty nodules. There were both cutaneous and subcutaneous nodules with the diameter of the largest lesion being not more than two centimetres. In thirteen patients, the lesions were situated on the lower limb and in one patient, on the forearm.

All the treatments were carried out under general anaesthesia though local anaesthesia is sometimes practical. Since most of the patients were elderly or lived a great distance away, they stayed in hospital overnight.

The area of treatment was first injected with a vasoconstrictor agent (adrenaline) to reduce bleeding prior to treatment. The area to be treated was then washed with betadine. Before treatment, the tissue was cooled with laparotomy packs soaked in a bowl of semifrozen saline slush to minimise thermal damage[344].

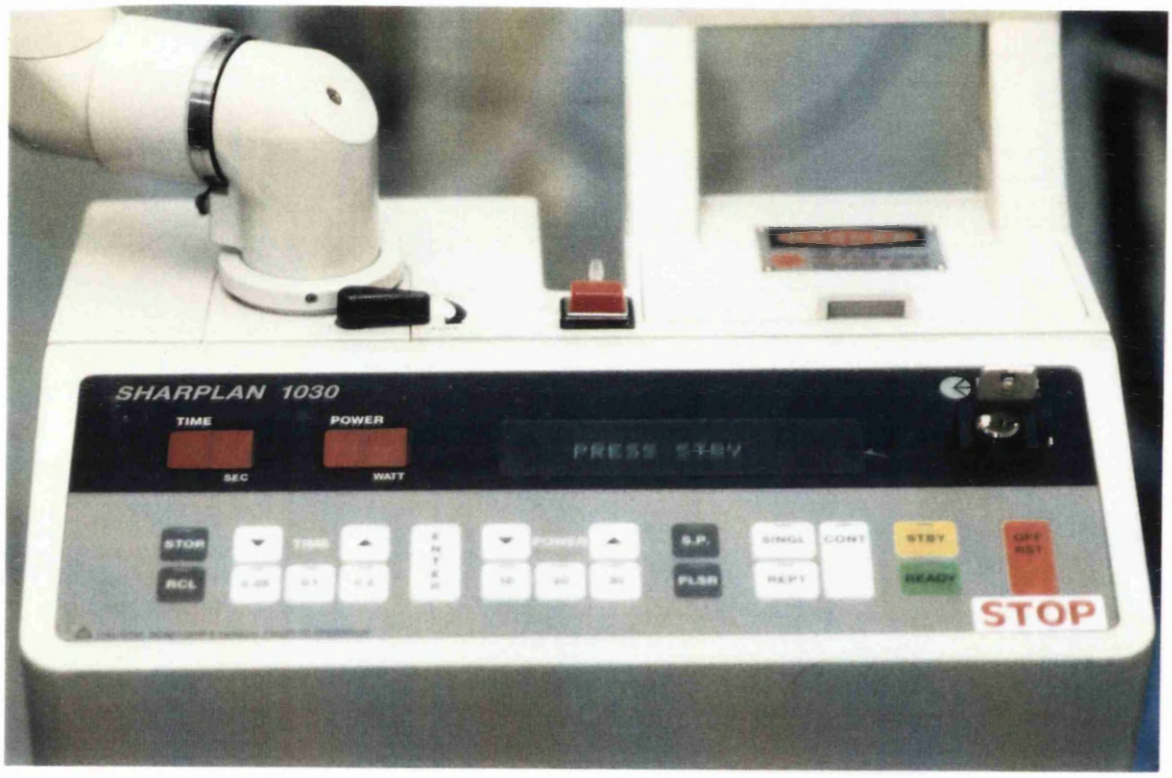


Figure 41 Sharplan 1030 Carbon Dioxide Laser

The operator and theatre personnel followed the laser code regulations issued by the Department of Clinical Physics and Bioengineering, University of Glasgow and this included the use of protective eye glasses and face masks. Once the appropriate precautions were taken, laser treatment was commenced.

The depth and diameter of the excision was variable and dependent on the size and the type of lesion. In small cutaneous lesions, a smaller spot size beam with a smaller output was used which resulted in a superficial burn with a shallow crater. In large subcutaneous lesions, a larger spot size beam was required at a higher energy output to completely ablate the lesion and in these cases, the depth of excision was greater and a full thickness burn resulted.

Ablation of the lesion was considered complete when a circumference of healthy tissue was seen. The tissues were also chilled at frequent intervals during the operation. Once all the lesions were treated, the area was then dressed with sofratulle and a dry gauze applied over.

Each lesion was ablated with a focus laser beam of spot size between 0.5 and 1.0 mm with an output between 10 to 20 watts in the continuous mode. The plume generated was removed by a smoke evacuator. Figure 42 shows a lesion being ablated. Figure 43 shows the appearance of the treated lesion in figure 42 as well as showing another lesion being treated.

5.4 RESULTS

The average time per treatment was 10-15 minutes and all the lesions were treated in one session. Since most of the lesions involved the full thickness of the skin, the resultant defects were multiple full thickness punctuate burns. Figure 44 shows a typical subcutaneous nodule and figure 45 shows the same nodule following laser treatment. Note that the treated lesion shows minimal bleeding with healthy surrounding tissue. Since the maximum diameter of lesions treated were not more than two centimetres, the wounds were small and healed without grafting.

Postoperative pain and bleeding were minimal or absent. Figure 46 shows the appearance of treated recurrences on day 1 postoperatively, the same lesions one week postoperatively (Figure 47) and six weeks postoperatively (Figure 48). Patients were reviewed at regular intervals postoperatively.

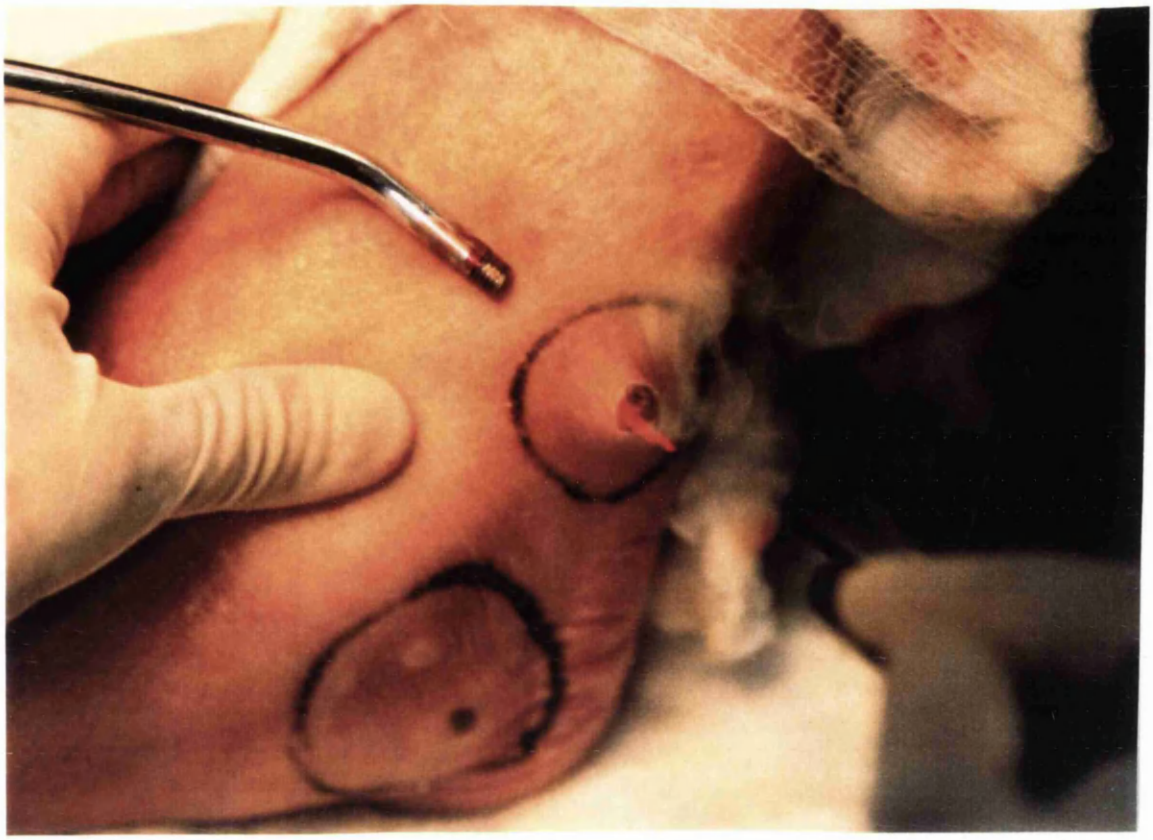


Figure 42 Laser ablation of a subcutaneous lesion

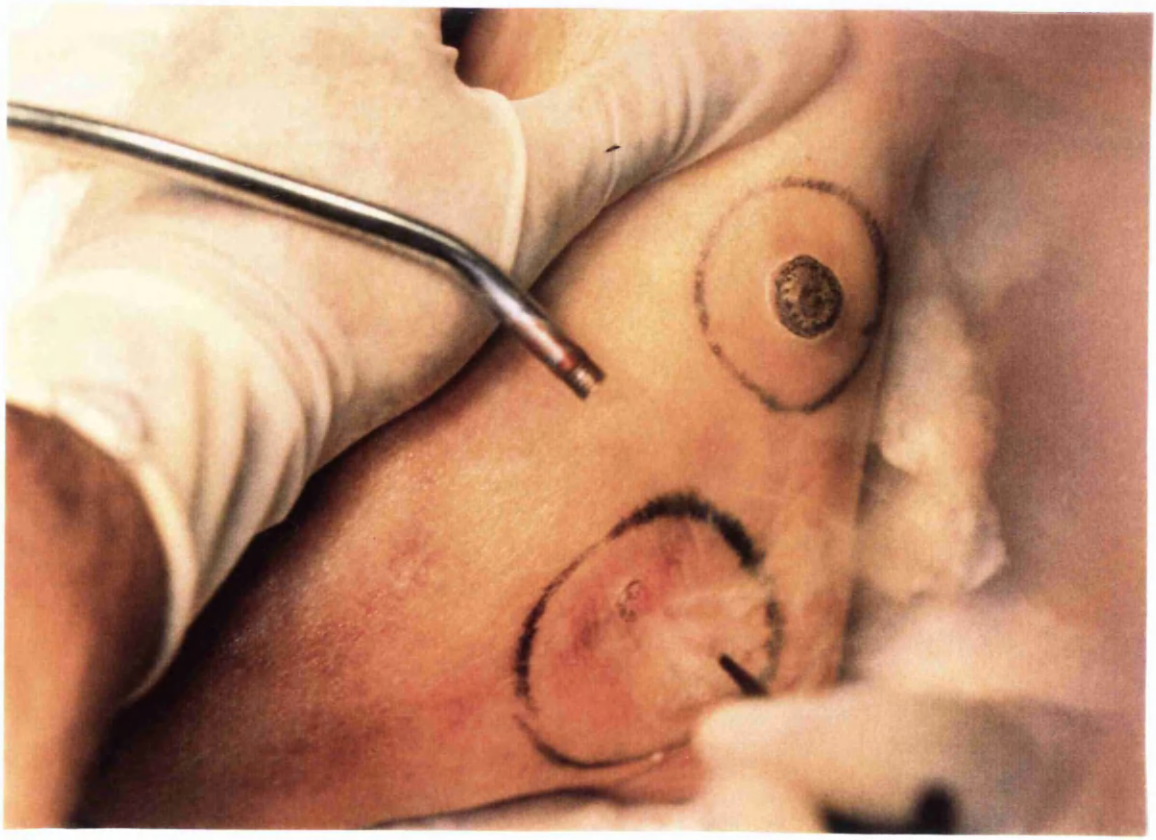


Figure 43 Appearance of the treated lesion from the figure above and laser treatment of the other recurrent nodules

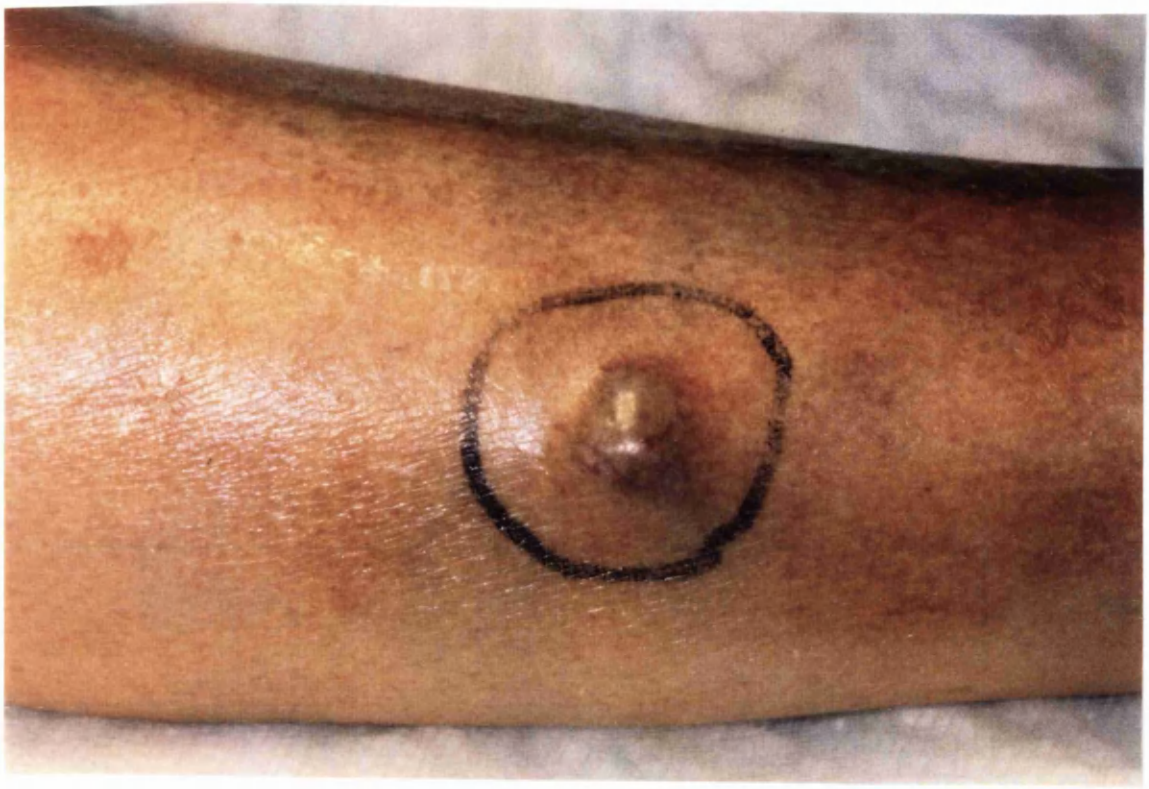


Figure 44 Typical subcutaneous nodule prior to laser treatment

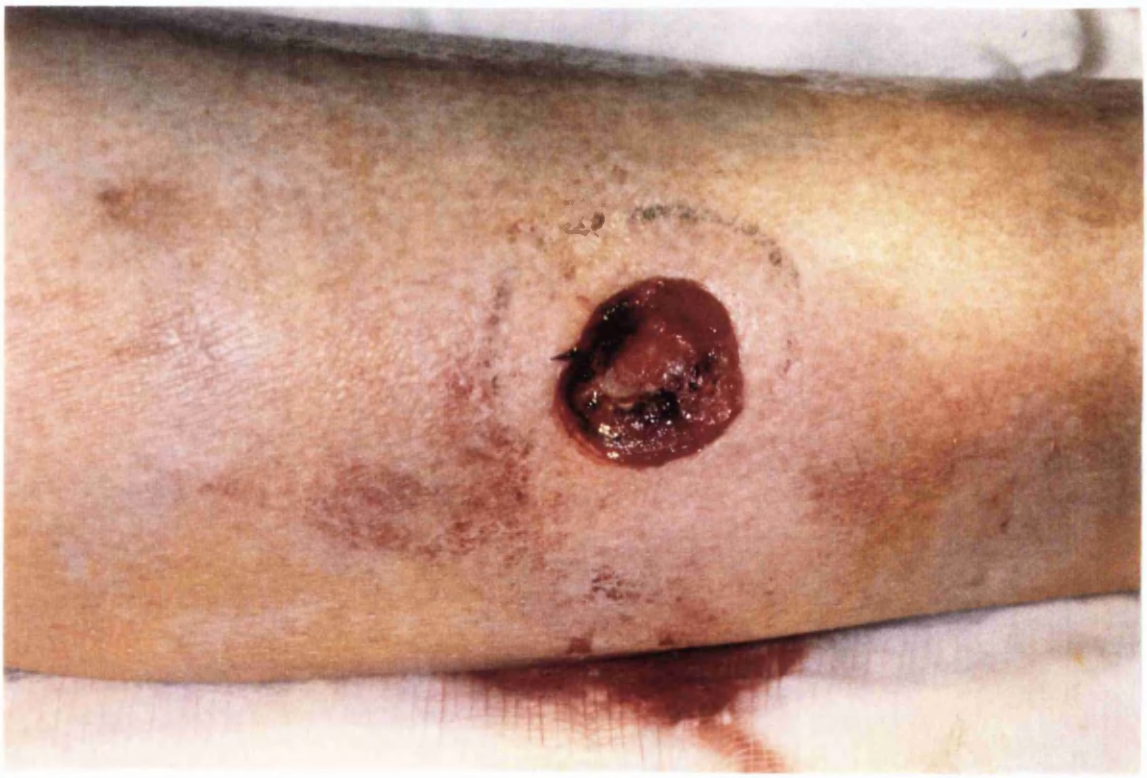


Figure 45 The same lesion immediately after laser ablation

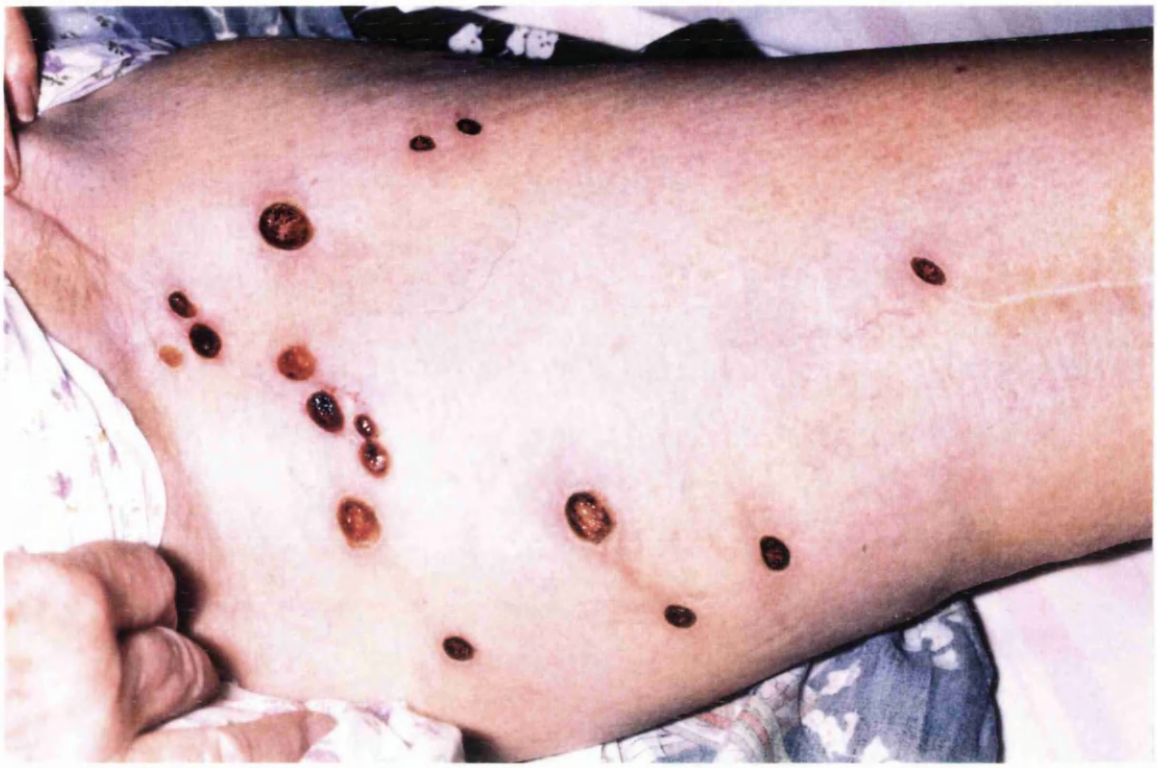


Figure 46 Appearance of the laser treated lesions on day 1 post-operatively

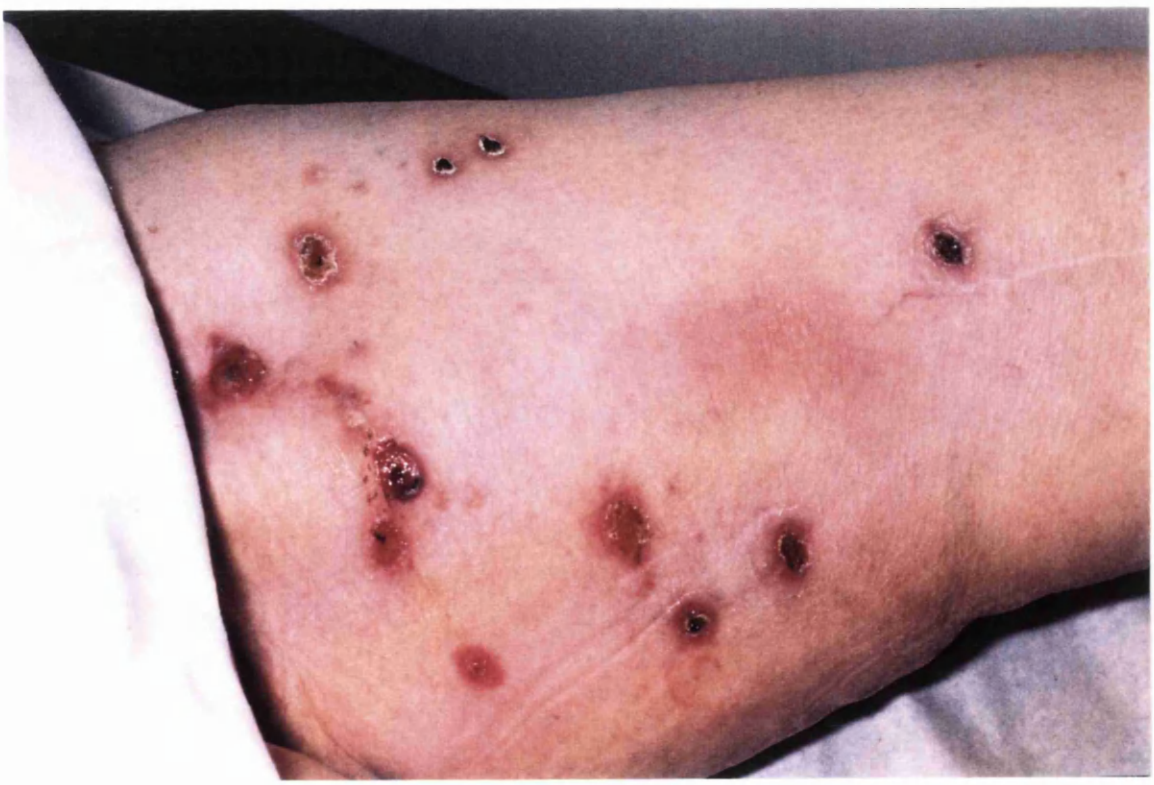


Figure 47 The same lesions at one week following laser treatment

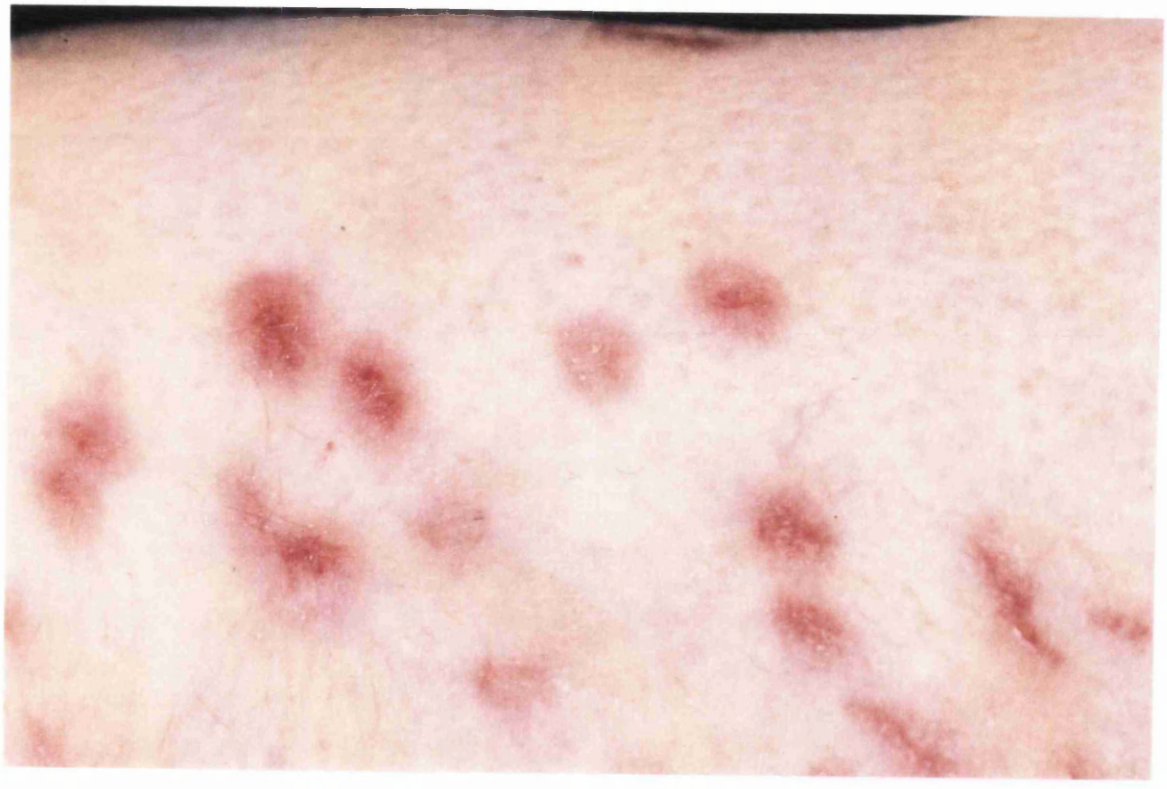


Figure 48 The same lesions at 6 weeks following laser treatment

At the end of six weeks, all the wounds were healing with acceptable cosmetic results. At three months follow up, only one patient had a recurrence at the treated site. This could be explained by the fact that this was the first patient to be treated and reflected the learning curve of the surgeon which probably resulted in failure to ablate the lesion fully. Median follow up of twelve months showed the incidence of recurrence at the treated site to be minimal. If new lesions developed, they were treated with further laser treatment. In total 27 treatments were required in the first fifteen months for the 14 patients.

