ASSESSMENT OF DELIVERY OF THERAPEUTIC AGENTS IN ISOLATED LIMB PERFUSION FOR MALIGNANT MELANOMA

A thesis submitted for the degree of M.D.

by

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to

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and

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DECLARATION

This thesis has been prepared and written entirely by myself. All aspects of the research described were performed by myself except for those areas where the help of colleagues has been specifically acknowledged. None of the contents of this thesis has been submitted in support of any application for another degree or qualification in this or any other university.

STATEMENT OF COLLABORATION

The studies presented in this thesis were performed by the author while working as the second Research Fellow in the Isolated Limb Perfusion team of Gartnavel General Hospital from 1988 to 1990, funded by a grant from the Cancer Research Campaign. During that time, he was personally involved in the treatment of 88 patients on 97 occasions by this approach. The patients included in the clinical studies described in this thesis were all treated within this period, with the author contributing directly to their management. In his assessment of the results of ILP in Glasgow, the author has included, in addition to those 97 cases, a further 65 cases treated prior to this. These patients have previously been discussed by Mr R.N. Scott, MD FRCS. Mr Scott's contribution to the assimilation of data concerning these patients and their treatment is gratefully acknowledged.

ACKNOWLEDGEMENTS

While engaged in research and in the preparation of this thesis, I have been fortunate to receive advice, encouragement and help from many friends and colleagues.

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In the course of my research, I have had to learn many new scientific methods and I have been fortunate in having good tutors. Dr Rob Mairs and Mr Steven Martin of the Department of Radiation Oncology at the CRC Beatson Laboratories taught me the techniques of cell culture with great clarity and patience. Discussions with Dr Mairs were always fruitful and I thank him for his lucid comments on many aspects of research. The melphalan HPLC assays were expertly performed, using a method which he developed and perfected himself, by Dr Robert Blackie, of the Department of Medical Oncology at the CRC Beatson Laboratories. Mr Colin Hughes, of the University Department of Surgery at the Western Infirmary, Glasgow, helped me to overcome my initial reticence in the care and handling of experimental animals. I also acknowledge the help of Mrs Jenny Stewart (Edinburgh) and Miss Evelyn Salt (Glasgow) of the Scottish Melanoma Group, who very

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SUMMARY

Advanced malignant melanoma is resistant to most forms of treatment. Even primary melanoma is often associated with a very poor prognosis. The incidence of the disease is rapidly increasing in all parts of the world from which data are available, including Scotland. Since its original description in 1957, Isolated Limb Perfusion (ILP) has become an established form of therapy for advanced or recurrent limb melanoma. Its use is associated with high response rates, but these are sometimes short-lived.

In Part A, an account of the epidemiology, aetiology and prognosis of malignant melanoma is presented, together with a summary of current standard melanoma treatment (Chapter I). The history of the development of the ILP technique is presented, including a review of published results.

The results of ILP in Glasgow are summarised in Chapter II. The technique has been associated with a low morbidity and no mortality. The results observed confirm the very high response rate of recurrent and advanced melanoma to therapeutic ILP. To date, only one small prospective randomized trial of adjuvant ILP for Stage I melanoma of the limbs has been reported. A case-control study of 63 patients treated by adjuvant ILP in Glasgow is presented. This reveals an overall and disease-free survival benefit for ILP patients of 20% and 15%

respectively at three years' follow-up. These differences are most apparent for patients with upper limb or poor prognosis (ie. $\geq$3.0mm thick) lesions.

Despite the fact that ILP has been available since 1957, many aspects of the technique vary from centre to centre so that, even in major reviews, the results quoted may not be consistent and are therefore difficult to compare. In particular, the control of the physiological parameters of extracorporeal circulation and the dosage schedule employed are highly variable.

Part B contains a description of clinical and laboratory studies performed by the author aimed at standardising and improving these aspects of ILP.

In Chapter III, the author presents a series of clinical studies performed to measure, for the first time, the effect of flow and pressure control on cutaneous perfusion in the isolated limb. These show that currently employed methods do not reliably ensure effective perfusion of skin despite an apparently stable circulation. Consequently, cytotoxic drug delivery to the skin may be suboptimal. The therapeutic outcome of the procedure thus becomes unpredictable. However, the studies reported here show that the extracorporeal perfusion parameters can be regulated by simple, non-invasive monitoring of the transcutaneous oxygen tension ($PtcO_2$) to ensure that the perfusion pressure in the isolated limb is sufficient to support an effective cutaneous circulation.

This approach should result in maximal exposure of the tissues of the isolated limb to the cytotoxic drug, although the adapted method does carry the theoretical risk of also increasing the leakage of cytotoxic drug from the isolated circuit to the systemic circulation. The effects of this modification of the perfusion technique have been quantified in a comparative study of melphalan pharmacokinetics, the "quality" of perfusion, uptake of melphalan by the skin, and leakage of melphalan to the systemic circulation in 38 ILPs. This reveals no direct correlation between the quality of perfusion and the concentration of melphalan measured in skin biopsies from individual patients. However, the skin melphalan concentration did correlate with the area under the curve (AUC) of melphalan concentration in the isolated circuit during ILP. Furthermore, this AUC was significantly higher in the group of patients whose ILP was controlled to ensure effective cutaneous perfusion even though there was no significant difference in the dose administered. As a result, the adapted perfusion technique was associated with increased skin melphalan concentrations. There was no evidence of increased leakage of melphalan to the systemic circulation using this technique.

In the final section of Chapter III, the effect of some commonly used vasodilators (which may lower the peripheral resistance in the limb sufficiently to allow effective perfusion at lower pressures) on the cytotoxicity of melphalan is tested in vitro against human melanoma multicellular tumour spheroids. Although verapamil and hydralazine have in the past been reported

to increase the cytotoxicity of several drugs (including melphalan) in vitro and in vivo, the experiments reported show no such effect against B0008 melanoma cells in vitro. More importantly, however, neither they nor papaverine were found to exert any adverse effect on melphalan cytotoxicity.

In Chapter IV, one of the other major sources of variation in the ILP technique - the choice of dosimetric schedule - is investigated. At present, three principal methods are employed, based on body weight, volume of perfused tissue and blood volume in the ILP circuit respectively. The simplest method to implement is that based on body weight. Measurement of total limb volume by immersion is an awkward technique. A study is therefore described from which a formula for the calculation of this volume based on only partial immersion of the limb has been derived. A comparison of the melphalan doses prescribed by each of these three schedules for 73 patients undergoing ILP shows that while the calculation of blood volume resulted in the prescription of consistently lower doses of melphalan, the doses prescribed by the two other methods were very similar. The simplicity of the method based on body weight therefore makes it the most appropriate.

ILP offers a means of localising treatment to the region of the body affected by the disease. The changes in the perfusion technique proposed in Chapter III may allow greater control of treatment by ensuring that the tissues most likely to harbour clinical and subclinical disease are effectively exposed to the cytotoxic agent. The

prospect of increasing the specificity of treatment still further by the introduction of anti-melanoma monoclonal antibodies into the ILP circuit to target melanoma cells selectively is presented for the first time in **Chapter V.**

In a study of 6 patients, successful accumulation of radiolabelled antibody at the sites of tumour deposits was demonstrated within the 60 minutes of ILP in 5 cases. This was associated with tumour:normal tissue uptake ratios of up to 3.5:1. Selection of a suitable radioisotope could therefore result in administration of more than 90Gy to tumour deposits without exceeding the radiation tolerance of the normal tissues of the limb.

This study also provides evidence of the value of $PtcO_2$ monitoring during ILP by revealing an association between cutaneous perfusion, successful targeting of tumour deposits by the radiolabelled antibody, and clinical response to melphalan.

Further studies into the targeted therapy of melanoma are required. To this end, an in vivo model has been developed by establishing human melanoma xenografts in the athymic nude rat. While this allows the study of melanoma targeting by systemically administered monoclonal antibodies, the technical problems posed by its small size make it an unsuitable model of ILP.

PART A

1 CHAPTER 1 - INTRODUCTION

1.1 The Melanocyte

Malignant melanoma is the tumour which results from the malignant transformation of melanocytes. Melanocytes are cells derived from the embryonic neural crest which migrate during development throughout the body (to skin, mucous membranes, nervous system, uveal tract) and which are responsible for the production of melanins, the pigments which give the skin its colour.

The development of the melanocyte starts with the migration of "**melanoblasts**" from the embryonic neural crest[1]; these then differentiate to melanocytes. Within melanocytes, characteristic organelles are formed, called "melanosomes", which assemble the various components for melanin synthesis. These include structural proteins formed in the Golgi apparatus (GA) and in the smooth endoplasmic reticulum (SER) and the enzyme **tyrosinase** which is believed to be synthesized on membrane-bound ribosomes and which is crucial to melanin production. The exact mode of incorporation of these two main components into melanosomes is not entirely clear and at least four theories have been advanced by various investigators[2-11]. Whatever the true mechanism, the resultant intracytoplasmic organelle is the site of the melanocyte's unique biochemical function - the synthesis of melanin. The enzyme tyrosinase is central to this biochemical pathway which involves the oxidation of tyrosine to dihydroxyphenylalanine (DOPA) and then to

dopaquinone (both of these steps being catalysed by the enzyme tyrosinase) and the polymerisation of the products of subsequent reactions to form melanin.

The cutaneous melanins fall into two main categories: the "**eumelanins**" which have a brown or black colour, and the "**phaeomelanins**" which are yellow to red in colour. Both of these are synthesized by biochemical pathways dependent on the enzyme tyrosinase.

Once melanised, the melanosomes are then transferred along the dendritic processes of the melanocytes to adjacent keratinocytes. This discharge of melanosomes from melanocytes is characteristic of epidermal melanocytes which have been described as "secretory" melanocytes to distinguish them from most melanocytes found at extracutaneous sites and in the dermis which are termed "continent" to describe their retention of synthesized melanin[12].

Epidermal melanocytes are large cells which rest on the basement membrane and, through multiple dendritic processes, contact in the region of 30 or 40 keratinocytes in the Malpighian layer of the epidermis (Figure 1). Melanosomes and melanin are distributed throughout the epidermis following their transfer to neighbouring keratinocytes by the upward movement of these cells, and human skin pigmentation is the result of this process. The relative proportions of eumelanins and phaeomelanins produced within the melanosomes are also important in determining the actual colour of the skin.

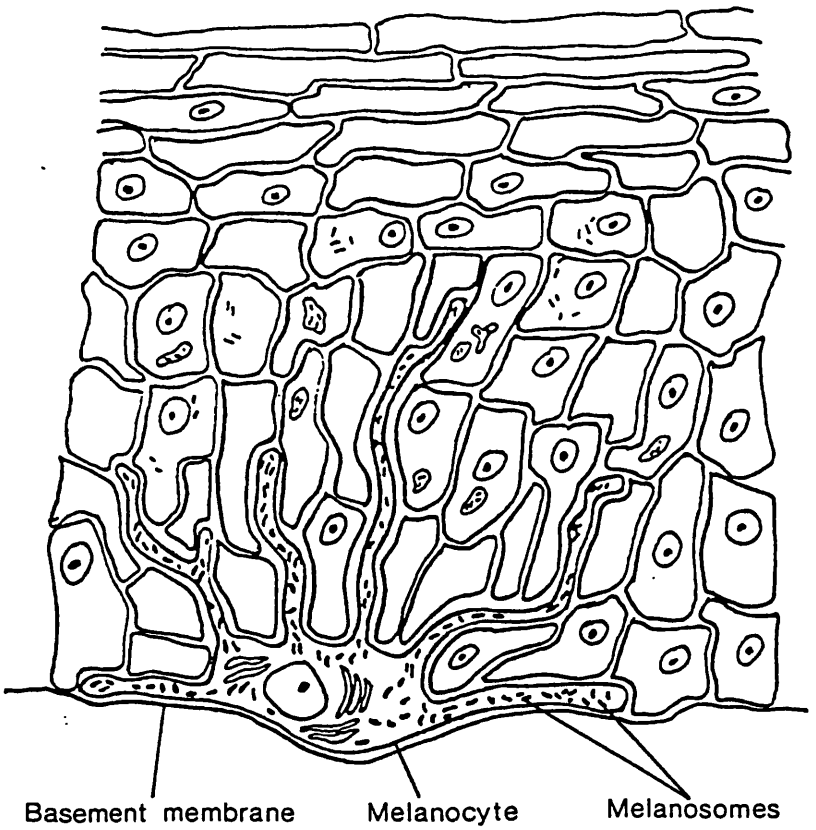


Figure 1 :
Diagrammatic representation of the "Epidermal Melanin Unit".

The large basal melanocyte rests on the basement membrane and contacts in the region of 30-40 keratinocytes in the malpighian layer through its dendritic processes. Melanosomes can be seen migrating along the dendritic processes. Some have already been transferred to neighbouring keratinocytes where they can be seen lying free in the cytoplasm or within membrane-bound vesicles.

It is interesting that while the different degrees of skin pigmentation are related to the number, size, type, and distribution of melanosomes, they are not related to differences in the numbers of melanocytes present. In negroid melanocytes, for example, melanosomes tend to be larger, more mature, and distributed in the dendritic processes, whereas in caucasoid skin, they tend to be smaller, less mature, and concentrated in the perikaryon. Although there are marked variations from one region of the body to another, the density of melanocytes or "epidermal melanin units" (the collective term employed to describe a melanocyte and its related keratinocytes) in the various regions shows remarkable similarity in all racial groups[13].

Genetic factors govern the constitutive skin colour of the individual, but environmental and hormonal influences can produce reversible changes of pigmentation by stimulating the melanocytes to produce increased numbers of more mature melanosomes which then become more peripherally distributed in the dendritic processes and are transferred to keratinocytes in greater numbers. Ultraviolet radiation (UVR) is the main stimulus to those changes which give rise to the tanning of skin seen after sun-exposure. Increased pigmentation can also be seen in certain conditions associated with alterations in hormone balance. Although their role in normal pigmentation has not been established, the pituitary hormones alpha-melanocyte-stimulating-hormone (alpha-MSH), beta-MSH, and adrenocorticotrophic hormone (ACTH) can all cause increased pigmentation. This is probably best

demonstrated in Addison's disease in which adrenocortical insufficiency is associated with increased production of ACTH and alpha-MSH by the pituitary gland, resulting in the well-reported hyperpigmentation typical of this disease. This feature is also seen in patients who have undergone bilateral adrenalectomy for Cushing's syndrome. Pregnancy is often associated with a particular pattern of increased pigmentation known as melasma. Although the exact aetiology of melasma has not been defined, progesterone, oestrogen and alpha-MSH are all thought to contribute to its development.

The function of melanin in the skin seems to be a protective one, for while white skin reflects more visible light than does dark skin, it in fact transmits more light in the ultraviolet range than dark skin. The melanin in dark skin is believed to act as a filter of ultraviolet light and to reduce the penetration of UVR by scattering the rays; it also absorbs light in the visible and ultraviolet range and dissipates its energy as heat[14]. Furthermore melanin can react with other intracellular substances such as reactive free radicals to neutralize the accumulation of potentially harmful molecules such as pyrimidine dimers, thereby reducing the damage to DNA caused by these molecules.

The protective effect of melanin is convincingly demonstrated by the great increase in solar changes such as keratoses, premalignant lesions and skin cancers (melanoma and non-melanoma) observed among albino negroes

compared with their normally pigmented counterparts living in the same environment.

1.2 Malignant Melanoma

1.2.1 Epidemiology and Aetiology

Melanocytes may appear in increased numbers in localised areas of skin where they form visible lesions known collectively as melanocytic naevi. Variations in the gross appearance of these lesions together with their histological characteristics based on the grouping of the melanocytes, their location in the skin (epidermis, dermis, or both), and the presence or absence of proliferative activity of the melanocytes, result in a great diversity of benign naevi.

Cutaneous malignant melanoma results from the malignant transformation of melanocytes either within one of these naevi or "de novo". Although this tumour causes 85% of deaths from skin cancer[15], it only accounts for 1-3% of all human cancers[16]. It has however attracted the attention of physicians to a much greater extent than this proportion would seem to justify. Several features of cutaneous malignant melanoma have contributed to the apparently inordinate interest which it generates.

[i] Incidence of melanoma

The incidence of melanoma is increasing in all parts of the world from which data are available[17,18]. In fact melanoma is one of the cancers showing the most rapid increase[19]. The highest incidence of the disease is seen

in Queensland, Australia where the age-standardised incidence is 39.6 per 100,000 population per year and is doubling every 15 years[20,21]. Other authors report a doubling incidence in Scandinavia[22-24] and a quadrupling incidence in Arizona and New Mexico[25,26] over a similar time scale. Data available from the British Isles show similar increases both in Scotland[27-30] and in England and Wales[31]. In particular, the incidence in Scotland has practically doubled between 1979 and 1989, from 3.4 to 7.1 per 100,000 for males and from 6.6 to 10.4 per 100,000 for females[30].

The consistency of this trend in all reports and the claim that it cannot be accounted for simply by better diagnosis of the cause of death suggest that this reflects a true increase in the incidence of the disease[18,32-35].

[ii] Age-incidence of melanoma

Melanoma appears to affect a younger section of the population than most other solid tumours. The peak age incidence of melanoma occurs in the fifth decade[16] and this naturally has serious implications in terms of morbidity and mortality for an active and productive section of the community.

[iii] Site and distribution

By its very presence on the skin, cutaneous malignant melanoma causes cosmetic defects and it is often this aspect of the disease which brings it to the patient's attention.

The relationship of melanoma to pigmentation and to pre-existing pigmented lesions is also interesting. While one might expect the distribution of melanomas on the body surface to resemble that of melanocytes or benign naevi, this in fact seems not to be the case. Neither are melanomas evenly distributed over the surface area of the body. In particular, the incidence of melanoma on the head and neck is proportionally higher than all three of these parameters[36]. Studies of benign naevi and melanomas suggest that the distribution of the benign lesions may be more closely related to melanocyte density in the various regions of the body[37], although not all studies are in agreement on this point[38].

The majority of melanomas occur in fair-skinned individuals and the incidence of melanoma seems to decrease with increasing pigmentation[19,36]. This can be clearly seen in studies of populations of mixed racial groups such as that in New Mexico where those of "anglo" descent have a much higher risk of melanoma than the hispanic, american indian and black members of the same community. In fact, the incidence among the anglos may be as much as six times that among the hispanics[26] and three times that among american indians[39].

The proportion of melanomas which arise in pre-existing naevi is not clear. Histologically, 20-40% may be shown to be associated with naevi[40-42]. This association is particularly strong with congenital or long-standing naevi[43], one report suggesting that the figure could be as high as 85% [44]. The importance of the association between melanoma and pre-existing naevi is not clear and in many cases may simply reflect the larger number of melanocytes which are at risk of undergoing malignant transformation within any of these lesions[45,46].

[iv] Dysplastic naevi

One particular type of naevus has been reported to be associated with an increased risk of melanoma - the dysplastic naevus. This association was initially suggested with reference to the numerous atypical benign naevi seen in some cases of familial melanoma[47]. The association was named the "B-K mole syndrome" after two young patients who had greatly assisted the investigating group[48]. It was later renamed the "**dysplastic naevus syndrome**"[49]. This was characterised by familial melanoma, often with multiple primaries; familial occurrence of large irregular moles on exposed and covered skin; histological evidence of melanocytic dysplasia and atypical patterns of growth. However in 1980 identical "dysplastic naevi" were recognised in sporadic cases of malignant melanoma also and were regarded as high risk factors for melanoma[50]. The term dysplastic naevus

syndrome is now used to describe families in which two or more members are found to have multiple dysplastic naevi. There may be an autosomal dominant pattern of inheritance in some families.

Dysplastic naevi are typically slightly raised lesions, 5-12mm in diameter with an irregular outline which itself is often not raised. The colour of these lesions can vary and may include pink areas, and it can be very difficult to differentiate them clinically from the superficial spreading form of melanoma, although the skin markings are usually preserved. Dysplatic naevi may be single or multiple, some patients bearing more than 100 such naevi. They start to appear in adolescence and continue to develop in adult life.

The risk of developing melanoma in patients with dysplastic naevi is difficult to ascertain, but seems to be highest in patients with the dysplastic naevus syndrome and with a strong family history of melanoma[51-53]. While some authors describe the progression from dysplastic naevus to malignant melanoma, others claim that most melanomas in patients with the dysplastic naevus syndrome develop on previously normal skin[54]. They suggest that the dysplastic naevus syndrome is a risk factor but that the dysplastic naevi are not themselves the precursors of melanoma.

Another type of naevus which is associated with an increased risk of malignant transformation is the **congenital melanocytic naevus**, in particular the giant or "garment" congenital naevus[53,55]. This form is associated

with a significant risk of malignant change, estimated to be between 4% and 10% [56-58], and it is generally recommended that such a naevus be surgically excised.

In recent years, evidence has accrued that quite independently of these specific instances, a large total number of benign melanocytic naevi is a strong personal risk factor for malignant melanoma[53,59]. Clinically atypical naevi - large diameter (>7mm), varied colouration, irregular margin - even when these do not show the histological features of dysplastic naevi, are also thought to be risk factors, albeit not as strong as the total naevus count[53,59]. This is not supported by the findings of one clinical study which examined the association of large numbers of benign melanocytic naevi with increased melanoma risk[60], although the statistical value of the study was limited by the relatively small number of patients. The combination of the number and size of benign naevi expressed as the total naevus density (TND) has been suggested as a measure of melanoma risk and is particularly high in families with the dysplastic naevus syndrome or familial melanoma[61].

[v] Hereditary and genetic factors

It has long been recognised that malignant melanoma can exhibit a familial distribution. Indeed, the first report of cutaneous malignant melanoma in the english literature is believed to a case report by Norris in 1820 which

clearly describes a case of familial melanoma[62]. Like many other hereditary malignancies, familial melanoma tends to affect rather younger patients and is more frequently associated with multiple primaries[63]. It is also associated with the premalignant "dysplastic naevus syndrome" in a significant proportion of cases. Reviews of several groups of patients with familial melanoma have shown a more even distribution of the primary lesions over the body surface and thus a slightly higher proportion on the poorer prognosis trunk area than in sporadic cases[63,64]. There may be a tendency for more lesions to be of the superficial spreading type and for the diagnosis to be made at an earlier stage[63]. The overall effect of these features is that hereditary melanoma is usually associated with a better prognosis than the sporadic form of the disease[65]. The mode of inheritance of hereditary melanoma is not yet clear. The dysplastic naevus syndrome shows some features of autosomal dominant transmission with incomplete penetrance, and while hereditary melanoma also shows some of these features, its mode of inheritance is likely to be multifactorial[63,65].

Several cases of transplacental transmission of melanoma from mother to foetus have been reported though these are very rare[66,67].

In recent years, a growing interest has been generated in the field of human genetics by the reports of alterations in one region of the short arm of chromosome 9 in a number of human malignancies including melanoma[68-71]. It has

been suggested that deletion of a gene or genes on chromosome 9p may represent an initial step in the malignant transformation of melanocytes[72,73].

[vi] Aetiology of malignant melanoma

Ultraviolet radiation (UVR)

Several factors have been implicated in the aetiology of the disease, the main one being solar ultraviolet radiation. Melanin is an effective barrier to UVR, and the body's natural defensive mechanism against sunlight is the production of mature melanosomes by the melanocytes and their transfer to the surrounding keratinocytes - tanning.

Deficient protection against the harmful effects of the sun's rays and/or excessive exposure to the sun are the most readily identifiable predisposing factors for malignant melanoma.

White skin is more commonly affected by the disease than the other types and this particularly affects fair-skinned or red-haired people, especially when they live in very sunny climates. Among these people, those who burn rather than tan or who develop large numbers of freckles when exposed to the sun are at highest risk[74,75].

Conversely, malignant melanoma is rare in negroes although exact incidence rates are difficult to ascertain. In addition, the anatomical distribution of melanomas among negroes is different, and in particular the

proportion of melanomas occurring on the relatively unpigmented plantar skin is much higher among negroes in both Africa and North America than among other ethnic groups[76-82].

In general, the incidence of melanoma among white-skinned people increases as one approaches the equator[18,19,33-35]. This effect is slightly masked by the variation in indigenous skin types according to geographical location so that in Europe there is a higher incidence of melanoma in Scandinavia than in the mediterranean region where skin type tends to be somewhat darker[83]. Similarly, while Denmark lies 15-20^{o} further north of the equator than Connecticut, the incidence of melanoma in both regions was practically identical around 1940 and again in the early 1970's[84]. The incidence in both regions had increased five-fold over this period.

In other areas, migrant populations can further accentuate the geographical difference. In Queensland, Australia, the very high incidence of melanoma is believed to be partly due to the large number of immigrants from Scotland and Ireland whose "celtic" skin is particularly fair and who are therefore at increased risk[85].

In addition, geographical latitude is not an independent determinant of sun exposure. Climatic conditions, altitude, and coastal location are also very important factors[21,25,26]. Within Queensland, cloud cover in the North reduces the sun exposure in this part; in addition, the reflectivity of sand for ultraviolet radiation is higher than that of water or grass (12% v 4% and 2%

respectively) so that coastal sunbathing results in higher exposure to UVR than, for instance, inland poolside sunbathing[21].

The anatomical distribution largely reflects the importance of sun exposure in the aetiology of melanoma. Over this century, fashions of dress have changed markedly to the extent that while revealing the skin of the arm might even have been considered shocking 50 years ago, this is now an acceptable feature of everyday dress in western society. In the same period, there has been a marked increase in malignant melanoma of the arm in particular[33] and a shift of distribution of melanoma by anatomical site in general towards the more heavily exposed body sites[32,86]. Sex differences in the disease also seem to follow the differences in dress pattern, a greater proportion of melanomas occurring on the leg in women and on the trunk in men[30,33]. This observation remains valid even after taking account of variations in the number of melanocytes or epidermal melanin units according to anatomical site[87].

The intensity of exposure to UVR is also important in the aetiology of melanoma and a positive correlation has been demonstrated between severe sunburn and the subsequent risk of developing melanoma on the sunburnt skin[88]. This is in contrast to the non-melanoma skin cancers which seem to be associated more with cumulative chronic exposure to UVR[17]. As a result, melanoma patients tend to be rather younger, to come from higher socio-economic groups than

patients suffering from non-melanoma skin camcer, and to have indoor occupations, their main exposure to sun taking the form of intense recreational sunbathing during short holidays[17,89].

Ultraviolet radiation occupies the wavelengths between 200nm and 400nm in the spectrum of light. The longer wavelengths in this range (320-400nm) are termed UV-A and are thought to be less potent inducers of actinic damage to cells in the skin. However they are believed to enhance the injurious effects of the UV-B rays (wavelengths 290-320nm)[17] which are mainly implicated in the aetiology of actinic skin lesions including melanoma and non-melanoma skin cancers.

The mechanism by which UVR induces these changes in the skin is thought to be the intracellular production of highly reactive free radicals which in turn produce pyrimidine dimers of DNA and lead to miscoding. This is supported by the observation that patients suffering from xeroderma pigmentosum have a much higher risk of cutaneous malignancy including melanoma than the general population[90] and indeed tend to die in the second or third decade of life from one of these cancers. This disease is very rare and is transmitted by autosomal recessive mode. The metabolic defect which characterises the disease is an inability to repair UVR-induced DNA damage by removing the pyrimidine dimers of DNA[91] and it has been suggested that this allows DNA coding errors to occur resulting in malignant change.

Our exposure to UV-B is substantially reduced by the layer of ozone (O_3) in the Earth's stratosphere which acts as a filter of the shorter UV-B wavelengths. The longer UV-B rays are also filtered though incompletely by ozone. It is therefore of major concern that this protective ozone layer has been and continues to be depleted by the release of chlorofluorocarbons (CFC's) into the atmosphere. These substances, which have a very long half-life in the atmosphere, are used as propellants in many aerosols and are also widely used for refrigeration. They release chlorine gas which destroys ozone and therefore results in greater penetration of UV-B to the Earth's surface[92,93].

The remainder of the ultraviolet radiation band (wavelengths 200-280nm) is termed the UV-C band and is totally absorbed by ozone so that no light in this range reaches the Earth. This block will continue to be effective even in conditions of extreme ozone-depletion and UV-C therefore presents very little threat.

The practice of sunbathing has become increasingly popular among caucasians in recent decades and this must result in a greater exposure to ultraviolet light including UV-B. An alternative method of obtaining a "cosmetic" tan which is gaining in popularity is the use of sunbeds. These mainly emit light in the UV-A band, but they may also deliver UV-B at rates similar to those of bright sunlight[94]. Initially, the only evidence of a causal relationship between the use of UV sunlamps and subsequent development of melanoma was in anecdotal case

reports[17,95]. However, recent case-control studies have demonstrated conclusively that the use of sunbeds and sunlamps is associated with a significantly increased risk of the disease[94,96]. This practice should therefore be discouraged.

Trauma

Skin trauma has been suggested as another possible factor in the aetiology of melanoma[86] and the high incidence of melanoma on the plantar surface of the foot in Asians and Africans accustomed to walking bare-footed was believed to support this theory. In fact the incidence of melanoma on the sole of the foot seems to be disproportionately high for such a small fraction of the total body surface in all racial groups[32] and this may reflect the chronic exposure of this skin to the trauma of weightbearing. Nevertheless, the exact role of trauma in the aetiology of melanoma is not clear and may, in many cases, simply be a mechanism for presentation of a pre-existing but unrecognised melanoma.

Immunological considerations

Patients with a primary malignant melanoma are known to be at a greater risk of developing another primary melanoma[97,98], suggesting that a transformation of the melanocyte system in general may be a primary step in the aetiology of the disease, possibly in response to some environmental or circulating carcinogenic substance[97]. However there is also an association between malignant melanoma and certain other malignancies such as breast

cancer and chronic lymphatic leukaemia[98,99] and an immunological mechanism could therefore be the common link in these diseases. This is further suggested by the observation that renal transplant recipients, who are medically immunosuppressed, have an abnormally high risk of both melanoma and non-melanoma skin cancer[100,101]. There have also been studies showing that exposure of animals to ultraviolet-B radiation and of humans to artificial solaria can result in changes in the immune system[102,103].

Hormonal influence

It has frequently been claimed that the endocrine system exerted some influence on the development and progression of melanomas, but this has mainly been based on confusing anecdotal reports of disease progression during pregnancy. In one group of patients, changes in size, shape and colour of lesions subsequently diagnosed as melanomas were first noted during pregnancy and seemed to be associated with a poorer prognosis[104]. Some authors have also reported cases of spontaneous regression of melanomas after parturition.

There seems little question that some melanocytes are sensitive to gonadal hormones since, in general, the number, size and pigmentation of melanocytic naevi increases at puberty and during pregnancy[37,38]. Receptors for oestrogens and other steroid hormones have been identified on naevus cells and melanoma cells[105-107] but the true significance of this is not clear.

A recent study showed that pregnancy itself, with its inherent hormonal changes, did not affect the prognosis of primary melanoma, although the melanomas diagnosed during pregnancy tended to be thicker and therefore of poorer outlook than those diagnosed outwith pregnancy[108].

[vii] Spontaneous regression of melanoma

Spontaneous regression is defined as the partial or complete disappearance of a tumour in the absence of any treatment or in the presence of therapy considered inadequate to significantly alter the natural course of the malignancy[109]. This has frequently been reported in relation to metastatic malignant melanoma; in fact, only hypernephroma and neuroblastoma exhibit this phenomenon more often than melanoma[109]. In order to maintain the accuracy of their observations, studies of tumour regression tend to concentrate on regression of metastases from histologically documented primaries[110]. In the case of melanoma, however, regression is probably more often a feature of primary lesions than of metastatic deposits. In one study of 361 cases of primary melanoma, 57 specimens (16%) showed histological evidence of regression[111]. By contrast, reviews of the reports of spontaneous regression of metastatic melanoma reveal a prevalence of only 0.2-0.3% of cases[110,112].

The mechanism leading to spontaneous regression of melanomas is as yet unclear but two principal theories are advanced, the first being that the regression may be a

response to hormonal influences. Support for this comes from the knowledge that other cancers can be responsive to hormonal manipulation (eg. breast, thyroid, prostate), that women tend to have a better prognosis for melanoma than men, that the disease is rare before puberty, and from several reports of changes occurring in melanomas during and after pregnancy including complete regression. Attempts to treat metastatic melanoma by hormonal manipulations have however not shown any benefit from hypophysectomy, orchidectomy, adrenalectomy, or androgen or oestrogen therapy[113,114]. The second theory attributes regression to an immunological process and is derived mainly from observations that as many as 60% of cases of spontaneous regression may be associated with a potential "immune-stimulating event" such as incomplete excision, infection, the use of "reticuloendothelial stimulants", or inadequate radiotherapy[110,115-117]. There is also evidence of a significant increase in cytotoxic lymphocyte activity during regression[118], and the histological appearances of spontaneous regression in melanoma sections closely resemble those of BCG-induced regression of metastatic melanoma deposits[119]. There is usually an intense lymphocytic inflammatory infiltrate and melanin-laden macrophages within the dermal layer[119,120]. These features are associated with an absence of melanoma cells in the basal layer of the epidermis, and in fact active phagocytosis of melanoma cells has been demonstrated in one report[121]. The naked-eye appearance of regressed melanoma is variable but usually takes the form of a depigmented area of skin which corresponds to

the size and shape of the previous melanoma[120].

While prognosis after spontaneous regression of metastatic melanoma is difficult to establish due to incomplete data presentation in the various reports, Bodurtha's review shows a 5-year survival of 66%, which is in excess of the expected survival rate for other patients with recurrent melanoma[110]. In the case of primary lesions, spontaneous regression does not seem to be associated with an improved prognosis, but it is probable that some patients in whom regression takes place before the disease has metastasised never seek a medical opinion. Consequently this feature of the natural history of melanoma is probably underdiagnosed.

Melanoma can also present as metastatic disease without evidence of a primary source being found, even after a very thorough search. This is estimated to be the case in 3-9% of diagnoses of melanoma[112,122-125] and of these 45-60% have lymph node metastases[126-128]. This is usually taken to be the result of metastasis from a primary lesion which has already undergone spontaneous regression[129], although in some cases the primary lesion may be undetected because of its benign appearance or its obscure location[130]. It has also been suggested in the case of lymph node deposits that these may have arisen from naevus cells occasionally found within lymph nodes[36,131-133].

The prognosis in these cases of occult primary melanoma with metastases seems to depend upon several factors such as whether the metastases are nodal or extranodal (the

latter carrying a poorer prognosis), the number and anatomical site of lymph nodes involved (multiple or cervical nodes being associated with a poorer outlook), and sex (females having a better prognosis than males)[120]. In general, it is recommended that these patients should be treated in the same way as patients with known or previously treated primary melanomas and that in the case of lymph node metastases in the groin or axilla this should include a formal lymph node clearance[120,134].

1.2.2 Classification by Histological Type

Initially, only three types of melanoma were described :

- superficial spreading melanoma
- nodular melanoma
- lentigo maligna melanoma

These were described by Clark in 1967 [135], and a fourth category of melanoma was added ten years later - acral (or plantar) lentiginous melanoma[136]. This classification of melanoma is based on both the histological and the clinical features of the disease.

Superficial spreding melanoma is characterised by radial, intraepidermal growth of the lesion - a feature which can proceed for several years. As a result, lesions of this type can become very large before the patients take any notice of them. During this time, tumour is confined to the epidermis and the papillary dermis and although this

is undoubtedly invasive tumour, it shows very little tendency to metastasise[137]. After this variable period of radial growth, the melanoma undergoes a transformation and begins to invade more deeply into the dermis. This is termed the "vertical growth phase" and is associated with the tendency of the disease to metastasise[138].

Typically, superficial spreading melanoma initially appears as a large (>1cm diameter), rather flat lesion, irregular in outline and in shade. While the main colour is usually brown or black, the lesion may have a pink or even blue appearance[36,139]. As the vertical growth phase develops, the lesion becomes thicker and may adopt a "nodular" appearance. Ulceration and crusting may also become apparent.

This type of melanoma is the most common and accounts for up to 70% of all melanomas[30,138]. Superficial spreading melanoma shows an approximately equal sex distribution and the mean age at diagnosis is 45.

In contrast, <u>nodular melanoma</u> does not exhibit a radial growth phase but shows vertical growth from the outset. As a result, the melanoma has usually invaded deeply into the dermis by the time of presentation and possesses a much greater potential for metastasis[140]. The histological changes in the skin associated with solar damage are not pronounced in this type of melanoma.

Nodular melanoma usually appears as a small raised nodule which may or may not be pigmented and can ulcerate.

This type accounts for 12% of all melanomas[138] but 20% of those in Scotland[29,30]. Men are affected about twice as often as women, and nodular melanomas seem to occur most commonly on the head and neck or on the back[138]. The mean age at diagnosis is 50.

Lentigo maligna melanoma is the name given to the melanoma which develops in a "Hutchinson's melanotic freckle" [141,142]. This benign lesion, which is also known as lentigo maligna, occurs commonly in elderly people and presents as an enlarging flat brown lesion on sun-exposed skin, usually showing areas of varying depth of pigmentation. This lesion is thought to result from chronic sun exposure and is premalignant. When lentigo maligna melanoma does develop in a lentigo maligna, it is characterised by a very long radial growth phase of 10-25 years before vertical invasion of the dermis occurs. These melanomas can grow to more than 5cm in diameter.

This type of melanoma is thought to account for about 10% of all melanomas and affects women twice as often as men. The mean age at diagnosis is 70[138].

Acral lentiginous melanoma occurs on the sole of the foot and palm of the hand or in a subungual location. This type of melanoma also tends to have a fairly prolonged radial growth phase, intermediate between superficial spreading melanoma and lentigo maligna melanoma. The lesions can exhibit variations in pigmentation and can grow to a large size. When vertical growth ensues, this can progress very quickly to metastatic disease. These

lesions are very similar to lentigo maligna melanoma and were in fact reported as such before being classed separately[143]. Two thirds of acral lentiginous melanomas occur on the volar surfaces of the foot and hand while the remaining third are subungual.

They present as irregular pigmented patches on the sole or palm, initially with preservation of the overlying skin markings. When they occur subungually, pigmentation is seen in the nailbed often with a "halo" of pigmentation spreading onto the eponychium.

This type of melanoma accounts for 4-9% of all melanomas[29,30,36].

1.2.3 Spread of Melanoma

The spread of melanoma is in many ways similar to that of most other solid tumours. Local invasion by the primary lesion, lymphatic spread, and haematogenous spread are all common features of the progression of the disease. The progression of melanoma, however, can also display features rather particular to this disease.

One of these is termed in-transit metastasis and results from the arrested progression of metastasising melanoma cells within lymphatic channels. This therefore usually presents as palpable subcutaneous tumour nodules along the path of normal lymphatic drainage of the primary tumour-bearing skin. This is a particularly common feature of extremity melanomas due to the relatively uniform pattern of lymphatic drainage of the limbs.

Satellitosis is the name given to the appearance of numerous cutaneous deposits of metastatic melanoma around a primary melanoma or the site of its previous excision. This is also believed to be due to lymphatic spread from the primary site.

It had been suggested that both of these features might be the result of the interruption of normal lymphatic drainage by surgical incisions, allowing lymph and metastasising cells to leak into the tissues or lymphatic drainage to proceed by abnormal channels and in a non-anatomical pattern[144,145]. This suggestion could be supported by the not uncommon co-existence of both of these patterns of locoregional spread with regional lymph node metastases, possibly reflecting obstruction of the normal lymphatic flow by the nodal deposits. Indeed, Stehlin reported from the M.D.Anderson Hospital that 90% of patients exhibiting this form of metastasis had proximal lymphatic obstruction due in the main to axillary or groin dissections or, less frequently, to clinically involved lymph nodes[146]. The main risk factors, however, for satellitosis and in-transit metastases are thickness and ulceration of the primary[145,147,148] and the exact cause for these characteristic metastases is not clear.

1.2.4 Prognosis

The principal determinant in assessing the prognosis of patients with cancer is the degree of spread of cancer at the time of diagnosis. This is termed "staging" and while

different cancers may exhibit different aggressiveness, in general, the more widespread the disease (ie. the higher the "stage") the worse the prognosis within each cancer. Many systems have been devised for staging cancer, each with its particular merits. However, to be of any true value, the staging system used to describe any particular cancer must have some relevance to the prognosis and/or treatment. Due to the variations in the preferential modes of spread of the various cancers, these systems are therefore not universally applicable.

In general, each system defines an early stage of disease - Stage I - characterised in the case of solid tumours by a localised primary lesion or limited local progression. The highest stage - usually termed Stage III or IV - describes widely disseminated cancer, while the intermediate stage(s) is used to describe degrees of local, regional and lymphatic spread. The TNM staging system presents this information in a rather different format, describing each of the three features of the cancer (primary Tumour status; Nodal status; Metastases) to give an overall description of the pattern of disease.

In describing melanoma, several staging systems have been used. The oldest system is the "original three stage system" [149] (Table 1) which is based entirely on the clinical features of the disease. In some ways, this has proved inadequate as it tends to stage together groups of patients now known to carry very different prognoses. The M.D.Anderson Hospital system was devised to take into account some of the features of the spread of melanoma, dividing the old Stage I into two separate categories for

Table 1 :

Original 3-Stage Classification of Melanoma

Stage I	-	Localised primary melanoma or local recurrence or satellites
Stage II	-	Regional lymph node metastases or in-transit metastases
Stage III	-	Distant metastases

Table 2 :

Clinical Staging System for Malignant Melanoma
M.D. Anderson Hospital and Tumor Institute

(Stage 0 - Superficial melanoma)*

Stage I - Localised primary melanoma
- A : primary intact
- B : primary locally excised
- C : multiple primaries

Stage II - Local recurrence
or
Primary lesion with local peripheral nodules ≤ 3cm distant

Stage III - Regional metastases
- A : in-transit metastases
or
satellite lesions > 3cm distant
- B : regional lymph node metastases
- C : in transit or satellite lesions
plus
regional lymph node metastases

Stage IV - Systemic metastases

* Stage 0 has now been abandoned, so that all invasive primary melanomas are included in Stage I.

Table 3 :

Staging System for Malignant Melanoma

American Joint Committee on Cancer Staging and End Reporting

Stage I - Localised primary melanoma
- A : thickness ≤ 0.75mm
  Clark level ≤ II
- B : thickness 0.76-1.50mm
  Clark level III

Stage II - Localised primary melanoma
- A : thickness 1.51-4.00mm
  Clark level IV
- B : thickness > 4.00mm
  Clark level V
  or
  Satellites

Stage III - Regional lymph node metastases
(nodes not fixed and < 5cm diameter)
or
In-transit metastases (n < 5)

Stage IV - Regional lymph node metastases
(nodes fixed or > 5cm diameter)
or
In-transit metastases (n ≥ 5)
or
Distant metastases

Table 4 :

UICC Staging System for Malignant Melanoma

Stage I	-	Localised primary melanoma thickness ≤ 1.50mm Clark level ≤ III
Stage II	-	Localised primary melanoma thickness 1.51-4.00mm Clark level IV
Stage III	-	Localised primary melanoma thickness > 4.00mm Clark level V or In-transit or satellite metastases or Regional lymph node metastases
Stage IV	-	Distant metastases

primary disease and locally recurrent disease, and subdividing stages to give a more detailed description of the tumour burden[150] (Table 2). This therefore includes information on whether or not the primary lesion is still present or has been excised, and on the apparent extent of locoregional recurrence or spread (ie. satellitosis more distant than 3cm from the primary site, in-transit metastasis, lymph node spread). More specific staging systems for melanoma have been devised as knowledge of the most significant prognostic factors has been assimilated, including the American Joint Committe on Cancer Staging System[151] (Table 3) and the classification recommended by the UICC (Union Internationale contre le Cancer) (Table 4). The main value of these systems is the inclusion in subdivisions of Stage I of the histological characteristics of the primary tumour now known to be of major prognostic significance.

General prognosis by Stage

The overall prognosis according to stage of the disease has been widely reported and for uniformity of understanding this is best done by the original three stage system. The 5-year survival figures quoted in the literature for melanoma are 72% (66%-80%) for Stage I disease, 29% (14%-39%) for Stage II, and 17% (0%-18%) for Stage III [45,152-155].

Within these broad classifications, however, several features pertaining to the patient or to the tumour are of additional prognostic significance.

The female sex carries a distinct survival benefit, particularly in Stage I [36,156,157] and although some authors have demonstrated an advantage to women even in the more advanced stages[158,159], this is not confirmed by the majority of the literature. It has been suggested that younger patients carry a better prognosis[156] but this is also disputed[157]. The anatomical site of the primary melanoma is believed to have some bearing on prognosis, limb lesions being associated with a better outlook than those on the head and neck, and trunk lesions carrying the worst prognosis[156]. An especially poor prognosis was claimed for melanomas arising on the **BANS** area (**B**ack, posterior **A**rm, posterior **N**eck, and posterior **S**calp)[160] but this too is disputed[161].

Certain histological features of the primary tumour are prognostically significant. The importance of depth of invasion of the primary melanoma into the dermis and subcutaneous tissue has long been recognised[162] and a classification of melanoma based on this was introduced by Clark in 1969 [163] (Table 5). This identified five levels of invasion by melanoma cells with increasingly poor prognosis. Later studies by Breslow showed that the absolute thickness of melanomas on histological section (measured in millimetres from the granular layer of the epidermis to the point of deepest vertical invasion) was a good prognostic indicator[164] and this has subsequently been confirmed and shown to be a more reliable indicator

Table 5 :

Classification of Malignant Melanoma by Histological Level of Invasion (Clark)

Level I	-	tumour confined to the epidermis (ie. melanoma in-situ)
Level II	-	tumour penetrating the basement membrane and extending into the papillary dermis
Level III	-	tumour filling the papillary dermis to the junction with the reticular dermis
Level IV	-	tumour invading the reticular dermis
Level V	-	tumour invading the subcutaneous fat

of prognosis than Clark's histological level[165-167]. It is now usual for primary melanomas to be placed in good, intermediate or poor prognostic categories on the basis of Breslow thickness. Recent data from Scotland indicate five year survival rates of 92.5%, 72.6% and 48.0% for lesions of <1.5mm, 1.5-3.49mm, and ≥3.5mm respectively[30]. The measurements of Breslow thickness and Clark's level are both rather observer-dependent. This criticism is however more applicable to Clark's method for this depends on a clear distinction between the papillary and reticular dermis to separate level III from level IV lesions which together form the majority of primary melanomas[168]. Breslow's measurement is somewhat more objective and therefore more reproducible as it requires only the identification of two points on the section. While there may be no value in routinely combining the two methods[166-168], Clark's level may give an indication of relative prognosis for primary lesions of equal Breslow thickness. Further, it would appear that for thin melanomas (Breslow thickness < 0.75mm) Clark's level may be a more accurate prognostic indicator[169].

The diameter of primary melanomas is reported by many authors to correlate with the 5-year survival[154,170-173], increasing diameter being associated with poorer prognosis.

The presence of ulceration of a primary lesion has also been shown to be a marker of poor prognosis[172,174,175], as have high mitotic activity on histological sections (mitotic index = highest number of mitotic figures seen in $1mm^2$) and microscopic evidence of vascular invasion by

tumour cells[163,167,168,176]. Naturally, a combination of these various factors together with many other less potent or as yet unrecognised factors will determine the prognosis for any individual patient. The contribution of each factor to the overall prognosis is complicated by the interdependence of many of these factors : for example, more "good prognosis" leg melanomas arise on women, who in any case appear to have a better overall survival than men. Attempts have been made to establish the relative importance of each of the main prognostic factors and in an analysis of a very large series of patients, the principal prognostic factors for Stage I melanoma were, in order of importance, (i) tumour thickness, (ii) ulceration, (iii) anatomical site, and (iv) sex [157].

Although many authors have suggested that in Stage II disease, the extent of lymph node involvement is significant in determining the prognosis, the evidence for this is disputed. The number of involved nodes may be important[177-179]. Some retrospective studies have shown better survival figures for patients with microscopic lymph node involvement only compared with those with clinically involved lymph nodes[36,180,181]. However, other reports including a large WHO study have also addressed this issue and have concluded that the incidence of occult lymph node metastases found in prophylactic lymph node dissections in clinical Stage I was similar to that of later lymph node involvement in patients in clinical Stage I treated by excision of the primary alone, and that the 5-year survival for the two groups of

patients was not significantly different[153,182]. The question of prophylactic lymph node dissection therefore remains a contentious issue [183]. Its value may be limited to patients whose melanomas are of intermediate Breslow thickness (0.75mm-4.0mm) with a prognostic advantage being conferred on women even within this group[184-186].

The depth of invasion of the original primary still bears some prognostic importance in Stage II disease, as may its anatomical site and the sex of the patient[156].

As discussed earlier in this chapter, metastatic melanoma may be found in lymph nodes in the absence of a detectable primary lesion. In these cases, the prognosis would appear to be similar to that of patients with known primaries[129].

When melanoma reaches Stage III, the prognosis is dismal (see above) but is modified slightly by the site(s) of spread[16,156,187]. Hepatic or cerebral metastases are associated with a particularly bad outlook compared to cutaneous or subcutaneous deposits, while pulmonary metastases hold an intermediate position.

Since this thesis is concerned mainly with melanomas of the extremities, the M.D.Anderson Hospital staging system is especially relevant (see Table 2). The significance of this staging system lies not only in its more accurate description of the pattern of locoregional disease but also in the prognostic implications of this description. Within the Stage III category, the group of patients with wide satellitosis or in-transit metastases (Stage IIIA) may carry a better prognosis than those with lymph node

involvement (Stages IIIB & IIIAB) although this is not universally accepted[179].

1.3 Current Standard Therapy of Melanoma

1.3.1 Surgical Treatment of Melanoma

The mainstay of treatment for malignant melanoma over the years has been surgical. However the applications of surgery to melanoma have been modified as the results of studies have become available and as the pattern of presentation of melanoma has changed. An increasing proportion of melanomas are presenting in Stage I and indeed there are reports that the thickness of melanomas at diagnosis is falling more and more into the better prognostic categories in many parts[30,188,189].

[i] Surgical treatment of Stage I melanoma

Surgical excision of primary melanoma is universally accepted as the treatment of choice and is the only option which offers the prospect of a cure[183,190]. How extensive this excision should be remains a matter of controversy. The historical recommendation was for a radial excision margin of as much as 5cm around the primary lesion[191-193]. The reasoning behind this approach was that local recurrence, which is associated with a very high mortality rate of 80%, could best be avoided by removing a wide margin of tissue in order to eradicate local microscopic extensions of the primary or microscopic satellite metastases as well as any areas of field change around the primary lesion. This was held to be the best approach

until recently when it has become clear that the risk of local recurrence is more closely related to the thickness of the primary lesion than to the width of the primary excision[147,194-200]. In this regard, the risk is almost negligible for lesions less than 0.75mm thick but rises to 10-20% when the thickness exceeds 4.0mm[148,166,168,195,201].

The first step in the management of melanoma is a biopsy to establish the histological diagnosis. In view of the prognostic significance of many of the histological features of melanoma, it would seem that a complete excision biopsy offers the best solution to an accurate assessment of the melanoma. This can be followed by a wider excision if indicated by the histological features of the melanoma. Nevertheless, some authors recommend the use of "punch" biopsies or of wedge-excision (or "incision") biopsies when technical problems of wound closure or significant cosmetic defects can be anticipated due to the size or site of the lesion[183]. Further removal of tissue can be undertaken once the diagnosis is established. The limitation of this approach, in the author's opinion, lies in the need to use a standard width of excision once the diagnosis is made since one cannot guarantee that a punch biopsy or incision biopsy will contain the area of deepest invasion of the melanoma. As a result, while the diagnosis may be made, the prognostic indicators may be incomplete and one risks excising less tissue than would be desirable, or having to perform a third excisional procedure to correct this. Moreover, in view of the suggestion that trauma may have a role in the

aetiology of melanoma[86], one could argue that incision biopsy could have a deleterious effect on the outcome of surgery for melanoma. In fact, there would appear to be no evidence for this argument[202], but the use of incisional biopsies should, in the author's view, be reserved only for lesions which, by their large size or awkward site, present very significant problems of skin cover after excision.

It would seem then that an excisional biopsy should be performed to establish the diagnosis and to assess the prognostic characteristics of the lesion (thickness, level of invasion, ulceration, mitotic activity) so that an informed decision can be made as to the extent of removal of surrounding tissue. In the case of thin melanomas (<1.0mm) which carry a very low risk of local recurrence, an excision margin of 1cm appears to be sufficient according to a large WHO study[203], and this is confirmed for lesions up to 1.5mm thick by another large study with extended follow-up[204]. In situ melanoma should be included in this group, for while it is a non-metastasising form of the disease, there is evidence that it may recur locally and that this recurrence can take the form of in situ melanoma or of frankly invasive melanoma[205]. For thicker lesions, a wider excision should be performed with a 3-4cm margin[183,186,206] where the Breslow thickness is less than 4.0mm. Above this thickness, opinions again diverge, some recommending no difference in approach, others favouring even wider surgical margins of 5cm.

The technique of wound closure employed is a purely practical question, split-thickness skin grafting being used when the defect cannot be sutured primarily. A case had been made in the past for using skin grafts in all cases so that small local recurrences could be detected more easily through this thin covering[206]. This recommendation is now discredited for several reasons : the incidence of local recurrence should be very low, especially in the thinner lesions; wide excision is therefore not always necessary and primary closure can often be achieved; there is no evidence that the slightly earlier recognition of local recurrences allows better treatment or offers improved rates of survival[206].

Melanomas arising on digits present particular problems since, in order to achieve excision with even only a 1cm margin, amputation of the digit will often be required. Subungual melanomas in particular have a notoriously high recurrence rate even after limited amputation[207]. This recurrence rate can be improved by amputation at the metacarpophalangeal (or metatarsophalangeal) joint[207] but the disabling effect of this procedure must be borne in mind especially for lesions of the thumb of the dominant hand. Selection of the most appropriate level of amputation should therefore be based on the histological features of the melanoma as well as on the anticipated disability caused by the treatment.

The depth of excision is also controversial in that opinions are divided on the necessity to excise the underlying deep fascia. In the past, "wide excision" was

taken to mean "three-dimensional wide excision" and therefore to include excision of the deep fascia[192,193]. However subsequent experience suggested that this might facilitate metastasis by removing a barrier to spread of the tumour[208]. Recent data suggest that it offers no benefit in terms of local control or survival[209]. Therefore excision of the deep fascia seems to add unnecessary morbidity to the primary treatment and can safely be omitted in most cases.

Lymph node dissection for Stage I melanoma

The place of lymph node dissection in Stage II disease is well established but its prophylactic role in the treatment of Stage I disease is much less clear.

Melanoma is believed to spread by a predictable stepwise progression from the primary lesion through the regional lymph nodes and then to distant sites[210]. The clinical involvement of lymph nodes must be preceded by a subclinical phase of microscopic disease. Once clinical evidence of metastasis to the lymph nodes is apparent, as many as 70-85% of patients will also have occult distant spread of their disease[177]. The rationale for prophylactic lymph node dissection, therefore, lies in the possibility of removing subclinical disease in the lymph nodes before the next step in the spread of the disease to distant sites has occurred. However, a significant proportion of patients with malignant melanoma do not follow this general rule. In a large study of Stage I melanoma treated by wide excision alone in which 516 of the 1164 patients developed recurrent disease within ten

years of treatment, as many as 22% of the first recurrences occurred at distant sites, and in 31% simultaneous regional lymph node and distant metastases were found[211].

It is claimed that since 56% of patients undergoing excision of melanomas with Breslow thickness 1.5-4.99mm later develop clinical involvement of their regional lymph nodes, this represents the true incidence of occult lymph node metastases at the time of the original excision[210]. However, since some of the lymphatic deposits may well have resulted from spread of tumour from other extralymphatic regional or distant sites, this is probably an overestimate of the true incidence. Several studies have shown that between 4% and 42% of melanoma patients with clinically uninvolved regional lymph nodes do have microscopic evidence of lymph node metastases[153,182,184,212-215]. Evidence of the value of lymph node dissection in these patients is mixed. While some studies show a 5-year survival advantage of 10-30% to certain subgroups of patients undergoing prophylactic lymph node dissection[153,181,184-186], most of these contain only very small numbers of patients. The subgroups in question comprise the intermediate thickness melanomas in both sexes and the thicker lesions in women, both on the trunk and especially on the extremities. Other studies which have specifically analysed the survival of patients according to the clinical and pathological stage of their disease have shown no significant difference in survival whether lymph node dissection is performed for <u>clinically</u> positive nodes

or electively for clinical Stage I but histopathological Stage II disease[182,214]. This is in contrast to the claims made by advocates of prophylactic lymph node dissection that the prognosis of patients found to have occult lymph node metastases at lymph node dissection is better than that of patients undergoing lymph node dissection for clinically involved nodes[184,210]. Given the difference in the tumour burden of these two groups of patients, it would not be surprising to find a survival benefit for the former over the latter. Comparison of these two groups does not seem appropriate.

Most of these studies are retrospective; indeed, to date, only two prospective randomized trials of prophylactic lymph node dissection have been reported[182,216-218]. Neither of these showed any survival benefit from the procedure. One can therefore argue that lymph node dissection can safely be reserved for the treatment of patients with clinically positive nodes, and that patients with clinical Stage I disease should not be submitted to this additional procedure.