Five patients died at a median follow up of 9 months from their first laser treatment; 3 were free of limb recurrences while 2 had recurrences at the time of death. Of the 9 patients who are alive, 4 have had no recurrences following the first treatment and 5 patients have required further treatments. In these five patients, 2 required two further treatments and 3 required three further treatments each. Six patients who at the first treatment had disease confined to the limb have since developed distant metastases.

5.5 DISCUSSION

Carbon dioxide laser ablation of cutaneous metastases of melanoma is an effective and safe alternative treatment to those currently used to control locally recurring disease. Since the aim of treatment is not to cure, the important consideration is that it can be carried out under local anaesthesia if necessary without much disruption to the patient's life. If these lesions are untreated they will enlarge, ulcerate and become painful.

The carbon dioxide laser with its high power density successfully ablates the lesions with minimal dissipation of heat to surrounding normal tissue and produces good healing. The use of a vasoconstrictor agent reduces intraoperative bleeding. It is important to apply iced packs following the laser treatment because cooling antagonises the continuing tissue damage by the vasoactive peptides released at the time of thermal injury. This has been shown to greatly diminish postoperative pain and swelling and also contribute to reduced healing time[344].

The CO_2 laser is a more precise method compared to diathermy and cryosurgery as it can accurately delineate the area of treatment without having to include normal tissue to ensure tumour destruction as occurs when using diathermy or cryosurgery. It is also a treatment that is simple to master.

The results of this study suggest that carbon dioxide laser treatment of cutaneous metastases should be considered as a serious alternative to the current modes of treatment available when ILP has failed or local surgical excision is not possible. Carbon dioxide laser has been shown to be effective in the control of local disease. Although some patients required several repeat laser sessions to control the disease, none required any other treatment. To date no patient has had to have an amputation to control the disease.

Carbon dioxide laser treatment has the advantage that it can be repeated several times without the high morbidity associated with isolated limb perfusion. It is however important to appreciate that laser treatment is not suitable for all patients particularly those with deep subcutaneous lesions, ulcerated lesions and lesions larger than 2 cm.

The treatment has very little morbidity and could be easily be carried out as a day case if necessary under local regional anaesthesia. Furthermore, with the lesions being infiltrated with a combination of lignocaine and adrenaline, there is very little postoperative pain or bleeding.

Following laser therapy, the time to recurrence may be short. This is not surprising as local recurrence is an indication of poor prognosis and locally aggressive disease. A single patient may need several treatments to control the disease. As seen in this study, almost half the number of patients developed distant metastases and 5 died, reinforcing the fact that prognosis is poor and cutaneous disease is a manifestation of almost inevitable distant metastases and eventual death.

Since cutaneous metastases from malignant melanoma is often the first stage of inevitable progression to widespread systemic disease, it could be argued that subjecting patients to ILP which is associated with moderate to high morbidity, a small but significant mortality may not be justified. There is a small (and difficult to define) percentage of patients, who develop cutaneous metastases but never go on to develop further disease and ILP treatment in this group may be difficult to justify.

In the light of this study and those of Waters and Clemens[346] and Hill and Thomas[347], a prospective randomised study is needed to compare isolated limb perfusion with carbon dioxide laser ablation in patients suitable for isolated limb perfusion with no other distant metastases.

In Glasgow, ILP is the treatment of choice for lesions confined to the limb. It not only deals with cutaneous recurrence but in a significant percentage of patients has been shown to provide long term control by eradicating local disease. By modifying the aggressive nature of the disease the technique also improves survival. From the experience of over 300 ILPs carried out in this unit, the morbidity of this technique has been minimal.

Although there has been only one death (gastrointestinal bleed) within 30 day following ILP, it is still a considerably greater undertaking for the surgeon and the patient compared with laser ablation. All the major studies of therapeutic ILP (chapter 3) indicate that a small but definite number of patients with cutaneous limb recurrence can achieve a long term disease free interval after ILP. It would seem appropriate, therefore, to offer ILP as the initial treatment of choice for these patients.

5.6 CONCLUSION

Carbon dioxide laser ablation only destroys the lesions locally. Longer follow up is required before it can be concluded if this treatment affects the disease free interval or survival or if it is comparable to other modalities of current treatment for control of local recurrence. Results of this study support the use of carbon dioxide laser as an effective alternative treatment for those patients in whom ILP fails or is inappropriate.

CHAPTER 6

INTRAOPERATIVE LYMPHATIC MAPPING USING PATENT BLUE DYE TO IDENTIFY REGIONAL MICROMETASTASES IN MALIGNANT MELANOMA

6.1 INTRODUCTION

Adjuvant treatment of stage I patients with ILP has not shown definite survival benefits over conventional surgical treatment in the form of wide local excision. The problem is reflected in the fact that in a large number of these patients, metastases may well have occurred by the time of diagnosis. Thus if long term control is to be achieved in these patients, the answer needs to be found in the biology of melanoma metastases and detection.

As early as the turn of this century, Handley[3] explained the mode of spread of melanoma. Many of his observations remain valid today. He commented that dissemination of malignant cells first occur to the fine lymphatics followed by centrifugal spread to the main lymphatics and invasion of the blood stream. In the later stages, the slow process of lymphatic permeation recedes into insignificance as the patient dies with widespread deposits resulting from blood embolism.

The process of cancer metastasis consists of a series of steps which the tumour cell must complete to produce clinically relevant lesions. These steps are shown in figure 49. The major steps involve progressive growth of the neoplastic cell with extensive vascularization. This is followed by invasion of the host's stroma. Thin walled venules and lymphatics offer little resistance to penetration and provide the most common pathways for the tumour cells to enter the circulation. Detachment and embolisation of small tumour cell aggregates occurs but rapid destruction of the majority of these cells follow.

Those tumour cells that survive aggregate in the capillary beds of the organs. Extravasation occurs next followed by proliferation within the organ. To produce detectable lesions, the metastases must develop a vascular network and evade the host's immune mechanism. Once they do so, the cells can invade host's stroma, penetrate blood vessels and enter the circulation to produce additional metastases.

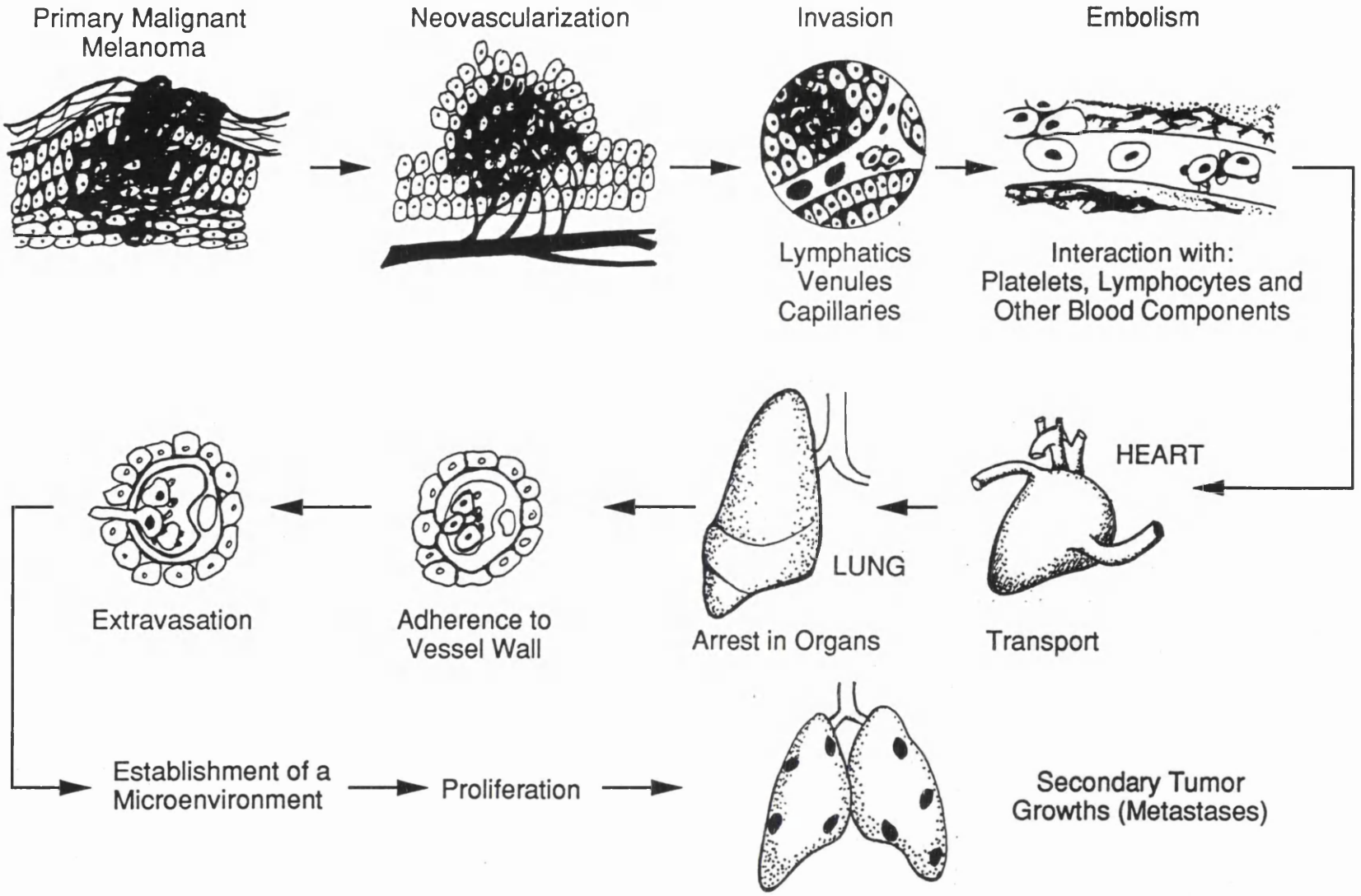


Figure 49 Process of cancer metastasis

If treatment could be directed towards disrupting any part of this process and destroying the tumour cells, then there is a possibility of cure. The process needs to be halted at the early phase of neovascularization and invasion of stroma into the lymphatics and vascular tree.

Intraoperative lymphatic mapping may be a technique which allows identification of the lymph nodes that harbour tumour cells. Removal of such nodes may increase the chance of a cure, as this removes those cells that invade the lymphatics. However melanoma not only spreads through the lymphatics but also through the vascular tree. If a method could be developed to target these cells in the blood system, then these single metastatic cells could be mopped up and destroyed before they establish and develop a vascular network within the distant organs. The author proposes that the use of labelled radioactive isotopes may be such a treatment. These isotopes could be used in the ILP circuit to selectively destroy metastatic cells in the limb. Chapter 7 gives the details and results of such treatment.

Radical lymph node clearance is generally recommended for melanoma patients with clinically suspicious nodes or biospy proven nodal disease. The role of lymphadenectomy in melanoma patients without clinical evidence of lymph node metastases remains controversial. Such patients present a therapeutic dilemma. Some surgeons advise immediate elective lymph node dissection[348,349] in all patients with high risk malignant melanoma (Breslow 1.5-4.0mm). They accept that many patients will undergo an unnecessary operation which is associated with significant morbidity. Others adopt a 'wait and see' policy[350,351] and remove lymph nodes if and when they become clinically palpable. Neither policy is ideal.

Proponents of elective lymphadenectomy claim that the risk of developing regional node disease increases with increasing thickness of the primary melanoma. They argue that lymphadenectomy would most apply to the group with an intermediate thickness lesion in the range 1.51mm to 4.0mm where the risk of regional node metastases is high at 57% but the risk of distant metastases is low at 15%[352].

Inevitably the argument against this policy is almost equally as strong and is mainly based on the fact that routine elective lymphadenectomy would subject large numbers of patients to an operation which carries a definite and quite considerable morbidity, a small but inevitable mortality, and who will ultimately prove to have no metastatic disease.

Clearly, therefore, if a technique could be developed that would allow positive identification of the subgroup of patients with clinically occult nodal disease, it may well be that such patients would be most likely to benefit from radical lymphadenectomy. **Lymphatic mapping may be such a technique.** The technique was first described by Morton[353] and Cochran[354] but several workers in Europe tried to reproduce the results without success. The method had never before been assessed in the United Kingdom.

The aim of this study was to answer the following questions:

1) Is the technique practical?
2) Is the technique of identification of a sentinel node using patent blue dye sensitive in detecting nodal metastases?
3) Does skipping of the first nodal basin occur?

6.2 ANATOMY OF LYMPHATIC DRAINAGE

6.2.1 LYMPHATIC DRAINAGE OF LOWER LIMB

Most of the lymphatic fluid from the lower limb drains to a terminal group of inguinal nodes in the groin which are divided into superficial and deep groups.

The superficial inguinal lymph nodes are arranged in an upper and lower group; the former usually consists of five to six nodes forming a chain immediately below the inguinal ligament and the latter group consists of four to five nodes disposed vertically along the terminal part of the great saphenous vein. The superficial inguinal nodes drain into the external iliac lymph nodes.

The deep inguinal lymph nodes vary from one to three and are on the medial side of the femoral vein. The lowest node is situated just below the junction of the great saphenous vein and femoral vein, the middle in the femoral canal and the highest in the lateral part of the femoral ring.

Lymphatic drainage of the superficial tissue of the lower limb occurs via superficial lymph vessels. There are two main groups (the medial and the lateral group) which both drain into the lower group of the superficial inguinal nodes. The lymphatic drainage of the deeper tissue of the lower limb is via the deep lymph vessels which accompany main blood vessels and drain into the deep inguinal nodes. Figure 50 shows a diagrammatic illustration of the lymphatic pathway of the lower limb.

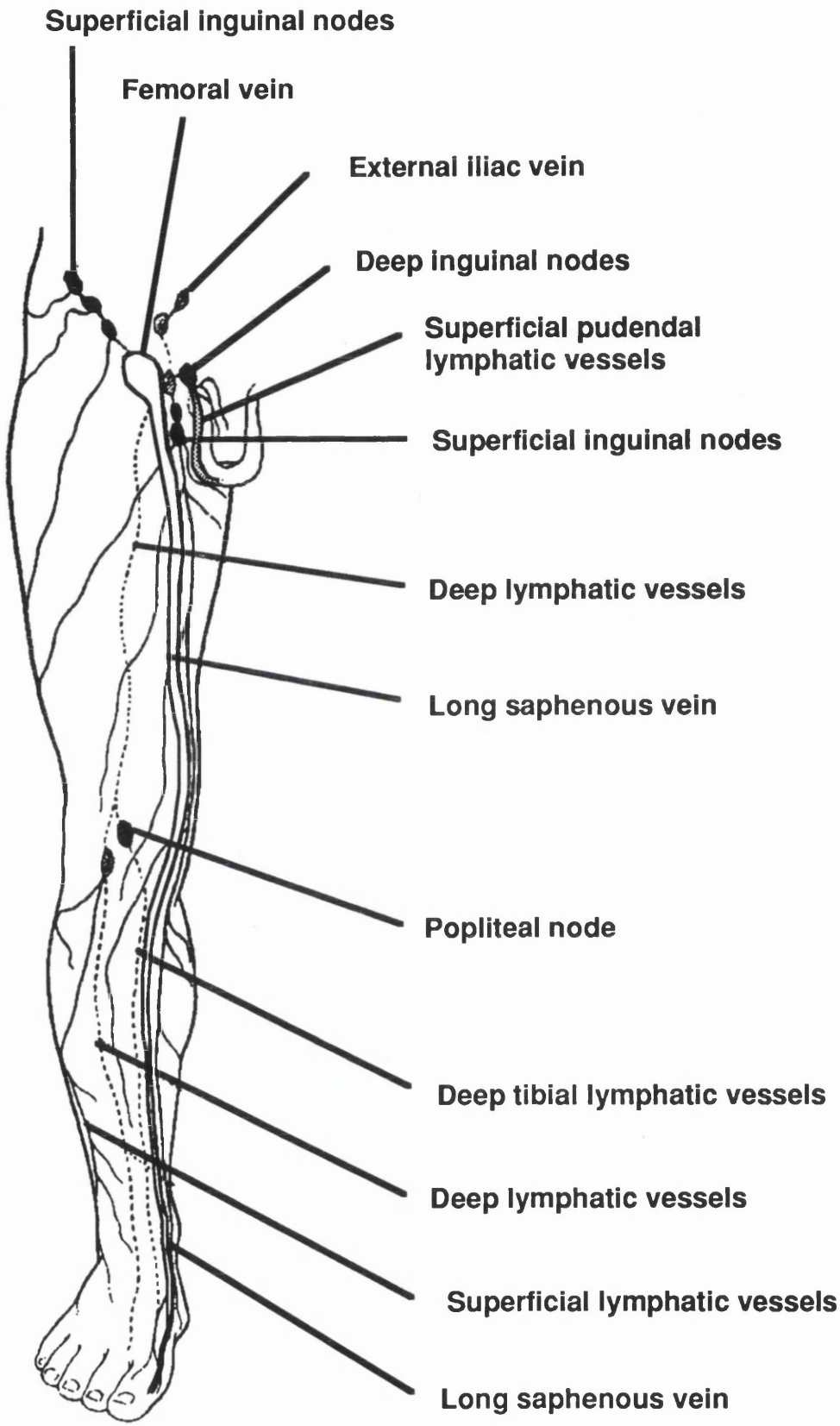


Figure 50 Diagrammatic illustration of the lymphatic pathway of the lower limb

6.2.2 LYMPHATIC DRAINAGE OF UPPER LIMB

All the lymph vessels of the upper limb drain into a terminal group of lymph nodes in the axilla, either directly or via outlying groups of lymph nodes.

The axillary lymph nodes are numerous, varying from twenty to thirty in number and may be divided into five groups. They are the lateral group, the anterior or pectoral group, the posterior or subscapular group, the central group and the apical group.

The superficial tissues of the upper limb drain by superficial lymph vessels which have two main branches. The lateral lymph vessel accompanies the cephalic vein and ends in the infraclavicular nodes. The medial lymph vessels accompany the basilic vein and end in the lateral axillary nodes.

The deep tissue of the upper limb is drained by deep lymph vessels which accompany blood vessels and join to form a terminal collecting trunk which lies alongside the brachial vessels and ends in the axillary nodes. Figure 51 shows a diagrammatic illustration of the lymphatic pathway of the upper limb.

6.3 PRINCIPLE OF INTRA OPERATIVE LYMPH MAPPING USING BLUE DYE

This technique is based on the theory that the initial route of metastases in most patients with melanoma is via the lymphatics to regional nodes. It also assumes that the spread occurs in an orderly pattern with the metastatic cells draining into the first node in the lymph node group and there is no skipping of the nodal basin.

The technique of lymph mapping allows identification of a sentinel lymph node, which is the lymph node nearest the site of the primary melanoma. By identifying and removing this sentinel node (which is the most likely site for early metastases) it can be examined for occult melanoma cells. The sentinel node is the first node to be stained blue by this technique. If the examination of the node shows metastatic cells, then the patient may benefit from a complete lymph node clearance.

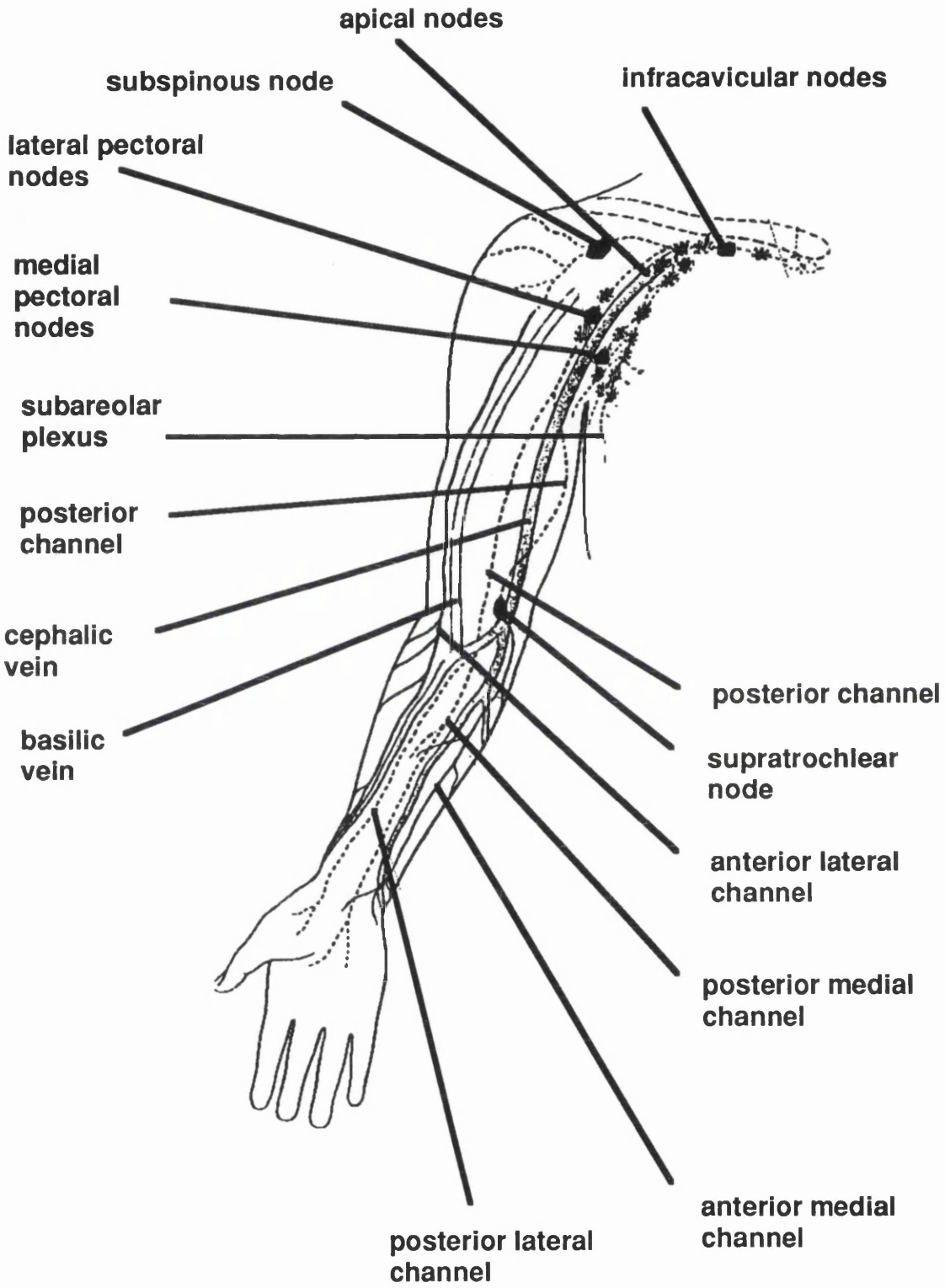


Figure 51 Diagrammatic illustration of the lymphatic pathway of the upper limb

6.4 PATIENT DETAILS

The procedure of intraoperative lymph mapping using blue dye was carried out on 30 patients between February 1992 and September 1993. The series included 22 females and 8 males whose mean age was 57 (range 25 to 80) years. According to the M. D. Anderson staging, 25 patients had stage I disease (primary disease), 1 patient had stage II disease (local recurrence) and 4 patients had stage IIIA disease (intransit metastases/satellitosis, skin recurrence). One patient had the primary lesion intact but presented with satellitosis. The primary lesions were situated on the lower limb in 22 patients and upper limb in 8. The mean Breslow thickness was 3. 4mm (range 1.5-8.1 mm). All patients had no evidence of distant metastases at other sites confirmed by ultrasound scan or CT scan and chest X-ray.

6.5 OPERATIVE TECHNIQUE

The procedure is carried out under general anaesthesia. The dye used is Patent Blue V. It comes in a prepacked vial containing 2.5% of Patent Blue V in a sterile isotonic solution. Patent Blue V can provoke an allergic reaction of varying degrees of severity. These reactions are rare and can be controlled with a corticosteroid. 0.5cc to 1.0cc of the dye is injected intradermally around the site of the primary melanoma using a 25G insulin syringe (Figure 52).