A novel technique has recently been developed which allows the intraoperative mapping of regional lymphatics and selective excision of the first lymph node(s) in the path of drainage of the primary lesion. This "sentinel" node is submitted for frozen section histology and rapid immunohistochemistry for immediate identification of metastatic deposits[215,219,220]. In a large study, the authors have found that the technique is highly sensitive, with a false negative rate of less than 1% when compared to the full lymphadenectomy specimens[215,220]. Patients

with subclinical lymph node involvement can therefore be reliably identified and offered a formal lymph node dissection without subjecting the majority of patients (whose lymph nodes are clear of tumour) to this procedure unnecessarily. In their series of patients, the authors found that melanomas less than 1.5mm thick were associated with lymph node metastases in 9.8% of cases but that this figure rose to 36.9% for thicker lesions[215]. While the current state of knowledge provides no convincing evidence of survival benefit following elective lymph node dissection, this new method should allow a more selective approach to the procedure by identifying those patients most likely to benefit from it[219].

Based on the evidence accrued so far, the following approach to the treatment of primary melanomas is suggested :

- **Breslow thickness <1.5mm** : excision with a 1cm margin (incl. in situ melanoma)

- **Breslow thickness ≥1.5mm** : excision with a 3-4cm margin

The author does not at present advocate prophylactic lymph node dissection for clinical Stage I disease.

[ii] Surgical treatment of regionally advanced melanoma

The most common first site of metastasis for melanoma is the regional lymph node chain and this occurs in some 20% of patients with primary melanoma[221]. As stated above, the overall 5-year survival of patients with clinical Stage II disease is about 29% (14-39%) in most reports. This however varies in relation to the extent of the metastases, from 50% when disease is confined to one macroscopic focus, to 30-40% when up to 3 nodes are involved, and to less than 20% if 4 or more nodes are involved[177,221]. Moreover, patients with only microscopic evidence of lymph node spread (clinical Stage I, but histopathological Stage II) have 5-year survival rates between 50% and 60% [153,177].

In the presence of clinically involved regional nodes, most authors agree that lymph node dissection is appropriate[217,218,222-227]. The clinical features are usually sufficiently suggestive that the diagnosis can be made on these grounds alone, but where doubt exists, valuable evidence of nodal involvement by tumour can be obtained from fine needle aspiration cytology of the palpable nodes[226]. In most instances, radical dissections are performed to provide clearance in the cervical and axillary areas, but the extent of dissection in the groin is debatable. Some authors recommend ilioinguinal dissection for all cases on the basis that a high proportion of patients with clinical involvement of the inguinal nodes also have iliac node involvement[228]. However the presence of metastatic deposits in the iliac

nodes is associated with a very poor prognosis and suggests that more proximal spread of the disease may already have occurred. One could therefore argue that the additional morbidity of this extensive surgery is unjustified if disease appears to be confined to the inguinal glands. In this situation, one should simply perform an inguinal node dissection to remove the superficial and deep nodes in the femoral tiangle. If there is clinical evidence of iliac node involvement, then ilioinguinal node dissection would be indicated in order to avoid the risk of ulceration of the tumour onto the skin or erosion into the adjacent vessels with resultant haemorrhage[221].

In the treatment of extranodal locoregional recurrence (local recurrence, satellitosis, in-transit metastases), most authors would advocate simple excision of the recurrences as an adequate form of treatment, particularly when the disease is confined to a small number of lesions. Many patients suffer repeated local recurrences without developing systemic metastases and in these patients local control is the main goal of treatment. However, for the majority of patients (as many as 70-90%), locoregional recurrences are widely regarded as the "harbingers of systemic disease" [148,221] and are associated with a poor prognosis. More radical approaches to their treatment have been performed including limb amputation and integumentectomy (the removal of all the skin, subcutaneous fat and deep fascia from the sole of the foot up to the inguinal ligament in the case of lower limb

lesions) [229,230]. Reports of the results of this type of treatment are based on small numbers of patients and do not really show any substantial benefit in terms of survival[148,229,231-234]. Nowadays, these operations are regarded by most as inappropriate since they merely inflict severe disability on patients who in any case have a high chance of already harbouring systemic disease and who therefore have poor survival prospects. Amputation may still have a role in the palliation of advanced, painful disease but even this is now regarded as inappropriate, most clinicians favouring a less disabling approach to palliation such as radiotherapy or isolated limb perfusion (see below).

[iii] Surgery for systemic disease

Systemic metastases from malignant melanoma are associated with an extremely poor prognosis (see Section 1.2.4) and surgery for this stage of disease can really only be regarded as palliative. However, in selected cases, this palliation can be prompt and prolonged depending on the site and extent of metastases.

Autopsy studies show that single organ involvement by advanced melanoma is extremely rare (0-1%) [16,235]. The most common sites of metastasis are the lungs, liver and gastrointestinal tract, followed by the central nervous system and bone and marrow. While there are no prospective randomised studies of surgical treatment of these metastases, several reports have suggested that

resection of lung deposits can produce effective palliation, especially if these are unilateral and few in number[16,236-238]. Resection of obstructing, bleeding or intussuscepting gastrointestinal lesions can provide effective symptomatic relief and in some cases may result in prolonged survival[16,237]. Similarly, surgical removal of solitary brain metastases, when possible, combined with radiotherapy can be beneficial[239-241]. Hepatic metastases are almost invariably associated with an extremely poor prognosis and patients are generally not regarded as candidates for surgery[16,242]. Melanoma metastases to bone are also associated with a very poor prognosis, but palliative treatment should nevertheless be considered as they cause severe pain and can result in pathological fractures. The pain due to these metastases can be treated effectively with radiotherapy, and pathological fractures of weightbearing bones can be stabilised by internal fixation and followed up with radiotherapy[243].

The place of surgery in the management of systemically metastasising melanoma is therefore in the palliation of the effects of these metastases. This should however be approached positively as, although the course of advanced melanoma tends to be progressive and unrelenting, some patients can benefit for prolonged periods from this type of palliation.

1.3.2 Non-surgical Treatment of Melanoma

As stated above, the treatment of primary melanoma is essentially surgical. Other therapeutic modalities may find a role in the treatment of advanced melanoma or as adjuncts to surgery for primary disease.

[i] Treatment of advanced melanoma

a) Chemotherapy

The principal non-surgical modality which has been used against melanoma is systemic chemotherapy. The results of this have been generally disappointing using both single agents and combinations of drugs. In single agent therapy, the best observed response rates are achieved by dacarbazine (DTIC) with overall response rates of 10-25% [244-246]. However this includes only about 5-10% complete responses[245,247]. Furthermore, responses tend to be rather short-lived lasting only 5-6 months on average[247], and while there does seem to be an improvement in survival among those who respond to the treatment, this too is fairly modest (median survival for responders = 10 months; median survival for non-responders = 5 months)[245]. Another disappointing feature of this treatment is that responses tend to be achieved in those patients whose prognostic characteristics are perhaps slightly more favourable than others' - for example, female patients respond better than males, and cutaneous,

lymphatic, pulmonary or soft tissue metastases tend to respond better than hepatic, cerebral or visceral deposits.

The other drugs which have elicited similar if somewhat lesser observed response rates include vindesine at 20-26% [248-252], the nitrosoureas lomustine (CCNU), carmustine (BCNU) and semustine (MeCCNU) at 10-20% [244,253], and the antimetabolites methotrexate (MTX) and cytosine arabinoside (ARA-C) at 10-15% [244]. However the higher morbidity associated with these drugs compared to that induced by dacarbazine means that the latter is still the standard against which all other chemotherapeutic agents are measured.

The use of most cytotoxic drugs is limited by their toxicity to normal tissues and in particular to vital organs such as bone marrow and liver. While the alkylating agents, such as melphalan, are known to be capable of producing modest responses in advanced melanoma, myelosuppression prevents their use in high doses. It was therefore suggested that autologous bone marrow rescue might provide a method for circumventing this problem[254], and in fact, using this technique, high dose melphalan alone or in combination with dacarbazine was found to produce response rates of 40-70% [255-258]. Early enthusiasm for this technique was however soon tempered by the finding that responses were once again short-lived[257-259] and that there was no improvement in survival rates[259].

Cis-platinum has been increasingly used alone or in combinations of drugs against melanoma. When used alone in high doses, it produces substantial response rates of 53-69% but with major toxicity in the form of peripheral neuropathy and no demonstrable survival benefit[260].

The lack of a clearly effective single-agent chemotherapy for melanoma has prompted several groups to try combinations of drugs in the hope that responses and survival rates might be improved and toxicity reduced. In fact the results of these trials have generally been disappointing, offering only marginal benefits over dacarbazine alone[244,253,261].

There are nevertheless a few combinations of drugs which have produced significant response rates of 40-55% and show signs of improved survival rates, albeit in small numbers of patients. These combinations all include dacarbazine or one of the more active nitrosoureas (BCNU, CCNU). The best results have been seen with the combination known as DBPT (dacarbazine, BCNU, cisplatinum and tamoxifen) with an overall response rate of 50-55% and complete responses comprising a third of these[262-268]. Of the other combinations, CBC (CCNU, bleomycin, cisplatinum) [269], BELD (bleomycin, vindesine, CCNU, dacarbazine) [270,271] and DVC (dacarbazine, vindesine, cisplatinum) [272] have all produced response rates of more than 40% with a similar proportion of complete responses to DBPT [261]. These studies also include details of patients showing longer-lasting responses than many previous studies and a few examples of hepatic metastases responding to treatment. Impressive results were

reported with the use of procarbazine, vincristine and CCNU (POC) which initially produced a 48% response rate, half of these being complete reponses (25%) [273]. This series however contained a very high proportion of patients with only cutaneous or lymphatic metastases (64%), in other words metastases which are known to be associated with better response rates to chemotherapy (see above). Another study in which fewer patients fell into this category failed to confirm such encouraging results [274].

New drugs

The other attempts to improve the chemotherapy of melanoma have centred around the development of new and sometimes more specific drugs. The new drugs which have shown encouraging activity against melanoma include **detrorubicin** which is a semisynthetic anthracycline differing from adriamycin only in one side chain (at C14) [261]. This however seems to be a critical difference since adriamycin has very little activity of its own against melanoma. The overall response rate with detrorubicin in one series is reported as 19%, but this includes seven partial responses and only one complete response in a patient with cutaneous metastases[275]. **Taxol** has also been shown to have some activity against melanoma. This drug has recently been developed from yew bark and when infused over 24 hours produces a response rate of 12-15%, including complete as well as partial responses[276,277]. It is worth noting that taxol produces responses in hepatic metastases, a supposedly very

resistant site[277]. **Dibromodulcitol** was reported as promising as long ago as 1978 [278] with a response rate of 16%. This was confirmed later with a response rate of 24% including responses in soft tissue and visceral metastases[279]. **Fotemustine** is a new nitrosourea which has been used to treat patients with cerebral melanoma metastases with a very high response rate of 28% (including 2 complete reponses) and a survivial rate of 21% at one year[280]. In subsequent studies, it has been found to produce overall response rates of 32% (and an especially high reponse rate in visceral metastases : 6/13) when used in combination with dacarbazine[281]. Another relatively new nitrosourea - tauromustine (TCNU) - has shown rather less encouraging results with overall response rates of 10-15% [282,283].

More specific approaches to melanoma chemotherapy have attempted to exploit the biochemical pathways of melanin synthesis in melanocytes by introducing substances which are converted to active metabolites by the enzyme tyrosinase, which itself is uniquely related to the melanocyte system (see Section 1.1). These substances have included levodopa, dopamine and 4-hydroxyanisole[283]. However, enthusiasm for this type of approach must be tempered by an awareness of its potential side-effects and limitations[284], and in particular the effects on the central nervous system of using levodopa or dopamine and on the the pigmented tissues of the eye. In addition, a method for ensuring release of the activated substance from the melanosome into the cytoplasm of the melanocyte

may be required in order to make it cytotoxic. A further problem to address would be the probable resistance of amelanotic melanoma to this form of treatment.

A point to remember is that, in spite of the development of these and many other drugs, no agent(s) alone or in combination has been shown to be convincingly more effective than dacarbazine alone.

b) Immunotherapy

The case for immunotherapy of melanoma is prompted by the natural history of the disease which reveals spontaneous regression of melanomas following "immune-stimulating events" such as infections or vaccinations, or for no apparent reason (see Section 1.2.1). Furthermore, the histology of primary melanomas shows that while the radial growth phase of a melanoma can last for several years without adverse effect on the host and is associated with a pronounced lymphocytic infiltrate, the vertical growth phase which follows is associated with a marked diminution in the number of lymphocytes and a much more aggressive course[138]. Several investigators have demonstrated the existence of tumour-associated antigens in melanomas[285-287], circulating antibodies to some of these antigens in patients with early melanoma[288,289], and specific lymphocyte-mediated cytotoxicity to melanoma cells among melanoma patients[290,291]. The specificity of this cell-mediated immunity is however disputed[292]. The initial efforts in immunotherapy of melanoma were therefore directed at methods to stimulate the patient's

own immune system to react against the disease. BCG (Bacille Calmette-Guerin) vaccine was injected directly into melanoma deposits and was found to induce tumour regression in immunocompetent patients[293-295]. The response rate of injected lesions was 66%, but in addition 22% of patients showed regression of uninjected cutaneous lesions and 27% of patients had complete regression of all cutaneous disease[292]. There have also been reports of regression of visceral metastases following cutaneous intralesional BCG injection, but generally the responses of patients with visceral metastases have been poor[292]. Several other nonspecific immunostimulants have been used in this way with similar results[292]. A more specific method has also been used in which irradiated melanoma cell vaccines were injected into patients in the hope that they might stimulate peripheral lymphocyte activity. These vaccines have been augmented by the addition of substances such as neuroaminidase which unmask tumour antigens[296] and the use of lysates of melanoma cells superinfected with oncolytic viruses[247]. Unfortunately, these measures have not proved particularly effective with an overall response rate of only 15% from this active immunotherapy[297]. Indeed, some studies have even suggested that immunotherapy of melanoma might have a deleterious effect on the course of the disease[298,299].

Modern technology has allowed the production of monoclonal antibodies to melanoma-associated antigens. While these have been used alone, they have also been conjugated to cytotoxic molecules (eg. ricin A chain)[297]

and used in combination with other agents to produce partial response rates of 20% [247]. This approach to therapy is still very much at the experimental stage however.

Recombinant DNA technology is now sufficiently developed that many cytokines can be produced for clinical use. Several interferons have been used in the treatment of melanoma with various dosage schedules. These have produced response rates similar to the other forms of therapy at about 20%, with responses tending to occur principally in the soft tissues and less frequently in pulmonary or hepatic deposits[247]. Interleukin-2 (IL-2) is another of the cytokines and is responsible for the growth and enhanced anti-tumour activity of the so-called lymphokine-activated killer (LAK) cells. In addition, it increases secretion of other cytokines such as interleukin-1 (IL-1), tumour necrosis factor (TNF) and interferon. IL-2 has now also been administered alone and in combination with cultured LAK cells for the treatment of melanoma and is achieving response rates in the region of 20% even in patients with melanoma deposits in unfavourable visceral sites[300-304]. Further studies of IL-2 in combination with various chemotherapeutic agents (cyclophosphamide, dacarbazine, flavone acetic acid), TNF, interferon or monoclonal antibodies have been reported[247,300]. Overall, IL-2 has been found to produce significant response rates in metastatic melanoma, comparable to those achieved with chemotherapy, and even in sites normally associated with a poor response rate (eg. lung, liver). Initial trials of IL-2 therapy were

associated with a very marked multisystem toxicity which resembled septicaemic shock, but as newer dosage schedules have been developed, so this problem has reduced, and the use of drugs such as ibuprofen have been found to markedly improve the symptoms of toxicity[247]. In future, other approaches to reducing the toxicity of IL-2 may include the use of monoclonal antibodies to TNF to scavenge this released cytokine and thereby limit its toxic effects.

c) Radiotherapy

Malignant melanoma has historically been considered as a "radioresistant" tumour. This term is slightly misleading as it is in no way an absolute term but merely reflects the experience of many radiotherapists who encountered difficulties in treating often very advanced disease at a time when the radiobiology of melanoma was ill-understood[305]. Most tumours are believed to have some capacity to repair damage caused by radiotherapy in the lower range of doses. The dose-response relationship for radiotherapy is therefore only exponential when this capacity is exceeded. A tumour's capacity for repair of sublethal damage is represented by the "shoulder" portion of the cell survival curves which precedes the exponential portion, and is often quantified as the Dq value. This is measured as the intersection of the exponential portion of the curve on the radiation dose axis (Figure 2). Therefore tumours such as melanoma which possess a large capacity for accumulation and repair of sublethal damage have a "broad-shouldered" cell survival curve and a high

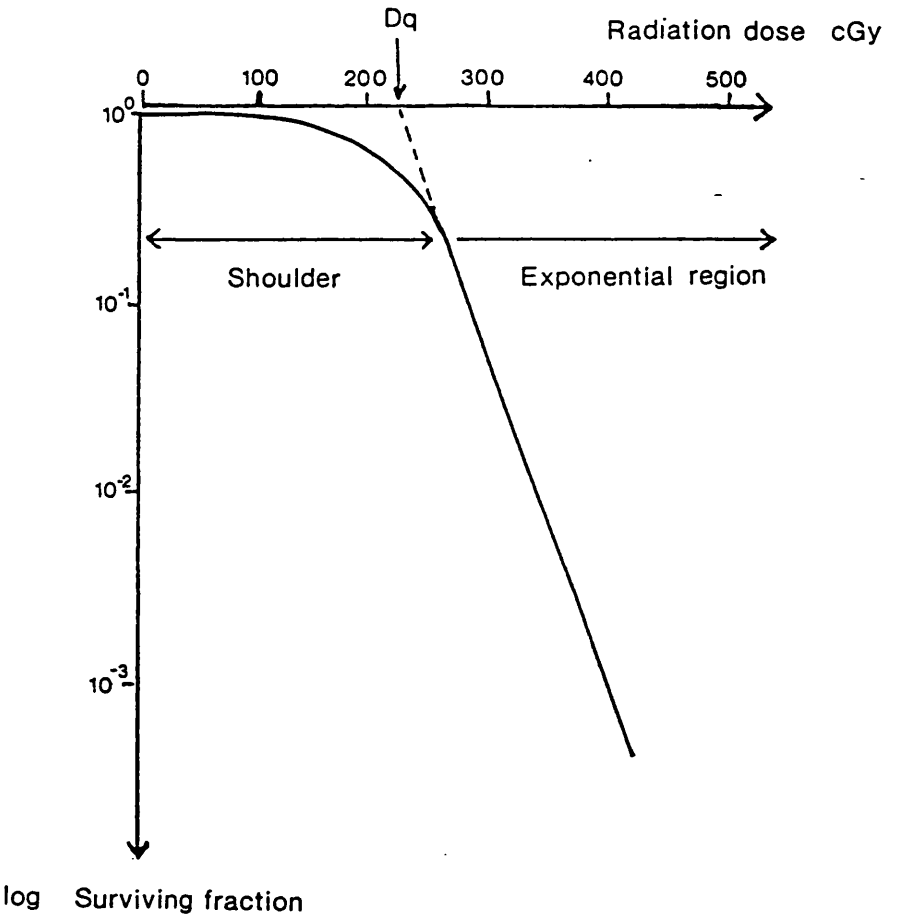


Figure 2 :
Example of a survival curve for cells exposed to radiation.

The initial "shoulder" phase represents the dose range within which sub-lethal, reparable damage is caused. Beyond the Dq value, the curve becomes exponential, indicating efficient cell sterilization.

Dq value and are relatively radioresistant[306]. In fact when radiotherapy has been administered in large fractions, several retrospective studies have shown an increased response rate compared with the more common dose-fractionation schedules[307-309]. Several factors may contribute to a tumour's capacity to survive radiotherapy including regions of hypoxia within the tumour, "contact resistance" of individual cells, and the ability to inactivate the intracellular free radicals which are thought to be responsible for much of the damage caused by radiation[310].

In spite of extensive research, the role of radiotherapy in the treatment of melanoma remains limited. There has been no convincing evidence of any survival benefit from prophylactic irradiation of primary melanoma sites or of lymph node beds. However, in combination with local surgery, similar survival rates and local control can be achieved as with radical and sometimes mutilating operations[305]. One should bear in mind that this surgery is now very rarely practised. There would therefore seem to be little place for radiotherapy in the prophylactic or adjuvant setting. As a palliative measure, radiotherapy may produce worthwhile symptomatic relief of pain due to bone metastases[311] or fungating cutaneous tumour masses[312]. Cerebral metastases can be palliated with radiotherapy and steroids, but the survival is so short for most of these patients that it is not clear whether this offers any advantage over the use of steroids

alone. Nevertheless, there is no reason to deny any possible symptomatic benefit from this treatment to this unfortunate group of patients.

d) Hormonal therapy

While some hormones may contribute to the aetiology of melanoma (see Section 1.2.1), there is little evidence to support a hormonal approach to treatment of the disease. Experience with the anti-oestrogen tamoxifen in the treatment of breast cancer has not been matched by results in melanoma[253,313]. Medroxyprogesterone acetate has similarly not been shown to be of any benefit[314].

It is of interest that tamoxifen does produce an advantage when added to the chemotherapeutic combination of dacarbazine, BCNU and cisplatinum (DBPT)[261-268]. This is believed to be due to a synergy between tamoxifen and cisplatinum, although the exact mechanism of this remains unclear[264,265,315].

[ii] Adjuvant treatment of melanoma

It is clear that while surgery offers the best chance of cure in primary melanoma, the disease is still associated with a generally poor prognosis. The combination of different cytotoxic agents and of therapeutic modalities has improved the efficacy of treatment of many other malignancies (lymphomas, leukaemias, sarcomas).

In the treatment of melanoma, patients with thick primary lesions or with lymph node metastases are at particular risk of developing systemic metastases. However several prospective randomised studies investigating these two groups of patients have failed to demonstrate any benefit from adjuvant chemotherapy (mainly dacarbazine) or immunotherapy (mainly BCG), used separately or in combination, in terms of disease-free interval and survival[316-322]. In fact in one of these studies the patients receiving adjuvant treatment with dacarbazine tended to fare worse than those in the control group[317]. Adjuvant radiotherapy to the area of lymph node dissections in Stage II melanoma has also failed to produce any advantage over surgery alone[323].

1.4 Isolated Limb Perfusion (ILP)

1.4.1 Regional Chemotherapy

Regional chemotherapy for cancer originated with the experience of Klopp and Bierman working independently who introduced the concept of intra-arterial drug administration in the treatment of cancer[324,325]. This had followed the observation that accidental administration of nitrogen mustard into the brachial artery rather than the antecubital vein of a patient with Hodgkin's lymphoma had resulted in erythema, then vesiculation and ulceration of the patient's hand and forearm, followed eventually by complete recovery. In addition, a number of cases of lung cancers responding to intravenous nitrogen mustard treatment had been reported, and simple anatomical consideration reveals that intravenously administerd drugs must pass directly through the right heart to the pulmonary arterial circulation, thereby delivering a higher concentration of drug to the tumour bed than in the diluted systemic circulation.

Klopp therefore employed the technique described by Donovan[326] to implant a plastic tube in a proximal branch of the desired nutrient artery. In animal studies, he demonstrated that by intra-arterial injection of nitrogen mustard he could produce tissue changes in the area supplied which could not be produced by intravenous injection of even lethal doses of nitrogen mustard. He also demonstrated that tumour tissue was more sensitive than normal tissue to smaller doses of drug and that this

property could be harnessed to produce tumoricidal effects by dose-fractionation as in radiotherapy without producing ulceration or necrosis in normal tissues.

Klopp also conducted a clinical study in which he achieved tumour responses following intra-arterial injection of nitrogen mustard in each of his first ten patients[324]. All but one of these patients had tumours of the head and neck or brain, the exception being a case of fibrosarcoma of the thigh. Biopsies of several of the tumours after treatment showed marked tumour destruction and changes "almost indistinguishable from those following cancericidal doses of roentgen or gamma rays". He did experience some difficulties with haematological toxicity and recorded tissue reactions to nitrogen mustard in the vascular fields adjacent to that supplied by the selected artery, but he noted, in the later part of his study using a fractionated regimen, that these adverse effects could be limited by applying some proximal venous compression in order to delay the systemic redistribution of the drug. This allowed a greater degree of detoxification of the drug to inactive metabolites prior to its systemic release. Similarly, by occluding collateral channels, notably on the scalp, he was able to limit the drug distribution to a narrower field of tissue. These manoeuvres had the added advantage of prolonging the period of exposure of the tumour bed to the cytotoxic agent.

Klopp suggested in 1950 that the use of a heart-lung mechanical pump, similar to those being developed in the field of cardiac surgery at that time, might allow the

circulation of an isolated region of the body to be maintained so that regional exposure to the cytotoxic drug could be prolonged, detoxification more complete, and subsequent systemic exposure to the drug minimised[324].

1.4.2 History and Development of ILP

The technique of isolation perfusion was introduced by Creech and his colleagues in 1957 [327]. This team of investigators had initially conducted animal experiments to establish the effectiveness and safety of the procedure[328]. These had confirmed that the limb, mid-gut and liver of the dog could be reversibly isolated from the systemic circulation and that the isolated region could tolerate perfusion at subnormal flow rates and pressures without ill-effect. They had also reported that blood provided the best perfusion fluid in their experience and that only after perfusion lasting more than 90 minutes was there a significant rate of postoperative oedema and ischaemic changes in the perfused region[329]. They studied the effect of this mode of drug administration on dosimetry and found the tolerable dose of cytotoxic to be significantly higher by this technique than by intra-arterial or systemic administration : in the case of nitrogen mustard (HN_2), the tolerable dose exceeded the whole body intravenous dose, and in the case of L-phenylalanine mustard (L-PAM, melphalan), the dose was equal to the whole body dose. They suggested that L-PAM remained active in the circulation for longer than HN_2 (

2 hours v 8 minutes) after measuring the LD_{50} of serial blood samples taken from the perfused dogs and injected intraperitoneally into mice.

Having treated 24 patients by isolation perfusion of various regions, they reported on 19 for whom follow-up data were available, including 6 patients with melanoma of the lower limb[327]. 18 cases had shown tumour regression, half of these of startling proportions. In particular, 5 of the patients with melanoma had shown significant responses to L-PAM, an observation of great importance as prior to this, systemic treatment of melanoma with this drug had only occasionally achieved modest reductions in tumour bulk but no convincing evidence of disease control[330]. The sixth patient with melanoma had undergone perfusion at the level of the aortic bifurcation in view of the advanced stage of his disease and had sustained a fatal myelosuppression as a result of the limited isolation achieved at this level. The early reports of Creech and his colleagues[327,331,332] stimulated other centres to adopt and develop the technique, and regional perfusion rapidly became an established form of cancer treatment. Experience revealed that limbs could be more effectively isolated than other regions of the body, and that malignant melanoma responded more consistently to this treatment than did other malignancies[332-334]. Modifications were introduced to increase the safety and efficacy of the technique : for example, radioiodine-labelled human serum albumin (RIHSA) was injected into the isolated circulation so that leakage to the systemic blood pool could readily be detected by

monitoring the patient's precordium with a gamma counter; flow rates in the isolated circuit were raised in an attempt to ensure a more uniform distribution of drug to the tissues; 100% oxygen was bubbled into the perfusate (rather than 95% oxygen and 5% carbon dioxide). This was based on the belief that since the biological changes induced by the alkylating agents were very similar to those induced by irradiation[335], and since increased tissue oxygen tension was known to potentiate the effect of irradiation[336-340], one could hope for a similar improvement in response to L-PAM by increasing tissue oxygenation. Indeed, Krementz and Knudson had confirmed this theory in animal experiments using nitrogen mustard to treat mice innoculated with sarcoma 37 or Ehrlich's ascites carcinoma cells[340].

The most significant change in the technique, however, was the addition of **hyperthermia**.

1.4.3 Hyperthermia

The effect of high temperature on tumour growth has been known for more than 100 years, ever since the reports by Busch (1866) and Bruns (1884) of complete tumour regression in two patients with advanced tumours (sarcoma and melanoma) following attacks of erysipelas with a high fever[341,342]. The patients remained well with no evidence of recurrence of their disease for 2 and 8 years respectively. These reports and his own experience of similar events prompted Coley in 1893 to attempt to

reproduce this effect deliberately. He reported 38 cases with histologically proven advanced malignancy who suffered incidental or deliberately induced erysipelas infections with high fevers[343]. 31 patients had shown marked improvement of their tumour, and in particular 12 had shown complete tumour regression. 10 of these had received the therapeutic innoculation, and Coley noted that the two patients in whom the fever had been the highest also enjoyed the longest survival (7 and 27 years). Thereafter came several reports of the use of heat in the treatment of inoperable malignancy of the cervix and uterus[344,345]. In 1953, Nauts and his colleagues published a review of the use of bacterial toxins as described by Coley ("Coley's toxin" being the name given to a combination of filtered extracts of Streptococcus haemolyticus and Serratia marcescens). They highlighted 30 patients with advanced cancer treated in this way of whom 25 were alive and disease-free 10 years later[346]. These observations led the authors to suggest that the increasing incidence of cancer coincident with the advent of aseptic practice and public health might be the result of the sharp decline in incidence of surgical infection and infectious diseases. They did not attribute the tumour responses to the fever but suggested that immunological mechanisms such as circulating inflammatory mediators might be responsible. In fact, a direct inhibitory effect of heat on cell growth in vitro and in vivo had already been reported, the degree of inhibition being related to the temperature and to the duration of exposure[347,348]. Furthermore, later studies demonstrated

that this effect was more promounced in tumour cells than in normal human tissues which are in fact highly resistant to raised temperature[349-351]. Following his in vitro studies, and having shown no ill-effect from hyperthermic perfusion of the hindlimb of dogs at 42-44°C, Cavaliere decided to treat human patients with large recurrent or single metastatic cancers localised to one extremity by isolation perfusion at raised temperatures (>40°C) but without the use of cytotoxic agents[351]. He made two very significant observations :

* 12 of the 21 cases with evaluable tumour at the time of surgery obtained complete regression of their tumour, including 6 of the 8 patients with melanoma.

* the morbidity and mortality of the treatment were very high. Only 8 of the 25 reported cases suffered no serious complication. 6 patients suffered second degree burns and 5 developed serious vascular complications (arterial thrombosis or necrosis) necessitating amputation in 3 cases; 6 patients died within 15 days of the operation including one from "crushed limb syndrome" and consequent renal failure, and one who, due to malfunction of the thermometer, was inadvertently perfused at 49°C and died within 24 hours.

Cavaliere concluded that heat produced a profound damaging effect on tumour cells. He claimed that this effect was selective, although the high morbidity and mortality rates

make this assertion highly debatable. Nevertheless, as a result of this work, many centres combined hyperthermia with the use of cytotoxic agents in isolation perfusion and this immediately created an impression of a greater response rate of tumour to treatment[352-355].

1.4.4 Technique of Isolated Limb Perfusion

The details of the technique of Isolated Limb Perfusion (ILP) vary from centre to centre, both in the surgical method and in the perfusion protocol. While this may be regrettable in that it prevents true standardisation of treatment and thus may limit the value of comparisons of published results, it is an unavoidable feature of such a complex therapeutic modality. In this section, the principal steps of the operation and the main components of the extracorporeal perfusion apparatus will be outlined. A brief description of the various surgical approaches employed will be included together with a general comment on the control of the isolated limb perfusion circuit, highlighting some of the most notable differences. A more detailed account of the surgical and perfusion techniques practised in Glasgow is included in a later section (Section 2.1.2).

Operative steps

The principles of vascular isolation are the same for all regions, although the method may differ slightly. The aim of surgery is to provide vascular access to the desired region of suitable calibre for cannulation, and to abolish all other routes of inflow and outflow of blood from that region. As a general rule, the best access will be offered by the most proximal approach as the vessels will usually be largest and least numerous at this level. These vessels should also provide control of even the most proximal branches of the vascular tree within the perfused region. The important preoperative steps include antiseptic preparation not only of the immediate surgical field but also of the entire region to be perfused, including the site of application of the tourniquet. The prepared area for iliac perfusion must therefore extend proximally to include the perineum and the ipsilateral buttock and lower quadrant of the abdomen; in femoral perfusion, the proximal limit of preparation will be the groin and buttock crease, while in popliteal perfusion it will be at the upper thigh. Preparation for upper limb perfusion includes the shoulder, posterior triangle of the neck, the pectoral and scapular areas, as well as the entire arm and axilla. The limb is then dressed in a light cotton stocking, wrapped in a heated blanket, and covered with sterile drapes leaving access to the operative field and the root of the limb.

The lower limb is most frequently isolated at the level of the external iliac vessels. These are usually approached via an oblique incision in the iliac fossa with retroperitoneal dissection. The branches of the external iliac vessels are all individually ligated. There is some debate over the significance of the internal iliac and obturator vessels in the collateral circulation of the lower limb. Some centres occlude the arterial and/or venous components of one or both of these groups. After systemic heparinisation (with doses of heparin ranging from 100iu kg^{-1} to 300iu kg^{-1}), the external iliac vessels are clamped. An arteriotomy and venotomy are then made and cannulae of suitable calibre are introduced distally into each vessel so that their tips lie below the level of the inguinal ligament. Balanced blood flow through the isolated circuit will usually demand that a wider cannula be placed in the low pressure venous side than in the high pressure arterial side. Once secured in place, the cannulae are connected to the extracorporeal part of the perfusion circuit (see below). A red rubber tourniquet is then placed tightly around the root of the limb to occlude potential subcutaneous and intramuscular collateral channels. This is then secured in place by anchoring it to a Steinmann pin inserted into the blade of the ilium. It is essential to position the tourniquet as high as possible in order to avoid restriction of the blood flow by pressure on or below the tips of the cannulae. At the end of the isolated perfusion, this surgical procedure is reversed by removing the tourniquet and cannulae, repairing the arteriotomy and venotomy, and

removing the temporary clamps on the external iliac vessels and any other vessels so controlled. The systemic heparinisation is then usually reversed using protamine sulphate.

Isolation of the leg at the femoral level is achieved through an infrainguinal incision in the femoral triangle, with direct exposure of the common femoral artery and its bifurcation and of the deep femoral vein. Collateral channels at this level are dealt with by temporary ligature. Secure placement of the cannulae and application of the tourniquet require that the arterial cannula be advanced into the superficial femoral artery. Since the profunda femoris artery is not directly perfused, one must bear in mind that perfusion of the thigh will only result from retrograde flow into the collateral vessels arising from the geniculate anastomosis. As a result, perfusion of the thigh may be incomplete, especially in its proximal part. In the most distal approach to lower limb perfusion, the popliteal vessels are dissected in the upper popliteal fossa through a medial incision in the lower thigh. At this level, there are very rarely any collateral vessels of note. The tourniquet is usually placed just above the incision so that collateral perfusion through the branches of the geniculate anastomosis is confined to the lower thigh only.

Two approaches to perfusion of the <u>upper limb</u> are described. The original reports describe an infraclavicular incision, splitting the fibres of

pectoralis major and exposing the first and second parts of the axillary vessels. Isolation of the upper limb circulation and mobilisation of the vessels require ligation and division of the branches of the artery and vein at this level, often including the thoracoacromial artery. The alternative and now more commonly employed approach is directly through the floor of the axilla displacing the branches of the brachial plexus to expose the third part of the axillary artery and the axillary vein. Cannulation of the vessels is often difficult in the infraclavicular approach due to the confluence of several veins in the axilla, many of which are of rather small calibre. The direct axillary approach circumvents this problem by approaching the vein below this confluence. The method of placing and fixing the tourniquet varies according to the position of the cannulae from being simply wrapped round the top of the arm (cannula tips at the lower deltoid level) to encircling the axilla and acromial part of the shoulder (more proximal cannula placement), being secured in this position with a single Steinmann pin inserted into the head of the humerus or several pins placed through the skin.

The operation is performed under general anaesthesia. While spinal anaesthesia could readily be applied to the operative field for lower limb ILP, the systemic heparinisation of the patient makes this inadvisable.

Extracorporeal circuit

The extracorporeal part of the perfusion circuit comprises a blood warmer and bubble oxygenator in series with a roller pump of the type used in cardiac surgery (Figure 3). The system is primed with whole blood or with a mixture of blood and crystalloid, although the proportion of these may vary from centre to centre. The perfusate in the venous reservoir of the circuit is bubbled through the heater/oxygenator, collected in the arterial reservoir, pumped through the arterial cannula, and, on its return from the patient, drains through the venous cannula back into the venous reservoir. The flow rate through the pump is regulated to maintain a "stable circuit". This may be based on the distribution of perfusate in the reservoirs or on the pressure in the arterial and venous lines. Typical flow rates reported are in the range 300-400ml min^{-1} for lower limbs and 100-200ml min^{-1} for upper limbs. The heat exchanger raises the temperature of the perfusate to the desired level. Most centres practise ILP at temperatures described as "controlled normothermia"(37-38^{o}C) or "mild hyperthermia"(38-41^{o}C), although these temperatures are sometimes exceeded for "true hyperthermia"(up to 43.5^{o}C). The perfusate may be oxygenated with a mixture of 95% oxygen and 5% carbon dioxide or with 100% oxygen (see Section 1.4.2).

Once the circulation in the isolated circuit is established, the completeness of isolation is checked by injecting fluorescein into the perfusate and observing its

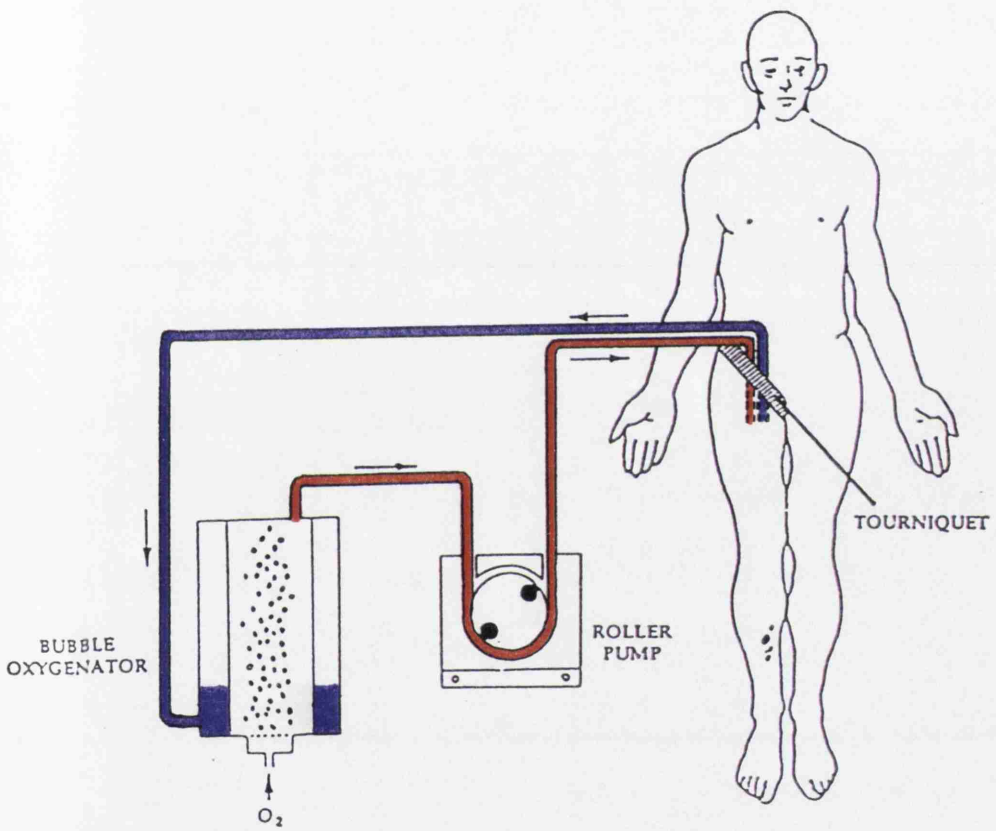


Figure 3 :
Diagrammatic representation of the essential components of the extracorporeal ILP apparatus.

distribution under ultraviolet light. Staining of the skin up to the tourniquet but not above it indicates satisfactory control of the collateral channels. Many ILP centres also add a quantity of radioiodine-labelled human serum albumin (RIHSA) to the perfusate in order to monitor leakage of perfusate to the systemic circulation throughout the procedure with a scintillation counter placed over the patient's precordium. The cytotoxic drug is then added to the perfusate either as a bolus into the venous reservoir or as a slow infusion into the arterial line in order to avoid excessively high peak concentrations of the drug. In Isolated Limb Perfusion for malignant melanoma, the most frequently used drug is **melphalan**, administered according to one of the recognised dosage schedules (see Chapter 4). Other drugs have also been used singly or in combinations including melphalan, and these are discussed in Section 1.4.5. After perfusion of the isolated limb with the drug for one hour, the whole circuit is rinsed with a physiological electrolyte solution which is not recirculated. This removes most of the residual drug from the perfusate so that the amount of cytotoxic released into the systemic circulation after reversal of isolation is minimised.

1.4.5 Results of Isolated Limb Perfusion

For several reasons, the results of Isolated Limb Perfusion for melanoma remain poorly defined. The original report of the technique was accompanied by a

description of an unprecedented response of advanced melanoma to chemotherapeutic agents[327]. In the decade which followed, several reports were published of larger series of patients treated by this modality, and although the results were less dramatic they remained impressive[356-358]. As a consequence of the high response rates of such an aggressive disease to isolation perfusion, the use of this operation spread very quickly in the treatment of all stages of melanoma without the clear definition of indications. Published results are therefore obscured by the grouping together of patients with varying stages of melanoma and varying tumour burden, poor reporting of the other surgical procedures associated with the perfusion, and incomplete information on the prognostic factors relevant to the patients (in particular, the significance of depth of invasion had not yet been shown by Clark[163] or Breslow[164]). Furthermore, no prospective controlled series were reported and results were generally compared to historical controls from the literature. These failings were all identified in a comprehensive review of the published results of ILP in 1979 [359]. Since then, many more authors have published their results but while they have tried to correct most of these failings, there is still very little information from prospective, properly controlled (ideally, randomised) trials.

The effects of cancer treatments are usually assessed in three ways :

[i] the **response** of visible (directly or indirectly using imaging techniques) tumour deposits to the treatment

[ii] where tumour is not visible, the duration of **control of the disease** (disease-free survival)

[iii] the **survival** of patients after treatment (overall survival).

Following isolated limb perfusion for locally recurrent or regionally confined metastatic melanoma (**Therapeutic ILP**), all three of these observations may be possible, while only the latter two are relevant in the treatment of primary (Stage I) melanoma (**Adjuvant ILP**). In this Section, the question of visible response of tumour will be addressed first, followed by consideration of the reported survival figures (disease-free and overall) achieved by therapeutic and adjuvant ILP respectively.

Objective response rate

In the early experience with isolated limb perfusion, typical response rates of visible tumour were in the region of 35-50% [146,357,360]. Little attention had been paid to the effect of temperature on the treatment at that time. In the last 15 years, however, most centres have performed isolated limb perfusion with varying degrees of hyperthermia (controlled normothermia, mild hyperthermia, true hyperthermia) and results have usually

shown response rates in excess of 70% [361-369]. The extent and duration of responses vary, and in some reports no comment is made on these aspects of the treatment. In an attempt to make the assessment of outcome more objective, a two-tier classification has been proposed and this system has now been adopted by the WHO (World Health Organisation) and the UICC (Union Internationale Contre le Cancer) to describe responses to cancer treatment[370,371] :

- COMPLETE RESPONSE (CR) - disappearance of all tumour.

- PARTIAL RESPONSE (PR) - decrease of ≥50% in measurable tumour deposits and improvement of non-measurable deposits, in the absence of progression of other lesions or development of new lesions.

Problems remain in defining just which parameter should be measured in the assessment of 50% reduction and in how long the response should be maintained[372] but, for the present, the general rule is that cross-sectional area of tumour deposits should be measured and that responses should be maintained for a minimum of four weeks.

The response rates reported using this system over the last decade are shown in Table 6. Most recent reports consistently quote overall response rates (CR + PR) of 80% or higher, and this is far in excess of the objective response rates achieved with any other modality (see Section 1.3.2). The duration of these responses is in

Table 6 :

Objective Response Rates of Melanoma to ILP with Melphalan

Author	Year	No. of patients	Response(n) Complete (CR)	Partial (PR)	Overall Response rate %
Lejeune[362]	1983	23	15	6	91
Jonsson[363]	1983	15	1	10	73
Vaglini[364]	1985	32	18	8	81
Storm[365]	1985	26	21	0	81
Minor[373]	1985	22	18	4	100
Cavaliere[366]	1987	72	26	43	96
Kroon[367]	1987	18	7	8	83
Santinami[368]	1989	56*	26*	27*	94
Skene[369]	1990	67	_**	_**	78

Notes:
CR and PR as defined by UICC (see Section 1.4.5)
* Numbers calculated from percentages quoted in text
** not stated

most cases not stated, and most authors report subsequent recurrence or progression of disease in 30-40% of patients showing an initial complete or partial response[362,369,374]. Two studies do give specific details of the recurrences[365,367]. In the first, regional control of the disease was maintained in 76% of the responders until the death of the patient (n=13; median time 15 months) or until the time of writing (n=3; mean follow-up = 6^{+} years). The other 24% of responders developed regional recurrences at follow-up times of 3-14 months. In the second study, 2 of the 7 patients who initially benefitted from a complete response to treatment later developed regional recurrences (after 10 and 28 months). Regional control of the disease was maintained to a mean follow-up time of 34.5 months in 4 patients, and until death from systemic disease in the other (17 months). These data confirm the general impression that most recurrences occur within the first 12-18 months after the initial response while at the same time highlighting the poverty of hard facts.

Survival after Therapeutic ILP

Many authors have claimed that isolation perfusion could significantly prolong the disease-free survival of patients with regionally confined recurrent or advanced melanoma. Almost without exception, they base their claims on comparisons with historical controls. In a large series published in 1972, Krementz, one of the

originators of the technique, reported disease-free survival rates of 42% after 5 years and 26% after 10 years[375]. The results were particularly impressive for patients with a solitary metastasis (77%). Hansson and his colleagues in 1977 reported a significant increase in the time to development of recurrences after limb perfusion in 16 patients compared with the pattern of their disease prior to perfusion[360]. Their series was very small, but they included a review of the literature and calculated that ILP offered a 5-year disease-free survival rate of 37%. This presented a distinct advantage over surgical excision, with or without lymph node dissection (21%) and limb amputation (24%). A paper from the Westminster Hospital in 1980 stated that regional disease control was maintained until the time of reporting or until death in 50% of patients, but it did not include information on the actual follow-up time[361]. A later publication from the same centre quoted regional disease control in 80% of patients after a median follow-up period of 55 months[369]. However, only 39% of patients remained free of systemic disease at that time; this is more in keeping with the generally quoted post-perfusion 5-year disease-free survival rates of 30-40% [360,375,376].

Only one prospective randomised trial of therapeutic ILP for locally or regionally recurrent or advanced melanoma has been reported to date[377-379]. An improvement of around 40% in the projected 5-year disease-free survival was demonstrated after limb perfusion, but unfortunately the study coordinators chose to terminate the trial early because of this difference. At termination of the

study, only 34 and 36 patients had been recruited to the perfusion and control arms respectively (divided into Stages II, IIIA, IIIB and IIIAB), and the trial has therefore been widely criticised for its small number of patients. Nevertheless, it remains the only trial of its kind so far, and while it does not provide conclusive evidence of survival benefit, it at least gives more weight to the claims of the earlier authors.

Most studies which report <u>overall survival rates</u> are afflicted by the same failings as the above; namely, they group together patients with different stages of disease and are often based on small numbers of patients. As a result, 5-year survival rates ranging from 28% to 74% are reported[368,369,374,376,380-384]. Breakdown of the patients according to stage (MDAH system) is only possible in a small number of these studies[380,383,384] but suggests that a 5-year survival rate of around 60-70% can be expected for Stage II, 30-50% for Stage IIIA, and 30-40% for Stage IIIAB. In fact, Krementz quotes 10-year survival rates 59%, 23% and 28% respectively for each of these stages[383]. The 5-year survival rates for patients with locoregionally advanced or recurrent melanoma who do not undergo isolated limb perfusion are in the range 14-39% (see Section 1.2.4) and it is therefore still questionable whether isolated limb perfusion confers any real survival benefit on this group of patients.

In summary, therapeutic isolated limb perfusion with melphalan is usually associated with a very high response rate of tumour to the treatment. The disease-free interval seems to be prolonged after this treatment,

although the effect on overall survival is still not certain. It is recognised that melanoma, whilst showing signs of local progression, can often remain confined to one region or extremity for long periods[373,385]. This group of patients should therefore benefit most from this type of treatment. In addition, isolated limb perfusion can offer useful palliation by destroying or shrinking large tumour deposits which would otherwise require mutilating surgery for their removal, and by relieving the pain which they may cause.

Survival after Adjuvant ILP

In view of the dramatic objective responses and prolonged disease-free interval which are observed after therapeutic ILP, one might expect that the disease-free interval interval should also be prolonged in patients with Stage I melanoma. In fact the evidence for this is very inconclusive, mainly due to the lack of reported studies with appropriate control populations on which to base comparisons of results or with details of the prognostic features of the patients treated.

The early evidence suggested a survival advantage to patients treated by isolated limb perfusion[353,357,386,387] although most of the patients included in these studies were treated before the prognostic significance of tumour thickness and level of invasion had been fully recognised. Later studies also showed a survival advantage of around 20% for patients with Stage I melanoma treated by

adjuvant ILP compared with non-randomised contemporary controls[388] or with the statistics quoted in the contemporary literature for patients treated by conventional surgery[383,389,390]. However, histological review of the patients in these studies showed that 37-50% of the melanomas were less than 1.5mm thick (Breslow), and the impressive survival figures may therefore be partly explained by the large proportion of the population with these good prognosis tumours.

In the last ten years, four very significant studies into the value of adjuvant isolated limb perfusion have been reported. In the first of these, patients undergoing ILP in Groningen (the Netherlands) were compared retrospectively to patients undergoing conventional surgery in Sydney (Australia), matched for sex and site of the primary tumour[355]. Females with melanomas of the lower leg formed the only group with sufficiently large numbers; the analysis was therefore restricted to this group and took into account most of the other major prognostic factors (tumour thickness, level of invasion, ulceration, site). The study unveiled a statistically significant advantage to the perfused patients in terms of survival (overall and disease-free) and regional disease control. Surprisingly, however, when the same group of investigators compared a larger population of perfused patients from Groningen (including those from the first study) with non-perfused controls from the Netherlands and neighbouring Westphalia (Germany), they found no statistically significant diference in the outcome of the two groups[391]. It was therefore suggested that the

differences observed in the first study might have been due to geographically-related factors such as skin complexion and sun exposure. This is challenged, however, by the findings of a third study performed at the M.D.Anderson Cancer Center (Texas, USA) which compared 151 patients treated by wide local excision of the primary melanoma and adjuvant isolated limb perfusion with 151 patients from the University of Alabama (USA) and the University of Sydney (Australia), individually matched for prognostic factors, treated by wide excision alone[392]. Melphalan was used in 85 perfusions (56%) and imidazole carboxamide in the other 66 (44%). Although the disease-free and overall survival rates were similar for perfused and non-perfused patients, those patients treated with melphalan showed a tendency towards better survival rates than those treated with imidazole carboxamide or not perfused which did not reach statistical significance. Subset analysis, however, revealed that perfusion with melphalan resulted in a statistically significant improvement in disease-free survival (70% v 42%) and overall survival (84% v 43%) for patients with primary melanomas thicker than 2mm. This difference was not apparent in the patients perfused with imidazole carboxamide who had similar survival rates to those treated by wide excision alone. The authors concluded that adjuvant isolated limb perfusion with melphalan conferred a real advantage on this subset of patients which could not be explained by geographical factors since, in effect, the imidazole-treated group served as an internal control population. It is interesting to note

that the survival rates after ILP with melphalan are similar in all three of these studies. The different conclusions are explained by the apparently better survival of non-perfused patients in the Netherlands and Westphalia (73%) compared with those in Alabama and Sydney (42-46%).

The only published prospective randomised trial of isolated limb perfusion for melanoma[377-379] included 37 patients with Stage I melanoma thicker than 1.5mm (Breslow) and more deeply invasive than Clark level III. A significant advantage was found for 19 patients treated by wide local excision, regional lymph node dissection and ILP over 18 control patients treated by wide local excision and node dissection alone. As noted earlier, this study has been criticised for its premature termination when only a small number of patients had been entered. In addition, there is very litle information on how closely the two groups of patients were matched, in particular for tumour thickness. The authors' only comment is that the distribution of patients into cohorts by tumour thickness (1.5-3.0mm, or >3,0mm) was comparable in the perfused and non-perfused groups[379,393], but this statement refers to all 107 patients and not to the subsets by stage of disease. This may be a vital factor since a disproportionate number of thin or thick lesions in one or other group might prejudice the results of the study.

Controversy remains over the value of adjuvant ILP for melanoma. While the studies described do suggest that a particular subset of patients might derive some benefit from this treatment, the results are anything but conclusive. A large multicentre prospective randomised study of adjuvant ILP with melphalan for primary melanoma thicker than 1.5mm (Breslow) is currently being conducted by the WHO and EORTC (European Organisation for Research into the Treatment of Cancer) [394]. Patients have been randomised on the basis of sex, age, tumour site, ulceration, level of invasion and Breslow thickness. In addition they have been randomised within the study group of each participating institution and a Quality Control Programme has been established in order to obviate the bias inherent in so many multicentre studies. Entry to the study has recently closed with approximately 800 patients recruited from centres in Europe, Australia and the USA. The value of such a large study is that it will allow subset analysis and should hopefully provide an answer to the question of whether adjuvant ILP is of benefit in Stage I melanoma, and, if so, to which patients. The results must be awaited with interest.

Other cytotoxic agents used in ILP for melanoma

Several other drugs have been used in isolated limb perfusion for melanoma. The response rates achieved by some of these drugs are shown in Table 7. On the whole, none of these agents, either alone or in combination with

melphalan or other drugs, has produced better response rates than melphalan alone. One study showed a 100% response rate to perfusion with the combination of vindesine, DTIC and cisplatinum, but this consisted entirely of partial responses with no observed complete responses[399]. Another paper reporting the use of DTIC claims a complete response rate of 100%, but it would appear from the text that visible tumour was excised at the time of perfusion[402]. If this is the case, then the UICC criteria for complete response cannot have been met and the results are not comparable. In the last few years, there has been a growing interest in the use of recombinant Tumour Necrosis Factor alpha (rTNFa) against tumours. This is thought to act by causing endothelial cell activation and vascular damage, possibly mediated by the release of elastase from neutrophils, resulting in haemorrhagic necrosis especially in intradermal tumour deposits. Lejeune and his colleagues have combined melphalan perfusion with administration of high dose rTNFa and recombinant gamma interferon on 31 occasions[403]. They have reported spectacular results with 90% complete response and 10% partial response rates, most responses being apparent after only a few days and being maintained for a minimum of 2 months. The complications of the treatment in this series were considerable, including two arterial thromboses, both requiring amputation, and two deep venous thromboses. Systemic side-effects include haematological toxicity in 52% of patients and an isolated serum bilirubin elevation in 33%. Furthermore, in pilot studies of perfusion with rTNFa alone for melanomas,

Table 7 :

Objective Response Rates of Melanoma to ILP with Agents Other than Melphalan

Author	Drugs	No. of patients	CR	PR	Overall Response rate %
Cox[395]	thiotepa melphalan	28	10	0	36
Golomb[396]	thiotepa actinomycin D melphalan	54	7	32	75
Aigner[397]	dacarbazine	4	1	0	25
Aigner[398]	cisplatinum actinomycin D	4	0	2	50
Aigner[398]	cisplatinum	12	2	4	50
Aigner[399]	vindesine dacarbazine cisplatinum	14	0	14	100
Shiu[400]	nitrogen mustard	19	6	6	63
Vaglini[401]	dacarbazine	24	3	7	42
(Pfefferkorn [402]	dacarbazine	15	15	0	100)*
Lienard[403]	rTNFa# rIFNgamma$ melphalan	29	26	3	100
Vaglini[404]	rTNFa# rIFNgamma$ melphalan	12	7	0	64**

Notes:
CR and PR as defined by UICC (see Section 1.4.5)
* Definition of complete response suspect
** 1 patient not evaluable
# rTNFa : recombinant tumour necrosis factor alpha
$ rIFNgamma : recombinant gamma interferon

sarcomas and carcinomas of the extremities, the authors had observed a "septicaemic shock-like" phenomenon on release of the tourniquet at the end of perfusion which proved fatal on two occasions[403]. In their larger study, they administered prophylactic treatment in the form of dopamine and fluid pre-loading to all patients in order to avoid this problem. This improved but did not completely abolish the haemodynamic instability observed in the pilot study. Other authors have since confirmed the high response rate but have also found toxicity a major problem[404]. These are early results from small series of patients. The impressive response rates must be weighed against the very high morbidity so far encountered.

Since universally high response rates are currently reported with melphalan, it would seem that other, as yet less effective, agents should probably be reserved for second-line treatment of melanoma resistant to, or recurrent after, ILP with melphalan.

1.4.6 Complications of Isolated Limb Perfusion

Isolated Limb perfusion is a complex procedure and can be associated with serious complications. These can be divided into three categories :

[i] complications of surgery - general or specific to this operation

[ii] complications in the perfused limb attributable to the cytotoxic treatment

[iii] systemic complications of the cytotoxic treatment.