If the primary melanoma has already been removed, the intradermal injection is made into either side of the excision scar. It is important that injection of the dye is intradermal as subcutaneous injection will result in passage of the dye into the deeper lymphatic channels along the veins, bypassing the nodes which drain the dermal plexus. As small a volume of dye as possible is used to prevent extravasation into the subcutaneous tissue. The tumour and surrounding skin will stain blue (Figure 53).

The injection site is gently massaged to encourage passage of the dye along the lymphatics. Immediately an incision is made over the lymph basin which is the site of the expected lymphatic drainage. The skin flap closest to the primary melanoma is then dissected free from the underlying tissue and lymphatic channels, taking care to remain superficial to the lymphatic channels to avoid dividing them. When a blue lymphatic channel is identified , it is followed through the fatty subcutaneous tissue of the lymph basin to the draining lymph node which turns blue.

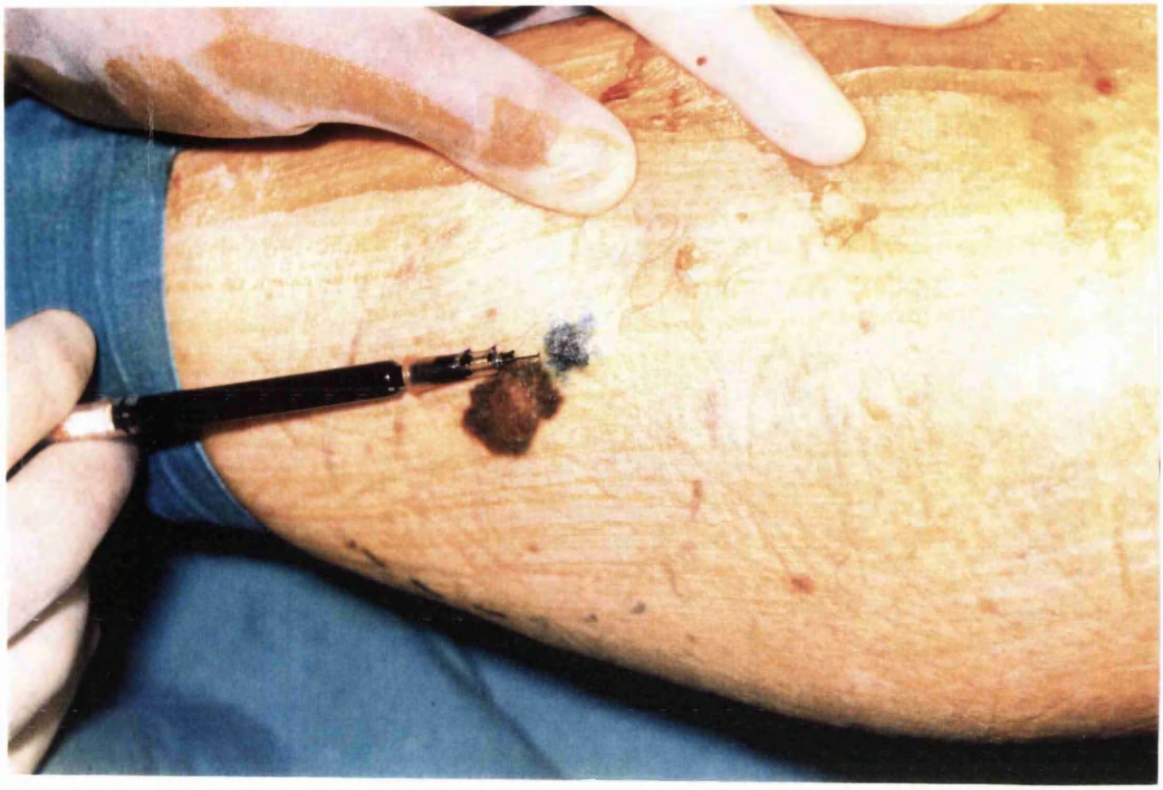


Figure 52 Intradermal injection of patent blue dye using a 25G insulin syringe around the site of the malignant melanoma

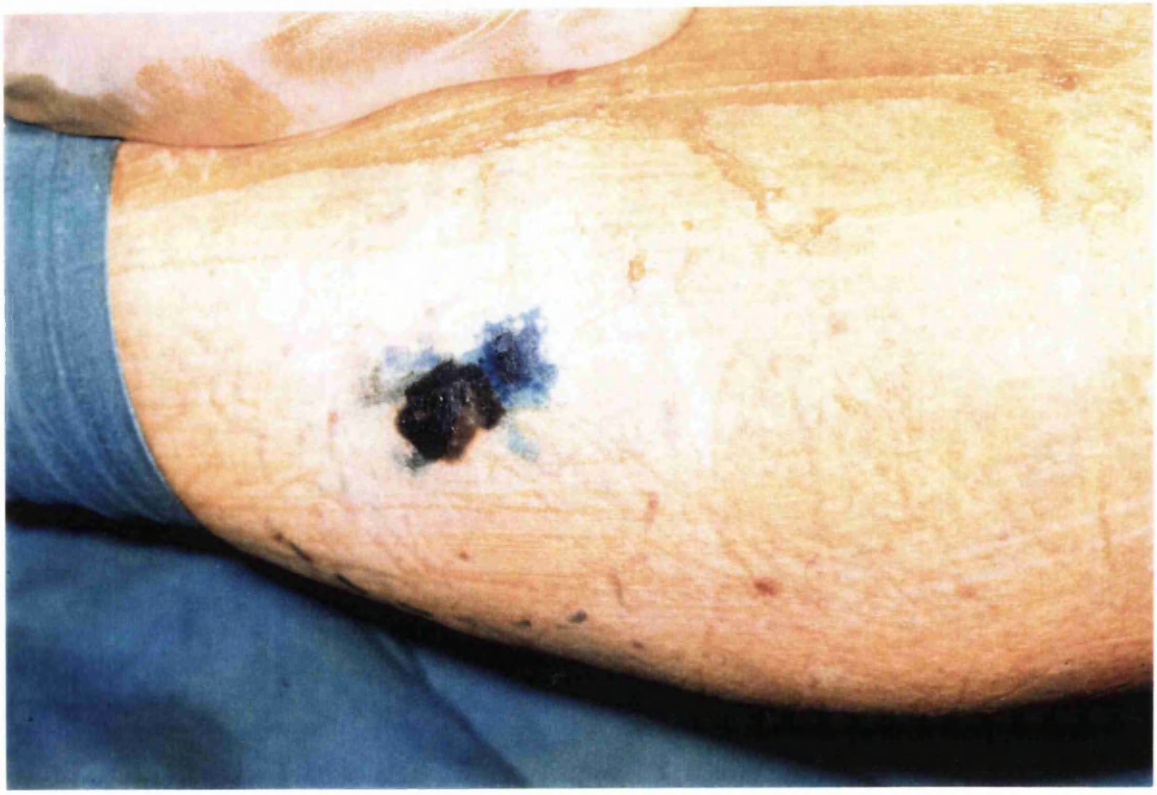


Figure 53 Appearance of the malignant melanoma and the surrounding skin after the injection of patent blue dye

Figures 54 to 57 illustrates the above technique. In figure 54, the earliest trace of the blue dye in the tiny lymphatics running parallel to the long saphenous vein is seen. In a very short time these lymphatics deepen in colour and the operator will gain an impression that they are draining towards a regional node, the so called sentinel node (Figure 55). Within a very few moments that staining increases and figure 56 shows afferent lymphatic channels approaching a node and an efferent channel heading away towards a second node. In some patients there can be more than one sentinel node. Two sentinel nodes are shown in figure 57.

Careful exploration is carried out around the sentinel node to identify any additional blue nodes. When the sentinel node has been identified it is carefully removed with control of all surrounding lymphatic channels. Two other non sentinel lymph nodes are also removed for examination. In the patients undergoing iliac limb perfusion, iliac nodes are also removed for examination.

6.6 PATHOLOGICAL TECHNIQUE

The fresh sentinel lymph node is bisected from the hilum to the periphery; one part is processed by routine haematoxylin and eosin (H&E) staining and the other part is processed for rapid immunoperoxidase staining. All the nodes are then fixed in formalin, embedded in paraffin, sectioned and examined by both H&E and immunohistochemical staining using S-100 protein antibody and melanoma reactive monoclonal antibody ($NKIC_3$).

The rapid immunoperoxide staining technique used is as follows:
The solutions used for the technique are:

1) Tris buffer (T.B.) pH 7.6	6.6 gm Tris Hydrogen Chloride (HCL) 1.39 gm Tris Base 1 litre distilled water
2) Tris buffered saline (T.B.S.)	900ml saline (8.5 gm Sodiun chloride/litre) 100 ml Tris buffer
3) Pronase	0.1% Pronase in Tris buffer (incubate for 15 minutes at room temperature)

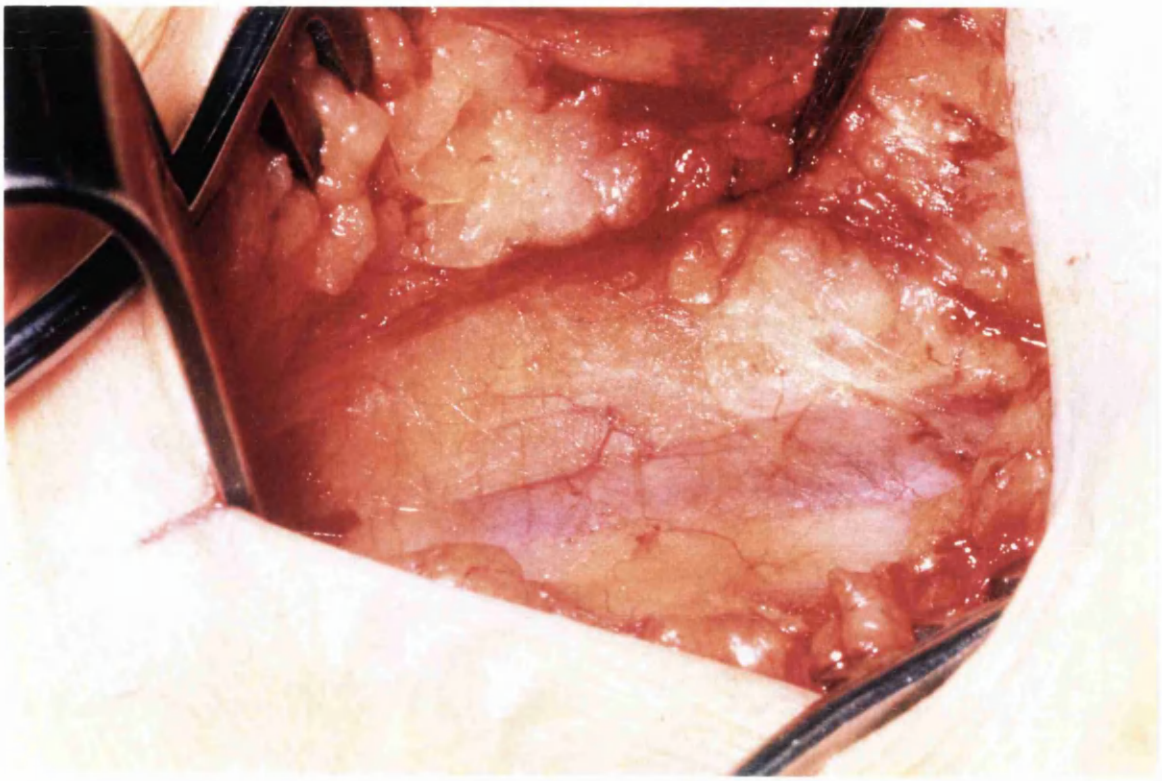


Figure 54 Earliest trace of dye in the tiny lymphatic channel parallel to the long saphaneous vein

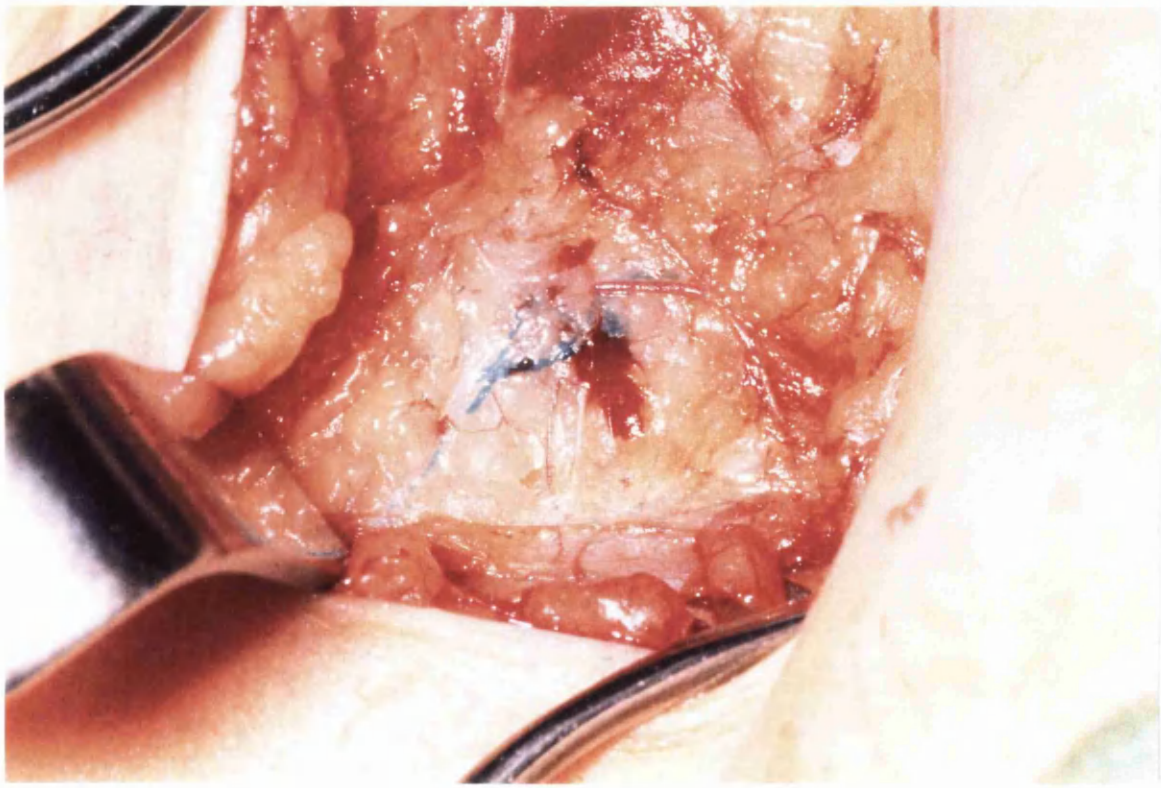


Figure 55 Blue dye becoming stronger in the lymphatic channel.

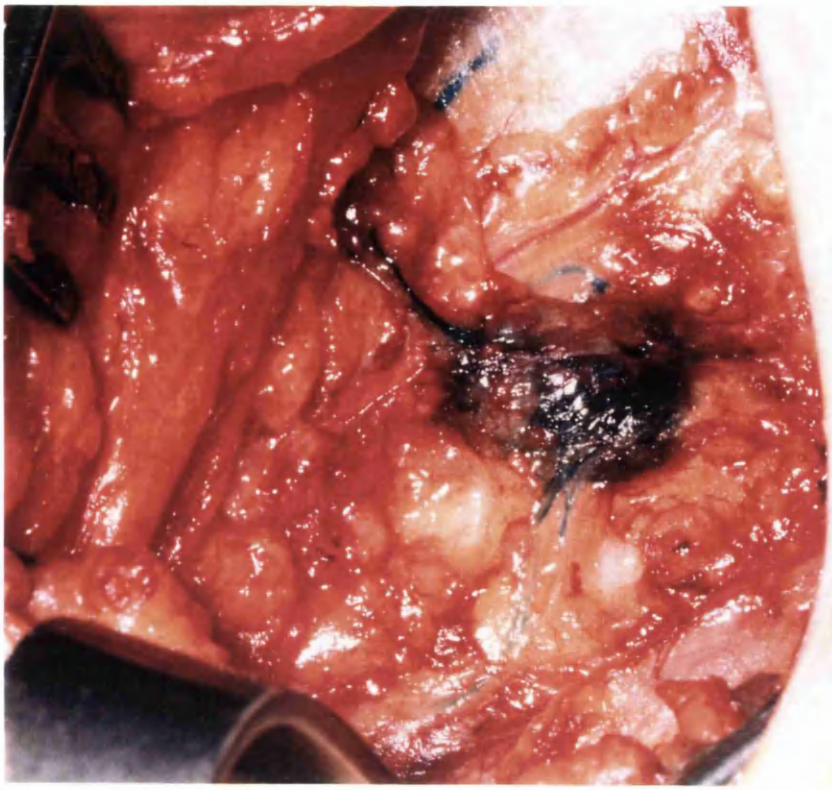


Figure 56 Afferent lymphatic channels are seen draining into the blue node with efferent channels draining away

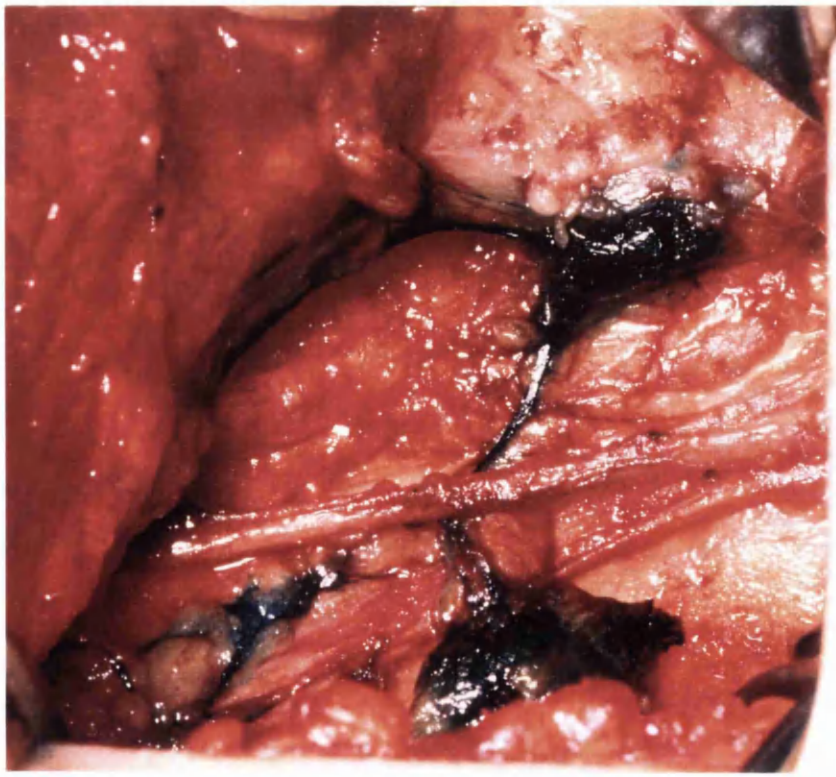


Figure 57 Two sentinel nodes stained blue

4) Diaminobenzidene (DAB)-Chromagen Substrate

Stock	0.6 gm of 3.3 Diaminobenzidene in 100 ml of Tris buffer aliquot into 5 ml amounts and stored at -20°C
Working solution	Add one 5ml aliquot to 95ml of Tris buffer, mix and add 1 drop of 30% w/v hydrogen and use immediately. (5 minutes at room temperature)

5) Vectastain Vasoactive Intestinal Peptide (VIP) Substrate Kit
To 5 ml of buffer add 3 drops of reagent 1 and mix well. Add 3 drops of reagent 2 and mix well. Add 3 drops of reagent 3 and mix well. Add 3 drops of the hydrogen peroxide solution and mix well.

Peroxide Anti-Peroxide (PAP) method for polyclonal antibodies
1) Take sections to water.
2) Block endogenous peroxidase by immersing in 3% hydrogen peroxide for 15 minutes.
3) Wash in T.B.S.
4) Carry out enzyme digestion with 0.1% pronase in tris buffer for 15 minutes at room temperature.
5) Incubate with normal swine serum diluted 1 in 5 for 10 minutes.
6) Pour off excess serum (do not wash).
7) Incubate with the appropriate primary antibody for 60 minutes at 37°C i.e. S-100 at 1:200 dilution in Tris buffer.
8) Wash in T.B.S.
9) Incubate with swine antirabbit Immunoglobin G diluted 1/50 for 30 minutes.
10) Wash in T.B.S.
11) Incubate with rabbit peroxide anti-peroxidase (PAP) complex diluted 1/100 for 30 minutes.
12) Wash in T.B.S.
13) Stain sections in Vectastain VIP substrate kit and incubate at room temperature until suitable staining develops: generally 2-15 minutes, alternately use DAB chromagen.
14) Wash in tap water.
15) Counterstain lightly in haematoxylin, blue in Scott's dehydrate, clear and mount.

N.B. All dilutions are made in Tris buffer (pH 7.6) and all washes are in Tris buffered saline (T.B.S.). Supplier for S-100 and all other immunochemicals are Delco. Figure 58 shows a positive lymph node using the H&E staining method and figure 59 shows a positive lymph node using the S-100 protein antibody.

6.7 RESULTS

OPERATIVE AND POST OPERATIVE COMPLICATIONS

There were no complications associated with the use of patent blue V dye for intraoperative mapping in this study. Five minutes following the injection of the dye, the skin of the patient becomes blue. This affects the anaesthetic monitoring of transcutaneous oxygen levels using pulse oximetry so the anaesthetist must be warned about the dye being used. All patients reported the presence of dye in their urine during the first 24 hours following the procedure. No hypersensitivity reactions were recorded.

PATHOLOGICAL EXAMINATION

A total of 32 sentinel node were identified. Micrometastases were found in 8 of the 32 sentinel nodes. Six of these metastases were detected by both routine H&E and immunohistochemical staining. In 2, metastatic tumour cells were detected only in sections stained by immunohistochemical techniques.

SENTINEL AND NON SENTINEL NODE ANALYSIS

In 28 patients, 32 sentinel nodes were identified with two patients having two sentinel nodes. There were 8 positive sentinel nodes of which 7 were from the inguinal basin and one from the axillary basin. A total of 96 non sentinel nodes were removed of which 56 nodes were removed from the same lymph basin as the sentinel node (axillary or inguinal basin). Forty nodes were removed from the iliac lymph basin in 22 patients undergoing iliac limb perfusion.

In relation to the Breslow thickness of the primary malignant melanoma, there were 4 positive sentinel nodes in the group with thickness between 1.5 - 2.99 mm, 3 positive sentinel nodes in the group with thickness between 3.00-3.99 mm and 1 positive sentinel node in the group with thickness of equal or greater than 4.00 mm. All the patients with positive sentinel nodes had been staged as having stage I disease using the M. D. Anderson Staging System.