[i] Complications of surgery

In addition to the general complications of surgery and general anaesthesia (wound infection, atelectasis), certain aspects of this operation may give rise to specific complications. Arterial and venous thrombosis have both been reported and this has resulted in limb amputations, especially in the early years of isolated limb perfusion[352,357,375]. It is possible that some cases of limb ischaemia have resulted from a toxic effect on the vascular endothelium rather than surgical trauma. In recent years, the need for amputation of limbs has become much less frequent with improved surgical technique, but it remains a very serious potential complication of this treatment. The systemic heparinisation can result in haemorrhage during and after the procedure. Restoration of the normal circulation at the end of the period of isolated perfusion can result in hypotension, sometimes profound, as the patient's blood is redistributed to the isolated extremity. For this reason, many centres routinely monitor central venous pressure during ILP,

although this situation can usually be avoided by adequate "pre-loading" of the patient's circulation with colloid solution or transfused blood.

[ii] Limb complications due to cytotoxicity

The effects of cytotoxic perfusion on the limb are well documented and all reports comment on oedema, erythema and sometimes blistering of the skin which tend to resolve after 2 weeks to 3 months, leaving a bronze discolouration of the skin which can be permanent. The cytotoxicity is not confined to the skin and subcutaneous tissues, however, and problems of nerve and muscle weakness are also reported together with several cases of peripheral neuritis. Some reports attribute the nerve problems to the pressure of the tourniquet[352], but since the incidence and severity of all of these complications increases with the temperature of the perfused limb[352,368,381], it is likely that a direct toxic effect is also involved. In some instances, tissue oedema has resulted in compartment syndromes, especially of the lower leg, requiring fasciotomy. In fact Schraffordt Koops recommends prophylactic fasciotomy for all lower limb perfusions[405], although most centres find this unnecessary. Skin burns have resulted from direct application of the heated blanket and it is now recommended that the limb be first wrapped in a thin protective bandage to reduce this risk[352]. The burns can be severe and in one reported case have necessitated amputation of the limb[389].

Table 8 :

Grading of Toxicity in the Perfused Limb after Isolated Limb Perfusion with Melphalan*

Grade	Description
I	No subjective or objective evidence of reaction.
II	Slight erythema or oedema.
III	Considerable erythema and/or oedema with some blistering; slightly disturbed motility permissible.
IV	Extensive epidermolysis and/or obvious damage to deep tissues, causing definite functional disturbance; threatened or manifest compartment syndrome.
V	Reaction which may necessitate limb amputation.

* Grading System according to Wieberdink[406].

It is generally held that a degree of local toxicity is unavoidable if an adequate cytotoxic effect is to be obtained. Wieberdink has described a classification of the toxic reactions seen in the normal tissues of the limb according to five grades of increasing severity[406]. This classification is shown in Table 8. Grades II and III are generally considered acceptable, while Grade I is taken to represent a relatively inadequate treatment. Grades IV and V describe excessive reactions and measures should be taken to avoid these if possible.

[iii] Systemic complications due to cytotoxicity

Myelosuppression due to leakage of melphalan into the systemic circulation is currently reported in about 5% of cases. Although initially there was a tendency to perfuse regions which could be only partially isolated, in modern practice the technique is usually only applied to limbs or regions whose isolation can be virtually complete - iliac, femoral, popliteal, axillary, and, more recently, hepatic. As a result, the problem is now encountered much less frequently than before. Similarly, alopecia has been reported but is a rare feature of the treatment.

During the circulation through the perfusion circuit, many blood cells haemolyse, possibly as a result of heat or the mechanical trauma of the pump. Some of this haemolysed blood may therefore be released into the systemic circulation at the time of restoration of the normal circulation to the limb. Current techniques of

rinsing the limb vasculature prior to reconnection should reduce the problems (eg. renal) which this might cause.

2 CHAPTER 2 - ISOLATED LIMB PERFUSION IN GLASGOW

2.1 Isolated Limb Perfusion in Glasgow

2.1.1 Introduction

Isolated Limb Perfusion for melanoma of the extremities has been performed in Gartnavel General Hospital since August 1984. This practice was set up at the instigation of the Scottish Melanoma Group (SMG) and the Scottish Home Office. Gartnavel General Hospital remains the only centre in Scotland to offer this form of treatment, and patients are consequently referred for ILP from the whole of Scotland and from further afield. The indications for ILP in this centre are:

[1] **primary melanoma (Stage I) of a limb with a Breslow thickness of $\geq$1.5mm**

[2] **advanced or metastatic melanoma confined to the limb of the original primary lesion**

[3] **palliative treatment of malignant melanoma of the limbs,** even in the presence of systemic metastases.

In this chapter, a detailed description of the ILP protocol and surgical technique employed in Glasgow is given, together with an account of the observed results and complications.

The role of ILP in the treatment of regionally advanced or recurrent melanoma is well established (see Section 1.4.5) but its role in the adjuvant treatment of primary melanoma is still unproven. A case-control study of this aspect of ILP is presented (Section 2.2.3).

2.1.2 ILP Technique

The technique originally employed in this centre was based on the reports of Krementz[354,375] and Schraffordt Koops[389,407] and on personal observation of their method. With increasing experience of this operation, and as a result of communication with the principal advocates of ILP, in Europe and in the USA, this technique has gradually evolved but is still based on the same fundamental principles.

Preoperative preparation

The suitability of patients for treatment is initially determined by confirming the histological diagnosis of malignant melanoma and the Breslow thickness of primary lesions, and by assessing the extent of disease and general health of the patient with a thorough clinical examination, chest radiograph, electrocardiogram and routine haematological and biochemical screens. This is followed, in those patients considered fit for treatment, by computed axial tomography (CAT) scan of the brain, thorax, abdomen and pelvis to exclude systemic spread of

the disease as far as possible. Having established the indication for ILP according to the three categories listed above (see Section 2.1.1), the treatment is then discussed with the patient so that fully informed consent for surgery can be given. The patient's blood is cross-matched so that packed red blood cells can be included in the priming fluid (see below) and in readiness for the intra- or postoperative need for blood transfusion.

On the morning of surgery, a foil "space blanket" (Universal Hospital Supplies Ltd) is wrapped around both legs (in lower limb perfusion) or the chest and affected arm one hour before transfer to the operating department in order to maintain the temperature of the limb.

Operative technique

Isolated limb perfusion is performed under general anaesthesia. Antibiotic prophylaxis in the form of Cefuroxime 1.5g IV (Zinacef, Glaxo Laboratories Ltd) is administered at induction of anaesthesia. A 20G teflon radial artery cannula (Viggo-Spectramed, UK) is then inserted and connected to a monitor (Model 1281, Siemens) to allow continuous direct monitoring of the patient's blood pressure throughout the procedure. The entire limb to be perfused is then washed with antiseptic solution and this process is extended proximally to include the area of the surgical isolation and tourniquet application. This demands that the patient be rolled onto his/her side to

allow the perineum, buttock and lower lumbar skin to be washed for perfusion at the iliac level. Preparation for upper limb perfusion includes washing the posterior triangle of the neck and the scapular and pectoral areas, as well as the shoulder and axilla. Sterile drapes are placed under the buttocks or shoulder before rolling the patient back into the supine position. The limb is kept elevated while drapes are placed round the surgical field. After securing thermistor probes on the skin, a loose stockingette is applied to the limb. This prevents direct contact between the skin and the heated water blanket (38-38.5^{o}C) which is next wrapped around the limb. This is now also covered with sterile drapes in such a way as to allow manipulation of the limb by the operators during the procedure.

Most isolated limb perfusions in this centre are performed at the external iliac or axillary level.

For lower limb perfusions, the external iliac vessels are approached via an oblique incision placed 2 cm above the lateral half of the inguinal ligament. The external oblique fibres are split, the internal oblique and transversus abdominis muscles are cut, and the peritoneum is then reflected medially to allow extraperitoneal exposure of the vessels. These are fully mobilised from the bifurcation of the common iliac vessels down to the inguinal ligament, ligating and dividing each branch or tributary individually and exposing the internal iliac vessels. The iliac lymph nodes are dissected during this

process and are sent for histological examination to assist in the staging of disease. The obturator vessels are identified on the lateral wall of the pelvis and are ligated in continuity. Heparin (150 i.u. kg^{-1}) is then administered intravenously and allowed to circulate for 2-3 minutes before applying arterial clamps to the external iliac artery and vein. The internal iliac vessels are also occluded using bulldog clamps. A longitudinal arteriotomy and venotomy are performed and the arterial and venous cannulae (William Harvey Arterial Perfusion Cannula, Bard; whistle-tip atrial cannula, Rusch UK Ltd) are inserted distally so that their tips lie below the level of application of the tourniquet. The cannulae are secured by doubly snaring them in the vessel using cotton tapes. The size of cannula is selected according to the calibre of the vessels. For iliac perfusion, 14-18FG arterial cannulae and 4-6mm venous cannulae are usually required. To complete the isolation of the limb, a tight red rubber tourniquet is applied as high as possible around the root of the limb. This lies in the perineal skin crease medially, includes the lower portion of the buttock posteriorly, passes laterally between the iliac crest and the greater trochanter, and is secured anteriorly around a Steinmann pin inserted into the anterior superior iliac spine through the lateral end of the skin incision. This configuration ensures that the entire femoral triangle is included in the perfused region and that the tip of the arterial cannula can lie in the common femoral vessel without being compressed by the tourniquet, thereby usually allowing perfusion of both the

superficial femoral and profunda femoris arteries. The tourniquet exerts substantial downward traction on the Steinmann pin, and this must therefore be firmly fixed in the pelvis to avoid loosening of the tourniquet and loss of isolation. The cannulae are then connected to the extracorporeal circuit (described below).

Upper limb perfusion is performed through the **axillary vessels.** These are approached directly through the floor of the axilla with the draped arm abducted and the scapula supported on a small sandbag to expose the axillary contents better. A transverse incision is made and the third part of the axillary vessels are exposed by separating the branches of the brachial plexus. Usually, two or three veins are present at this level. The principal vessel is preserved for cannulation and the remainder are ligated as are any tributaries in this area. The artery is mobilised sufficiently to allow safe manipulation and cannulation, and this usually necessitates the ligation and division of only one or two branches. Full heparinisation is used as in lower limb perfusion and vascular clamps are then applied. A proximal longitudinal arteriotomy and venotomy are made and the vessels are typically cannulated using 10-14FG arterial and 14-16FG venous cannulae (William Harvey Arterial Perfusion Cannula, Bard). Cotton tapes are used to snare the cannulae in place, introducing them only as far into the vessel as is necessary to ensure that all the side-holes are distal to the snares. The tourniquet is placed proximal to the level of cannulation and is wrapped

tightly round the shoulder, being maintained on the acromion process by a Steimann pin inserted axially into the head of the humerus.

In this centre, a standard extracorporeal circuit is used for all limb perfusions (Limb Perfusion Set, Model S6830C, Bard). This consists of arterial and venous tubing connected to a Harvey bubble oxygenator (Model H1700). The oxygenator is combined with a heat exchanger which functions by circulating hot water, pumped by an adjustable heater-cooler (Normo-hypothermia module, Cobe), in close proximity to the blood. The arterial tubing is threaded through a simple roller pump (Multiflow Bloodpump, Stockert Instrumente) which has a flow rate control dial and continuous display flow meter. A bubble-trap manometer is incorporated into the arterial line distal to the pump. The extracorporeal circuit is primed with a mixture of 750ml Hartmann's solution and 350ml matched packed red cells to which are added 3000i.u. of heparin. This is thoroughly mixed and warmed by circulation in the extracorporeal circuit for 15-20 minutes prior to connection of the arterial and venous tubing to the vascular cannulae. Once connected, the isolated perfusion circuit is established. The blood is oxygenated throughout the procedure with 100% oxygen at 0.5-1.0 l min^{-1}. Satisfactory isolation is confirmed by adding 5ml of 20% fluorescein (Martindale Pharmaceuticals Ltd) to the perfusate and demonstrating the absence of fluorescence in the skin above the level of the tourniquet when examined under ultraviolet light. In the course of

the studies described in this thesis, the control of the perfusion circuit in this centre has been modified (see Chapter 3). Originally, the flow rate was adjusted to achieve and maintain a stable distribution of perfusate between the oxygenator and the patient. The normal practice is now to adjust the flow rate until effective cutaneous perfusion is demonstrated by a rising transcutaneous oxygen tension ($PtcO_2$) on the perfused limb. Once this is achieved and the skin temperature exceeds 37°C, melphalan is added to the perfusate by bolus injection into the venous compartment of the oxygenator. The dose regularly employed for iliac perfusion is 1.75mg kg^{-1} body weight and for axillary perfusion 40 to 60mg, depending on the patient's morphology.

Perfusion is continued for one hour after the addition of the melphalan. At the end of this hour, the perfusate is replaced with 2 litres of Hartmann's solution (or 1 litre for upper limb ILP) which drains into the oxygenator reservoir after rinsing the limb and is not recirculated. The cannulae are then disconnected from the extracorporeal circuit and the tourniquet is released. After removing the snares, the cannulae are withdrawn from the vessels which are then repaired using a continuous polypropylene suture. The arterial and venous clamps can then be released together with any temporary clamps on collateral vessels and the systemic circulation to the limb restored. The heparinisation is reversed at this stage using an appropriate dose of protamine sulphate, and the wound is closed, suturing the muscular and aponeurotic layers separately.

Fasciotomy is not performed as a prophylactic measure in this centre and has so far never been necessary for declared or impending compartment syndromes.

Postoperative care

Postoperatively, the patients are reviewed on a daily basis to observe any toxic reactions and to monitor the response to treatment. Early (day 1-2) mobilisation is encouraged. Full haematological and biochemical screens are performed on alternate days and blood transfusion administered if required. Patients are detained in hospital until the observed toxicity (clinical, biochemical, and haematological) has passed its peak and mobility is satisfactory. Following discharge from hospital, patients are reviewed on a monthly basis until complete resolution of all toxicity is observed, and thereafter three-monthly for the first 2 years, and 6 monthly for the next three years.

2.1.3 General Observations

Between August 1, 1984 and October 31, 1990, 140 patients underwent 162 Isolated Limb Perfusions for histologically proven malignant melanoma. The patients were referred for adjuvant treatment of a primary melanoma on 66 occasions. The remainder (96 cases) had locoregionally advanced or recurrent melanoma at the time of treatment. The sex

ratio was 2.7:1 (f:m) which is similar to the sex ratio of melanoma at diagnosis in Scotland[29]. Patients were aged between 16 and 86 years with a mean of 54.9 years (SD=15.8). Patients were referred for treatment from all over Scotland (133 patients), from 2 centres in England (5 patients) and from Ireland (2 patients). The referral pattern of Scottish patients according to their local Health Board is shown in Table 9.

Isolated Limb Perfusion was performed at the iliac level on 121 occasions. Other lower limb perfusions were performed at the femoral (5 cases) and popliteal (10 cases) levels. The upper limb was isolated at the axillary level on 22 occasions and at the subclavian level on 4 occasions. The subclavian approach presented technical difficulties in cannulation of the axillary vein on 3 occasions. This approach has now been abandoned in favour of the direct axillary approach. A technique of mild hyperthermia is routinely employed, and in this series the mean perfusion temperature (measured on the skin surface) was 38.3^{o}C (SD=1.2).

Post-operative hospital stay varied widely, especially during the initial experience of ILP. The median post-operative stay was 10 days (range 5-42) following lower limb perfusion and 7 days (range 4-22) following upper limb perfusion.

Table 9 :

Referral Pattern of Scottish Patients for ILP for Melanoma

Health Board	No. of Patients Adjuvant	Therapeutic (*)	Total
Greater Glasgow	30	23 (11)	53
Ayrshire & Arran	15	5	20
Argyll & Clyde	10	9 (3)	19
Lothian	1	9 (3)	10
Forth Valley	2	5	7
Lanarkshire	2	4 (2)	6
Dumfries & Galloway	3	2 (1)	5
Highland	3	2	5
Fife	0	4	4
Tayside	0	2	2
Grampian	0	2	2
Total	66	67 (20)	133

* no. of patients undergoing repeat ILP in brackets.

2.1.4 Complications

The complications encountered following 162 isolated perfusions are summarised in Table 10.

[1] surgical complications

This procedure is associated with a risk of similar complications to those seen after many other major elective operations. 2 mild wound infections have been observed (ie. cellulitis only), both of which resolved with oral antibiotic therapy. 2 patients developed early postoperative haematomas which required exploration and ligation of a small branch of the external iliac artery. A third patient developed a wound haematoma 24 hours after a popliteal perfusion; this was treated conservatively. One case of lymphocoele developing at the site of the external iliac dissection has been documented. This was only diagnosed on CAT scanning two years after treatment during routine follow-up assessment.

The most serious complication observed in this series was pulmonary thromboembolism (3 patients). In 2 cases, this was associated with clinical signs of deep venous thrombosis in the perfused limb, confirmed radiologically. The third case presented two weeks after discharge from hospital (22 days post-ILP). In this case, no source of embolism was identified. One further patient developed an uncomplicated deep venous thrombosis.

Table 10 :

Complications Encountered in 162 ILPs

Surgical		
	Wound infection	2
	Deep venous thrombosis(DVT)	1
	DVT & pulmonary thromboembolism(PTE)	2
	PTE (no evidence of DVT)	1
	Post-operative bleeding (re-exploration)	2
	Wound haematoma	1
	Lymphocoele (late)	1
	Hepatitis B	1
Systemic		
	Alopecia	2
	Myelosuppression	16
	leukopenia - 12	
	leukopenia & anaemia - 1	
	leukopenia & thrombocytopenia - 1	
	pancytopenia - 2	
	Angina/postural hypotension	1
	Postoperative confusion	1
Regional		
	Postoperative neuromuscular disability	8
	Elevated serum transaminases/creatine kinase	3
	Ulceration of pre-existent skin graft	4
	Failure of new skin graft	3
	Flexion contracture (elbow)	1
	Cutaneous burns	1

One patient is known to have suffered an attack of hepatitis-B eight weeks after ILP. This is presumed to have been transmitted by post-operative blood transfusion. The patient has acquired immunity to the virus and is not believed to be a carrier of the $HepB_sAg$.

Two other patients suffered from post-operative complications thought not to be specifically related to the isolated perfusion: one elderly patient who had suffered a myocardial infarction a few months earlier complained of angina during the first 24 hours after the operation and of postural hypotension for a few days thereafter; serial electrocardiograms and cardiac enzyme measurements showed no evidence of acute myocardial infarction. The other patient became very confused and disorientated for three days; he was later found to have cerebral metastases and died within 2 months of treatment.

[2] systemic complications of ILP

The most common systemic side-effect of isolated limb perfusion has been nausea. This occurs in most patients, noticeably more often than after other major vascular procedures, and is mainly restricted to the first 12 hours after operation. The cause of this is not clear but it is likely that the sustained release into the circulation of even low concentrations of melphalan from the perfused limb is a major contributor.

A more serious effect is the myelosuppression which has been noticed in around 10% of cases. This mostly takes the form of a mild leukopenia (white cell count 1.0 - 3.0 x 10^9/L) but has been associated with anaemia in one case (Hb 5.6 g/dl) and thrombocytopenia in another (platelet count 39 x 10^9/L). Furthermore, two cases of pancytopenia have been observed (case 1 : Hb 8.6 g/dl, WCC 1.0 x 10^9/L, platelets 23 x 10^9/L; case 2 : Hb 9.8g/dl, WCC 0.6 x 10^9/L, platelets 12 x 10^9/L). While these effects have often resulted in a longer stay in hospital, they have all resolved without complication.

Two patients have reported a mild alopecia which developed about three weeks after treatment. One of these had also been noticed to have a mild leukopenia. Both cases resolved completely within three months.

[3] regional complications of ILP

Since a degree of toxicity to the tissues of the perfused limb is a desirable feature of this treatment, this is described separately in Section 4.1.5. In eight cases, however, the patients' post-operative recovery has been complicated by excessive toxicity, resulting in temporary muscle weakness and peripheral neurological deficits. This was associated with markedly elevated serum transaminases and creatine kinase in three cases, indicating severe muscle damage. As a result of this, one patient developed a flexion contracture of the elbow and another showed obvious atrophy of the thigh and calf

muscles. Three patients complained of sensory disturbances attributable to one peripheral nerve, and while compression by the tourniquet may have been a contributory factor, the pattern of the combined sensory and motor loss in one patient indicated an ulnar nerve lesion in the forearm. In this patient at least, the symptoms were probably due to a direct neurotoxic effect. The relative contributions of melphalan and hyperthermia to these complications is unclear. Fortunately, full function was restored in all cases within 4 months of treatment.

Many patients undergoing ILP had previously been treated by wide excision of primary and/or recurrent melanomas and application of skin grafts. In four cases, the skin grafts ulcerated after perfusion. These all healed satisfactorily without the need for further grafting.

One patient sustained first degree cutaneous burns due to overheating of the water blanket when the thermistor probes failed.

2.1.5 Regional Toxicity

The toxic effect of isolated limb perfusion on the normal tissues of the limb is well recognised. A degree of erythema and oedema are common but in some cases this is very marked and can be associated with blistering of the skin. The grading system devised by Wieberdink[406] (see Table 8) is now widely used to classify the observed toxicity in each case. Although initially in this centre

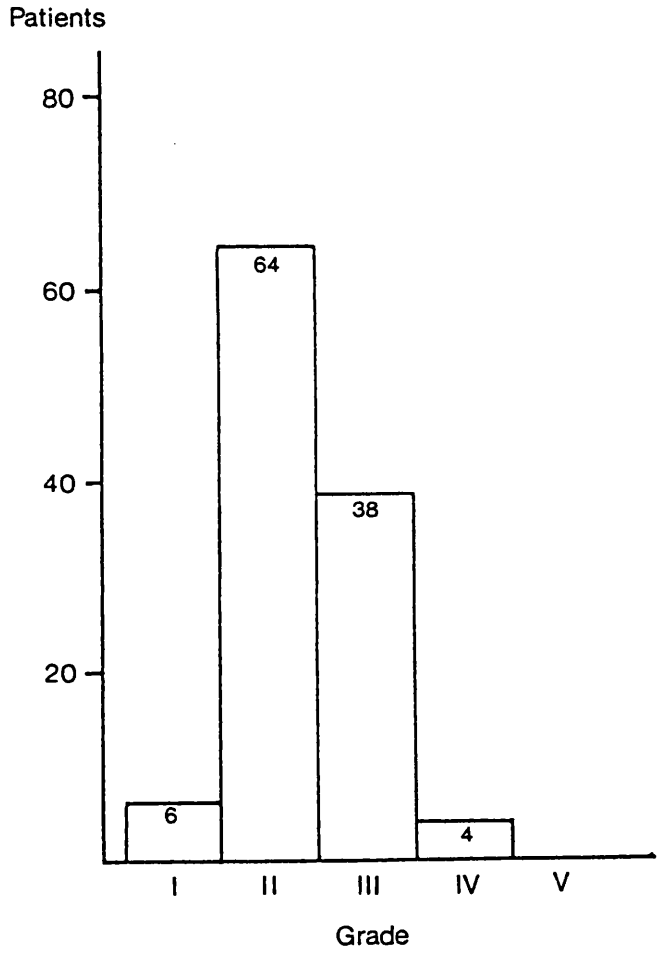
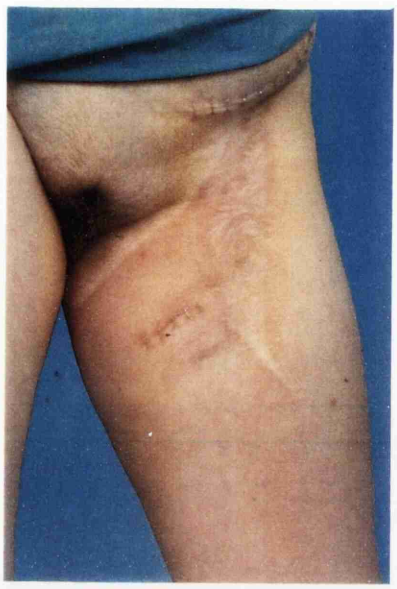
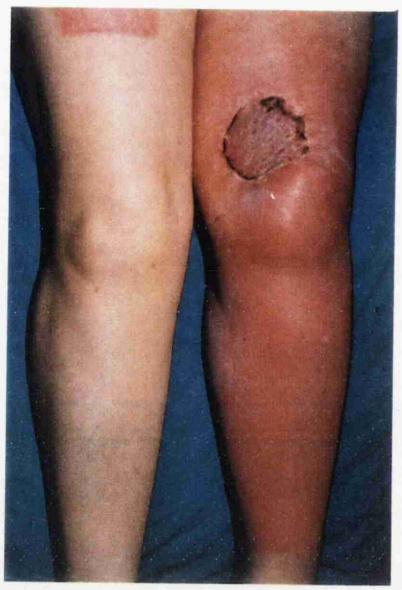


Figure 4 :
Toxic reactions registered after 112 ILPs.

(Wieberdink grading system[406], see Table 8)



a.



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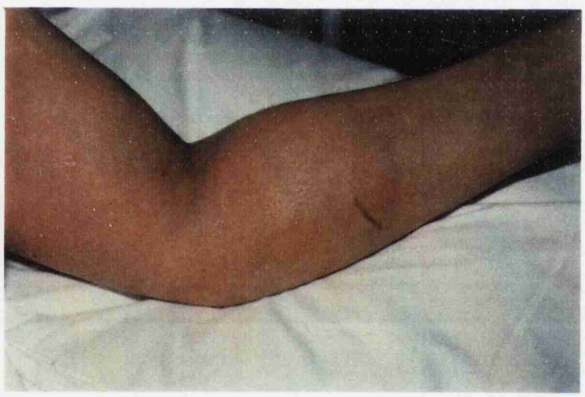


c. (arm)

Figure 5 :
Examples of the regional toxicity encountered after ILP.

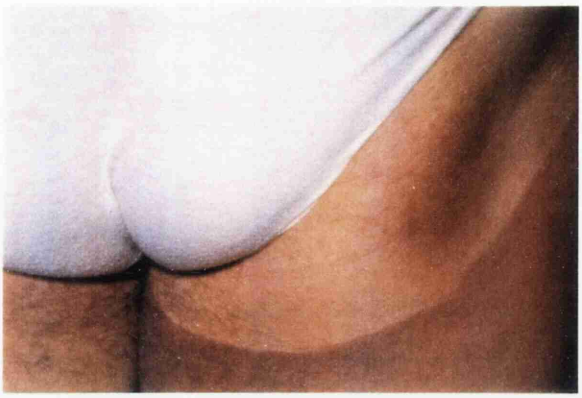
(a) Grade II (b) Grade III (c) Grade IV.

(cases 1-65) only the most severe toxic reactions were documented, the peak toxicity is now routinely recorded in every case. The observed reactions in 112 cases classified according to Wieberdink are shown in Figure 4. Photographic examples of these reactions are shown in Fig 5.

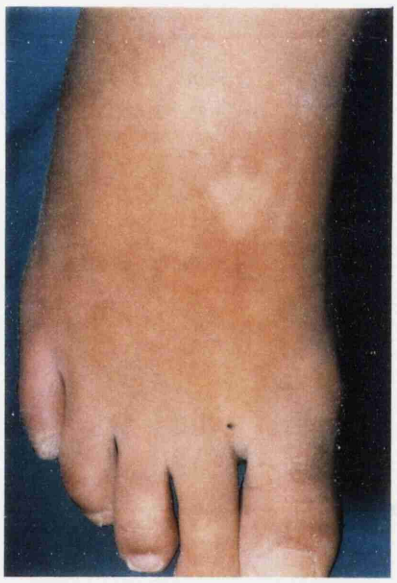
More than half of the patients treated in Glasgow have experienced Grade II reactions, and more than 90% fall into the "desirable" range of toxic reactions (Grades II and III). Grade IV reactions have only been recorded on four occasions, equivalent to 2.5% overall, since failure to record the reaction in 50 of the first 65 patients implies that it was less severe than Grade IV in all of these (see above). These four patients all had muscle weakness in the perfused limb and are included in the group described above (Section 2.1.4).

The relative contributions of melphalan and hyperthermia to the observed toxicity are not clear. Each is capable of independently inducing the typical tissue reactions. Furthermore, it is possible that ischaemia occurring during the period of dissection and cannulation or during the isolated perfusion itself may contribute to this, either directly or by sensitizing the tissues to the effects of the melphalan and heat. No direct correlation has been demonstrated between the grade of reaction and the skin temperature during ILP, but all four cases which resulted in Grade IV reactions were associated with temperatures in excess of 38.8^{o}C. Based on this observation, a maximum skin temperature during ILP of 39^{o}C is now imposed in this centre.

The sequence of erythema and oedema followed by a prolonged or permanent bronze discolouration of the skin would appear to be specific to treatment with melphalan. This is demonstrated by the clinical photographs in Figure 6 which clearly show the "tanning" reaction of the perfused region. The area of nonpigmented skin on the dorsum of the foot corresponds exactly to the outline of the dorsalis pedis artery cannula. This must have been caused by compression of the skin at that point by the firmly secured cannula so that, although the temperature could be raised by transfer of heat from the surrounding skin and the heated blanket, no melphalan could reach this skin.



a.



b.

Figure 6 :
Pigmentation of the skin after ILP with melphalan.

The very clear cut-off in the gluteal fold (a) corresponds to the level of the tourniquet. In (b), the non-pigmented silhouette of the dorsalis pedis arterial cannula is easily recognised.

2.2 Adjuvant ILP

2.2.1 Patient details

Since January 1985, 66 patients have undergone Isolated Limb Perfusion in Glasgow as adjuvant treatment of Stage I melanoma. One further patient, originally included in this group, was found to have iliac lymph node involvement at the time of ILP. Accordingly, her staging has been amended to Stage IIIB, and she is now considered in the group of patients undergoing "therapeutic" ILP for regionally advanced or metastatic melanoma.

The demographic details of this group of patients are shown in Table 11 together with details of their primary tumours. Two thirds of patients were female, this sex difference being almost entirely due to the preponderance of lower leg lesions in women. The patients' mean age was 52.11 years. The primary tumours were evenly distributed into the intermediate (Breslow < 3.0 mm) and poor (Breslow $\geq$ 3.0mm) prognostic categories, the median Breslow thickness being 2.98mm. Three patients referred for adjuvant ILP had primary melanomas less than 1.5mm thick. Although they did not meet the normal inclusion criteria for this treatment (see Section 2.1.1), ILP was performed at their own request. They are included in this description of results but, in view of the better prognosis associated with their primary disease, they have been omitted from the later analysis of survival and disease-free survival after adjuvant ILP (Section 2.2.3).

Table 11 :
Details of Patients Treated by Adjuvant ILP

		Female	Male	Overall
Number		46	20	66
Age	mean	53.6	48.6	52.1
	range	(17-77)	(16-81)	(16-81)
Thickness (Breslow)	mean	3.37	3.06	3.28
	range	(1.3-7.5)	(0.9-5.0)	(0.9-7.5)
Site				
Upper limb	arm	7	1	8
	forearm	3	4	7
	hand	3	0	3
	palm	0	0	0
	subungual	0	4	4
	total	13	9	22
Lower limb	thigh	3	4	7
	leg	23	3	26
	foot	4	3	7
	sole	2	0	2
	subungual	1	1	2
	total	33	11	44

Initially, most patients were referred for adjuvant ILP after the definitive surgical treatment of their primary melanoma. However, in order to avoid any delay in treatment caused by the time taken for skin grafts to heal, referring surgeons and dermatologists have been encouraged through the Scottish Melanoma Group to refer patients for treatment as soon as possible after their diagnostic biopsy. Consequently, wide excisional surgery of the primary site has been performed at the time of ILP on 25 occasions (upper limb 6, lower limb 19). This was done at the end of the procedure, once the systemic heparinisation had been reversed, and consisted of a 3cm lateral clearance of the primary lesion, excising all the tissue down to, but not including, the deep fascia. Primary closure of the defect was possible in 6 cases (upper limb 5, lower limb 1); in the remainder, split-thickness skin grafts were applied. The skin grafts were all harvested from the contralateral thigh, fenestrated and secured with sutures or staples. All but three of the skin grafts resulted in a greater than 50% take, even in the presence of Grade III or IV toxic reactions of the surrounding perfused skin (see Figure 5).

2.2.2 Results

All patients are followed up regularly after ILP in Glasgow. Because of the great distances involved in travelling from their towns of origin, some of the

patients elect to attend their local dermatologist or surgeon, and follow-up data are collected by regular correspondence for these patients. All other patients are seen at Gartnavel General Hospital by a member of the perfusion team. In this way, no patients have been lost to follow-up after adjuvant ILP.

Disease control and survival

Of the 66 patients in this group, 17 have suffered recurrences of their melanoma. The site of first recurrence for these patients is shown in Table 12, together with the timing of the recurrences and their outcome. Almost all the recurrences have taken the form of regional lymph node or systemic metastases, or a combination of the two. Only two patients have developed recurrences in the perfused limb, and indeed one of these also had lymph node involvement at that time.

All patients with systemic metastases have since died of their disease, as have 3 patients whose initial recurrence was in the regional lymph nodes. The mean time to first recurrence for these 10 patients was 14.9 months (range 3-32 months) and their mean survival time was 26.1 months (range 6-51 months). One patient who developed lymph node metastases 26 months after ILP died of other causes 17 months later, but the other 6 patients remain alive at a mean follow-up of 35.8 months (range 26-53 months). The mean disease-free survival for these patients was 22.2 months (range 8-34 months).

Table 12 :

Site and Time of First Recurrence after 66 Adjuvant ILPs

Site	Limb		Total	Time (months)	Outcome
	Upper	Lower			
Limb	0	1	1	15	alive
LN	2	6	8	16,27,33,34 7,10,30 26	alive dead (melanoma) dead (other)
Limb & LN	0	1	1	8	alive
Systemic	2	2	4	3,7,18,32	dead
Systemic & LN	0	3	3	3,8,31	dead

LN - regional lymph node

At present, 48 patients remain alive with no evidence of recurrent disease 47.1 months (mean; range 25-97) after ILP. 10 patients have died of metastatic melanoma. One patient with lymph node metastases and another with no evidence of recurrent melanoma have died of other causes at 42 and 18 months respectively. Six patients are still alive with recurrences of their melanoma 35.8 months (mean; range 26-53 months) after ILP.

2.2.3 Adjuvant ILP - a Case-Control Study

Adjuvant isolated limb perfusion for melanoma remains of unproven value, mainly due to the lack of randomized controlled trials (see Section 1.4.5). In Glasgow, it has proved impracticable to randomize patients, often referred from distant centres, into a trial of adjuvant ILP. One such trial was initiated by the Medical Research Council but closed prematurely due to poor patient accrual. Since then, the policy in this centre has been to consider all patients with primary melanomas $\geq$1.5mm thick for adjuvant ILP. The patients treated here form a group in whom a standard technique has been employed by a single perfusion team. This therefore avoids some of the variability which is inherent in most multicentre studies.

For the last fifteen years, the Scottish Melanoma Group (SMG) has been collecting clinical and pathological data prospectively on all melanomas diagnosed in Scotland, and a very large and detailed database has now been established.

In order to study the results of adjuvant ILP in Gartnavel General Hospital, a closely matched population of non-perfused control patients was selected from the SMG database.

Matching criteria

In order to exclude the bias created by the selection of certain patients for Adjuvant ILP in the years since 1985, controls were picked from the SMG database relating to the five-year period immediately preceding this (1980-1984). Only patients with primary melanomas $\geq$1.5mm thick were included in this study. Patients were matched on the basis of site (excluding laterality) and Breslow thickness of the primary tumour. Site matching was absolute (using the SMG 3-digit site code, but grouping the dorsum of the foot and hand with the lower leg and forearm respectively) and the closest thickness match was selected within this classification. Sex, level of invasion (Clark), and age were defined as secondary criteria where two or more equally matched controls were identified on the basis of site and thickness.

A description of the two matched populations is shown in Table 13. The thickness of lesions was very closely matched, with differences in the range -0.6 to +0.55 mm (mean=0.00mm, SD=0.16). This is demonstrated graphically as the plot of the difference in thickness (patient - control) in Figure 7. The greatest differences occurred

Table 13 :

Details of Matched Populations of ILP Patients and SMG Controls

		ILP	SMG
Number		63	63
Sex ratio (m:f)		19:44	12:51
		matched: 42/63	
Age	mean(SD)	52.0(17.2)	55.5(16.4)
	range	16-81	18-93
	median	52.0	58.0
Thickness (Breslow)	mean(SD)	3.37(1.59)	3.37(1.61)
	range	1.5-7.5	1.5-8.0
	median	3.00	3.00

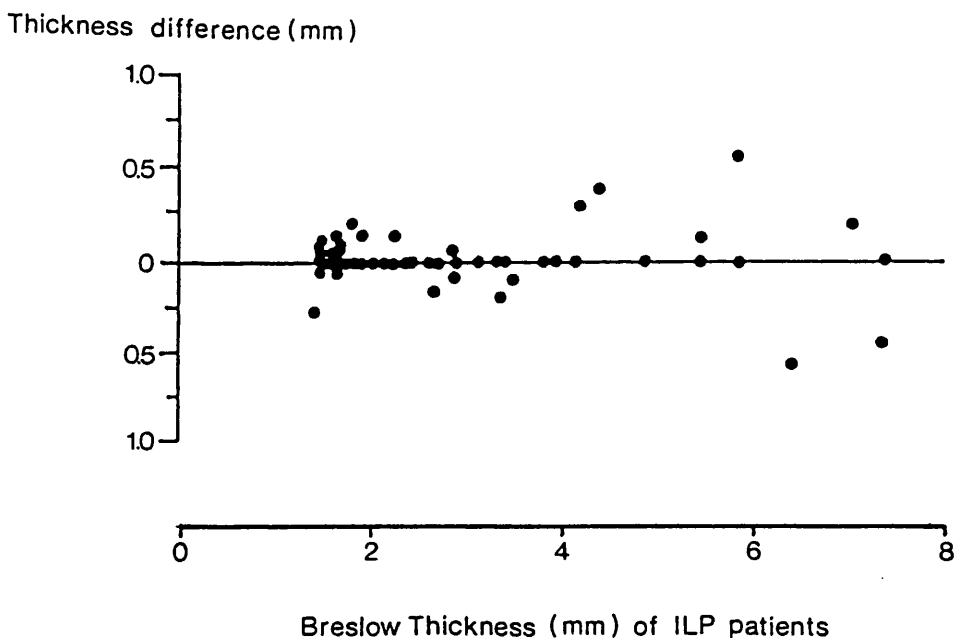


Figure 7 :
Graphic representation of the matching of ILP patients and their SMG controls by Breslow thickness.

The difference between each pair of subjects (ILP-SMG) is plotted against the thickness of the ILP patient's melanoma.

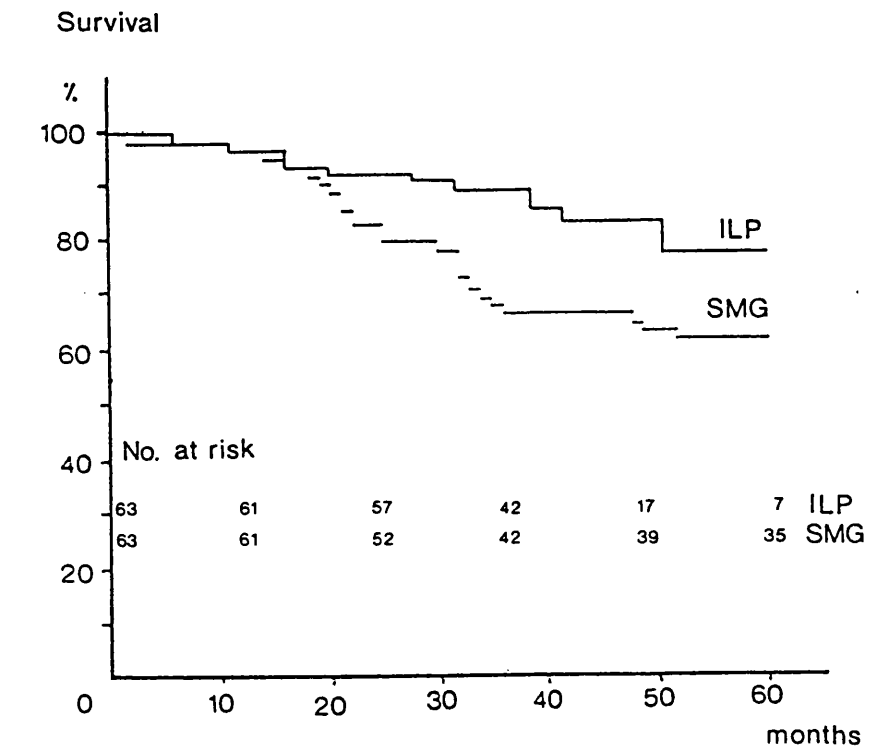
in the matching of patients with the thickest lesions and in whom this degree of mismatching would be least significant.

Up-to-date follow-up data were obtained for all patients and controls up to 31st October 1993. Details of disease recurrence and death (including cause of death) were recorded. These results were analysed by constructing life tables for each of the two groups of patients[408] which were then compared using the "logrank" test[408] and a proportional hazards model. More detailed analysis of the results was performed by match analysis using first recurrence of melanoma and death caused by melanoma as the end points of the study.

Results

No patients were lost to follow-up. In all, 7 patients are known to have died of causes other than melanoma (ILP 2, Control 5). These patients are included in the analysis up to the date of their death.

Figure 8 shows (a) the survival and (b) the disease-free survival curves drawn from the life table analysis of both groups of patients. Separation of the survival curves for the two groups can be seen in both plots with apparent survival and disease-free survival benefits to the ILP group of approximately 20% and 15% respectively at 36 months. Comparison of the curves using the logrank test



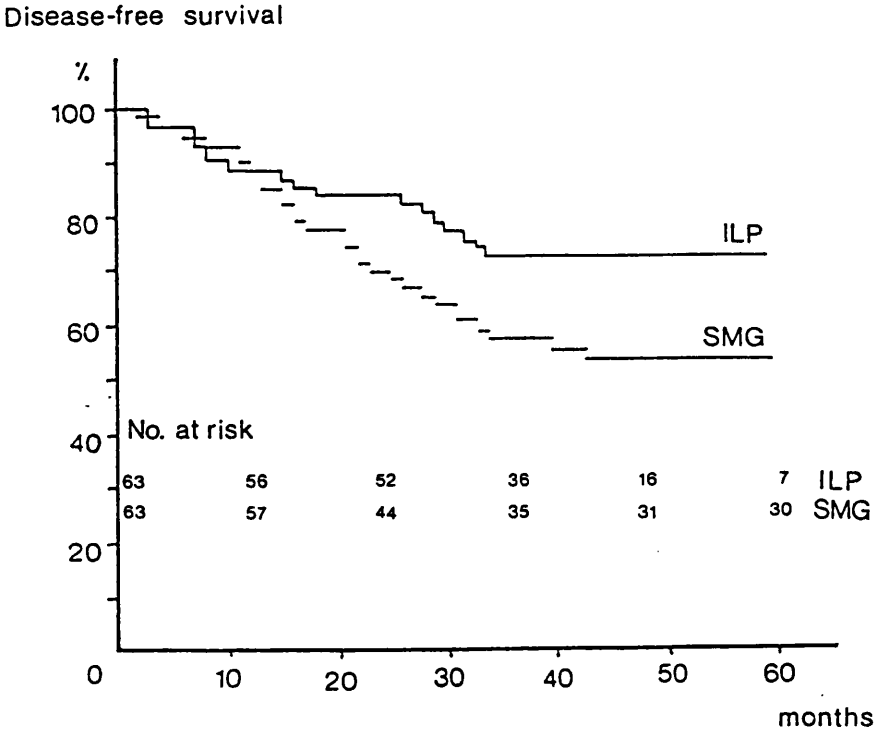


Figure 8 :
(a) Survival and (b) disease-free survival curves for 63 patients undergoing Adjuvant ILP and their matched controls from the SMG register.

revealed a statistically significant advantage to the ILP group in terms of both survival ($p = 0.029$) and disease-free survival ($p = 0.042$) (see Table 14).

The patients were divided into subgroups according to (1) the region perfused ([i] upper limb or [ii] lower limb) and (2) the thickness of the lesion ([iii] Breslow <3.0mm or [iv] Breslow $\geq$3.0mm). The survival curves for each of these subgroups are plotted in Figures 9(i)-9(iv) and the results of the analysis by subgroup are also shown in Table 14. The most significant improvements in both survival and disease-free survival after ILP were for the subgroups of patients with lesions $\geq$3.0mm thick or with upper limb melanomas, although the differences did not achieve statistical significance at the 5% level.

The "relative risk" of survival or disease-free survival up to three years after ILP, calculated using a proportional hazards model (ie. relative risk = x, where post-ILP survival = control survival^x), was slightly greater than 1 for all groups and subgroups but was greatest for the thicker melanomas (survival 1.12, disease-free survival 1.12) and for disease-free survival after upper limb ILP (1.10).

Match analysis revealed that twice as many ILP patients outlived their controls as did the reverse in terms of both survival and disease-free survival (18:9 and 22:13 respectively), although these differences did not achieve statistical significance at the 5% level ($p = 0.083$ and $p = 0.129$ respectively).

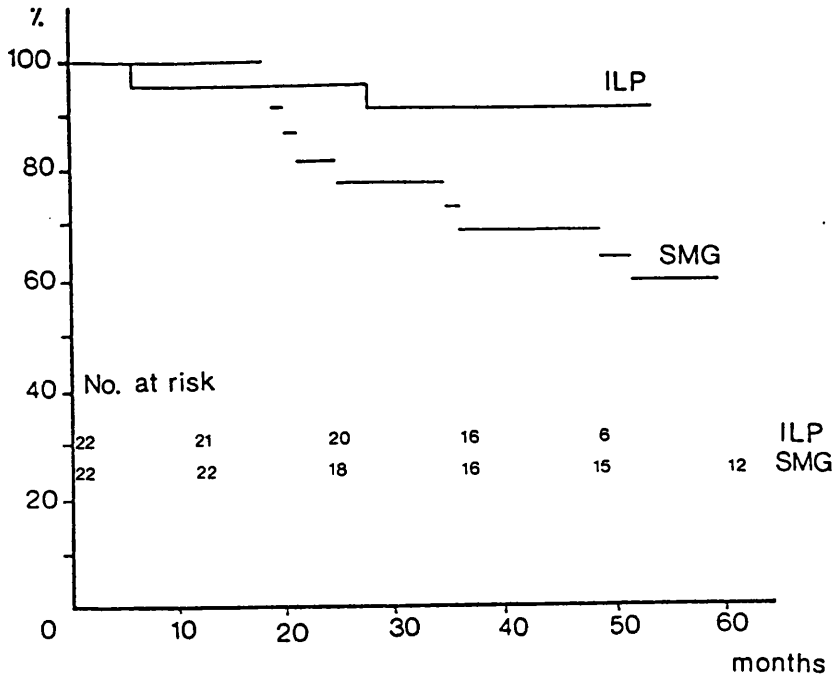
Table 14 :

Analysis of Survival and Disease-free Survival for ILP Patients and SMG Controls

		% Survival (3-year)	Relative* Risk	Logrank test Obs.	Exp.	X^2	p
SURVIVAL							
Overall	ILP	88.6	1.07	10	16.53	4.77	.029
	SMG	66.4		26	19.47		
Upper limb	ILP	90.7	1.07	2	4.95	3.21	.073
	SMG	68.2		9	6.05		
Lower limb	ILP	87.6	1.07	8	11.62	2.10	.147
	SMG	65.4		17	13.38		
lesions < 3mm	ILP	89.7	1.03	3	5.06	1.45	.229
	SMG	79.3		9	6.94		
lesions ≥ 3mm	ILP	87.9	1.12	7	11.50	3.38	.066
	SMG	55.2		17	12.50		
DISEASE-FREE SURVIVAL							
Overall	ILP	72.3	1.06	17	24.17	4.12	.042
	SMG	56.9		33	25.83		
Upper limb	ILP	81.3	1.10	4	7.36	3.23	.073
	SMG	54.6		10	6.64		
Lower limb	ILP	67.7	1.04	13	17.00	1.78	.182
	SMG	58.1		23	19.00		
lesions < 3mm	ILP	82.9	1.02	5	6.71	.79	.374
	SMG	75.9		10	8.29		
lesions ≥ 3mm	ILP	62.5	1.12	12	17.60	3.59	.058
	SMG	40.4		23	17.40		

* relative risk = x, where
ILP survival = $(\text{SMG survival})^x$

Survival



Disease-free survival

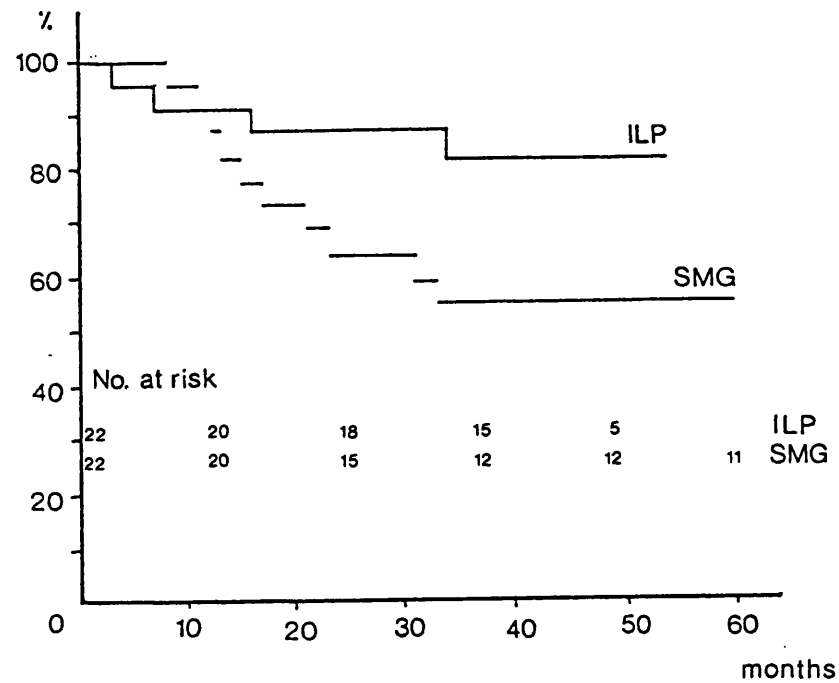
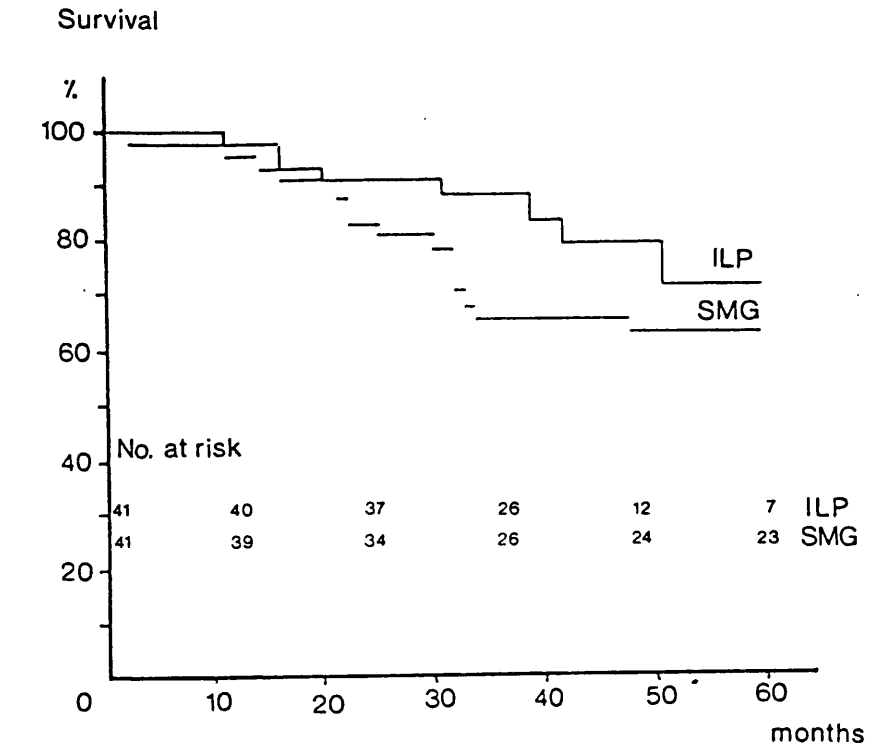


Figure 9(i) :
(a) Survival and (b) disease-free survival curves for 22 patients with upper limb lesions undergoing Adjuvant ILP and their matched controls from the SMG register.



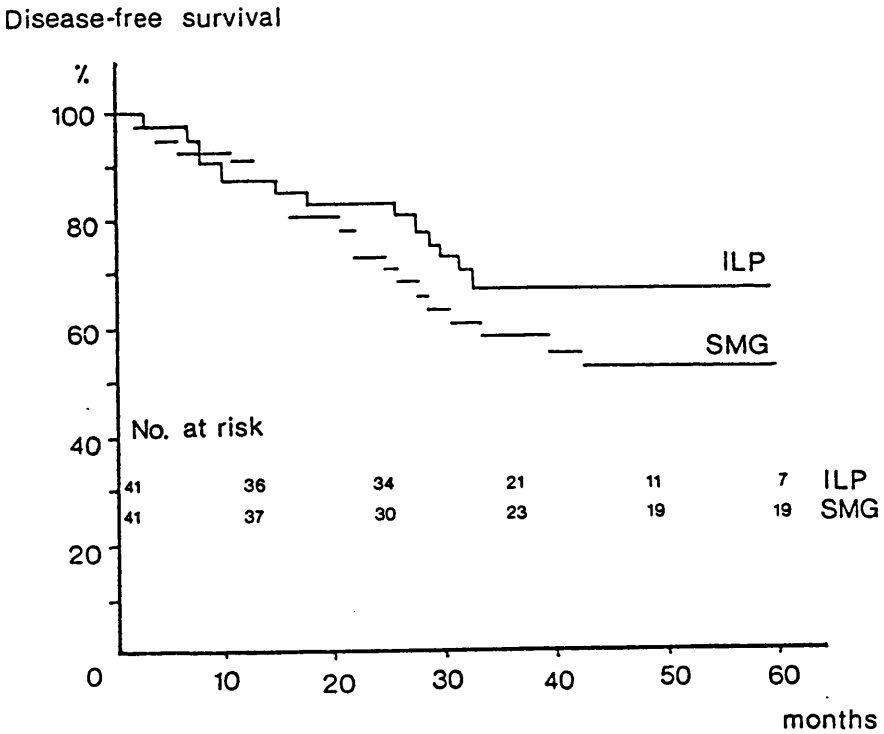
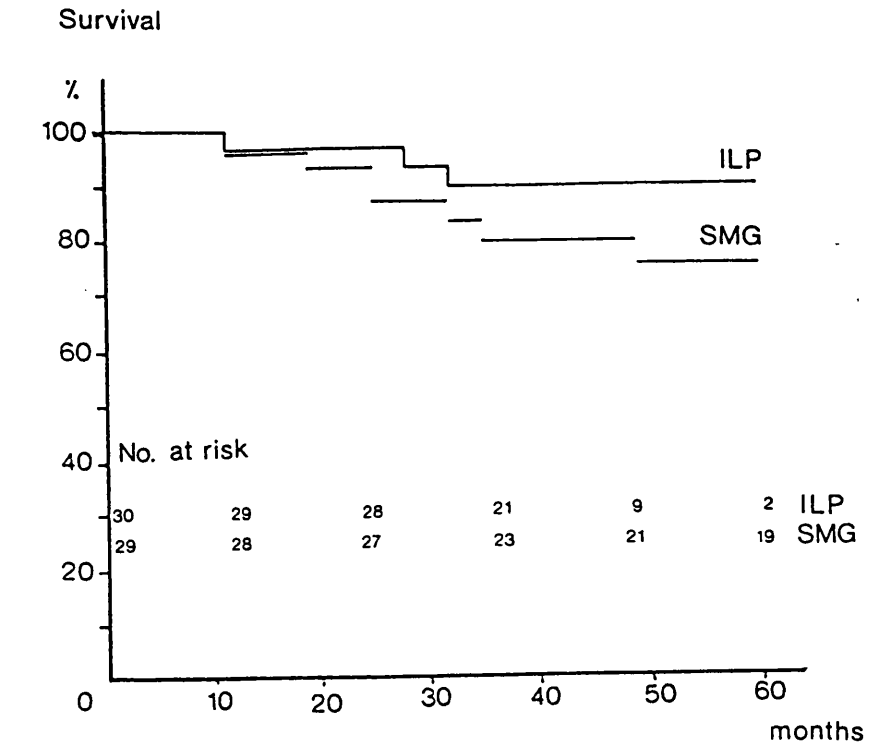


Figure 9(ii) :
(a) Survival and (b) disease-free survival curves for 41 patients with lower limb lesions undergoing Adjuvant ILP and their matched controls from the SMG register.