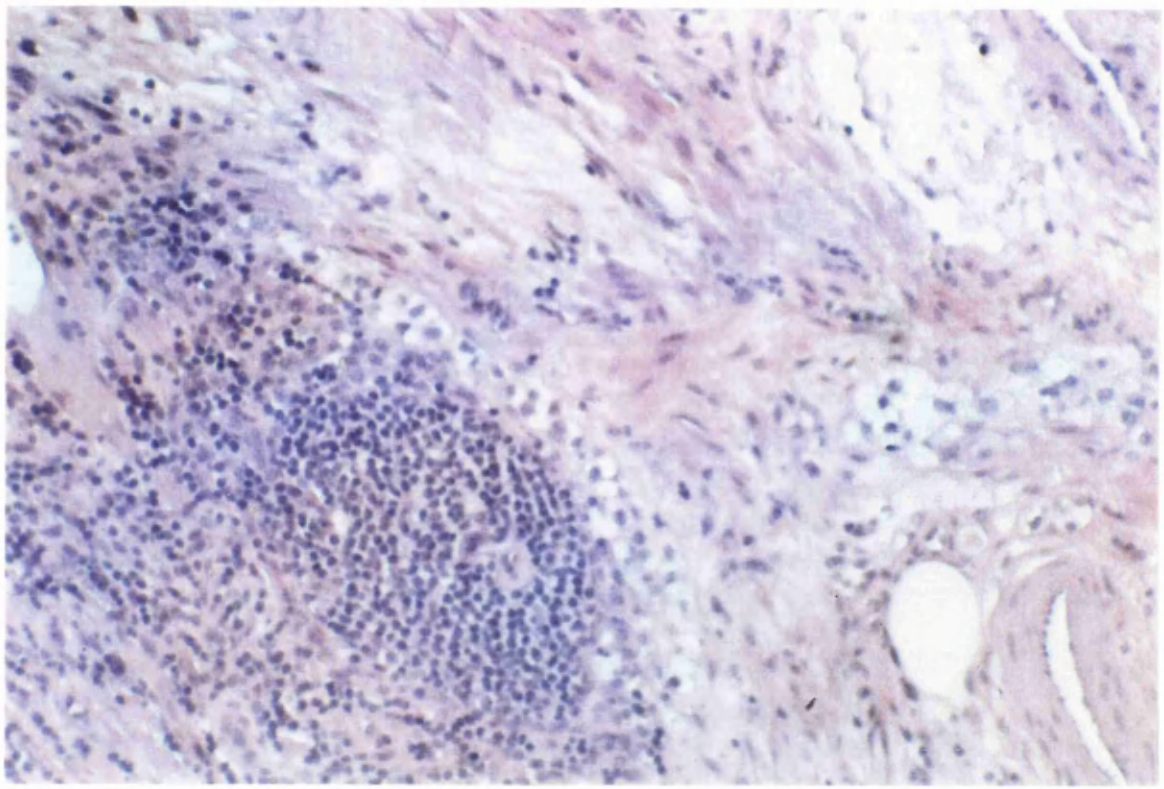


Figure 58 H&E staining of a sentinel lymph node showing the presence of melanoma cells
(courtesy of Professor R M MacKie)

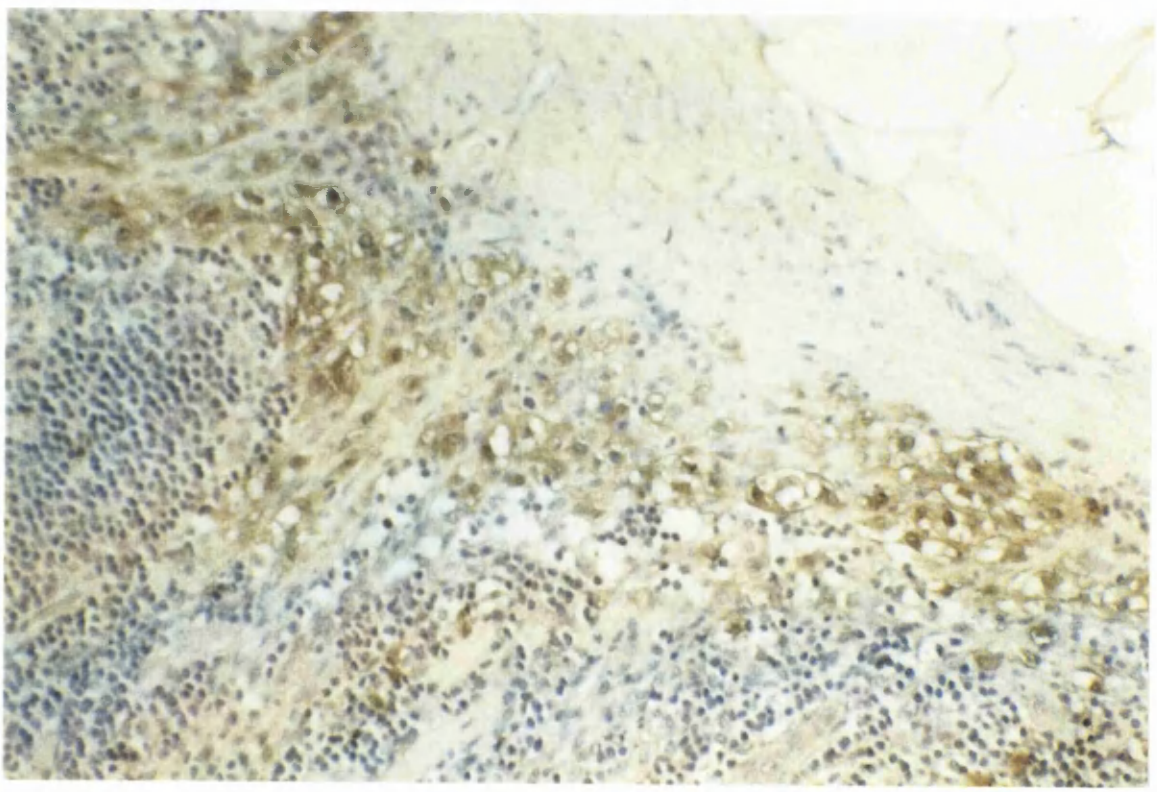


Figure 59 S-100 protein antibody staining of a sentinel lymph node showing the presence of melanoma cells
(courtesy of Professor R M MacKie)

Of the eight patients with a positive sentinel node, 6 showed ulceration in the primary tumour. In the 22 patients with negative sentinel nodes, only one had ulceration in the primary lesion. This was in the patient with Breslow thickness of 8.0mm with a subungual melanoma of the right thumb.

When the non sentinel nodes were examined, no micrometastases were found in non sentinel nodes in the first draining lymph basin. However in the 40 iliac nodes removed from the 22 patients undergoing iliac perfusion, one of the iliac nodes was positive for micrometastases in a patient with negative sentinel node. She had a nodular melanoma of the lower leg of Breslow thickness 2.58mm and no ulceration.

Of the 8 patients with positive sentinel nodes, 7 underwent en bloc lymphadenectomy. One patient did not undergo lymphadenectomy due to the her clinical condition. A total of 47 lymph nodes were removed. They were all negative both by H&E and immunohistochemical staining for metastastic melanoma cells.

Of the 30 patients who have had sentinel node biopsy, 22 patients with negative nodes have had no recurrence of melanoma. In 8 patients where micrometastases were identified, 2 have since developed recurrence, one of these patients having now died.

6.8 DISCUSSION

To date, the surgical world has had considerable difficulty in knowing how to deal with lymph nodes which may or may not contain metastatic malignancy. Most would agree that involved lymph nodes should be removed, but what if nodes are not clinically known to be involved and yet contain micrometastases? Such a situation is probably more common than realised and has important implications for the staging of disease and interpretation of therapeutic trials.

In 1992, Morton[353] and Cochran et al[354] at the John Wayne Institute for Cancer Treatment at UCLA School of Medicine, Los Angeles described the technique of lymphatic mapping. They detected micrometastases in 18% of sentinel nodes removed from patients with high risk (1.5mm-4.0mm) stage I disease. In the non sentinel nodes removed simultaneously, less than 1% were found to contain micrometastases giving a very low false negative rate. Although their results showed that this technique appeared to be the answer to the dilemma as to which patients would benefit from elective lymph node clearance, there were several flaws in their study and questions still remain to be answered.

Recently ELND has also been challenged by those who believe that lymph node metastases are primarily blood borne and occur in a random fashion to regional or distant lymph node groups via vascular lymphatic communication, thus challenging the conventional view of the orderly spread of metastatic cells along regional lymphatic channels to the draining nodes. Despite this fact, it has been shown that the five year survival of patients with multiple involved and clinically enlarged nodes after lymphadenectomy is only 15-25%[351,355,356,357] whereas the survival of patients with one or two microscopically involved nodes after lymphadenectomy is as high as 80%[76,355]. This difference may appear substantial but the benefit of ELND is probably much less because some of these patients have subclinical distant haematogenous metastases in addition to nodal metastases.

Intraoperative lymph mapping may provide an alternative method of management without having to subject patients to unnecessary lymphadenectomy and reserve lymphadenectomy for patients in whom metastases are positively identified.

This technique may also be beneficial in those patients with clinically palpable lymph nodes which are suspicious of metastases. In a minority of these patients, examination of the removed nodes may show only reactive changes and no evidence of metastatic melanoma cells. This prevents unnecessary lymph node dissection in these patients whose sentinel nodes may contain no metastases.

It could be argued that fine needle aspiration cytology may be used for such a purpose but this is not ideal as the sentinel node may be missed or in nodes with partial micrometastases occupying the node, the sampling may miss the micrometastases resulting in false negative findings.

There is still a small group of patients who would benefit from removal of the sentinel node. These are patients who are at the very early stage of lymph node involvement and detection of these few micrometastases may be missed by the current immunohistochemical techniques and are classed as being node negative. But in reality their nodes are diseased. This is important as even a single node involvement has been shown to reduce survival.

Our experience with this technique leads to several conclusions. Intraoperative lymphatic mapping is a user friendly technique that is easy to master. We have not encountered allergic reactions to the dye. An important aspect of this technique is to warn the anaesthetist that the dye effects the transcutaneous oxygen monitoring as the skin of the patient becomes blue and to warn patients that it is natural to have staining

at the site of injection and that for 24-48 hours, their urine will be blue and these reactions are temporary and will resolve spontaneously.

The technique of identification of micrometastases in the lymph node must be accurate. In this study both routine H&E and immunohistochemical staining were used. The two immunohistochemical stains used were S-100 protein antibody and NKIC3 antibody. S-100 protein antibody can identify melanocytic tumour even of a differentiation degree lacking melanin versus other soft tissue. Almost all human benign and malignant melanocytic tumours of the skin and metastases of human malignant melanoma stain for S-100. It also retains its reactivity in formalin fixed paraffin fixed processed material. In several studies using S-100 protein antibody to detect primary and secondary melanoma tissue, it has been shown that positive staining patterns are seen with 80%-90% of melanoma tissue[358].

NKIC3 antibody also shows strong reactivity to paraffin processed tissue. Using NKIC3 on tumour cells with a conventional immunoperoxide technique, virtually all metastatic melanoma show positive staining. NKIC3 antibody staining pattern has been compared to S-100 protein antibody and was shown to give stronger staining of a higher percentage of cells and is a useful addition to the other antibodies for diagnostic purposes[359]. Neither of these antibodies should be used alone for detection of melanoma cells because these antibodies are not specific for melanoma cells and must be used in a package of antitumour antibodies.

The results in this study show that routine staining with H&E alone is not sensitive and specific enough to detect all micrometastases in lymph nodes. Cochran[360] in a recent study showed that the number of lymph nodes demonstrating melanoma cells is significantly higher when S-100 protein antibody label is used in comparison with conventional H&E stained sections. It is therefore important to combine routine H&E staining with a battery of immunohistochemical staining which includes S-100 and NKIC3 antibodies for improved sensitivity in the identification of micrometastases.

In this study, it was the practice to identify and remove the sentinel lymph node at the time of the perfusion. Radical lymphadenectomy was carried out as a separate procedure at a later date. However, it is possible to carry out frozen section analysis of the sentinel node and proceed to performing a radical lymphadenectomy if necessary at the same operation.

Until the present study, the original results of Morton[353] and Cochran[354] had not been reproduced elsewhere. Our experience of this technique appears promising with detection of metastases in 25% of sentinel nodes. There was no technical difficulty in identifying the sentinel node, reinforcing the fact that the technique is practical.

The non sentinel nodes removed from the same lymphatic basin did not reveal any micrometastases in patients when the sentinel node was negative. This suggests that the sensitivity of the technique is high.

If this technique is to have a wide application it is critical that the theory of metastatic spread is sound and that lymphatic fluid drainage occurs in an orderly and non randomised fashion to the nearest draining node and lymphatic basin. If micrometastases can skip an entire nodal basin, then the sentinel node theory will not be applicable widely in clinical practice.

In this study, the author was in a unique position to sample not only the inguinal nodal basin but also the iliac node basin that was exposed at the time of routine limb perfusion surgery. In one patient in whom the sentinel and non sentinel nodes in the inguinal basin were negative, by the method described above, a positive node was encountered in the iliac region. This shows that skipping of the sentinel node and the first nodal basin can occur. This skipping was not shown in the original study.

When the prognostic factors in this group of patients were analysed with respect to predicting the sentinel node status, several interesting points arose. Firstly, it was not possible to predict the status of the sentinel node from the Breslow thickness of the original tumour. This is perhaps surprising as the proponents of elective lymphadenectomy claim that the risk of developing regional node disease increases with the thickness of the primary melanoma and propose that elective lymph node dissection is beneficial in those patients with intermediate thickness tumour (1.5-4.0mm).

Secondly, ulceration did appear to be significant in the prediction of sentinel node status. Six of the eight patients with positive sentinel nodes had ulceration in the primary tumour. In contrast, only one of the 22 patients with negative sentinel nodes had ulceration in the primary tumour. This was a patient who had the subungual melanoma. Although numbers in this study are small, it appears that ulceration as a prognostic factor is more sensitive in predicting sentinel node status and survival.

This study has shown that in patients found to have no evidence of nodal involvement by clinical and radiological methods, 26% in fact have microscopic metastases in the regional lymph nodes. In all the current staging systems, the presence of involved nodes reflects more advanced disease. In this study positive sentinel nodes were seen in 8 of the 25 patients with stage I disease. In the other 5 patients with stage II and IIIA disease the sentinel node was negative. Thus it appears that 32% (8/25) of patients with stage I disease have regional node involvement.

If the sentinel node status is taken into account in these patients, their accurate staging would be stage IIIB according to the M. D. Anderson Staging. Of the seven patients who underwent radical lymphadenectomy, in 2 patients a total of 5 further positive nodes were identified. Thus in 5 patients, the sentinel node was the only node containing micrometastases. At follow up, the two recurrences and one death occurred in the patients who had further positive nodes.

Clearly current staging techniques such as CT scanning and ultrasound are not sufficiently sensitive in identifying nodal involvement. Intraoperative lymphatic mapping is a tool that the surgeon can use not only to identify patients who may benefit from lymphadenectomy, but also to obtain a more accurate staging of the disease.

The other observation from this study is the suprising fact that there was no evidence of nodal disease in the patients with stage II and IIIA disease. This suggests that in melanoma although lymphatic invasion occurs earlier, the more important mode of spread appears to be haematogenous. This echoes the long standing finding of the early pioneer, William Handley, that as the disease advances, lymphatic permeation regresses and it is the vascular embolism of metastases that eventually kills.

Ulceration is an indication that the tumour has gained access to the blood stream and in such cases lymphadenectomy may be too late. If a cure is to be found in these patients, targeting and destroying subclinical melanoma metastases would be needed.

If 32% of patients with clinically stage I disease have nodal involvement, then this has important implications in the design and interpretation of therapeutic trials as a quarter of the patients will be understaged. The author suggests that to improve the accuracy of the current staging of melanoma, the sentinel node status should be included. The author suggests that the M. D. Anderson staging be altered as follows:

STAGE	CRITERIA
I	Primary melanoma Sentinel node negative
II	Local recurrence or local metastases within 3cm of the primary. Sentinel node negative.
III	Regional metastases
IIIA	Only sentinel node positive and skin recurrence.
IIIB	Sentinel node and one or more positive non sentinel nodes
IIIAB	Local recurrence and sentinel node positive and/or one or more positive non sentinel
IV	Distant metastases

The staging outlined above may more accurately predict survival.

6.9 CONCLUSION

Intraoperative lymphatic mapping is an easy technique to master and appears to be highly sensitive in the identification of patients with nodal disease. This technique is applicable in all patients with no clinical or radiological evidence of nodal disease. These include patients with M. D. Anderson stage I, II and IIIA as unsuspected metastases may be identified.

It appears that if the sentinel node status could be incorporated into the current staging system, then a more accurate staging of the disease can be achieved with more accurate prognosis. Furthermore, if 32% of patients with cutaneous malignant melanoma of a limb who are clinically stage I have nodal disease, this has considerable importance for the design and interpretation of therapeutic trials.

In conclusion, lymphatic mapping presents itself as a tool for the identification of melanoma dissemination by the lymphatics. This not only gives a more accurate staging of the primary lesion, but also selects patients with nodal disease for radical lymphadenectomy which removes the involved lymphatic pathway and may improve survival.

CHAPTER 7

TARGETING RADIOTHERAPY IN THE TREATMENT OF MALIGNANT MELANOMA

7.1 THE ASSESSMENT OF THE BINDING ABILITY OF METHYLENE BLUE TO PIGMENTED MALIGNANT MELANOMA IN NUDE MICE.

7.1.1. INTRODUCTION

Malignant melanoma has a dismal prognosis once metastases occurs. It is known that even at the time of diagnosis of primary malignant melanoma, metastases to lymph nodes, liver, lung and central nervous system may have occurred. The current treatment modalities for dealing with micrometastases are limited. It is thus conceptually attractive to think of using targeted radiotherapy to treat such metastases. In theory, this could be done by using a compound that has a high binding affinity for melanin. This compound could be labelled with a radioisotope to target, scavenge and destroy these metastases.

Compounds with selective affinity for melanin have been known for many years and were first described by Potts[361,362,363]. These selective melanin "seekers" either bind to preformed melanin[364] or are incorporated into the growing melanin[365]. The compounds originally used were the phenothiazine derivatives. Chlorpromazine was the first to be used for this purpose but it did not prove to be effective. Several other phenothiazines were tried and eventually methylthionine bromide (commonly known as methylene blue MTB) was chosen because of its great affinity for isolated natural melanin[362], selective accumulation in pigmented tissues, and low toxicity. Link[366,367] and colleagues showed that methylene blue was incorporated at least five times more effectively by cultured pigmented melanoma cells than by non pigmented tumour cells. She also showed that its distribution in vivo showed the highest and most stable level in pigmented melanomas[368].

The experiments described in this chapter were designed to answer the following questions:

1) Can MTB effectively bind to melanoma cells?

2) Does MTB have any tumouricidal effect?

All the animal experiments described in this chapter were carried out with the approval of and in accordance with the Home Office rules and regulations.

7.1.2. MATERIALS AND METHODS.

EXPERIMENT 1

The experiment was carried out in vivo using melanotic B16/F10 mouse melanoma. A primary melanotic B16/F10 mouse melanoma cell line was obtained by courtesy of Professor R. M. MacKie, Department of Dermatology, University of Glasgow. The sub-line had been constantly maintained by serial subcutaneous injections of animals with chips of tumour tissue. This pigmented subline was passaged every three weeks in the C57 mouse. A single cell suspension was isolated by dispersing 2-4 excised melanoma tumour fragments of $1mm^3$ with 0.25% Trypsin in a phosphate buffer saline (PBS) which by the process of digestion eventually results in a dilution of $1x10^6$ cells in 0.2mls of saline. For the experiments, Eagle's minimum essential medium (MEM) supplemented by 10% bovine serum was used.

Non radioactive methylene blue (MTB) was obtained from a prepack manufactured by Martindale Ltd. The MTB was then diluted with saline to give a dilution of one microgram in 0.2mls saline.

Athymic nude female mice of 50-60 days old were used in all the experiments and were obtained from Charles Rivers Ltd. The animals were kept in sterile cages and fed sterilised food and water. All the experiments were performed under strict aseptic techniques using a laminar flow cabinet. Inoculation of the melanoma cells and MTB was carried out on mice anaesthetised using intraperitoneal hypnovel. The animals were divided into three groups :

1 Group A (control group) received only tumour cells (6 animals).
2 Group B received a mixture of MTB and tumour cells (6 animals).
3 Group C received tumour cells with MTB being administered at the first sign of tumour growth (6 animals).

According to the protocol submitted to the Home Office prior to the issue of the animal licence, due to the time and cost involved in the care of the nude mice, application for a maximum of 20 nude mice were made for the use in each experiment. Hence the maximum number of animals in each of the subgroups was six.

B16/F10 mouse melanoma cells ($1x10^6$ cells) were incubated for three hours in test-tubes containing 1ml of MEM and then centrifuged. The cells were then incubated either in a medium containing MTB or in a medium containing no MTB. Group A mice were injected with $1x10^6$ cells in 0.2mls saline and 0.2mls saline, Group B mice were injected with a mixture of $1x10^6$ cells in 0.2mls and 1microgram MTB in 0.2mls saline and Group C mice were injected with $1x10^6$ cells in 0.2mls saline followed by 1 microgram MTB in 0.2mls saline intravenously into the tail vein when tumour growth was first noted.

All tumour cells were injected subcutaneously into the right thigh as the rate of growth of tumour at this site is more gradual and less likely to ulcerate. The animals were sacrificed when the tumour became larger than 1.5cm or when the tumour ulcerated or interfered with the locomotion of the animals.

The tumour growth was recorded daily at the same time each day using a venier calliper. Measurements of the greatest dimension (a) and the length perpendicular to this dimension (b) were recorded. Tumour volume was calculated using the formula below:

$$\textbf{Tumour Volume(V)} = (a)^2 x\ b\ x\ 0.5$$

EXPERIMENT 2

Experiment 2 was a repeat of experiment 1 using the same technique as outlined above. This experiment was carried out to confirm that the results obtained from experiment 1 were not due to chance.

1) Group A (control group) received only tumour cells (4 animals).
2) Group B received an admixture of MTB and tumour cells (4 animals).
3) Group C received tumour cells with MTB being administered at the first sign of tumour growth (12 animals).

There were more animals in group C than in group A or B because the results from experiment 1 had showed that there appeared to be a greater difference in the tumour growth rate in group C when compared to the control. Thus a larger number was needed to be studied in this group before any conclusion about the action of MTB could be made.

7.1.3 RESULTS

The results of experiments 1 and 2 were analysed as described below:

1) Tumour volume for each animal in each group was calculated and a mean tumour volume and rate of growth derived. The average tumour volume on day 12 in each group was also calculated.

2) The mean tumour volume, rate of growth and tumour volume on day 12 was compared between the groups.

3) To confirm if there was a difference in the groups with respect to the variables shown above, the statistical analysis outlined in the paragraph below was used:

Statistical Analysis

For each group of animals, the results were subjected to the following statistical analysis. A mean and standard deviation of tumour volume was first calculated and plotted against time. The results were then linearised and plotted against time and the rate of tumour growth was calculated from this graph. To check that the rate of tumour growth was linear, a Pearson Test was used. In each group the tumour volume on day 12 was also compared and plotted.

One Way Analysis of Variance (95% confidence limit) was used within the groups in each experiment to look for differences in the tumour growth. Friedman's Test for the specific comparison was used to analyse the difference in tumour growth rate and tumour volume on day 12 in each group.

Experiment 1

Tumour growth was documented in all the 18 nude mice injected with tumour cells. Each animal developed tumour at the injected site with a total of 18 tumours in the 18 animals. The first sign of tumour growth in group A was evident on day 5 following inoculation. The animals were sacrificed on day 15. In group B tumour growth was first seen on day 9 and the animals were sacrificed on day 15. In group C the first sign of tumour was seen on day 5 and the animals were sacrificed on day 15.

The results calculated using the One Way Analysis Variance showed that the rate of growth was significantly different in group A, B and C ($p = 0.001$). Using the Friedman's Test, the rate of growth was shown to be different with the greatest growth in group A followed by group C and then in group B ($p = 0.001$).

Experiment 2

Tumour growth was documented in all 20 animals with each animal showing a solitary tumour at the site of injection with a total of 20 tumours. The first sign of tumour growth in group A was evident on day 6 and the animals were sacrificed on day 14. In group B tumour growth was first recorded on day 11 and the animals were sacrificed on day 17. In group C tumour growth was first evident on day 6 and the animals were sacrificed on day 15.

When the Friedman's Test was used, the rate of growth was shown to be different with the greatest growth in group A followed by group C and then in group B ($p = 0.001$). These findings were similar to those of experiment 1.

Figures 60 and 61 show the rate of tumour growth and tumour volume on day 12 in experiments 1 and 2 respectively.

Since group A animals in experiment 1 and 2 were the same, a One Way Variance analysis was used to see if these groups could be pooled to provide a larger sample size. When this was carried out, there was a significant difference between the controls ($p = 0.01$). This meant that the control groups could not be pooled. This however did not affect the analyses in the individual experiments.

If MTB has only a delaying effect and is not tumouricidal, then once the MTB molecule is displaced from the tumour cells, they should be free to multiply and growth should return to a pattern similar to that shown by the control group. In order to investigate this, the data were dealt with in segments of 7 days length. Group A (the control) group covered days 6 to 12. Groups B and C were systematically lagged to see how far behind the tumour growth was from the control group. Day 6 to 12, 7 to 13, 8 to 14, etc were compared to day 6 to 12 in the control group and examined for any similarity.

In experiment 1 in groups A and B there was a lag of 5 days. The groups were similar but discrepancies were apparent. Between groups A and C the lag was 1 day and again there were some discrepancies. Similar results were also seen in experiment 2.