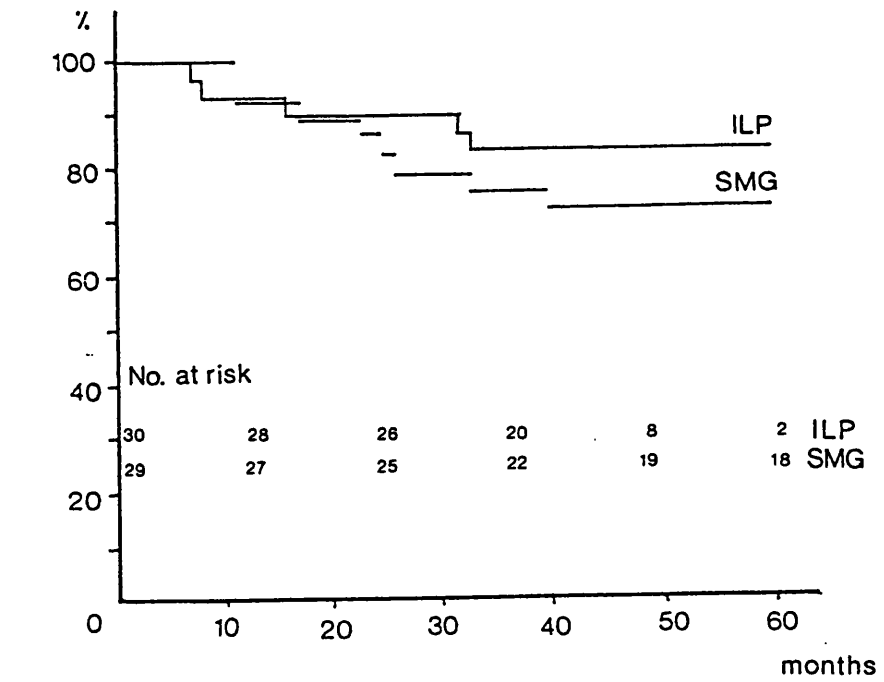
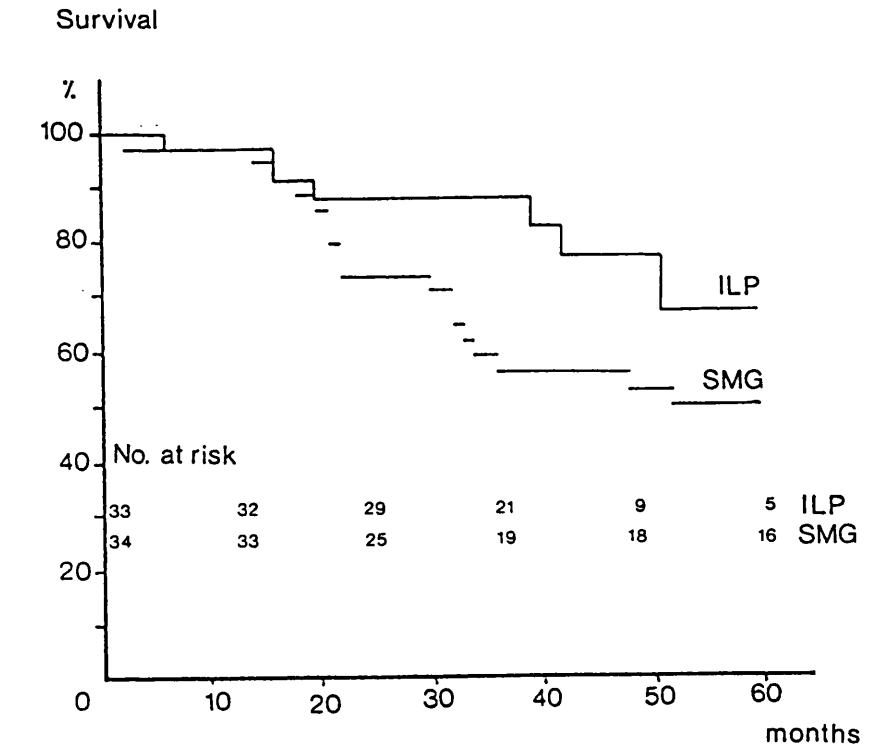


Figure 9(iii) :
(a) Survival and (b) disease-free survival curves for 30 patients with lesions less than 3.0mm thick undergoing Adjuvant ILP and 29 controls from the SMG register.



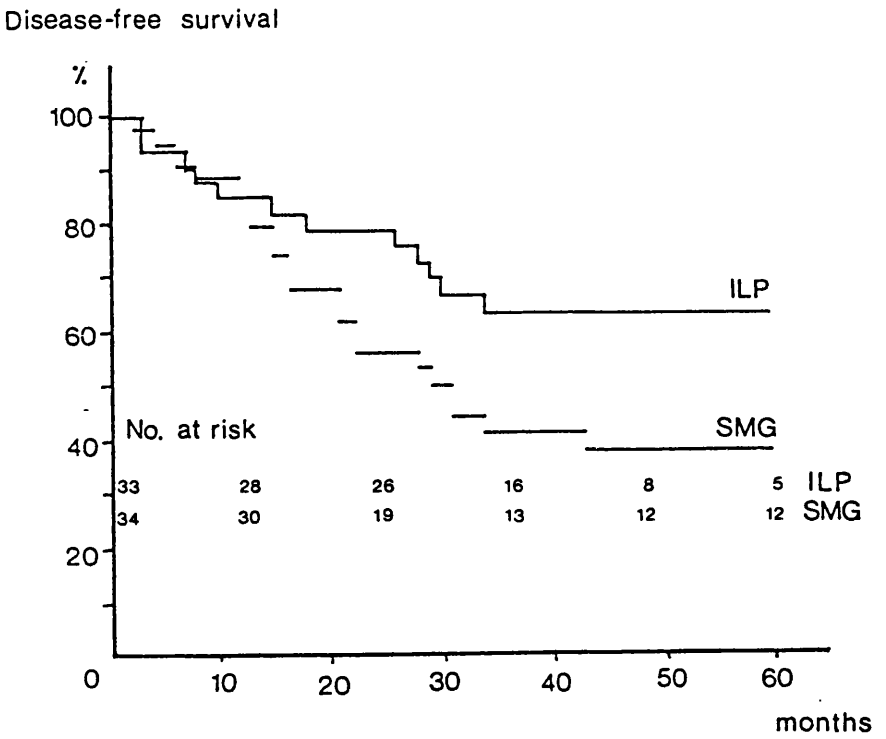


Figure 9(iv) :
(a) Survival and (b) disease-free survival curves for 33 patients with lesions more than 2.99mm thick undergoing Adjuvant ILP and 34 controls from the SMG register.

Discussion

This study has compared the outcomes of two closely matched groups of patients undergoing **wide excisional surgery alone** or **wide excisional surgery plus adjuvant Isolated Limb Perfusion** for Stage I melanoma of the limbs (Breslow thickness ≥1.50mm). The mean difference in Breslow thickness between the ILP patients and their non-ILP controls was 0.00mm (SD. 0.16mm), and although the range was from -0.6mm to 0.55mm, the greatest differences occurred in the group of patients with the thickest melanomas. Thus the ratio of the Breslow thicknesses for each matched pair (ILP/control) had a mean of 1.0 (SD. 0.04) with a range of 0.83 to 1.12.

The analysis of the results of this study consistently shows a survival and disease-free survival benefit to the group of patients treated with adjuvant ILP. This is most obvious in the overall analysis but is also apparent for the subgroups of patients with lesions thicker than 3.0mm or with upper limb melanomas. The trend in all the analyses is similar, the ILP and Control curves tending to diverge after about 18 months.

In interpreting these results, one must bear in mind that the follow-up of ILP patients is shorter than that of the controls and that, consequently, the number of ILP patients included beyond 36 months decreases very rapidly. The division of the study population into subgroups results in rather small numbers of patients so that only very large differences in outcome for these subgroups

will achieve statistical significance. One might therefore predict that the most significant differences in this study would be found in the overall analysis. It is interesting that the subgroups showing the greatest differences at this time are not those with the largest numbers of patients.

Thicker melanomas are known to carry a significantly worse prognosis than thinner lesions and it is perhaps not surprising therefore that this group of patients should be the first to show a significant benefit. One can hope that prolonged follow-up might uncover a similar benefit to those patients with thinner melanomas.

As described in Section 2.2.2, the disease control within the perfused limb after adjuvant ILP appears very good. In this assessment of disease-free survival, the site of first recurrence was not specifically analysed. However the improvement in the survival of the ILP patients observed in this study suggests that spread of disease to the lymph nodes or systemic organs (normally associated with worsening prognosis) may, to some extent, also be better controlled.

Conclusion

While it does not represent a randomized controlled study, this comparison of 63 patients undergoing wide excisional surgery and adjuvant ILP for Stage I melanoma ($\geq$1.5mm Breslow thickness) between 1985 and 1990 in a single centre with a very closely matched group of

control patients from the same geographical area, first registered in the immediately preceding 5-year period and undergoing wide excisional surgery alone, does provide early evidence of a benefit to the ILP patients. This applies both to survival and disease-free survival, the differences after three years being 20% and 15% respectively. The benefits seem most marked for patients with primary melanomas $\geq$3.0mm (Breslow thickness) or with melanomas situated on the upper limb. It is encouraging to note, however, that at this time all subgroups of the study population show an improvement in the survival and disease-free survival of the ILP-treated patients. The significance of this apparent improvement will only be determined by prolonged follow-up.

2.3 Therapeutic ILP

2.3.1 Patient details

Since August 1985, 77 patients have undergone 96 therapeutic ILPs for recurrent or advanced melanoma. The upper limb has been perfused on 4 occasions (4 patients) while the lower limb has been perfused on 92 occasions (73 patients). Details of these patients are listed in Table 15. The sexes were represented in a ratio of approximately 3:1 (f:m) and the mean age was 56.8 years. Technical difficulties were encountered in the first two upper limb ILPs which prompted a change of technique from the infraclavicular approach to the direct axillary approach for this region. Lower limb ILP was performed at the external iliac level in 70 of the 73 patients. The other 3 patients, including one lady who had previously undergone adjuvant iliac ILP, were elderly and had disease confined to the lower leg. The isolation in these cases was performed at the popliteal level.

ILP has been repeated on 22 occasions for 18 patients whose disease advanced after the initial perfusion, including 3 patients originally treated by adjuvant iliac ILP. In order to avoid the serious potential complications of a second exposure of the same vessels, a more distal approach (femoral 5, popliteal 8) was employed if the disease was believed to be confined below the knee. In 9 cases, the iliac route was re-employed. One patient has now undergone a total of 4 ILPs (2 iliac,

Table 15 :
Details of Patients Treated by Therapeutic ILP

Number	patients(ILP)		male	20 (22)
			female	57 (74)
			TOTAL	77 (96)
Age			mean	56.8
			range	(17-86)
Region	First Therapeutic ILP (n=77#)	Lower limb	iliac	70**
			femoral	0
			popliteal	3*
		Upper limb	subclavian	2
			axillary	2
	Repeat ILP (n=22#)	Lower limb	iliac	9**
			femoral	5
			popliteal	8*

\# includes 3 patients previously treated by Adjuvant ILP.
** includes 2 patients previously treated by Adjuvant ILP.
* includes 1 patient previously treated by Adjuvant ILP.

1 femoral, 1 popliteal) while two others have been treated on 3 occasions (1 iliac, 1 femoral, 1 popliteal).

The stage of disease of the patients at the time of therapeutic ILP is shown in Table 16. This classification takes account of the status of the iliac or axillary lymph nodes excised at the time of ILP and which were found to contain tumour deposits in 15 cases (iliac 14; axillary 1). In 60 cases, the disease was confined to the limb with no evidence of lymphatic or systemic spread. In 36 cases, there was evidence of more advanced disease with regional lymph node involvement (clinical, cytological or histological) in 34 cases and known systemic spread in 2 cases. These last 2 patients were given palliative treatment to control extensive, painful, ulcerated limb deposits of melanoma.

At the time of referral for ILP, most patients had already undergone treatment for recurrent melanoma : 61 patients had been treated surgically (excision 55; lymph node dissection 4; therapeutic ILP 19) and 6 had received systemic chemotherapy. The number of previous surgical procedures ranged from one or two in most cases to as many as 19 in one case. In addition, 3 patients had earlier been treated by adjuvant ILP in this centre. However 15 patients, referred at the time of their first recurrence or because their disease was already locally or regionally advanced at the time of diagnosis, had received no other treatment than the initial excisional surgery.

Table 16 :

Stage of Disease (MDAH*) at the Time of 96 Therapeutic ILPs

	Upper limb	Lower limb	Total
Stage II	-	16	16
Stage IIIA	3	41	44
Stage IIIB	1	14	15
Stage IIIAB	-	19	19
Stage IV	-	2	2

* M.D.Anderson Hospital Staging System[150] (see Table 2).

2.3.2 Results

Objective response of visible tumour deposits

The observed response rates of melanoma deposits in this centre have been very similar to those of the other major perfusion centres with an overall response rate (complete response + partial response) of 83%, according to the UICC criteria (see Table 6, Section 1.4.5). Table 17 lists the responses observed in these patients and in those undergoing repeat ILP. At the time of their first therapeutic ILP, 37 patients had visible tumour deposits on the perfused limb. Complete or partial responses were observed in 30 of these cases (CR = 15; PR = 15). It is worth noting that the response rate after repeat ILP was very similar to this. Furthermore, of the 10 patients in this group who had evaluable tumour at the time of both their first and subsequent ILPs, 9 had a complete or partial response to the first perfusion and all responded to the second. This suggests that fears that recurrence of melanoma after ILP might be due to the selective survival of clones of relatively melphalan-resistant melanoma cells are ill-founded.

Recurrence of melanoma after therapeutic ILP

In the assessment of disease control after therapeutic ILP, it is important not only to observe recurrence of melanoma but also progression of existing disease, since many patients do not have their disease excised.

Table 17 :

Objective Response Rates after Therapeutic ILP in 53 Evaluable Patients

	n	CR	PR	NR	PD
First ILP	37	15 (41)	15 (41)	3 (8)	4 (11)
Repeat ILP	16	6* (38)	8# (50)	-	2 (13)
Total	53	21 (40)	23 (43)	3 (5)	6 (11)

CR - complete response
PR - partial response
NR - no response
PD - progressive disease

Figures quoted are numbers(%).

* includes 3 previous CR and 1 previous PR
# includes 3 previous CR, 2 previous PR and 1 previous PD

Table 18 :

Recurrence or Progression of Melanoma after 96 Therapeutic ILPs

Site	No.	Time
Locoregional (extra-nodal)	16	7.0 (2-21)
Nodal	6	8.5 (4-14)
Locoregional and nodal	6	7.0 (2-13)
Systemic	21	8.0 (2-28)
Nodal and systemic	6	9.0 (2-50)
Locoregional, nodal and systemic	4	5.0 (1-9)
TOTAL	59/96	7.0 (1-50)

Site describes site of first recurrence after ILP.
Time expressed in months as a median (range).

Following the 96 therapeutic ILPs performed in Glasgow, recurrence or progression of melanoma have been observed in 59 cases. These occurred at a median time after ILP of 7.0 months (range : 1-50). Details of these recurrences are listed in Table 18. It is interesting to note that half of these cases had evidence of systemic disease at the time of first post-ILP recurrence. The appearance of these recurrences so soon after ILP lets one suppose that many of these patients harboured undetected systemic metastases at the time of ILP and that their fate was not likely to be affected by regional treatment of their disease.

Although such a high recurrence rate is disappointing, if not unexpected, it is encouraging to note that a third of patients (n=27) are alive with no evidence of recurrence or disease progression at a median follow-up time of 20.5 months (range : 1-67).

Survival after therapeutic ILP

Of the 77 patients treated by therapeutic ILP, 31 have since died of their melanoma. The median survival of these patients was 13.0 months (range : 2-46). Three patients have died of other causes (at 7, 15, and 24 months follow-up).

Of the 43 surviving patients, 27 have had no further recurrence of their melanoma at a median follow-up of 20.5 months (range : 1-67) while 16 are alive despite progression of their disease at 24.5 months (range : 6-67).

These data are presented in Fig 10 in the form of life tables which show that the overall survival and recurrence-free survival at 2 years are of the order of 60% and 35% respectively.

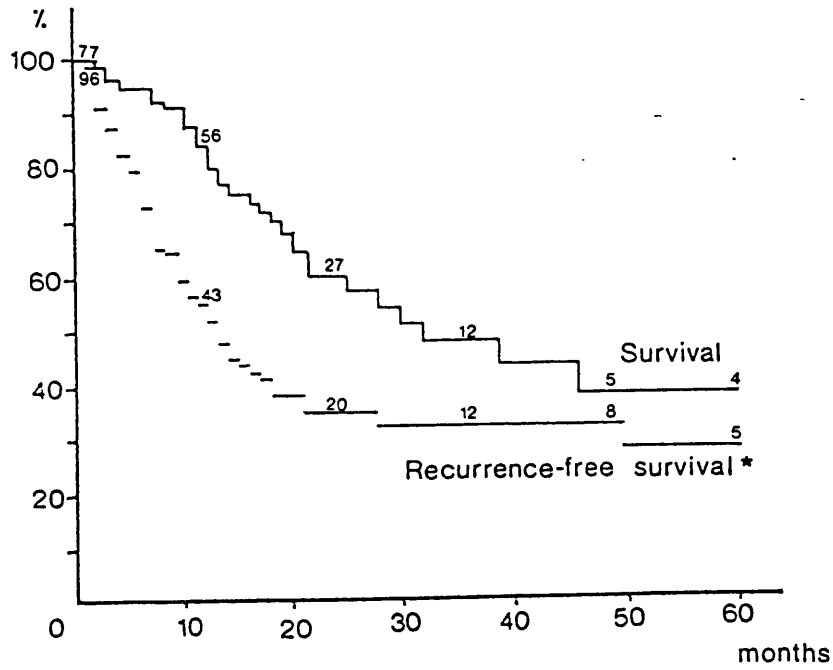


Figure 10 :
Survival (n=77) and recurrence-free survival* (n=96) curves for patients undergoing Therapeutic ILP.

*** Recurrence-free survival signifies no new recurrences and/or no progression of existing disease.**

2.4 Conclusions on ILP in Glasgow

In assessing the results of isolated limb perfusion in Glasgow so far, it is reassuring to observe that while a degree of toxicity is almost inevitable, serious complications are very rare. The regional toxicity has been almost entirely limited to the "desirable" grades (II-III), and the haematological and pharmacokinetic data have shown that the surgical technique employed is associated with minimal systemic distribution of melphalan during and after ILP.

The results of adjuvant ILP give early evidence of a possible improvement in survival, especially for the thicker melanomas and lesions located on the upper limbs. More prolonged follow-up of patients is necessary to confirm these early results. They do, however, provide some justification for the continued use of adjuvant ILP for Stage I melanoma while the results of the large multicentre EORTC/WHO randomized study are awaited.

After therapeutic ILP, the very high objective response rate observed (83%) is similar to the best response rates quoted in the literature (see Table 6, Section 1.4.5). The disease control provided by therapeutic ILP is of limited duration in many cases. However, there is evidence that prolonged control can be achieved in up to a third of patients. Furthermore, the high response rates observed after a first ILP can be reproduced on repeating the procedure. Therapeutic ILP therefore offers a means

of controlling a disease which often runs a relentless course of successive recurrences in the limb, as well as useful palliation of intractable regional tumour deposits in the presence of systemic disease. In the treatment of malignant melanoma of the extremities, mutilating excisional surgery and limb amputations can now be regarded as things of the past.

PART B

PART B

Introduction

The details of the surgical technique of isolation and cannulation for ILP have changed remarkably little since the original description in 1957, so that a more or less standard approach is used in most of the major centres. There is, however, a surprising lack of conformity with regard to several aspects of the technique of perfusion of the isolated circuit. This is especially true of the measurement of the important physiological parameters governing the perfusion, and of the dosage of cytotoxic drug. The effect of this variability on the clinical outcome of the procedure is not known, but the interpretation of results from various centres must be coloured by the knowledge that the technique employed in these centres is often substantially different. In order to make comparisons of results more valid and to improve the reliability of multicentre studies, as many aspects of the treatment as possible should be standardised.

Isolated Limb Perfusion offers the possibility of confining cytotoxic treatment to the region of the body harbouring the disease and thus of minimising the toxicity to vital organs while maximising the dose of drug administered. The therapeutic ratio could be increased further by manipulating the physiological parameters of perfusion to ensure that, within the perfused region, those tissues most likely to contain tumour are

preferentially exposed to the drug. Even greater specificity may be achieved by targeting treatment to the cells responsible for the disease - in this case, melanoma cells. In the past, attempts to target therapy have been directed at characteristic biochemical reactions or hormonal influences. More recently, immunological approaches to targeting have resulted in the development of monoclonal antibodies to tumour-associated antigens which can then be used as vectors for the delivery of cytotoxic agents to the malignant cells. Several antibodies to melanoma-associated antigens are now available.

Having reviewed the early Glasgow experience with ILP (1984-1988), and finding that several important questions had never been addressed by other perfusionists, the author devised a series of experiments to quantify and evaluate certain specific aspects of this therapeutic modality with the following aims :

AIMS :

1. Physiology

to standardise the regulation of the perfusion parameters in the isolated circuit so that effective delivery of cytotoxic drug to the tissues of the limb can be ensured, reliably and safely (Chapter 3).

2. Dosimetry

to study the relative merits of the currently used melphalan dosage schedules, and to establish which schedule most consistently leads to the administration of the highest tolerable dose of melphalan (Chapter 4).

3. Targeting

to assess the possibility of increasing the specificity of ILP by targeting treatment to melanoma using monoclonal antibodies (Chapter 5).

3 CHAPTER 3 - PHYSIOLOGICAL ASPECTS OF ILP

3.1 Variations in the ILP Technique

While the surgical techniques of isolation and cannulation for ILP are similar in most reports, several aspects of the technique of perfusion of the isolated circuit still vary widely between perfusing centres. These relate to the priming fluid, the cytotoxic dosage, and the physiological parameters governing the perfusion.

[i] The Priming Fluid

The choice of priming fluid has been influenced over the years by the practice of cardiac surgeons. In many reports of Isolated Limb Perfusion, particularly in the early years, the priming fluid consisted of whole blood[382,384,388,390,409] in accordance with the experimental work of the originators of the technique[327-329]. In the field of total cardiopulmonary by-pass for cardiac surgery, perfusionists have adopted a mixture of whole blood and electrolyte solution to produce a less viscous fluid than whole blood alone. This has the advantage of allowing the blood to pass more easily through the extracorporeal part of the circuit at the rapid flow rates which are required to support an adequate systemic circulation. Moreover, since cardiac surgery is generally performed under hypothermic conditions, the reduced oxygen carrying capacity of the dilute mixture is

sufficient to ensure adequate tissue oxygenation. Nowadays, the majority of isolation perfusion centres also use this type of mixture[360,361,369,373,375,376]. The exact proportions of the various constituents vary, as does the total volume of the priming fluid in the circuit. In fact, some centres use only balanced electrolyte or colloid solution and rely on the blood trapped in the limb at the time of isolation to provide the oxygen carrying capacity of the perfusate[376,402,403,410]. Recent reports seem to indicate that most centres aim to produce a haematocrit of about 25% in the perfusate[411], although one of the major European perfusion centres still uses whole blood as its priming fluid as a result of experimental work which demonstrated less toxicity using this method[377-379]. Many reports in fact give no indication of the composition or volume of the perfusate[365,367,368,386,389,392,412].

While little comment is made of the effects that this variability may have on the outcome of isolated limb perfusion, one should bear in mind the following points :

- the volume of the priming fluid can contribute substantially to the volume of distribution of the administered drug. Some authors make no mention of the volume of priming fluid used, while others quote volumes ranging from 500ml to 2 litres[361,375]. This lack of uniformity must result in substantial variations in the concentration of cytotoxic drug achieved in the perfusate by similar doses. The effect of this on the clinical outcome is, as yet, unknown.

- large fluid shifts are often observed by perfusionists during isolated perfusion; these are thought to reflect the movement of fluid between the intravascular and interstitial fluid compartments of the limb and will be partly determined by the protein concentrations in these compartments and their respective oncotic pressures.

- isolated limb perfusion is now usually performed under normothermic or hyperthermic conditions; the oxygen requirements of the tissues of the limb are therefore higher than would be the case in cardiac surgery. The reduced oxygen carrying capacity of a dilute perfusate may therefore result in inadvertent hypoxic injury to the tissues of the limb.

These are important issues and surprisingly little has been done to try to resolve them.

[ii] Cytotoxic Dosage

Several dosage schedules are used in isolated limb perfusion, some of which include subjective judgements of individual patients' build and prognosis. While it does seem desirable to tailor the dose of cytotoxic drug to the patient, current thinking still holds that the highest

tolerable dose of drug offers the greatest therapeutic potential. The three most popular dosimetric methods are based on

(a) a fixed dose of drug per kilogram of total body weight

(b) a fixed dose of drug per litre of perfused tissue

(c) the dose of drug required to achieve a predetermined concentration in the estimated total perfusate volume.

These methods are assessed in a later section of this thesis (Section 4.3-4.5).

[iii] Physiological Parameters of Perfusion

Our current knowledge of the pharmacokinetics of cytotoxic drugs is principally based on studies of systemic treatment. Isolated Limb Perfusion offers the unique advantage of being able to exclude from calculations the limits imposed by toxicity of the drug to central organs (eg. bone marrow, liver, kidneys) and its detoxification and excretion by them. The management of the extracorporeal circuit also allows many of the physiological properties of the blood in the limb to be controlled or manipulated to advantage, including oxygenation, temperature and pH. Much has been written of these aspects of perfusion management. However the

potential benefit of any such manipulations can only be realised if the physiological parameters of perfusion are maintained so that optimal delivery of the cytotoxic drug to the tissues of the limb is ensured.

In this chapter, some of the aspects of the perfusion technique which can result in variable drug delivery to the tissues will be outlined. A series of studies performed by the author and aimed at developing a method of ensuring optimal drug delivery to the tissues of the isolated limb will then be described.

3.2 Assessment of Perfusion in the Isolated Limb

3.2.1 Introduction

The two essential criteria for Isolated Limb Perfusion, as for any other treatment, must be its safety and its efficacy. The former objective demands that systemic toxicity be avoided by eliminating the potential routes of leakage of drug from the limb to the systemic circulation, and by washing out as much of the unbound drug from the vasculature as possible before reversing the isolation. This is discussed in the description of Isolated Limb Perfusion (see Section 2.1.2). It also requires the protection of the tissues in the isolated limb from ischaemic injury and excessive toxicity.

The efficacy of the treatment is dependent on the delivery by the perfusate of effective concentrations of the cytotoxic drug to the appropriate tissues. This may be improved by manipulation of the physiological conditions in the limb such as temperature and pH, but only if the first condition is satisfied.

The composition of the perfusate and control of the perfusion circuit are thus of paramount importance in ensuring both the safety of the limb and the efficacy of the treatment. It seems rather surprising, therefore, that perfusion centres should persist in using different perfusion techniques in terms of both the composition of the perfusate and the regulation of the perfusion.

In many centres, the principal concern of perfusionists is for safety and the avoidance of leakage of cytotoxic drug to the systemic circulation. In these centres the flow rate is usually regulated to maintain the perfusion pressure below the systemic arterial pressure in the belief that the resultant positive pressure gradient can prevent leakage of perfusate to the systemic circulation[357,360,361,369,412]. Some centres deliberately use lower than normal blood flow in the isolated limb[146,357,402]. However, several authors have claimed that the aim of perfusion should be to mimic the normal physiological conditions in the limb[411]. For this reason, many perfusion centres have now adopted the practice of regulating their perfusion parameters according to those observed in normal limbs. In particular, many seek to achieve similar blood flow rates to those seen in the lower limb at rest[355,361,369,375,409,412]. Although this approach may seem very appealing at first, it does not take into account the decreased viscosity and oxygen-carrying capacity of the perfusate, nor the effect on the peripheral vasculature of hyperthermia and of the period of interrupted flow to the limb during isolation and cannulation. All of these factors would, in the "physiological" situation, result in an increased blood flow in the limb. It therefore seems illogical to assume that physiological "normality" can be maintained by normal flow rates when the perfusate and conditions of the limb are themselves so abnormal.

This aspect of Isolated Limb Perfusion has been studied with four particular aims in mind :

[1] To establish whether current practice reliably ensures effective perfusion of the tissues of the limb.

[2] If this is not the case, to determine whether an alternative method can be used which does achieve this.

[3] To assess whether control of adequate perfusion could ensure more effective delivery of the cytotoxic drug into the tissues.

[4] To measure the effect of this type of perfusion control on the leakage of cytotoxic drug to the systemic circulation.

In the treatment of malignant melanoma of the extremities by Isolated Limb Perfusion, be it adjuvant or therapeutic, effective perfusion of the cutaneous and subcutaneous vascular beds should be regarded as critical since the spread of melanoma tends to occur primarily in these layers. Unfortunately, the circulation to these very tissues tends to be the first to be compromised by the sympathetic vasoconstrictor response to conditions of hypotension[413]. Therefore it would be crucial to know whether the blood flow rates routinely encountered during

isolation perfusion and the pressures which they generate are sufficient to support the circulation to these tissues.

3.2.2 Arterial Pressure in the Perfused Limb

In most reports of isolated limb perfusion, the pressure in the arterial side of the isolated circuit is monitored throughout the procedure. This is usually done by placing a pressure transducer at a point on the circuit between the pump and the arterial cannula (see Fig.3). The flow rate is then adjusted to keep this pressure below the systemic arterial pressure in order to minimise the risk of leakage. However the pressure recorded at this position does not accurately reflect the intra-arterial pressure in the perfused limb. This is in fact dependent not only on the pressure exerted by the pump but also on the resistance to flow exerted by the rigid tubing and the narrowest part of the circuit, which in this case is usually at the tip of the arterial cannula.