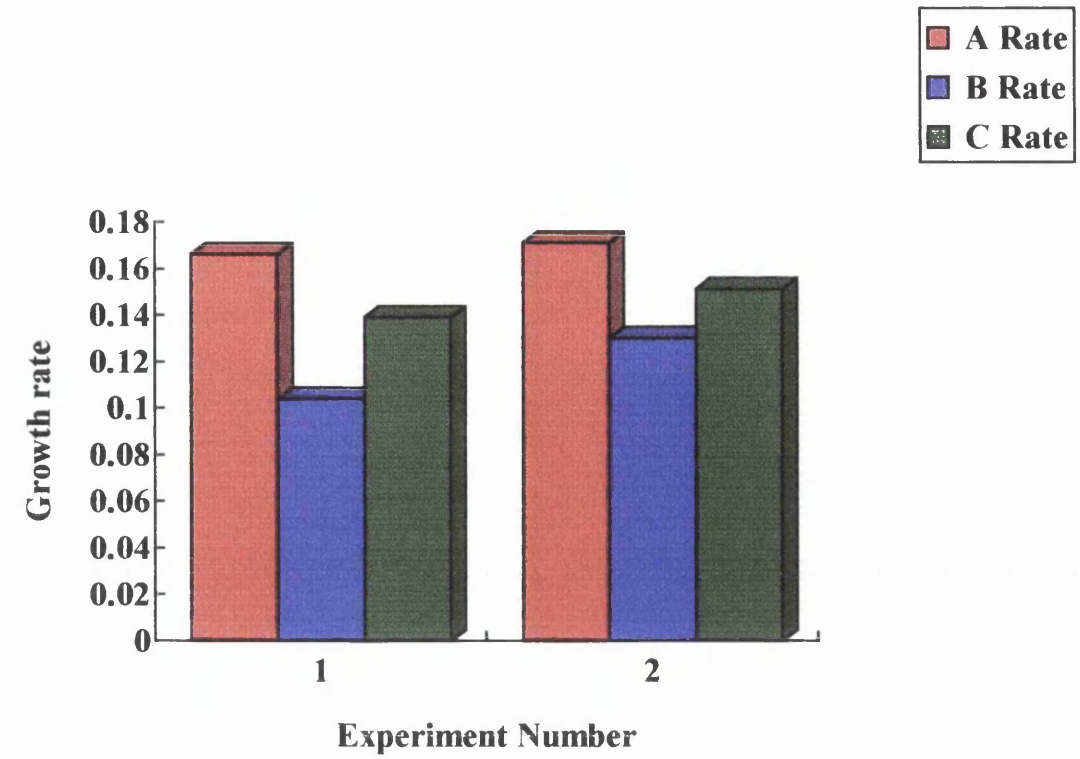


Figure 60 Rate of tumour growth in groups A, B and C in experiments 1 and 2

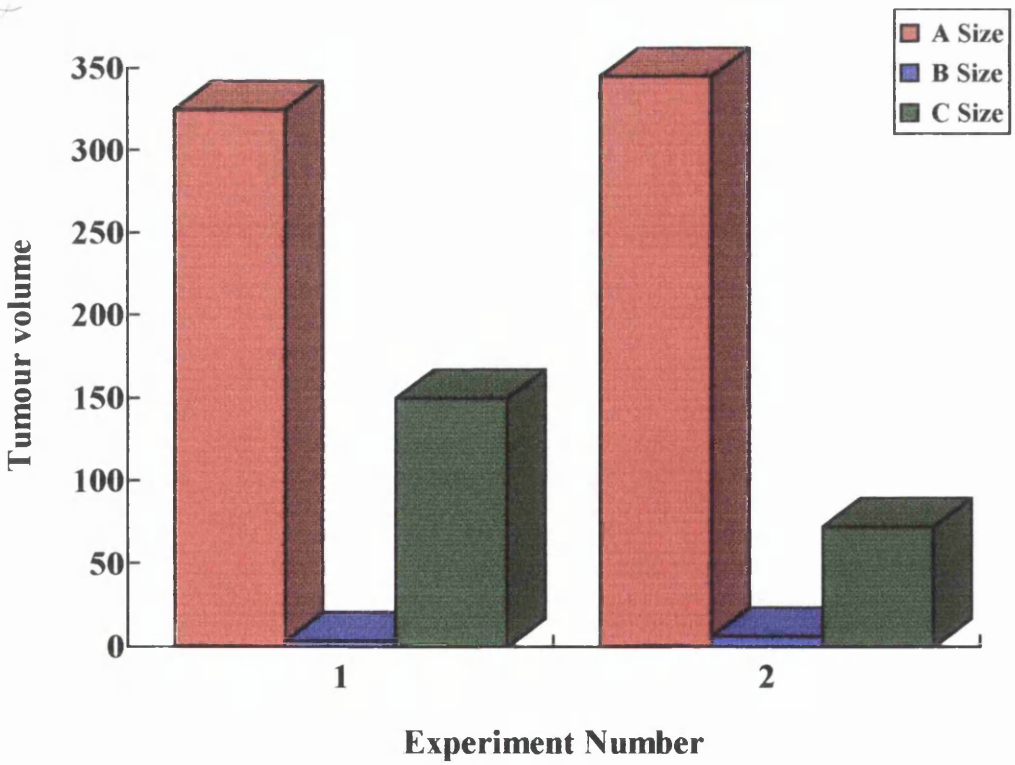


Figure 61 Tumour volume on day 12 in groups A, B and C in experiments 1 and 2

Formal analyses of the data was carried out using Repeated Measures Analysis of Variance. A significant group effect indicates an overall difference between the groups. The group day interaction measures the pattern across days for each group, and a significant interaction means the pattern differs for the two groups. When the results were lagged, there was a significant group effect between group A and B and also between groups A and C but there was no significant interaction between the groups. Thus although the growth rate was different, there was no difference in the growth pattern.

In summary, there was a significant difference in the growth rates in all the groups of animals. The growth rate was greatest in group A followed by group C and then group B.

7.1.4 DISCUSSION

Analysis of the results obtained in experiments 1 and 2 showed that there was a significant difference between the groups with the growth rate of group A greater than those of groups B and C. The addition of MTB therefore appeared to delay the onset of tumour growth.

If MTB has only a delaying effect and not a tumouricidal effect on the melanoma cells, when the results are lagged to that seen in the control, there should be no difference in the growth rate i.e. no significant interaction. When the results were lagged, there was a significant group effect between group A and B and between groups A and C. However no significant interaction between the groups were noted. This meant that although the growth rate was different, there was no differences in the growth pattern between the groups.

This clearly shows that MTB has no effect on tumour growth but does have a delaying action. These results provide confirmatory evidence that in order to achieve this delaying action, MTB must bind to melanin in the tumour cells. The MTB-melanin complex may inhibit the use of the melanin needed in the multiplication of the tumour cells. When experiment 1 was repeated (experiment 2) similar results were shown confirming that this was not a chance finding.

The delay in time to growth should reflect the half life of MTB. This is known to be 2.3 days. If radioactive isotopes are to be attached to the MTB molecule for targeted therapy, then the radioactive isotopes should have a half life shorter than that of MTB so that effective treatment can be delivered before MTB is displaced from the melanin in the tumour cells.

There was also a significant difference in growth in groups B and C. In group B MTB was incubated with the tumour cells prior to inoculation whilst in the group C MTB was injected intravenously only when the tumour became macroscopically visible. Thus the amount of tumour cells in the two groups were different at the time of MTB adminstration but both the groups received the same amount of MTB.

This meant that for the same volume of MTB used the proportion of cells that were bound to MTB was higher in group B than in group C and thus the number of cells available for multiplication was greater in group C than in group B animals.

7.1.5 CONCLUSION

Experiments 1 and 2 confirm that MTB binds to melanin in the tumour cells. MTB has no specific tumouricidal effect. If radioisotopes are to be labelled to MTB for targeted therapy, then the chosen isotope should have a similar half life to the MTB molecule.

7.2 THE EFFICACY OF TARGETED RADIOTHERAPY USING RADIOACTIVE IODINE IN THE TREATMENT OF MALIGNANT MELANOMA IN NUDE MICE.

7.2.1 INTRODUCTION

Targeted radiotherapy is defined as a treatment selectively directed at a particular tissue, by using either a radioisotope exhibiting a high affinity to this tissue or a compound with such affinity used as a carrier for a suitable radioisotope. The selectiveness of the radioisotope uptake enables deposition of high radiation doses in the targeted tissue with only minor exposure to surrounding structures, unlike classical beam radiotherapy where both the targeted and adjacent tissues are irradiated.

The idea of targeted radiotherapy was first conceived when the Hungarian scientist, G. Hevesy[369] together with F. Paneth[370] reported the use of lead as a radioindicator in chemistry. The first clinical studies using radioisotopes compared the velocity of blood circulation in healthy individuals with that in patients with heart disease[371]. This led to several other studies with the eventual use of phosphorus in the treatment of leukaemia[372] and the use of radioiodine for the diagnosis and treatment of thyroid disease[373,374].

The main difficulty for the clinician treating malignant melanoma is that early random metastases can occur. If a melanoma specific compound could be labelled with an appropriate radioisotope, this compound could be used as a scavenger to "mop-up" single melanoma cells and tumours below the limit of clinical detectability. Methylene blue may be such a compound as it possesses great affinity for melanin. Potts[361] showed that the binding affinity of methylene blue to melanin was 87%. Link[368] and colleagues showed that its distribution in vivo was highest and most stable in pigmented melanoma but that uptake was also seen in normal organs which are pigmented such as the eyes and liver.

It has earlier been seen that adjuvant ILP with melphalan does not significantly improve the survival of patients with stage I disease. This failure may be related to the chemotherapeutic agent rather than the technique of ILP itself. However if a different agent can be found that is suitable for use in the ILP circuit, then a survival benefit may be demonstrated.

Such an agent could be a labelled radioactive isotope with methylene blue. To test this hypothesis a series of experiments were conducted using several radioactive labelled isotopes in the nude mouse model used in experiments 1 and 2. These experiments were designed to answer the following questions:

1) Is targeted therapy possible?
2) Is there an optimal radioactive isotope?
3) Is MTB an effective carrier molecule for the chosen radionuclide?
4) What is the most effective route in the delivery of this treatment?
5) Can this therapy be delivered without detrimental effects on other organs in the body?

7.2.2 BIOLOGY OF RADIOISOTOPES

Ionising radiation is radiation which when incident upon matter can cause changes in the atomic or nuclear structure of the matter. One form of ionising radiation is electromagnetic which can be regarded as a wave form composed of transverse electric and magnetic fields propagated at the speed of light. This type of radiation includes X-rays (electronic in origin) and gamma rays (nuclear in origin) and is highly penetrative in matter. The second type of ionising radiation is particulate and includes the proton, the neutron, the alpha and the beta particle. These particulate radiations are relatively much less penetrative than electromagnetic radiation.

Atomic nuclei are characterised by two numbers, the atomic number (Z) which is the number of protons in the nucleus and the mass number (A) which is the total number of protons and neutrons in the nucleus. A third number sometimes quoted is the neutron (N), $A = N + Z$

Nuclides can be stable or unstable, the latter undergoing radioactive decay to become stable. Nuclides having the same proton number are termed isotopes. Such a group comprises different nuclei of the same element. Since isotopes differ only in neutron number, they have the same chemical properties. Most elements have a mixture of stable and unstable isotopes. Isotopes containing an unstable arrangement of protons and neutrons will transform to a completely stable or a more stable combination of nucleons. These isotopes are said to be radioactive and are termed radioisotopes.

Radioactive decay occurs with the emission of a charged particle from the nucleus or the capture of an electron from one of the shells surrounding the nucleus. This results in a change in the balance between the protons and neutrons.

When radioactive decay releases an electron or positron, the emission is termed beta decay; when it involves emission of a helium nucleus, it is termed an alpha particle. It is common for gamma radiation to accompany emission of a charged particle.
The range of beta particle penetration in tissue is a few millimetres and can be useful in selective therapeutic treatment. Alpha particle radiation gives a much higher localised radiation dose in tissues.

When cells are exposed to ionising radiation a proportion of them eventually die or lose their ability to divide. Some develop abnormal sets of chromosomes or transmit their chromosomes abnormally, while others exhibit heritable changes (i.e. gene mutations). Exposure of cells to ionising radiation therefore sets off a chain or network of reactions giving rise first to chemical and subsequently to metabolic or physiological changes.

Although different kinds of ionising radiation interact with matter in a basically similar manner they vary with regard to the spatial distribution of ionisation and other energy absorption events within the irradiated material. Differences in radiation quality are expressed in terms of linear energy transfer (LET), which can be defined as the average rate of energy deposition per micron of track of the incident particle or ejected electron.

Sparse ionising radiation has low, and dense ionising radiation high, LET. Radiation with different LET's kills cells with varying efficiency, some requiring greater dose to kill a certain proportion of cells than others. The ratio of the effectiveness of a given radiation to that of a standard is termed its relative biological effectiveness (RBE). Isotopes with high LET have high RBE.

Therapeutic radionuclides should emit radiation with a high LET in order to destroy tumour tissue. Radionuclides with potential for therapy fall into three main categories:
i) Beta emitting radionuclides.
ii) Alpha emitting radionuclides.
iii)Electron capture and internal conversion decaying radionuclides.

Beta emitting radionuclides have been and remain the only type of radionuclide to be used clinically. Beta particles are negatively charged and are relatively lighter than an alpha particle and thus travel through tissue more quickly and have less densely ionising properties and a lower LET.

Beta emitters may be subdivided into those with low particle range (less than 200micrometers), those with a medium range (200-1000micrometers) and those with a relatively long range (> 1mm).

Alpha emitting radionuclides produce alpha particles. Alpha particles are positively charged, relatively bigger, move slowly through tissue and penetrate only a short distance (50-90micrometers). Alpha particles are very densely ionising and have a high LET (80 keV per micron). Since tissue damage produced by alpha emitters is extensive, it is essential that the radionuclide label is firmly bound to the targeting pharmaceutical to avoid damage to normal tissue from dissociated radionuclides.

The electron capture and internal conversion decaying radionuclides release Auger electrons of low energy X-rays. The range of these low energy particles is extremely short (< 1micrometers). In order to deliver a lethal dose to the tumour cell, it is essential that the radionuclide is internalised within the cell ideally within the nucleus.

For local intracellular treatment alpha emitting isotopes should therefore be better than beta emitting isotopes but they may not be as effective for radiotherapy of solid tumours as tissue penetration is poor. More densely ionising radiation like alpha particles have a greater probability of hitting the target. Alpha emitting radionuclides that have therapeutic potential are few and many have complex decay schemes. If the target is as complex as a mammalian cell then some critical part of the cell such as the nucleus may need to be hit before any damage will be achieved in the biological sense.

7.2.3 MATERIALS AND METHODS.

Three iodine isotopes were used in the following experiments. Iodine was chosen for these experiments because not only is iodine readily available, there are two different radioactive isotopes with varying properties, one an auger electron emitter, the other a beta emitter allowing for comparison. There is no iodine isotope that emits alpha radiation. I^{127} is stable and was used as the control. I^{125} is an auger electron emitter with high LET and mean range in tissue of one micrometer and a half life of 60 days. I^{131} is a beta emitter with a mean range in tissue of eighty micrometers and a half life of 8 days.

Figure 62 shows the chemical structure of methylene blue. Methylene blue has a formula of C_{16} H_{18} S $3H_2O$ and a molecular weight of 373.9 Dalton. Figure 63 shows the site to which methylene blue binds to melanin in the melanosome. The sites R1, R2 and R3 represent the possible sites of iodine attachment.

Iodinated methylene blue was obtained by courtesy of Professor A. T. Elliott, Department of Clinical Physics and Bioengineering, University of Glasgow.

The electrophilic substitution method was used to make iodinated methylene blue. 1% methylene blue, sodium iodide, potassium iodide, sterile water for injection and concentrated hydrochloric acid (10N) were mixed using the following method as described by Link:

1) 1.5 ml concentrated hydrochloric acid (HCL) was added to 100 ml water for injection.
2) 11.7 mg sodium iodide (NaI) and 77 mg potassium iodide (KIO_3) were weighed into a vial.
3) 20ml of sterile water was added and agitated until dissolved.
4) The following reagents were added in the stated order mixing thoroughly each time:
a) 0.5ml methylene blue
b) 1ml NaI/KIO_3
c) 0.5ml HCL
The labelling procedure was carried out as below:
1) 0.2ml of the above mixture was taken and added to sodium iodide (i.e. I^{125}, I^{131}, I^{127}).
2) The vial containing the radioisotope and the mixture was incubated at 110° C for an hour.
3) On completion of the incubation, the reaction vial was cooled briefly prior to the separation on a C18 semi-preparative column. Solvents used were 40% acetonitrile and 60% citric acid buffer.
4) The relevant fraction was collected and the acetonitrile component removed via a stream of free nitrogen gas.
5) The citric acid component was buffered with bicarbonate.
6) The methylene blue obtained should be radiochemically stable for up to two weeks. The amount of radioactivity used was 0.5 KBq per mouse.

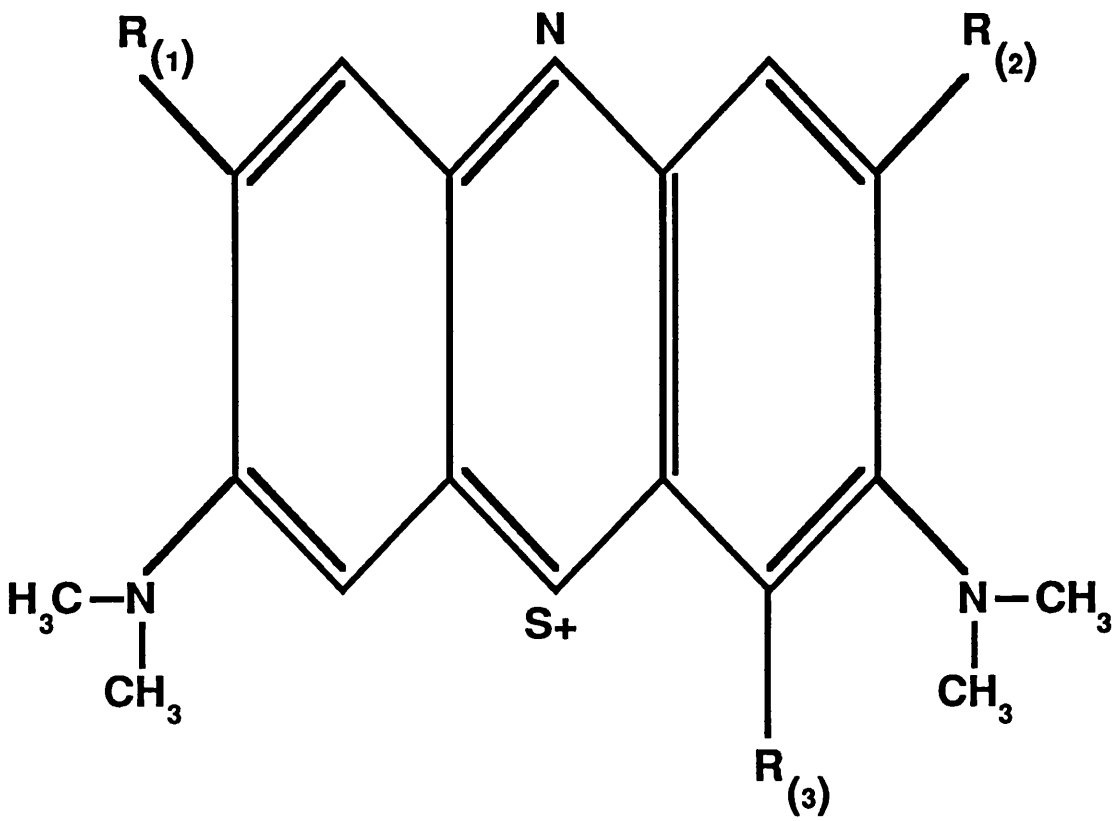


Figure 62 Chemical structure of methylene blue

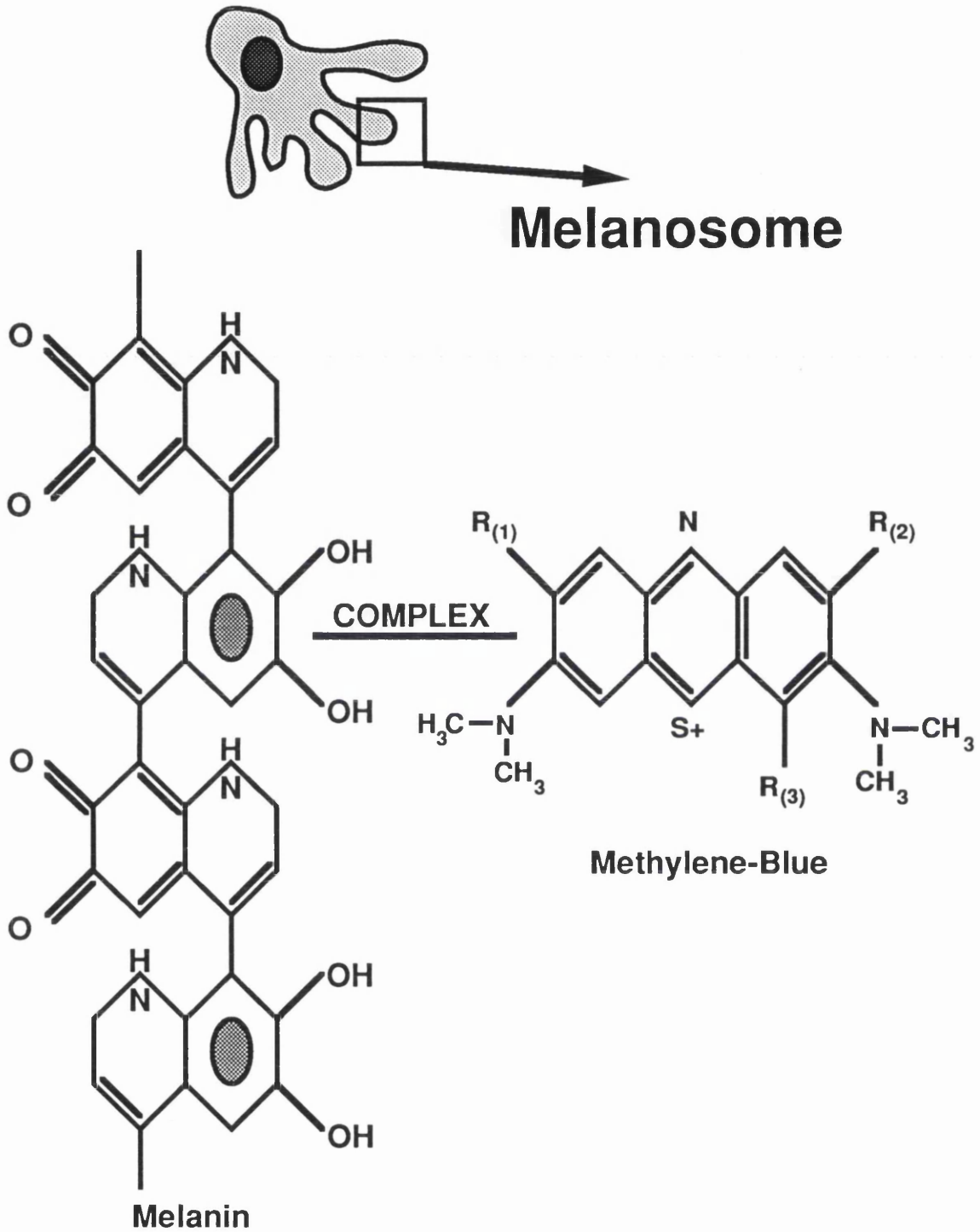


Figure 63 The site at which methylene blue binds to melanin in the melanosome

The experiments were carried out in vivo using a single cell line ($1x10^6$) of pigmented melanotic B16/F10 mouse melanoma. This pigmented subline was passaged every three weeks in the C57 male mouse. A single cell line ($1x10^6$) was obtained as follows:

1) Excised tumour was washed in serum containing Ham's F-12 medium and was cut into very small fragments.
2) The supernatant was removed and the remaining fragment of tumour was placed in a flask containing 30ml of Ham's F-12 medium and three enzymes (0.02%DNase, 0.05%Pronase and 0.02%Collagenase).
3) The mixture was carefully stirred and then incubated for 15-20 minutes at 37° C.
4) The cells were recovered by centrifugation and washed 2-3 times with the serum containing F-12 medium before filtering through a cotton gauze.
5) The cells were counted using a haemocytometer, further centrifuged and then diluted to the density of $1x10^6$ in the phosphate buffer as described in experiments 1 and 2. Experiments utilising radioactive material were conducted in accordance with local rules formulated by Professor A. T. Elliott in conjunction with the Radiation Protection Adviser.

There were three groups of animals:
1) Group A mice were injected subcutaneously over the thigh with a single cell line of pigmented melanoma cells.
2) Group B mice were injected subcutaneously over the thigh with a mixture of $1x10^6$ melanoma cells and stable or radioactive iodine labelled methylene blue.
3) Group C mice were injected subcutaneously over the thigh with $1x10^6$ melanoma cells and once tumour growth was evident, the mice were innoculated with either stable or radioactive iodine labelled methylene blue via the tail vein (intravenously).

The tumour growth was recorded daily by measuring the greatest dimension (a) and the length perpendicular to the greatest dimension(b). Figure 64 shows the typical appearance of tumour growth. Figure 65 shows the intravenous injection. The formula used to calculate the tumour volume is:

$$\textbf{Tumour volume (v)} = (a)^2 \times b \times 0.5$$

Radioactivity measurements were carried out on alternate days examining the areas over the tumour, thyroid and liver using the Neoprobe Model 1000 Portable Radioisotope Counter (Figure 66). This counter is designed to detect and quantify gamma radiation. It consists of a sensitive collimated gamma ray detector and a

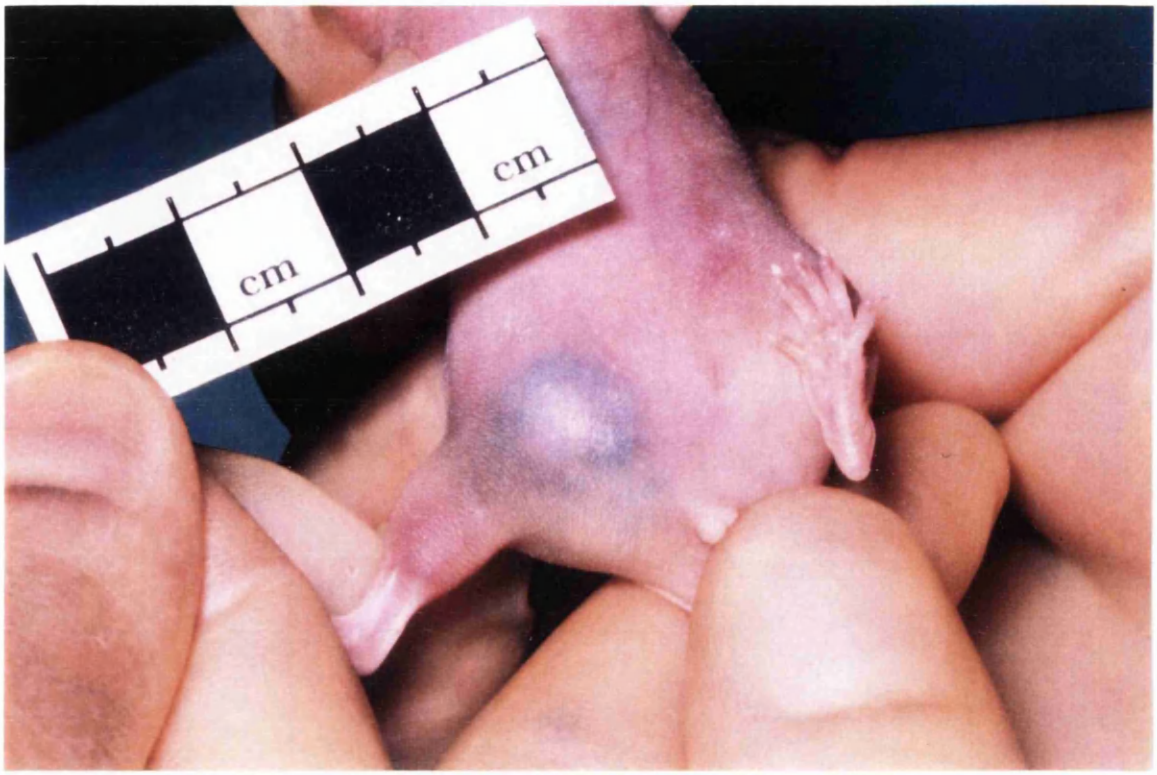


Figure 64 Typical appearance of tumour over the thigh in the nude mice

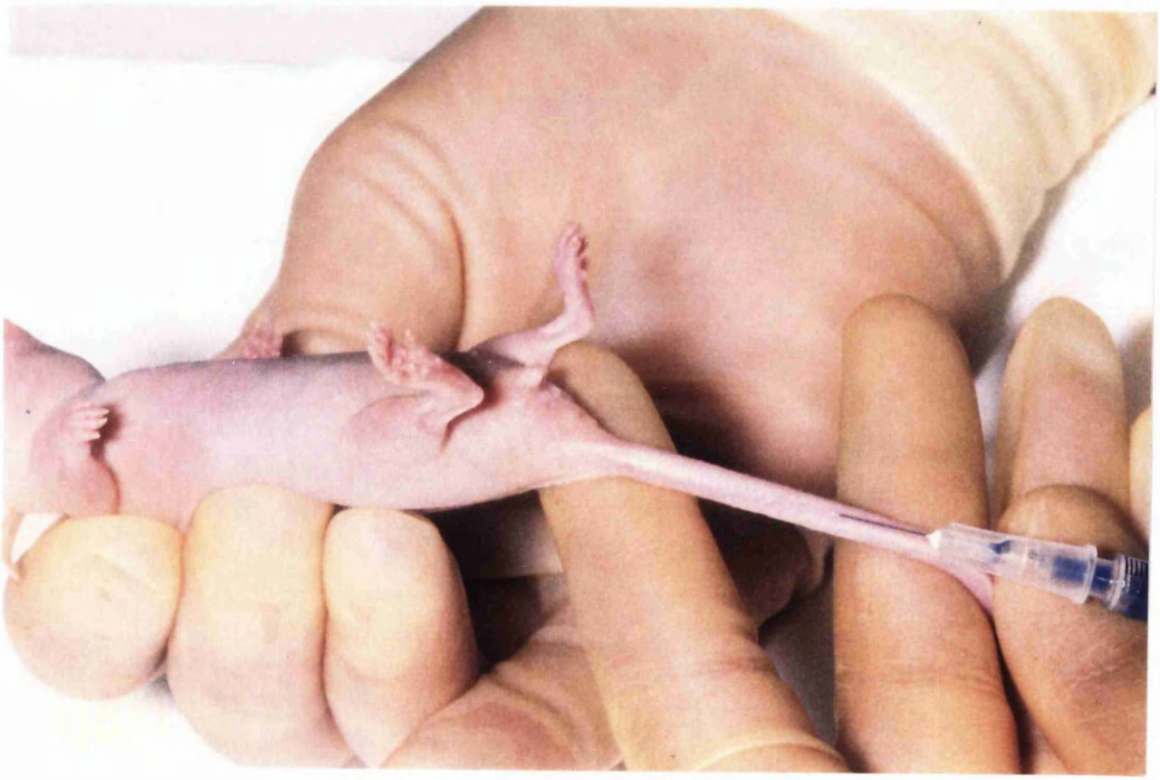


Figure 65 Intravenous injection of labelled methylene blue into the tail vein

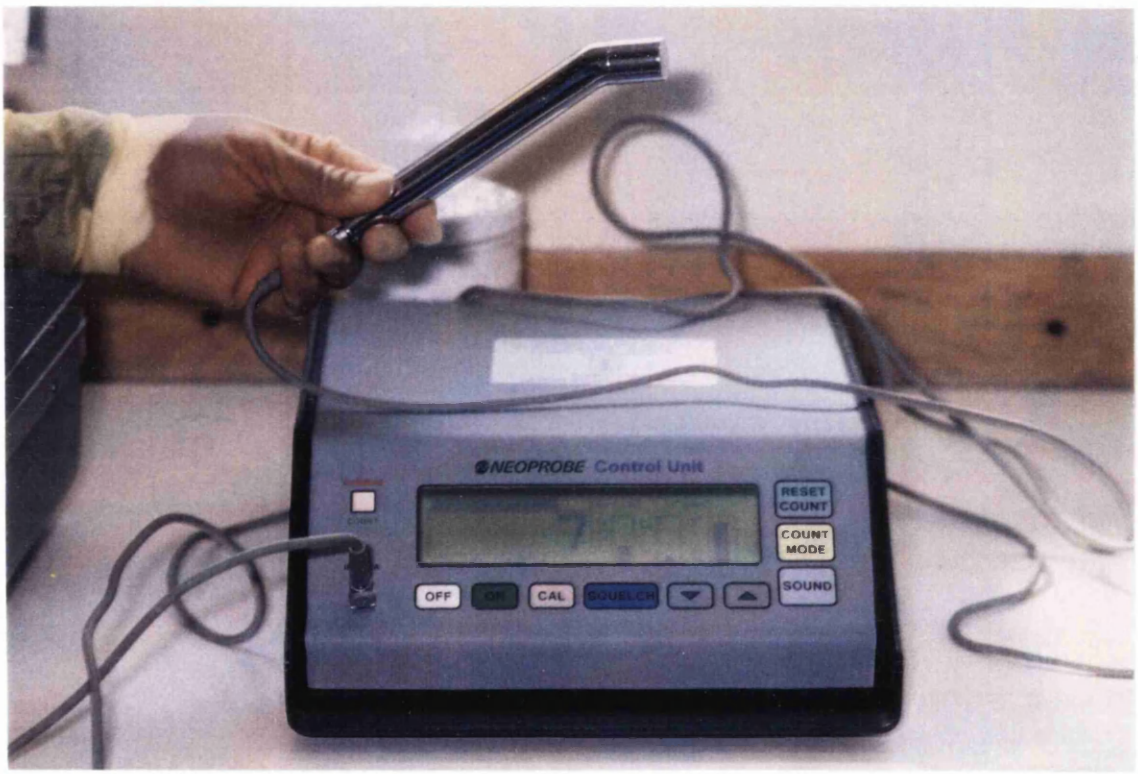


Figure 66 Neoprobe model 1000 for measuring radioactivity (courtesy of Mr P O'Dywer, Dept of Surgery, Western Infirmary, Glasgow)

microcomputer based control unit for fast, easy and positive location of gamma emitting isotopes.

A hand held detector probe allows the detector to be positioned close to the source, yielding reliable resolution and detection efficiency. It also has an audible response which enables the user to scan tissue and "listen" for areas of elevated radioactivity. A digital count display screen displays the count in either a continuous read out mode or for a selected time interval.

Radioactivity measurements were carried out in the liver, thyroid and tumour to assess the quantity of the labelled isotopes in the liver and the thyroid in relation to that seen in the tumour. The time taken to achieve peak levels were also gauged.

The thyroid was examined because iodine is normally taken up by this organ. No thyroid blocking agent was used so that the amount of iodine that accumulated in the thyroid gland could be studied. This gave the indication of the amount of free iodine in the preparation at injection. Any increased activity in thyroid during the experiment would thus indicate breakdown of the labelled methylene blue in vivo. Since the liver is the biggest organ which is pigmented, in theory, labelled isotopes would also target this organ.

When the animals were sacrificed, radioactivity in the tumour, liver and thyroid were measured.

EXPERIMENT 3

In experiment 3, stable I^{127} was used. The experiment was carried out according to the protocol outlined above. The experiment consisted of 3 animal groups:
Group A (the control group) received only tumour cells subcutaneously (7 animals).
Group B received an admix of tumour cells and I^{127} labelled MTB subcutaneously (6 animal).
Group C received tumour cells subcutaneously and on tumour growth becoming evident received I^{127} labelled MTB intravenously (7 animals).

EXPERIMENT 4

In experiment 4, the radioactive iodine used was I^{125}. Three groups of animals were used:
Group A (control group) received only tumour cells subcutaneously (6 animals).

Group B received an admix of tumour cells and I^{125} labelled MTB subcutaneously (6 animals).
Group C received tumour cells subcutaneously and when tumour growth was evident, received I^{125} labelled MTB intravenously (6 animals).

EXPERIMENT 5

In experiment 5, the isotope of iodine that was used was I^{131}. Similarly three groups of animals were used:

Group A (control group) received only tumour cells subcutaneously (6 animals).
Group B received an admix of tumour cells and I^{131} labelled MTB subcutaneously (6 animals).
Group C received tumour cells subcutaneously and once tumour was macroscopically visible, the animals received an intravenous dose of I^{131} labelled MTB (6 animals).

IN VITRO CELL CULTURE USING IODINE 125

Since there was no growth seen in the animals in group B in experiment 4, it was not possible to deduce whether the treatment was effective in destroying all the melanoma cells or the mixture that was injected into the nude mice in this group contained no melanoma cells. To assess what had happened, an in vitro cell plating experiment was designed and carried out.
B16/F10 melanoma cells were exposed to the radioisotope I^{125} under in vitro conditions. Cell plating was carried as below:
1. B16/F10 cells were grown to near confluence, harvested and counted. Two aliquots of 1x 10^6 cells were taken, centrifuged and resuspended in PBS.
2. One aliquot of cells was exposed to 1 Mbq of I^{125}, while the other was kept as a control.
3. After exposure to I^{125}, both sets of cells were centrifuged, then washed several times to remove exogenous iodine.
4. The concentration of the cell aliquots was adjusted by dilution with complete medium such that either 500 or 1000 cells were plated into 25 cm^2 tissue culture flasks.
5. Cultures were incubated at 37^0C for 6 days after which the formed colonies were fixed with methanol, stained with trypan blue and counted.
6. The numbers of colonies formed by the cells exposed to I^{125} were compared to the numbers formed by the control cells, and the results expressed as % plating efficiency.

7.2.4 RESULTS.

Statistical Analysis

All the results from experiments 3, 4 and 5 were analysed as described below:

1) Tumour volume for each animal in each group was calculated and a mean tumour volume and rate of growth derived. The average tumour volume on day 12 in each group was also calculated

2) The rate of growth and tumour volume on day 12 between different groups were compared.

3) To confirm if there was a difference in the groups with respect to the variables shown above, the statistical analysis as outlined in experiments 1 and 2 was used.

4) The mean tumour volume and standard deviation on each day was calculated and plotted. The rate of growth was then obtained by linearising the results. The One Way Analysis of Variance was used to assess whether the results obtained in each group were statistically different. Friedman's test was used to assess whether the growth rate in the groups was significantly different.

5) Similar to experiments 1 and 2, the results were lagged. Formal analyses of the data was carried out using Repeated Measures Analysis of Variance. A significant group effect indicates an overall difference between the groups. The group day interaction measures the pattern across days for each group, and a significant interaction means the pattern differs for the two groups.

EXPERIMENT 3 (nude mice treated with iodine 127)

In group A, tumour growth was observed 5 days after inoculation of the tumour cells, just as had been seen in experiment 1, group A. The animals were sacrificed between days 12 and 14.

In group B growth of tumour became evident 9 days following inoculation of tumour cells with iodine 127 labelled MTB. The animals were sacrificed on day 15.

In group C tumour was evident on day 5. The animals were sacrificed on day 14.

Analysis of the results showed that the rate of growth was maximal in group A and that this rate was statistically different to that in groups B and C ($p = 0.001$).

Analysis of the tumour volume on day 12 showed that the tumour bulk was largest in group A and least in group B.

When the results were lagged in group B with respect to group A, there was a 4 day lag with a significant group effect ($p < 0.01$) but no significant group interaction ($p = 0.09$). When A and C were compared, there was no lag with no overall group effect ($p = 0.09$). A lag of zero indicates that the drug has no effect in reducing tumour growth.

EXPERIMENT 4 (nude mice treated with iodine 125)

In group A, tumour growth was observed 5 days after the inoculation of the tumour and the animals were sacrificed between days 14 to 20. There was minimal background radioactivity in the tumour, thyroid and liver.

In group B mice, there was no tumour growth. The animals were sacrificed on day 20 after inoculation. Radioactivity was maximal at the site where the tumour cells were inoculated followed by the thyroid and then the liver and similar results were obtained when the animals were sacrificed and the organs examined. The radioactivity at the site of injection was three and a half times and nine times that seen in the thyroid and liver respectively.

In group C, tumour growth was evident on day 7 and the animals were sacrificed between days 18 to 21. Maximum radioactivity was seen in the thyroid, followed by the liver and then the tumour. When the animals were sacrificed, maximum radioactivity was demonstrated in the liver, followed by the thyroid and then the tumour.

One Way Analysis of Variance showed that tumour growth was maximal in group A and that the rate was significantly different ($p = 0.001$) to that seen in group C. Using the Friedman's Test the rate of growth was significantly greater in the group A animals than in group C ($p = 0.001$).

Analysis of tumour volume on day 12 again showed that maximal growth was in group A.

When the results in group C were lagged and compared to group A, there was no overall group effect ($p = 0.58$) but a significant interaction was seen.

Analysis of radioactivity showed that in group B, radioactivity at the site of injection was maximal and that this was significantly different ($p < 0.001$) to that in the thyroid and liver. In group C, radioactivity was greater in the liver and thyroid and this was significantly different ($p < 0.01$) to that at the tumour site.

IN VITRO CELL CULTURE (melanoma cells treated with iodine 125)

The colonies were counted and expressed as plating efficiency. For each of the experiment, there were 3 plates and the plating efficiencies for the control cells were 78%, 88.5% and 86%. The mean value was 84.17% +/- 4.48. In the tumour cells exposed to I^{125}, the plating efficiencies were 76%, 75%, 83% and 77.25% with a mean of 79.0% +/- 2.84. The colonies formed by the tumour cells exposed to the radioactive $Iodine^{125}$ was significantly less than in the control group.

EXPERIMENT 5 (nude mice treated with iodine 131)

In group A, tumour growth was evident on day 5 after the inoculation of the tumour cells. The animals were scarified between days 13 and 15. There was minimal background radioactivity in the tumour, liver and the thyroid.

In the group B, tumour growth was evident on day 9 following inoculation. The animals were sacrificed on day 18. Radioactivity was maximum in the tumour followed by the thyroid, then the liver. When the organs were examined, maximum radioactivity was seen in the thyroid followed by the liver and then tumour.

In the group C, tumour growth was seen on day 5 and the animals were sacrificed on day 15. In group C the radioactivity was maximal in the liver followed by the thyroid and then the tumour. When the animals were sacrificed and the organs examined, radioactivity was maximal in the thyroid, followed by liver and then tumour.

Analysis showed that the rate of tumour growth was maximal in group A and that this rate was significantly different to that in groups B and C ($p < 0.001$). Using the Friedman's Test, the rate of growth was significantly greater in group A followed by group C and with the least growth in group B animals ($p = 0.001$).

Analysis of the tumour volume on day 12 showed that the tumour bulk was greatest in group A with the least in group B.

When the results were lagged in group B and compared to group A, there was no overall group effect ($p = 0.13$) or interaction ($p = 0.09$). Again lagging group C and comparing it against group A, there was also no overall group effect ($p = 0.37$) but a significant interaction ($p < 0.01$) was seen.

Thus in group B, it seemed the effect of the drug was to delay the tumour growth for 4 days, and thereafter the pattern of growth was the same as that shown from the start by the control group.

In groups A and C the delay was around 2 days but although there was no overall difference between the groups, their pattern of growth varied from day to day and so it was not simply the case that group C followed the same pattern as the control group after this time.

Analysis of radioactivity showed that in group B, radioactivity at the site of injection was maximal and that this was significantly different ($p < 0.001$) to that in the thyroid and liver. In group C, radioactivity was greater in the liver and thyroid and this was significantly different ($p < 0.01$) to that at the tumour site.

Figure 67 shows the rate of tumour growth in experiments 3, 4 and 5. Figure 68 shows the tumour volume on day 12 in experiments 3, 4 and 5. Figures 69-74 show the radioactivity of the various organs in experiments 4 and 5.

7.2.5 DISCUSSION.

The aim of these experiments was to test if targeted therapy was effective and to identify the most suitable isotope. The results obtained in the experiments confirm the hypothesis that targeted radiotherapy using radioactive iodine is effective in the control of tumour growth in an animal model.

The rate of tumour growth in the treated groups were significantly different to that of the control, suggesting that the radioactive isotopes were having some effect on the tumour growth. If radioactive isotopes (iodine 125 and 131) had only a delaying action, then when the results are lagged to the control group, there should be no significant difference. However, when the results were lagged to the control groups, the pattern of tumour growth was significantly different. This suggests that the

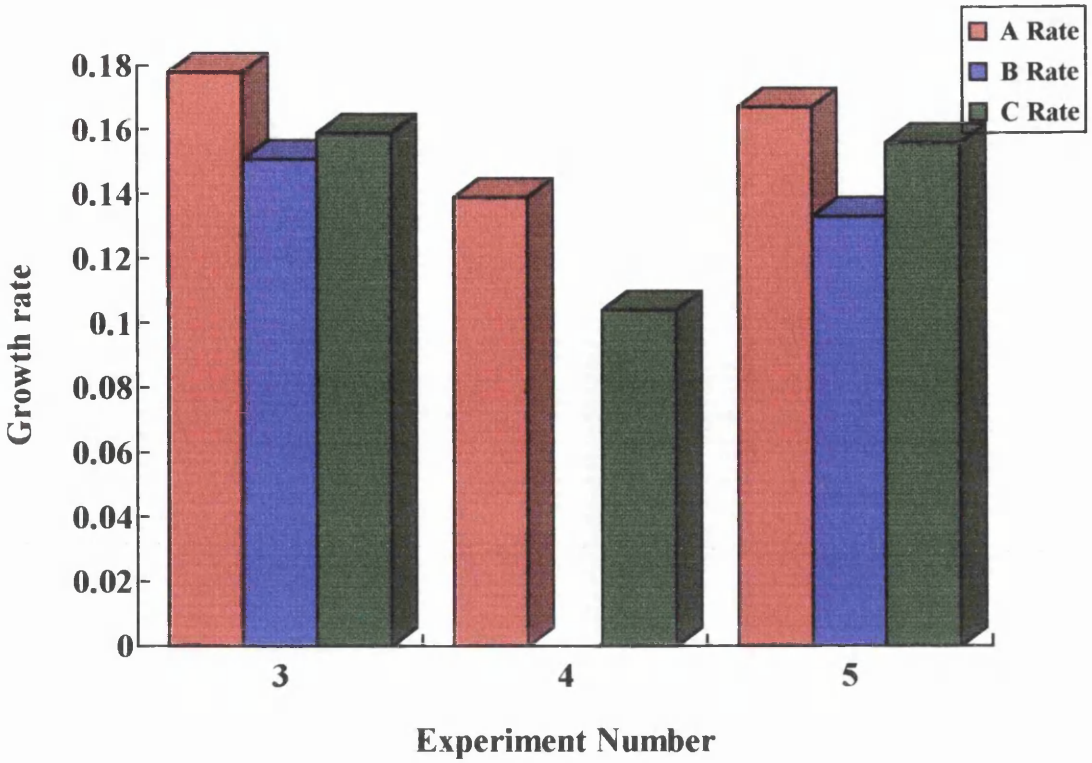


Figure 67 Rate of tumour growth in groups A, B and C in experiments 3, 4 and 5

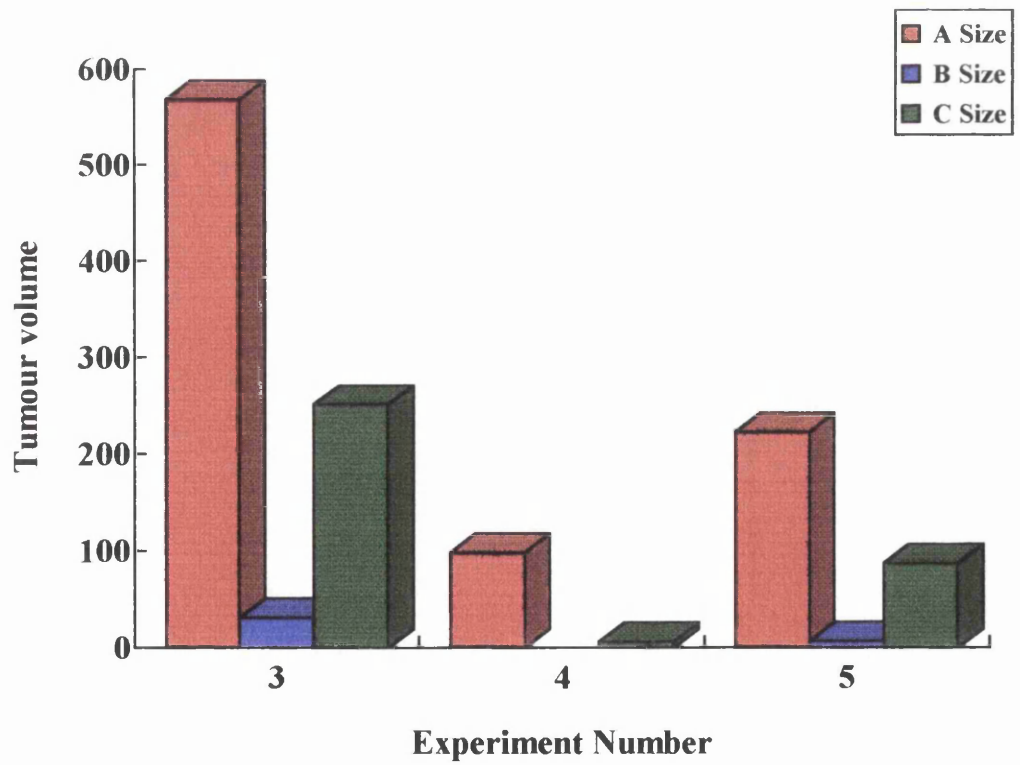


Figure 68 Tumour volume on day 12 in groups A, B and C in experiments 3, 4 and 5

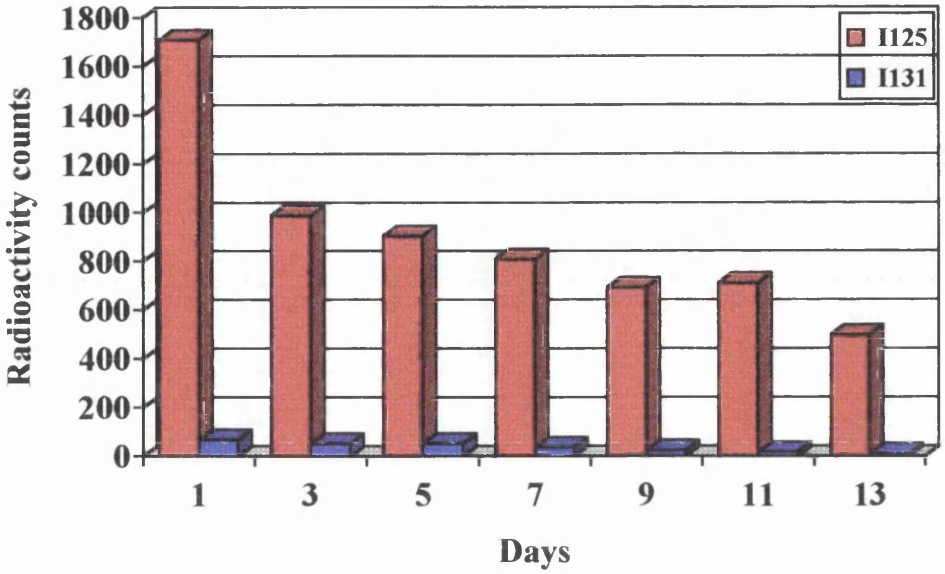


Figure 69 Radioactivity in the tumour in group B in experiments 4 and 5

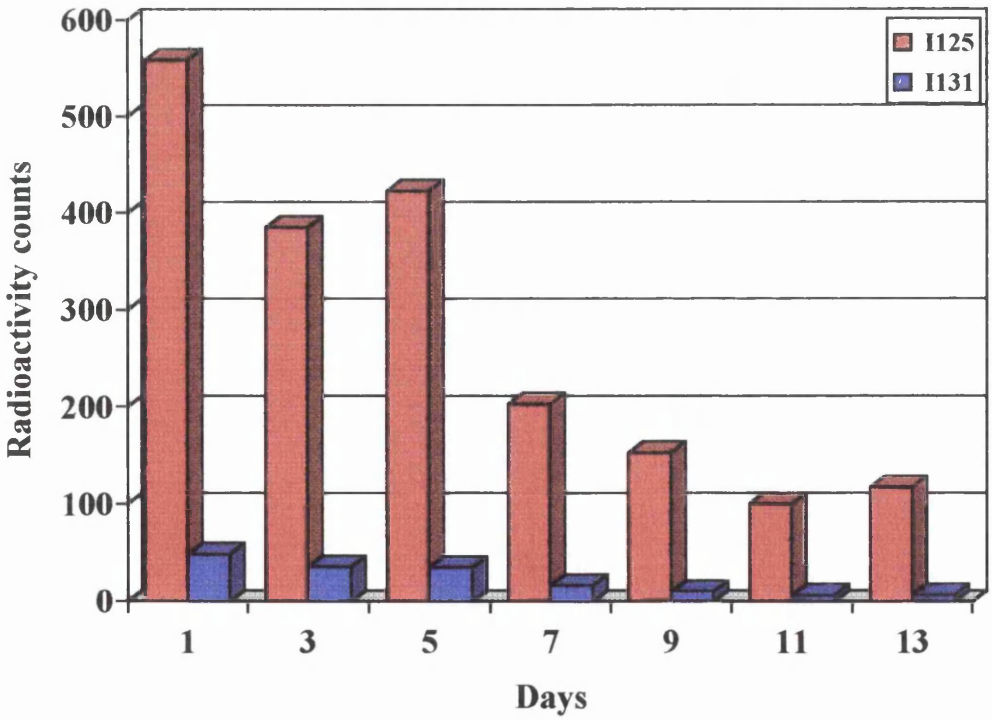


Figure 70 Radioactivity in the thyroid in group B in experiments 4 and 5

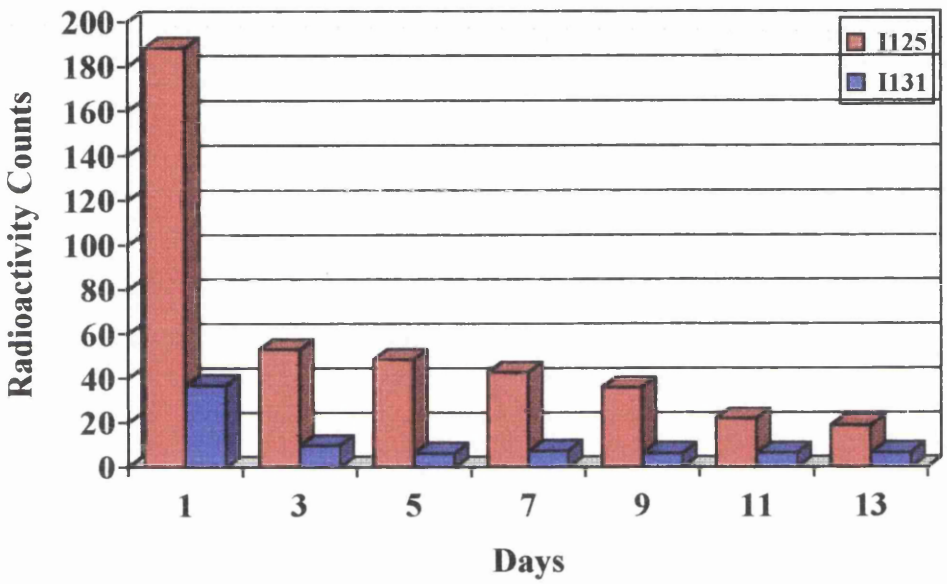


Figure 71 Radioactivity in the liver in group B in experiments 4 and 5

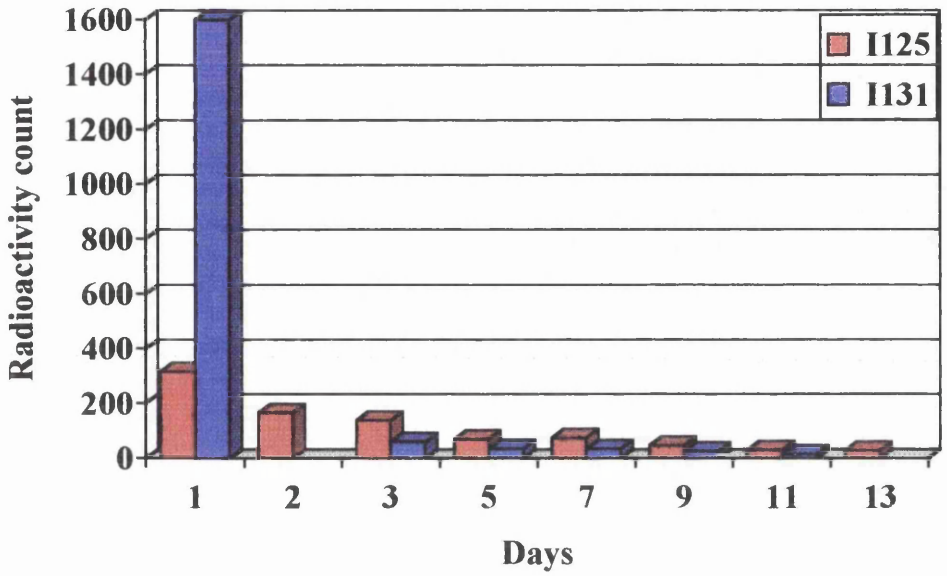


Figure 72 Radioactivity in the tumour in group C in experiments 4 and 5

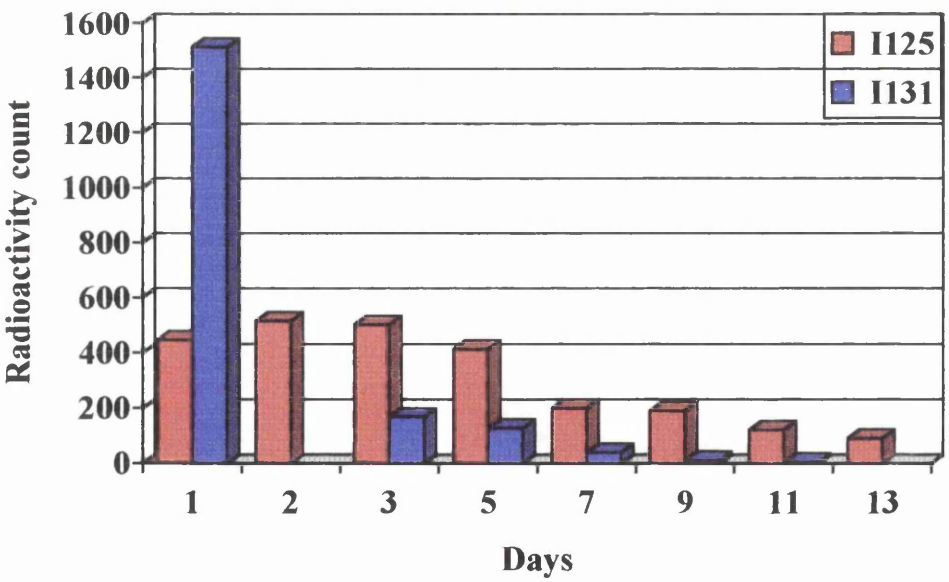


Figure 73 Radioactivity in the thyroid in group C in experimentss 4 and 5

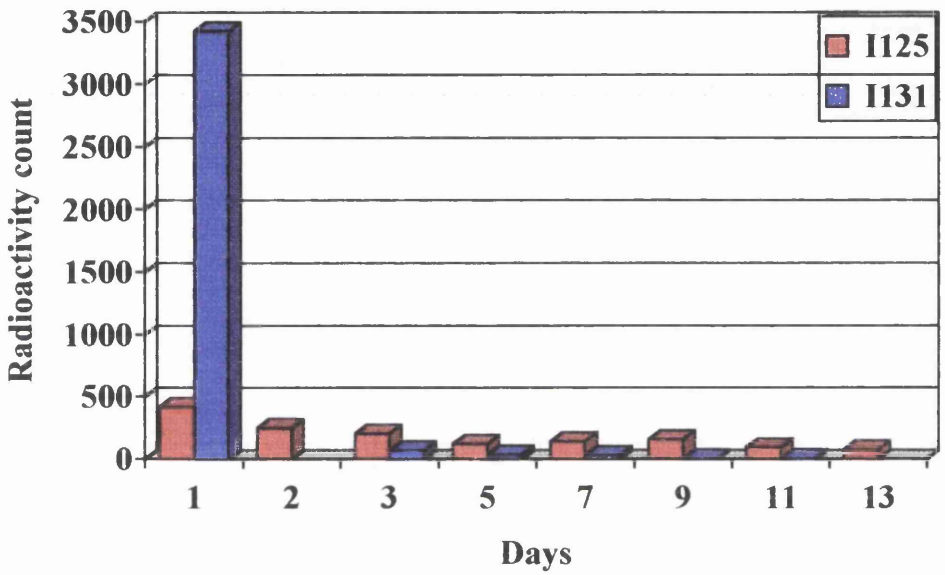


Figure 74 Radioactivity in the liver in group C in experiments 4 and 5

treatment with the radioactive iodine was having an effect on reducing tumour growth and this effect was not simply one of delaying action.

Since there was no growth in group B in experiment 4, the experiment was repeated in vitro. The plating results showed that the growth of tumour cells was significantly retarded by I^{125}.

From this animal model, it can be concluded that both the radioactive isotopes of iodine (I^{125} and I^{131}) significantly effect the tumour growth. Iodine127 has no effect on tumour growth.

However it was not possible to reach a definitive conclusion as to which was the more effective radionuclide. This was because different batches of animals and cells lines were used, making direct comparison of I^{125} and I^{131} not possible. Therefore in order to make a direct comparison, the experiments had to be repeated using animals from the same batch and using the same cell line simultaneously. This was carried out in section 7.4.

Results obtained in experiment 3 using stable I^{127} labelled with MTB were similar to the results obtained when MTB was used alone. Here there was no significant group effect and there was no lag confirming that I^{127} has no tumouricidal effect. It also showed that iodine binds effectively to methylene blue without altering it's affinity for melanin. This is an important finding. It shows that MTB is a suitable carrier molecule for radionuclides of iodine.

The only spurious results that were obtained in these experiments were in group B in experiment 4. There was no growth observed in these animals. The radioactivity in the animals was high from day 1. This could be explained if there had been some contamination resulting in a different dose of radioactivity being used that was sufficiently large to kill all the melanoma cells or that there were no melanoma cells in the mixture that was inoculated into the animals.

In order to address this problem, a cell culture was set up using the same method in vitro to assess the effectiveness of iodine 125. This experiment showed that there was less tumour growth in the cell line isolated with MTB labelled iodine 125 than in the control. This suggests that a higher dose of I^{125} was possibly used (as the radioactivity was higher than that seen in group C) and this was sufficiently large to kill all the melanoma cells during the incubation period prior to inoculation.

The results of these mouse melanoma studies raises several questions. High levels of radioactivity were recorded in the thyroid gland and the liver of the mice. These levels were greater in group C mice who received the isotope via the tail vein than in group B mice where the isotope was injected subcutaneously after incubation with the tumour cells.

This difference can be explained. Group B animals received a mixture of tumour cells and radioactive iodine which had already been incubated for three hours before subcutaneous injection thus giving the iodine labelled methylene blue time to bind to the melanoma cells. In this circumstances, there may have been less labelled methylene blue available to enter the circulation.

The greater activity in group C mice could be because the radioactive isotope was injected intravenously and gained immediate access to the systemic circulation. It would perfuse through organs such as the lung and liver prior to reaching to the skin. If pigmented cells are present as in the liver, the isotope will attach to the pigmented cells. This is reflected in the finding that the radioactivity count in the liver in all group C experiments was higher than in the tumour.

Despite the fact that the cells of the thyroid gland are not pigmented, there was still radioactivity detected in the thyroid gland. This meant that the labelled methylene blue may not be as stable in vivo with free iodine generated actively taken up by the thyroid cells. Thus if this treatment is to be used in the human situation, the thyroid would have to be blocked using oral potassium iodide and potassium perchlorate to minimise the radiation dose to the thyroid. Furthermore, the stability of the compound has to be assessed before it is applied to the clinical situation.

These experiments have shown that targeted radiotherapy using I^{125} and I^{131} is possible and that MTB is an effective carrier molecule. There is significant accumulation of the radionuclides in non targeted organs and this appears to be related to the mode of delivery.

7.3 COMPARISON OF IODINE 125 AND IODINE 131 IN THE TREATMENT OF IN VIVO AND IN VITRO B16/F10 MELANOMA

It was not possible to conclude from the earlier experiments which of the two isotopes of iodine was superior. A further series of experiments were thus designed to address the following unanswered questions:

1) Which isotope is more effective?
2) Is the accumulation of the isotopes in other organs a constant feature?

7.3.1 MATERIALS AND METHODS

The materias and methods used to carry out these experiments were as described in 7.2 unless otherwise as stated below. Both in vivo and vitro experiments were carried out to confirm that the effect of radioactive iodine was not just a chance event and that the results observed in these experiments were seen in both the in vivo and in vitro situation. The experiments in this section were carried out using the same isotopes of iodine as in experiments 4 and 5. Both in vivo and in vitro experiments were carried out simultaneously using the same batch of tumour cells (from the same cell line) and the same batch of iodinated methylene blue. Since the number of animals used in these experiments were greater than the agreed number in the original protocol, written permission was obtained from the Home Office before the experiments described below were carried out.

IN VIVO EXPERIMENTS USING IODINE 125 AND 131

There were three groups of animals:
1) Group A mice were injected with a single cell line $1x10^6$ of pigmented melanoma cells subcutaneously (6 animals).

2) Group B was divided into two subgroups:
B1 mice were injected with a mixture of $1x10^6$ melanoma cells and I^{125} labelled methylene blue subcutaneously (6 animals).
B2 mice were injected with a mixture of $1x10^6$ melanoma cells and I^{131} labelled methylene blue subcutaneously (6 animals).

3) Group C mice were divided into two subgroups:

C1 mice were injected with $1x10^6$ melanoma cells subcutaneously and once tumour growth was evident, the mice were innoculated with the I^{125} labelled methylene blue intravenously via the tail vein (6 animals).
C2 mice were injected with $1x10^6$ melanoma cells subcutaneously and once tumour growth was evident, the mice were innoculated with the I^{131} labelled methylene blue intravenously via the tail vein (6 animals).

Radioactivity measurements were carried out on alternate days using the Neoprobe Model 1000 Portable Radioisotope Counter. The tumour growth was recorded daily by measuring the greatest dimension (a) and the length perpendicular to the greatest dimension(b). The formula used to calculate the tumour volume was:

Tumour volume (v) = (a) x b x 0.5

When the animals were sacrificed, the tumour, liver and thyroid glands were examined for radioactivity. The amount of radioactive used was 0.5 KBq per mice.

IN VITRO CELL CULTURE USING IODINE 125 AND 131

Culture of B16/F10 cells were exposed to the radioisotope in in vitro. Cell plating was carried as below:
1) B16/F10 cells were grown to near confluence, harvested by trypsinization and counted. Three aliquots of $1x\ 10^6$ cells were taken, centrifuged and resuspended in PBS.
2) One aliquot of cells was exposed to 1 KBq of I^{125}, one to I^{131} and one was maintained as a control. The cells were exposed to each iodine compound for 30 minutes at room temperature.
3) After exposure, the cells were centrifuged (1000rpm for 5 minutes), then washed with PBS several times to remove exogenous iodine.
4) The concentration of the cell aliquots was adjusted by dilution with complete medium and approximately 100 cells were plated into 25 cm^2 tissue culture flasks.
5) Cultures were incubated at 37°C for 7 days, with a medium change after 3 days. On day 7, the formed colonies were fixed with methanol, stained with methylene blue and counted.
6) The colonies were fixed and stained as below:
i) The monolayer was rinsed with PBS, and the rinse discarded.
ii) Fresh PBS was added, 5ml per 25 cm^2.

iii) Methanol was added slowly to the PBS with mixing. The bottle was tilted so that the PBS was on the side opposite to the monolayer. 1 ml methanol was added and the mixture was run over the cells. The bottle was tilted back and 1 ml added and the monolayer rinsed, and this was continued until 50% methanol was reached.
iv) The 50% methanol/PBS mixture was discarded and replaced with fresh methanol. This was left for 10 minutes.
v) The methanol was then discarded and replaced with fresh anhydrous methanol. The monolayer was rinsed and the methanol discarded.
vi) To stain, methylene blue was added , 2 ml per 25 cm^2, making sure that the entire monolayer was covered and remained covered.
vii) After 2 minutes, the stain was diluted with 8 ml water and was agitated gently for a further 2 minutes.
viii) The stain was discarded and the monolayer washed vigorously in running tap water until any blue cloudy background stain was removed but not leached out of the cells.
ix) The water was poured off and the monolayer rinsed in deionised water and examined under the microscope while still wet.
7) For each isotope and the control, 3 platings were performed. The numbers of colonies formed by the cells exposed to I^{125} and I^{131}were compared to the numbers formed by the control cells, and the results expressed as % plating efficiency.

7.3.2 RESULTS

IN VIVO EXPERIMENTS

The mean tumour volume and standard deviation was calculated for each animal group. These results were then linearised and plotted against time to obtain the rate of tumour growth for each animal group. Since earlier experiments (1,2,3,4,5) had already shown that the radionuclides had a significant effect in slowing growth rate and not just a delaying effect, lagging of the results was not done. The statistical tests used were One Way Analysis of Variance and Friedman's Rank Comparison.

In the group A mice, tumour growth was evident on day 5 after inoculation of the tumour and the animals were sacrificed between days 10 and 12.

In group B mice, tumour growth was evident first in group B2 mice on day 7 followed by B1 on day 8. The animals in this group were sacrificed between days 12 and 16.

In group C mice growth was evident on day 5 in both the subgroups C1 and C2. The animals were sacrificed between days 12 and 15.

When the rate of tumour growth was compared in the three groups, the rate of growth was greatest in group A (Figure 75).

When the rates of growth in group B animals were compared to the control, the growth rate was significantly different both by the One Way Analysis of Variance ($p = 0.001$) and Friedman's rank comparison test.

When the rates of growth in group C animals were compared to the control, the growth rate was also significantly different in C1 and C2 ($p = 0.001$).

When intra group growth rates were compared, the growth rate was slower in subgroup B1 than B2 and this was statistically significant ($p = 0.002$).

Similarly, when the intra group growth rate was compared in group C, although the growth rate of C1 was slower than C2, this did not reach statistical significance ($p = 0.228$).

When inter group growth rates were compared, the growth was significantly slower in B1 than C1 ($p = 0.018$) and in B2 than in C2 ($p = 0.001$).

In summary, the addition of radioactive I^{125} and I^{131} labelled to methylene blue produces slower tumour growth when compared to no treatment. In group B animals those treated with iodine 125 had a slower tumour growth than iodine 131. However in group C, the growth rate of tumours in mice treated with iodine 125 and 131 was not significantly different. Thus it would appear that iodine 125 is superior to iodine 131 in retarding tumour growth.

Radioactivity in the tumour, liver and thyroid of the Group A was similar to the background radioactivity. However in subgroups B1 and B2 radioactivity was maximal in the tumour followed by the thyroid and then the liver. In the subgroups C1 and C2, radioactivity was maximum in the liver, followed by the thyroid and then the tumour

Similarly, when the animals were sacrificed, maximal radioactivity was recorded in the tumour in groups B1 and B2. In groups C1 and C2, radioactivity was maximal in the liver and thyroid.

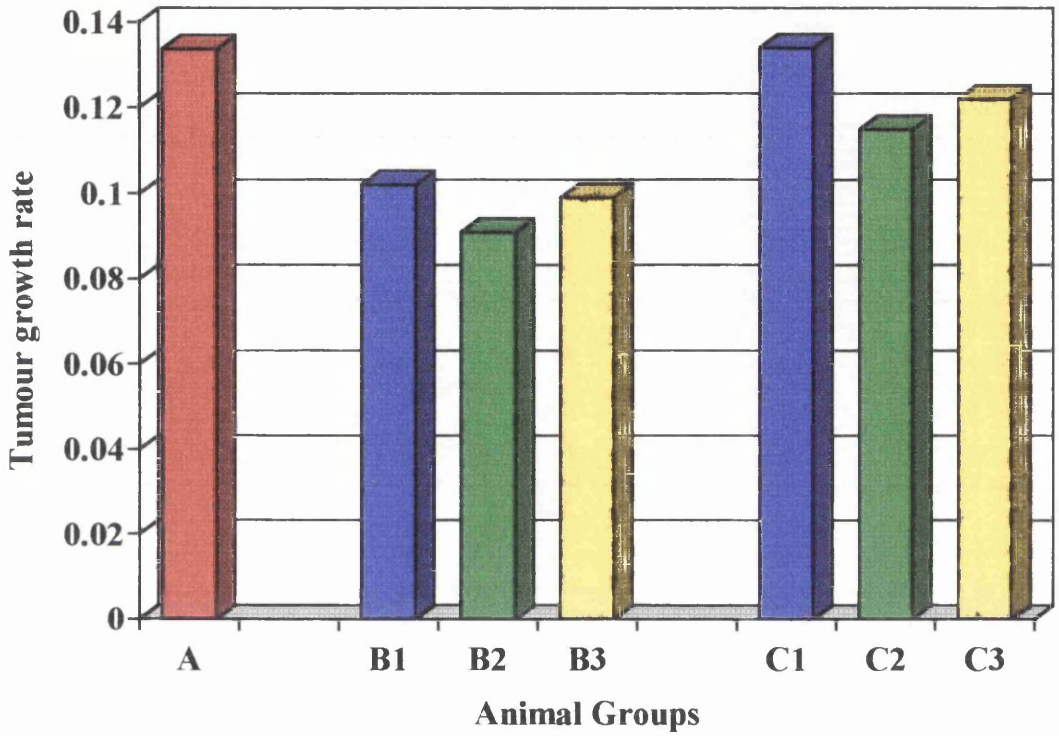


Figure 75 Rate of tumour growth in groups A, B and C in vivo

IN VITRO EXPERIMENTS USING IODINE 125 AND 131

Figure 76 shows the appearance of the melanoma cells in the control plate when examined under the microscope and figure 77 shows the melanoma cells treated with iodine 125 when examined under the microscope. Here it can be clearly seen that in a single field, there were less melanoma cells in the treated plate than in the control.

The colonies were counted and the results expressed as a percentage of the control. The results of the number of colonies and the percentage in relation to the control are tabulated in Table 13. Figure 78 shows a graphical illustration of the percentage efficiency of the plates treated with iodine 125 and 131 to the control. The results show that growth of the melanoma cells incubated with the iodine isotopes is significantly reduced when compared to the untreated control ($p < 0.001$). There was approximately a reduction of 66% and 33% in the tumour growth in the melanoma cells exposed to iodine 125 and iodine 131 respectively when compared to the control. When iodine 125 was compared to iodine 131, the reduction in tumour cells treated with iodine 125 was twice that of tumour cells treated with iodine 131 ($p < 0.001$). Thus iodine 125 was also superior to iodine 131 in vitro.

7.3.3 DISCUSSION

Cellular DNA is the main target for ionising radiation. Radiation with high LET causes more permanent damage than that with low LET. Low LET radiation may cause initial damage which is subsequently modified by post irradiation cellular processes such as repair.

The factors that must be taken into account when selecting a therapeutic radionuclide include its chemistry, half-life and availability. The physical half-life of the radionuclide must be matched to the half-life of the prepared radiopharmaceutical in the tumour, namely the biological half-life.

Radiosensitivity is a significant factor affecting the response of tumour cells to radionuclide therapy. Hypoxic cells are recognised as being relatively radioresistant.

The results obtained in these experiments showed that when nude mice with melanoma were treated with I^{125} and I^{131} labelled methylene blue, the growth rate of the tumour was significantly reduced when compared to the nude mice that did not receive treatment. These experiments reinforce the results that were obtained in

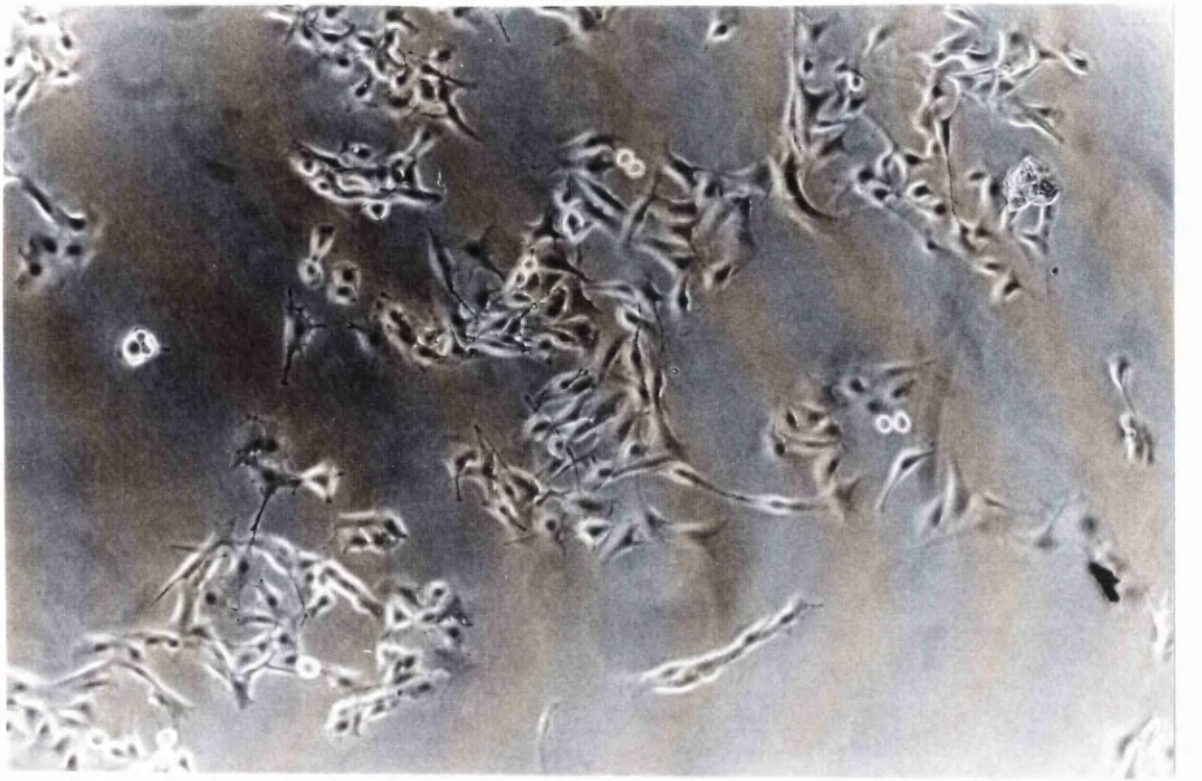


Figure 76 Control plate showing tumour cell growth in the in vitro culture

Figure 77 Tumour cell plate treated with iodine 125 in the in vitro culture

		NUMBER OF COLONIES		MEAN+ S D	% OF CONTROL
B16/F10 CONTROL	102	91	87	93.33 +/- 6.34	
B16/F10 + I^{125}	28	36	36	33.33 +/- 3.77	35.72
BI6/F10 + I^{131}	59	68	52	59.67 +/- 6.55	63.93

Table 13 Results of in vitro cell plating

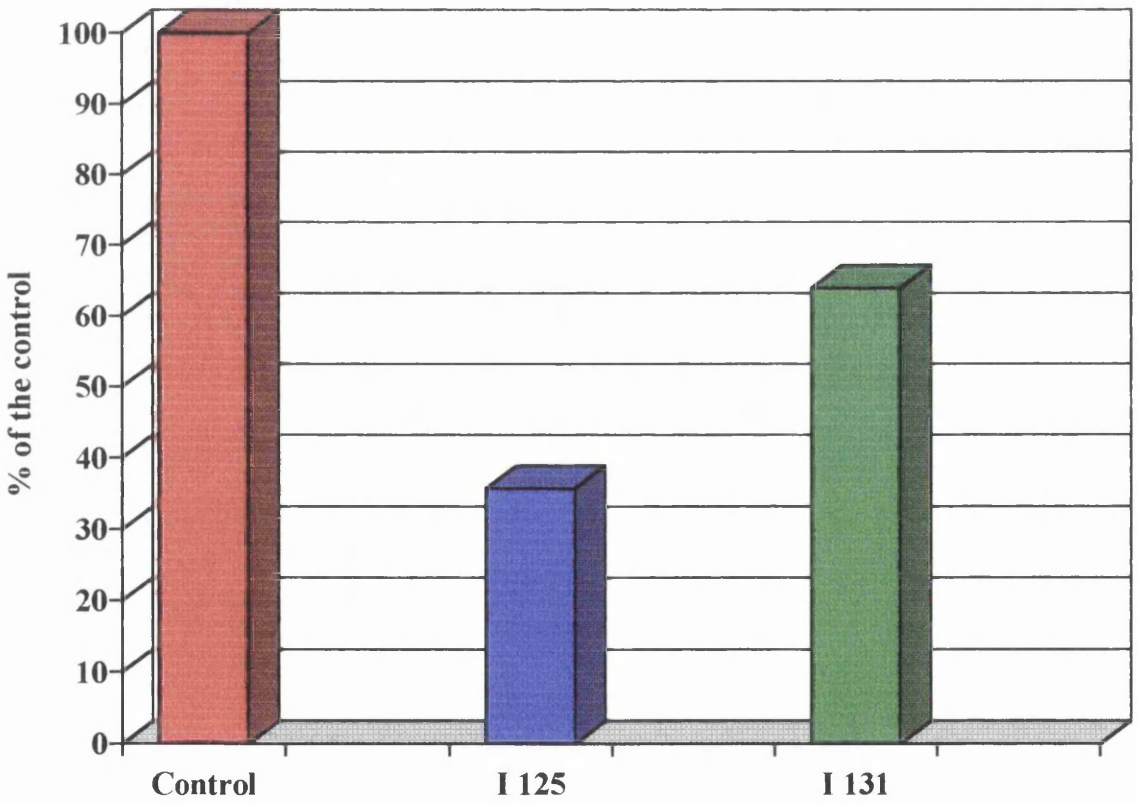


Figure 78 Tumour growth in the in vitro experiment

experiments 4 and 5 that targeted radiotherapy using radioactive I^{131} and I^{125} labelled methylene blue is effective in reducing the growth of melanoma.

If the in vivo and in vitro experiments could be compared, then the group that should be compared to is group B. In in vitro experiments, the reduction on the rate of tumour growth was significantly greater to that seen in group B animals. This reinforces the fact that it is not sufficient to demonstrate and conclude from in vitro experiments that a certain therapy is effective. In vivo studies are important as they reflect the problems that are encountered in the clinical situation more accurately. There may be several interacting factors in the host that may modify the treatment and reduce the efficacy of the treatment. The results of these experiments demonstrate that the reduction of rate of tumour growth is different in the in vivo and the in vitro situation although the same amount of tumour cells and radioactivity were used.

In the in vivo experiments, the growth rate of the tumour in the group B animals which received iodine 125 was slower than those that received iodine 131. There was no statistical difference in the growth rate in experiment C between animals treated with iodine 125 and 131. This difference is probably explained by the considerable difference in tumour bulk at the time of inoculation of the labelled methylene blue. This reinforces the principle of adjuvant therapy (section 2.1.2) that the effectiveness of the treatment is inversely proportional to the tumour burden, i.e. the smaller the tumour the better the response.

Furthermore, the route of adminstration was different in the groups B and C. In group B, the treatment was given subcutaneously but in group C, it was given intravenously. Thus in group C, the iodine labelled methylene blue gained access to the systemic circulation and became incorporated into the cells of organs that are pigmented. This resulted in less labelled methylene blue being available to target the melanoma cells.

Link[367] and colleagues have compared the therapeutic effectiveness of iodine 125 and astastine 211 in the treatment of pigmented melanoma in nude mice. Both these isotopes are auger electron emitters but astastine has a shorter half-life than iodine 125. Experiments were performed both in vivo and in vitro. The effectiveness of the treatments were determined by lung colonogenic assay. They concluded that iodine 125 was ineffective when incorporated into melanosomes distributed in the cytoplasm whilst astastine 211 was effective in the treatment of the tumour.

However the results from the experiments reported here, contradict Link's findings. Both the in vitro and in vivo experiments show that iodine 125 significantly effects tumour growth. These differences could be explained by the different route of adminstration of labelled methylene blue.

When the animals were injected intravenously, the highest radioactivity count was not found in the tumour but in organs of the body that are pigmented and also in the thyroid. The reason that Link reported no effect was probably related to the fact that a significant amount of radioactive iodine was in the thyroid and not in the targeted tissue. Astatine does not normally accumulate in the thyroid. Thus, thyroid blocking should be carried out before such a conclusion can be reached.

From this series of both in vivo and in vitro experiments, it can be concluded that Iodine 125 and 131 have a significant effect on the tumour growth of melanoma in nude mice. Iodine 125 appears to be more effective in the control of tumour growth than iodine 131. This may be reflected in the chemistry of the isotope as iodine 125 has a higher LET energy than iodine 131.

The route of adminstration is important if therapy is to have clinical application. As seen in experiments 4 and 5, radioactivity in group B was maximal in the tumour whilst in group C radioactivity was greater in the liver and the thyroid. If the treatment is to be used to destroy limb metastases, then the isolated limb perfusion circuit may be a route of delivery for this treatment. It would allow direct delivery of a radiopharmaceutical into the vascular bed perfusing the tumour and would results in high concentrations of the drug in the cells targeted without the systemic complication. Furthermore, since oxygenation can be maximised as already shown in chapter 4, hypoxia in the targeted cells can be prevented and treatment effect maximised. This localised form of treatment has already been shown to be effective in the treatment of unresectable liver tumours.

Metastatic deposits from melanoma tend to be found in the liver and the lung. It has already been shown that when the therapy is administered intravenously, a significant amount of iodine accumulates in the liver. Thus if this therapy is to be used to target and destroy blood borne metastases, then intravenously administered therapy is appropriate. It is however important to appropriately block the thyroid to prevent any free radioactive iodine from being taken up

Another possible clinical application for targeted therapy is its use in direct intradermal injection of recurrent nodules similar to that seen with BCG. This may not only have the effect of reducing the size of the lesion but may also modify the disease process and ultimately improve disease free survival and the overall survival.

In all the experiments there were three groups of animals. The first group or group A is analogous to the patient with untreated tumour. The second or group B is analogous to the patient with primary melanoma receiving adjuvant therapy and the third or group C is analogous to the patient receiving therapeutic treatment. Thus in theory, targeted radiotherapy could be used after as an adjuvant or in the therapeutic setting as outlined above.

Before using the ILP circuit to deliver such treatment, further experiments are needed to examine the effects of combining radioactive iodine with melphalan in the isolated limb perfusion circuit in larger animals. This will involve the monitoring of time dependent bio-distribution of the isotope and calculating the optimal dose and time. The circuit could be used to assess the effect of tumour growth, appearance of distant metastases and side effects of treatment.

This treatment may have therapeutic potential to improve survival in the high risk stage I melanoma patient. The cure of metastases requires total destruction of all the tumour. Nothing short of that can produce long term remission. The presence of undetectable micrometastases will result in rapid tumour cell proliferation. If a cure is to be found for the patient who may have no evidence of clinically detectable tumour, a treatment aimed at identifying and targeting minute metastases is necessary as these cells will go on to form recurrencs of the tumour.

Again applying the adjuvant principles, when animals were inoculated with a known tumour dose and randomly allocated to single treatment modalities or combination modalities, the latter produced superior results. Thus in patients with high risk primary melanoma, the use of combined targeted radiotherapy and melphalan in the optimised ILP circuit may be beneficial. This treatment will selectively target and destroy those in transit metastatic cells that have gained access not only to the lymphatics but also to the blood stream. This may reduce the tumour sub-population that survive thus producing a longer remission and hopefully a cure. The use of a repeat perfusion or a double perfusion schedule technique may also be beneficial in stage I patients ensuring that all possible melanoma cells have been eradicated.

7.3.4 CONCLUSION

Targeted radiotherapy using iodine 125 and 131 is effective in slowing the growth of B16/F10 malignant melanoma in the nude mice model. It appears that iodine 125 is superior to iodine 131. These results were also seen in vitro. Methylene blue is an effective carrier molecule to which the iodine radionuclides can be attached. Intravenous adminstration of the targeted therapy may result in accumulation of the radiopharmaceutical in non targeted organs. The use of local targeted therapy appears to be a solution and ILP may be the vehicle for such a treatment.

CHAPTER 8

CONCLUSION

The incidence of malignant melanoma is increasing worldwide at the rate of 6%-7% per year which is faster than any other cancer. In Scotland, 588 cases were registered in 1992, an increase from 507 in 1991. Melanoma is a curable malignancy if identified and surgically excised at an early stage. To quote Neville Davis "melanoma writes its message on the skin for all to see. But the eye cannot see what the mind does not know and so some see the writing but do not comprehend the message. Those who understand the message will save many lives by taking the appropriate action". Professional and public education campaigns therefore play an important part in programmes aimed at early detection.

In Scotland, melanoma is being actively monitored by the Scottish Melanoma Group (SMG). This group consist of physicians and surgeons whose aim is not only to improve the treatment of the disease but also to create an awareness amongst the general public of a malignancy with a potential cure if detected at an early stage. Gartnavel General Hospital in Glasgow is the only centre in Scotland and the North of England to provide isolated limb perfusion treatment for malignant melanoma of the extremities.

Surgery has long been the cornerstone in the treatment of malignant melanoma. Wide local excision of an early malignant melanoma can confer a 95% cure rate in certain patients. However, even as early as the 19th century, it was appreciated that local excision alone was not sufficient. At the turn of the century, elective lymphadenectomy became established in the treatment of melanoma. Handley showed that although melanoma spread first to the lymph nodes, it was blood borne spread that finally kills the patient. There were no changes in the treatment of melanoma for over fifty years. Primary and recurrent disease of the limb was treated surgically and in intolerable disease, amputation was the only option.

In 1957, isolated limb perfusion using melphalan was introduced for the treatment of limb melanoma. In patients with melanoma of the extremities, treatment with isolated limb perfusion has now been established for locally inoperable melanoma and as an adjunct to surgery in recurrent melanoma. ILP has been available in Glasgow since 1983. It was first used in the treatment of limb recurrences that failed to respond to local surgery and where amputation was the only option available.

Since 1983, 103 patients (78 female, 25 male) with recurrent limb melanoma have undergone ILP. A total of 122 perfusions have been carried out. Although the 5 year survival of the group was only 26%, a high objective response was seen in the patients. 75% showed complete and 23% showed partial response to the first treatment of ILP with 47% showing complete and 53% showing partial response to repeat perfusion. It was interesting to see that when treatment was repeated, a significant response was seen even with the same chemotherapeutic agent. The poor survival seen in this unit when compared to those published, reflects the timing of referrals. Most of the patients referred had advanced disease and the most important consideration then was control of the disease and avoidance of amputation. This was successfully achieved as no patient underwent amputation either as a result of complications from ILP or from failure to control the disease.

From analysis of these results, the important message is that physicians should refer patients at an early stage of the disease. ILP can be used not only to control recurrences but also to modify the course of the disease, thus improving survival. There may however be another option for the treatment of limb recurrences, i.e. surgical excision of the recurrences followed by ILP with melphalan. This uses the principle of adjuvant therapy in that agents with activity against moderately advanced disease are more active against residual metastatic disease after debulking the tumour by surgery (section 2.1.2).

What is now needed is a randomised trial comparing surgical excision followed by secondary adjuvant therapy to ILP alone. At the moment, results from the Glasgow unit show that ILP is the most effective means of palliation for recurrent limb melanoma when compared to other treatment modalities such as radiotherapy or chemotherapy (section 2.2, 2.3).

The role of ILP in the treatment of limb recurrence has been clearly established. However, the use of ILP as an adjuvant to surgery in primary melanoma remains controversial. In the Glasgow unit, between 1985 and 1993, patients with primary lesions greater than 1.5mm underwent adjuvant ILP. Ninety nine patients, 31 males and 68 females with a median age of 58 (range 21-84 years) and with a mean Breslow thickness of 3.2mm were treated by wide excision and ILP using melphalan. To date, the overall survival of the patients treated with ILP does not significantly differ from non perfused patients (from the SMG data base). However, for patients with lesions of 1.5mm to 2.99mm, analysis of both time to first recurrence and survival suggests a consistent trend towards benefit in the ILP group.

Subungual melanoma is rare and experience in treating this condition with isolated limb perfusion is limited. From 1985 to 1993, 24 patients were treated with local amputation and ILP using melphalan and mild hyperthermia. The overall 2 year and 5 year survival was 77% and 46% respectively. Sex and site did not influence survival. No difference in survival could be demonstrated between this group of 24 patients and a group of 111 patients with subungual melanoma treated without ILP from the Scottish Melanoma Group. Subungual malignant melanoma is known to carry a poor prognosis. No added benefit was seen by using ILP. The routine use of ILP for patients with subungual melanoma is thus not recommended.

The results from the Glasgow unit suggest that the use of adjuvant ILP does not appear to confer survival benefits. The exact reason for this remains unclear. It is possible that the patients were either staged incorrectly or the "type" of melanoma seen in this part of the world is different from that seen elsewhere. Before defining the role adjuvant ILP several questions needed to be answered. Firstly, could the ILP circuit be improved further to maximise the efficacy of the chemotherapeutic agent? Secondly, could prognostic factors for survival be identified in patients with primary melanoma?

Having reviewed the results of ILP in Glasgow, experiments were designed to answer the question "How can the current management of limb malignant melanoma be improved?"

Isolated limb perfusion has been available for over 30 years. The main advantage of this treatment is that high doses of chemotherapeutic agents can be delivered locally without producing generalised toxicity. However since it's introduction in 1957, few changes have been made to the operation of the circuit. There are certain facts relating to the ILP circuit.

Firstly, the ILP circuit operates in a continuous blood flow mode whilst blood flow in normal blood vessels is pulsatile in nature. Secondly the ILP circuit is a confined space in which heated blood is circulated thus predisposing to the development of compartment syndrome. This has led to the practise by several investigators of performing routine prophylactic fasciotomy after ILP. Lastly, several studies have shown that the effects of chemotherapeutic agents can be enhanced by increasing the tissue oxygen tension.

With the above facts in mind, several studies were designed and carried out to investigate if the ILP circuit could be improved. Twenty-two patients undergoing ILP were recruited into the first study to assess the physiological advantages of introducing pulsatility into the ILP circuit. Pulsatility was introduced by using a Stockert Frequency Control Module applied externally to the circuit and synchronised to the patient's electrocardiogram. Twenty-two patients matched for sex, age and site undergoing ILP in the standard continuous flow ILP circuit were used as controls.

Using pulsatile blood flow, the time taken to reach physiological oxygen saturation was shorter and there was less fluctuation in levels of oxygen tension. There was also less fluid shift in the pulsatile circuit compared to the non pulsatile circuit. However, there was no significant difference in the maximum oxygen levels achieved between the two groups. Introduction of pulsatility is advantageous as it produces a more stable circuit and thus better oxygenation.

The next study examined how the compartment pressures in the limbs were affected by ILP and to assess if there was a difference when the circuit was operated in the pulsatile and non pulsatile mode. Eight patients had pulsatile ILP and 8 had non pulsatile ILP. Pressures were measured using the wick technique.

Results of this study showed that with time there was a rise in the compartment pressure and the rate of rise in the non pulsatile group was significantly greater than in the pulsatile group. The lower rate of rise in compartment pressures in the pulsatile group is less likely to predispose this group to the compartment syndrome. Since the introduction of pulsatility into the ILP, neither routine prophylactic fasciotomy nor therapeutic fasciotomy has been needed to be performed.

The third study was designed to assess the use of vasoactive drugs in patients in whom satisfactory tissue oxygenation could not be achieved despite high flow rates. In theory, by reducing the peripheral arterial resistance using a vasodilator agent, the flow rate could be increased further with a resultant rise in the tissue oxygenation. The drug chosen was verapamil hydrochloride. Twenty-one patients undergoing ILP were recruited, with 16 undergoing non pulsatile and 5 undergoing pulsatile perfusion. After adminstration of the drug, a significant fall in the peripheral pressure and oxygen tension were seen. Although this fall was not sustained, there was no increase in the local oxygen. The effects were similar in both the pulsatile and the non pulsatile blood flow modes. Thus verapamil hydrochloride could not be used to improve local oxygen in the perfused tissues.

From these studies it was clearly seen that the ILP circuit could be improved, optimising the tumouricidal action of the chemotherapeutic agent. The ILP circuit in the Glasgow unit is currently being operated in the pulsatile blood flow mode.

ILP is the treatment of choice in this unit for the management of recurrent limb malignant melanoma. However, in some patients ILP is not possible or fails to control the disease. The cutaneous metastases from melanoma, if left untreated, will grow, ulcerate and become painful. Other treatment options such as radiotherapy, immunotherapy and cryotherapy can be used to control the disease and are largely ineffective. These modes of treatment are also not without significant morbidity. A study using carbon dioxide laser to ablate these cutaneous lesions was carried out.

Results of this study showed that carbon dioxide laser is an effective alternative to ILP when ILP has failed or is not possible. It has the advantage over ILP in that a series of repeat treatments can be administered with little or no significant morbidity unlike ILP which is associated with moderate morbidity. The drawback of this treatment is that all underlying tumour cells are not being treated. This treatment is ideal in patients with terminal disease and allows control of the local disease without subjecting the patient to major surgery and at the same time maintains the quality of the patient's life. There may also be a small subgroup of patients who may benefit from carbon dioxide laser treatment as the first line of management.

As seen earlier, there were no prognostic factors predicting survival in the patients with primary melanoma and survival benefits were not demonstrated in these patients following adjuvant ILP. The answer may lie in the fact that these patients were not properly staged in the first instance. It is known that 20%-40% of patients with stage I melanoma harbour clinically undetectable micrometastases in the regional lymph nodes. Since none of these patients had lymph node sampling, a significant number of patients may thus be harbouring metastases in the regional lymph nodes. If this is so, then the staging and prognosis will be affected. If the role of adjuvant ILP therapy is to be defined, then proper staging is necessary.

Intraoperative lymphatic mapping may be such a technique that will allows selective sampling of the sentinel lymph node for metastases. This technique has been developed on the assumption that metastases embolise via the lymphatic channels to the regional lymph nodes. The technique is based on identifying the first or sentinel lymph node and examining it for micrometastases. The sentinel node is the draining lymph node nearest the primary melanoma.

By using patent blue dye which is injected around the site of the primary melanoma, the regional lymphatic basin is explored and the blue lymphatic channels are followed to the first stained blue lymph node. This technique was performed in 25 patients with stage I disease and sentinel nodes were identified in all patients. Eight nodes were found to contain micrometastases. All these patients underwent adjuvant ILP. In those with negative sentinel nodes, there has been no recurrence or distant metastases. However in the patients with positive sentinel node analysis, 2 developed recurrences and 1 developed distant metastases and died of the disease at a mean follow up of 18 months.

The results from this study suggest, that if these 8 patients who were originally staged as being stage I are removed from this group, adjuvant ILP may be shown to improve the disease free interval and overall survival in the true stage I patient. It is now the practise in this unit to perform lymphatic mapping on all patients to try and select those patients with microscopic nodal disease, for whom nodal clearance may be more appropriate than ILP.

It was seen that patients with nodal metastases also had a higher risk of developing distant metastases. This is not suprising as once lymphatic invasion has occurred, it is only a matter of time that blood borne metastases will arise. This was clearly seen in the ulcerated tumour in the "stage I" patients with positive sentinel nodes.

If a cure or long term remission is to be found in these patients, the subclinical metastases in the blood stream need to be destroyed before they become established in distant organs. A treatment modality that may be able to destroy such metastases is targeted radiotherapy.

An animal model was designed to assess the possible use of targeted radiotherapy in patients with nodal metastases. Targeted radiotherapy is a treatment in which selective uptake of a radionuclide allows deposition of a high radiation dose in the targeted tissue with little exposure of surrounding structures. Methylene blue has been shown to have a high affinity for melanin. By adding labelled radioactive iodine to methylene blue, melanoma cells which are rich in melanin might selectively take up the compound and be destroyed.

Radioisotopes of iodine were injected into 3 groups of nude mice with B16/F10 mouse melanoma. Group A (controls) received tumour cells subcutaneously, Group B received a subcutaneous mixture of tumour cells and radioactive labelled methylene blue and Group C received tumour cells subcutaneously and on macroscopic evidence of tumour growth received intravenous radioactive labelled methylene blue into the tail vein.

Parallel experiments were performed on B16/F10 melanoma cells grown as plate cultures. In both the animals and the cell cultures, the effects of different isotopes were compared. It was observed that the tumour growth was delayed in the treated groups and the effect was maximal using iodine 125. Targeted radiotherapy in an isolated circuit using iodine 125 labelled methylene may now be clinically possible. From the results of the above studies, targeted therapy for micrometastases is a realistic possibility.

In conclusion, how will the results obtained from these studies alter the current management of melanoma of the limb? Surgery should no longer be just confined to wide local excision of the primary melanoma or recurrent disease. Adjuvant and secondary adjuvant ILP are necessary if improvement in both disease free survival and overall survival is to be achieved.

In patients with high risk primary limb melanoma, a wide local excision and lymphatic mapping followed by ILP with melphalan is indicated. If the sentinel node is positive, the patient should undergo radical lymphadenectomy. In the small subgroup of patients with stage I subungual melanoma, intraoperative lymphatic mapping with radical lymphadenectomy if necessary should be performed. ILP with melphalan and TNF may be beneficial in patients with subungual melanoma.

In patients with stage II and IIIA limb melanoma ILP with melphalan should be offered at the first recurrence. The patients should also have intraoperative lymphatic mapping to identify the sentinel node for micrometastases. Patients with stage IIIAB should undergo ILP with melphalan and also therapeutic lymphadenectomy. However in the patients with failed ILP or when ILP is not possible, the patient should be treated with carbon dioxide laser to control the cutaneous recurrences with the treatment repeated as often as needed.

The introduction of the pulsatile blood flow mode in the perfusion circuit has improved ILP. With this mode of blood flow, no prophylactic or therapeutic fasciotomy has been performed following ILP. Pulsatility, thus not only improves the circuit, but also reduces the morbidity from the procedure.

Targeted radiotherapy using isotopes of iodine in an animal model has been shown to be effective in the treatment of melanoma. There have been reports of combination radiotherapy and chemotherapy producing improved control of tumour recurrences. Targeted radiotherapy could now be applied in the ILP circuit together with melphalan both in the adjuvant and therapeutic treatments. Targeted radiotherapy could also be administered intravenously to mop up the melanoma cells in the systemic circulation thereby reducing and delaying the development of metastases in distant organs. The other possibility is the use of intradermal targeted radiotherapy to treat local recurrences.

The role of surgery in the management of local and regional limb malignant melanoma is no longer confined to local excision or amputation. It has now been established that ILP offers improvement in survival and disease free survival in patient with limb malignant melanoma. The new challenge to the surgeon is now in improving prognosis in stage I disease. The answer may lie in the combined therapy of lymphatic mapping and targeting metastases in the limb using a combination of chemotherapy and targeted radiotherapy using the ILP circuit as the vehicle.

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