The relationship between the drop in pressure across a rigid tube and the flow through the tube is expressed by Poiseuille's equation which states that

$$Q = \frac{(P_1 - P_2).pi.r^4}{8u.L}$$

where Q = the flow through the tube, (P_1-P_2) = the drop in pressure across the tube, u = the viscosity of the fluid, L = the length of the tube, and r = the radius of the tube. This equation can be re-written to give

$$P_1-P_2 = \frac{8u.L.Q}{pi.r^4}$$

So for a fluid of given viscosity "u" flowing through a rigid tube, the pressure drop across the tube is directly proportional to the length of the tube and the flow, and inversely proportional to the fourth power of the radius of the tube.

This relationship can be modified to apply also to localised narrowings or stenoses which, in effect, can be regarded as short sections of rigid tubing. Rodbard[414] expressed this as follows:

$$Q = C.A\sqrt{(P_1-P_2)}$$

or

$$P_1-P_2 = \frac{Q^2}{C^2.A^2}$$

where A = the cross sectional area of the stenosis $(pi.r^2)$ and C is a factor dependent on other features of the narrowing, termed the Coefficient of Contraction.

In other words, while the pressure drop across an orifice is inversely proportional to the fourth power of the radius, it is directly proportional to the square of the flow through it (cf. pressure drop across a rigid tube, above).

In considering the isolated perfusion circuit, we can therefore predict that the true arterial pressure may be considerably lower than that measured in the arterial tubing, the difference being dependent on the length and calibre of the arterial tubing and cannula, the radius of the orifice at the tip of the cannula, and the flow rate. Furthermore, at very high flow rates the pressure difference increases disproportionately due to the onset of turbulent flow.

Wieberdink[415] has derived curves to predict the difference between the inflow and outflow pressures across the arterial cannulae which are commonly used for isolated limb perfusion in one centre. This method gives a better estimate of the true intra-arterial pressure, but the curves would have to be recalculated for each perfusion centre according to the viscosity of the perfusate and the commercial brand of cannula used. A simpler and more reproducible method would be the direct measurement of the intra-arterial pressure in the perfused limb. Fontijne and colleagues reported the use of the smaller channel of a double-lumen arterial cannula for this purpose[416]. They confirmed that the pressure measured in the extracorporeal circuit did not accurately reflect the true intra-arterial pressure and that it always grossly underestimated this

value, the difference ranging from 62mmHg to as much as 153mmHg. Another method of achieving this direct arterial pressure measurement would be the placement of a peripheral arterial cannula connected to a pressure transducer.

3.2.3 Assessment of Cutaneous Perfusion

Effective assessment of skin perfusion during ILP requires a technique of continuous measurement and cannot, therefore, rely on the measurement of substances which accumulate in the tissues with the possibility of saturation being reached. Several potential markers exist within the isolated circulation but most of these would be unsuitable for this purpose. These include fluorescein which accumulates in the tissues, and radioisotope-labelled proteins (often used in ILP as markers of leakage of perfusate to the systemic circulation) which tend to remain within the intravascular compartment and whose exact location in the vascular tree (artery, arteriole, capillary, venule, vein) would be difficult to establish. A reliable marker of capillary circulation must have the ability to diffuse through the capillary walls in order to distinguish it from other purely intravascular substances. In hyperthermic isolated limb perfusion, the temperature of the skin should vary in response to changes in the cutaneous perfusion. However the perfusate is not the only source of heat, the other being the heated water

blanket wrapped round the limb. The relative contributions of these two heat sources to the final temperature of the skin would be difficult to determine, especially at tissue temperatures lower than that of the blanket.

Oxygen diffuses out of the blood via the capillaries and through the tissues, and because of its consumption by the tissues does not accumulate significantly. Although the skin is also exposed to oxygen from the atmosphere, only the outer dead layer of the skin is exposed to this second source. The conductivity of this layer for oxygen is normally so low that measurement of the oxygen tension on the skin surface using a technique which excludes the atmosphere from the immediate vicinity of the measurement site should reflect diffusion of oxygen from the capillaries only. This is known as the transcutaneous oxygen tension measurement ($PtcO_2$) and is further discussed below (see Section 3.2.4).

In order to understand the significance of transcutaneous oxygen tension measurement in the assessment of cutaneous perfusion, it is important to appreciate the anatomical structure of the cutaneous circulation.

Blood supply of the skin

The blood supply of skin comes from subdermal and subpapillary arteriolar plexuses. These give rise to "hair-pin" capillary loops which in turn drain into dermal venous plexuses. The capillary loops penetrate into the

dermal papillae and thus bring blood into close proximity with the cells of the living layer of the epidermis (Figure 11). The muscle of the arteriolar plexuses controls the inflow of blood to these capillary loops by vasoconstriction or vasodilatation. This is normally determined by neural, chemical and hormonal influences. However heat can cause maximal vasodilatation to occur independently of these other factors[417].

The anatomy of the capillary loop allows it to function as a countercurrent exchange system so that the oxygen tension at the apex of the loop is somewhat lower than the incoming arterial oxygen tension[418]. The magnitude of this difference will depend mainly on flow through the loop : the slower the flow, the greater the difference. However, in conditions of maximal vasodilatation, flow through the loop can increase to such an extent that the exchange of oxygen between the limbs of the loop is minimal, and in this situation the oxygen tension at the apex of the loop approaches the incoming arterial oxygen tension (PaO_2).

From the apex of the loop, oxygen can diffuse out through the tissues and towards the skin surface. As it does so, it is consumed to some extent by the cells of the living layer of the epidermis thus lowering its partial pressure in the tissues. This is further reduced by the resistance of the tissues to the diffusion of oxygen, especially in the dead layer of the epidermis, the stratum corneum.

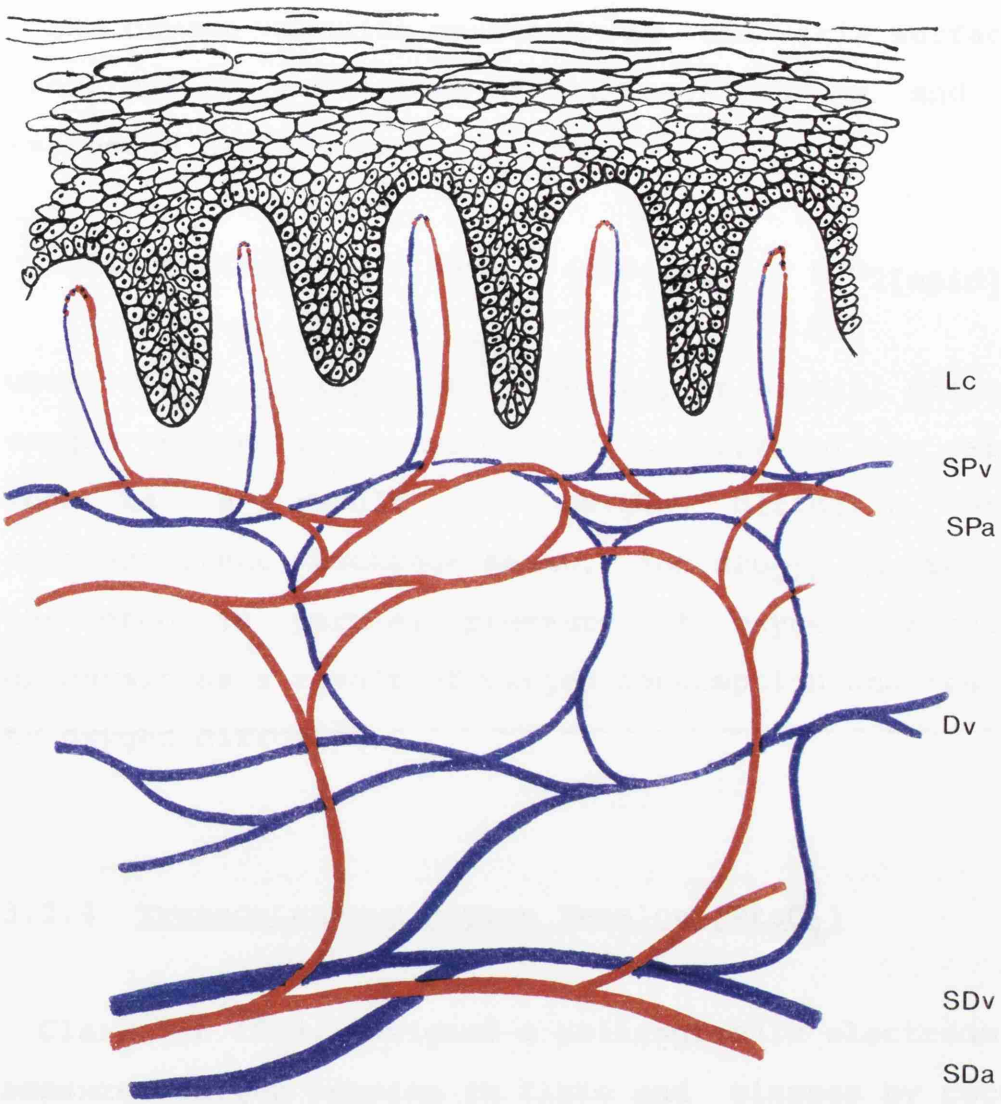


Figure 11 :
Diagrammatic representation of the skin vasculature.

The subdermal arteriolar plexus (SD_a) supplies the subpapillary arteriolar plexus (SP_a) which in turn gives rise to the network of "hair-pin" capillary loops (L_c). These project into the dermal papillae and drain via the subpapillary (SP_v), dermal (D_v) and subdermal (SD_v) venous plexuses.

The oxygen tension measured at the skin surface will therefore be a function of all these factors and can be expressed as follows :

$$\text{Skin surface } PO_2 = PaO_2 - (dPO_{2[cap]} + dPO_{2[epid]})$$

where $dPO_{2[cap]}$ represents the drop in partial pressure of oxygen between the arteriole and the apex of the capillary loop as a result of oxygen diffusion and the countercurrent exchange shunt, and $dPO_{2[epid]}$ represents the drop in partial pressure of oxygen across the epidermis as a result of oxygen consumption and resistance to oxygen diffusion.

3.2.4 Transcutaneous Oxygen Tension ($PtcO_2$)

Clark, in 1956, designed a polarographic electrode which measured oxygen tension in fluid and tissues by recording the current generated by reduction of the oxygen at the cathode[419]. This now forms the basis of most blood gas analysers. The "Clark's cell" was also incorporated into a tissue probe in the hope that it might allow non-invasive estimation of the arterial oxygen tension but, in the initial studies of this approach, it was found that the measurements made on the skin surface were much lower than the true arterial oxygen tension[420,421]. On the other hand, when vasodilatation was induced chemically or by ultraviolet light the difference was reduced. Thereafter, other investigators used a heated probe to

induce vasodilatation and found that by "arterialising" the capillary blood flow in this way, they obtained a good correlation between the oxygen tension measured at the skin surface and that in arterial blood[422,423]. The principal effects of heat on the oxygen tension measured at the skin surface are believed to be fourfold[417,424-428] :

[1] it induces **maximal vasodilatation**, thereby increasing the flow through the capillary loop. As a result, the effect of the countercurrent mechanism of the capillary loop is negated and the capillary oxygen tension approaches the inflowing arterial oxygen tension.

[2] at raised temperatures, the **lipid structure** of cell membranes is altered, allowing oxygen to diffuse more readily through tissues, especially in the relatively impermeable stratum corneum of the skin.

[3] heat is one of the factors which cause the **oxygen-haemoglobin dissociation curve** to shift to the right. In other words, a constant oxygen content in blood exerts a higher partial pressure at higher temperatures. This increase has been estimated to be in the region of 1.0-7.4% per degree C [417,424,427].

[4] **respiration** by normal cells is increased at raised temperatures leading to a greater consumption of oxygen by the skin.

The first two effects tend to reduce the difference between the arterial and skin surface oxygen tensions. The third effect results in an "artificial" increase in the oxygen tension; this is balanced by the increased oxygen consumption in the tissues. The overall balance is therefore dependent on the blood flow through the capillary network and the resistance of the tissues to oxygen diffusion[427]. This is a function of skin thickness and all investigators have shown a much better agreement between arterial and skin surface oxygen tensions in children and neonates than in adults[417,424,426,428,429]. However, if the skin is heated to 43-44^{o}C a good correlation is obtained, even in adults. It has therefore been possible to use this technique for continuous monitoring of blood gas tension in neonatal and paediatric medicine as well as in adult intensive care units.

In haemodynamically compromised adults, the correlation between transcutaneous oxygen tension ($PtcO_2$) and arterial oxygen tension (PaO_2) is poor. This has sometimes been quoted as a limitation of the applicability of this technique[430]. However, since this method measures the PO_2 diffusing through the tissues (in this case, skin), it must depend on both the oxygenation of the arterial blood and the distribution of blood to the tissues. In haemodynamically stable patients, conditions of maximal vasodilatation such as those produced by heating the skin to 44^{o}C produce complete arterialisation of the capillary blood, so that the PO_2 at the apex of the capillary loop is equal to the incoming PaO_2. In this situation, the

$PtcO_2$ is "flow independent" [417] and can be regarded as a measure of PaO_2. However, if circulatory disturbances occur, the heat-induced vasodilatation will be maintained so that, in the absence of any change in the blood oxygenation, changes in the $PtcO_2$ will reflect the perfusion of the papillary and subdermal plexuses and will therefore act as a measure of peripheral blood flow and blood pressure[417,425,428,429,431,432,433]. In addition, most currently available $PtcO_2$ monitors record a second measurement : this is the power supplied to the heating element to maintain the preselected temperature. Blood flow under the probe will tend to dissipate heat away from the site of the probe. Increases and decreases of blood flow will therefore be reflected by similar changes in the heating power recorded[424,427,434,435].

Rather than limiting its usefulness, these features have increased the applicability of $PtcO_2$ measurement in other fields. The technique has been advocated for the continuous assessment of haemodynamic status in conditions of low flow shock[426] and for the assessment of viability of skin flaps in plastic surgery[436]. Furthermore, the technique has proved useful in assessing the severity of peripheral vascular disease[432,437-439] and has been recommended as a method for selecting the best level for limb amputation with reduction of stump healing problems[440,441].

The structure of the currently used transcutaneous oxygen tension probes is shown diagrammatically in Figure 12. In the early reports of the technique, one author suggested heating the cathode itself to generate the desired

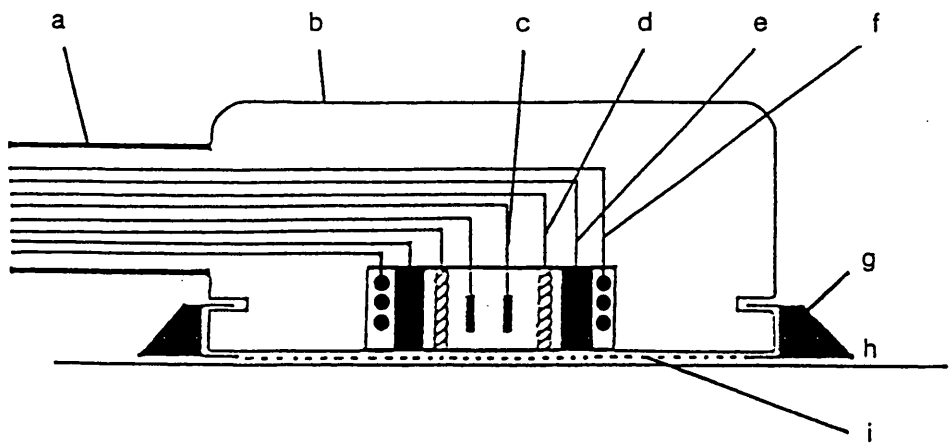


Figure 12 :
Diagrammatic representation of the transcutaneous oxygen tension ($PtcO_2$) probe.

a. cable
b. probe casing
c. thermistor
d. cathode (Pt)
e. anode (Ag)
f. heating coil
g. fixation ring
h. skin
i. teflon or cellophane membrane

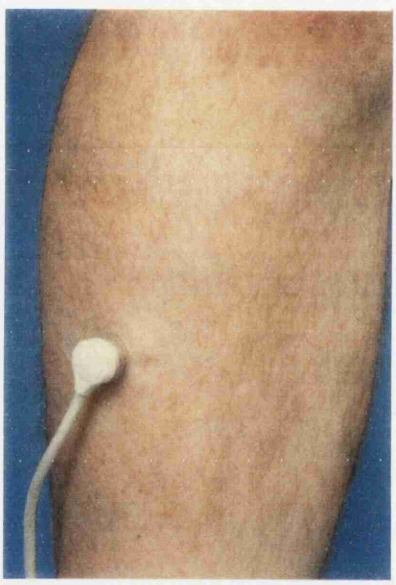
hyperthermia[424]. The disadvantage of this suggestion, however, was that the larger surface area of the cathode necessary for this also resulted in greater consumption of oxygen by the electrode. Most probes now incorporate a separate heating element (as shown in Fig. 12). The electrodes are bathed in fluid enclosed by a cellophane or teflon membrane. The probe is fixed to the skin by means of an adhesive fixing ring, and air is excluded from the probe-skin interface by application of contact fluid. This has the additional effect of hydrating the stratum corneum, thereby making it more permeable to oxygen. The various influences of heating and hydrating the skin on the measured PO_2 (see above) tend to cancel each other out, and the measured $PtcO_2$ is therefore dependent on the arterial PO_2 and the capillary perfusion.

While measuring $PtcO_2$, it is important to avoid pressure on the probe as this can result in decreased local perfusion through compression of the capillaries[421,424,442]. Traction on the electrode wire can also result in decreased capillary perfusion. Both of these potential influences can be avoided by lightly securing the probe and wire with tape.

In the studies described in this thesis, the E5242 $PtcO_2$ electrode connected to the TCM2 $PtcO_2$ module (both Radiometer Ltd, Copenhagen) were employed for all measurements of the transcutaneous oxygen tension (Figure 13).

a.

b.



c.

Figure 13 :
The TCM2 monitor and E5242 $PtcO_2$ probe (Radiometer, Copenhagen).

(a) Each monitor provides a continuous display of the $PtcO_2$ measurement (left) and of the power supplied to the heating coil of the probe to maintain the preselected temperature (right).

(b&c) The probe is fixed to the skin by screwing it into an adhesive fixation ring, applying a few drops of contact fluid to seal the probe-skin interface.

3.3 A Study of Cutaneous Perfusion during ILP

3.3.1 Introduction

The importance of ensuring that the skin and subcutaneous tissues are adequately perfused during isolated limb perfusion with cytotoxics for malignant melanoma of the extremities lies in the belief that the spread of melanoma occurs primarily via the cutaneous and subcutaneous lymphatics. It is not clear which circulatory parameter(s) can most effectively be used to control this. In most centres the perfusion technique is aimed at achieving fixed flow rates or at maintaining a "stable circuit", the flow rate being altered in response to changes in the extracorporeal blood reservoir volume while at the same time ensuring that the extracorporeal circuit pressure does not exceed the systemic arterial pressure[357,365,369,375,376,409,412].

To date, none of the perfusion centres has developed a method which guarantees effective perfusion of the tissues of the limb. This study was therefore performed in order to establish whether the current technique ensures that the skin and subcutaneous tissues of the isolated limb are adequately perfused during Isolated Limb Perfusion using the $PtcO_2$ as a measure of skin perfusion.

3.3.2 Patients and Methods

During a period of six months, 18 successive patients underwent ILP at the iliac level according to the technique normally used in this centre. After surgical isolation of the limb, cannulation of the external iliac vessels and application of the tourniquet, isolated perfusion was initiated and, after allowing for an initial loss of up to 300ml of fluid from the reservoir, the stability of the circuit was maintained by increasing or decreasing the flow rate as required. The perfusate was oxygenated with 100% oxygen at 0.5 litre min^{-1}. After confirming satisfactory isolation by injection of fluorescein into the perfusate and demonstration of its presence only within the limb, melphalan was injected into the reservoir (in a dose of 1.75mg kg^{-1}) and perfusion was continued for one hour. Thereafter, the limb was rinsed with 2 litres of non-recirculated Hartmann's solution and the isolation procedure reversed.

In accordance with normal practice, the systemic arterial pressure was monitored directly via a 20G teflon cannula (Viggo-Spectramed, UK) inserted into one radial artery and connected to a pressure transducer.

For this study, an additional arterial cannula was inserted into the dorsalis pedis artery of the perfused leg and connected to a pressure transducer to allow direct measurement of the perfusion pressure. A transcutaneous oxygen tension ($PtcO_2$) probe (E5242; Radiometer Ltd, Copenhagen) heated to 43°C was attached to the skin of the lower leg over the proximal half of tibialis anterior

and lightly secured with tape. This was connected to a continuous display $PtcO_2$ monitor (TCM2; Radiometer Ltd, Copenhagen). $PtcO_2$ was also measured on the other leg using identical equipment in order to act as a control during the period of stabilisation of the $PtcO_2$ measurement. Both arterial pressure traces were projected on a continuous display monitor (Model 1281, Siemens).

Throughout the preparatory period of the operation and the isolation procedure, the mean systemic blood pressure (radial artery cannula) and the mean dorsalis pedis artery pressure were recorded at five-minute intervals together with the PtcO2. After the commencement of isolated perfusion of the limb, these parameters were recorded at two-minute intervals for the first 20 minutes of perfusion and at two and a half or five minute intervals thereafter until the end of perfusion, together with the blood flow rate through the pump, the volume of perfusate in the reservoir and the skin temperature (mean of the two measured values) at each of these times. A specimen of the intra-operative data recording sheet is shown in Figure 14.

The data were analysed to investigate the relationships between these parameters.

Samples of "arterial" perfusate were withdrawn at regular intervals throughout the procedure in order to measure the PaO_2 of the perfusate during the isolated perfusion.

ILP Time (min)	Mean BP Sys. (mmHg)		Flow rate (ml/min)	Reservoir volume (ml)	$PtcO_2$ R. (mmHg)		Temp. Perf. Limb (^{o}C)	
-15	.	.	.	.	.	.	.	.
-10	.	.	.	.	.	.	.	.
-5	.	.	.	.	.	.	.	.
0	.	.	.	.	.	.	.	.
2	.	.	.	.	.	.	.	.
4	.	.	.	.	.	.	.	.
6	.	.	.	.	.	.	.	.
8	.	.	.	.	.	.	.	.
10	.	.	.	.	.	.	.	.
12	.	.	.	.	.	.	.	.
14	.	.	.	.	.	.	.	.
16	.	.	.	.	.	.	.	.
18	.	.	.	.	.	.	.	.
20	.	.	.	.	.	.	.	.
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25	.	.	.	.	.	.	.	.
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30	.	.	.	.	.	.	.	.
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35	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.
40	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.
45	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.
50	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.
55	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.
60	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.
65	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.
70	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.
75	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.
80	.	.	.	.	.	.	.	.

Figure 14 :
Specimen of the intra-operative data recording sheet used in the studies of cutaneous perfusion during ILP (Sections 2.1.3-2.1.5).

3.3.3 Results

Three of the patients entered into this study were excluded due to failed cannulation of the dorsalis pedis artery of the perfused limb (2 cases) or to malfunction of the $PtcO_2$ electrode (1 case). Data collection from the remaining 15 patients was complete. 12 patients were female and 3 were male, with an age range of 16-78 years (mean = 48 years). The surgical isolation technique was uncomplicated in most cases, although one patient had undergone isolated limb perfusion previously at the same level and therefore presented considerable technical difficulies with the vascular dissection and identification of collateral vessels. Before dissection of the iliac vessels was performed, good agreement was obtained between the blood pressure recordings of the radial and dorsalis pedis artery cannulae (mean difference = 4.3 mmHg, SD = 2.9) and between the $PtcO_2$ measurements from perfused and nonperfused legs (mean difference = 14.9 mmHg, SD = 15.9). During the dissection of the iliac vessels, the dorsalis pedis pressure fluctuated according to the traction applied to the vessel and spasm of the vascular smooth muscle. These effects resulted in a lowering of the $PtcO_2$ in the ipsilateral leg. On application of the arterial clamp, the pulsatile trace from the dorsalis pedis artery cannula was lost and the pressure almost immediately dropped to a stable level of 25.7 mmHg (mean; SD = 7.4). At this time, $PtcO_2$ decreased steadily and continuously, in most cases reaching 0 mmHg in less than 10 minutes.

On commencement of isolated perfusion, the flow rate was set at 170-300 ml min^{-1}. Following the initial period of stabilisation (10-15 minutes), the mean blood flow maintained in the isolated circuit in each case ranged from 106.7 to 593.0 ml min^{-1}, with a mean of 337.3 ml min^{-1} (SD = 144.8). The mean PaO_2 in perfusate samples taken throughout the procedure was 408 mmHg (SD = 102). The mean systemic pressure throughout the period of isolated perfusion was calculated in each case. This ranged from 62.1 mmHg to 93.5 mmHg (mean = 77.4 mmHg, SD = 7.7).

On one occasion, perfusion was terminated prematurely after only 40 minutes due to impairment of the venous return from the limb resulting in excessive fluid loss from the reservoir. In all other cases, the flow rate was adjusted throughout the period of isolated perfusion according to the perfusion pressure and the fluid volume in the reservoir. In view of the wide variability of this aspect of the perfusion, the recordings of the perfusion parameters (flow rate, perfusion pressure, $PtcO_2$) were not pooled, but were assessed separately for each patient.

Curves of the variation in the flow rate, perfusion pressure and $PtcO_2$ with time were drawn for each case. These plots are shown in Appendix A. In most cases (1,2,4,7,9,10,11,13,14,15) the $PtcO_2$ curve follows a similar general outline to the flow rate and perfusion pressure profiles, albeit on a different scale. Figure 15 demonstrates one of the best examples of this. In two

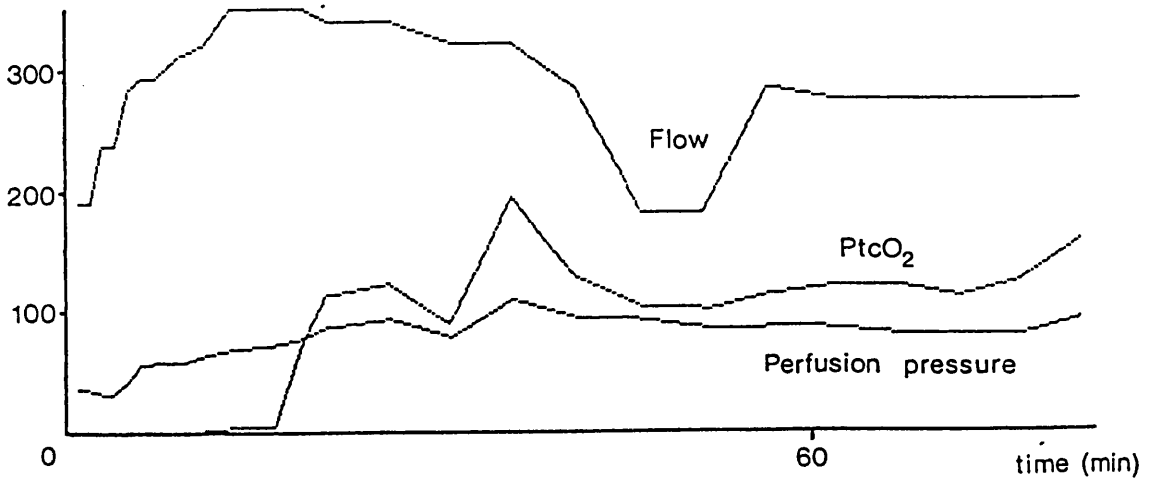


Figure 15 :
Graph showing the variations in $PtcO_2$ and perfusion pressure observed during ILP and their relation to the flow rate in the isolated circuit (patient 9).

cases, the similarity seems limited to only pressure (case 5) or flow (case 8). In the three remaining cases, the $PtcO_2$ remained constant throughout the perfusion period. In two of these (cases 6 and 12), $PtcO_2$ fluctuated between 0 and 1 mmHg during isolated perfusion and only rose above this level on reversal of the isolation. In case 3, the $PtcO_2$ only dropped to 35 mmHg during the isolation procedure, even after clamping the external iliac artery, and remained between 42 and 83 mmHg thereafter in spite of substantial increases in the flow rate (250-450 ml min^{-1}) and variations in perfusion pressure (22-48 mmHg).

The relation between $PtcO_2$ and perfusion pressure was then assessed independently by plotting the $PtcO_2$ values against the corresponding pressures. A typical example of these plots is shown in Figure 16. This reveals that below a critical "take-off" pressure, the $PtcO_2$ was zero, but that as perfusion pressure rose above this critical level the $PtcO_2$ rose rapidly. Figure 17 shows the lines of best fit drawn through these plots for all patients, and demonstrates that these features were present in all but three cases (3,6,12). Furthermore, several of the curves show a plateau at high perfusion pressures, where the $PtcO_2$ approached the arterial PO_2.

The "take-off" pressure in each case was estimated by extrapolation of the straight line part of the slope down to the pressure axis. Values ranged between 30 and 75 mmHg with a mean of 51.1 mmHg (SD = 12.8). This was lower

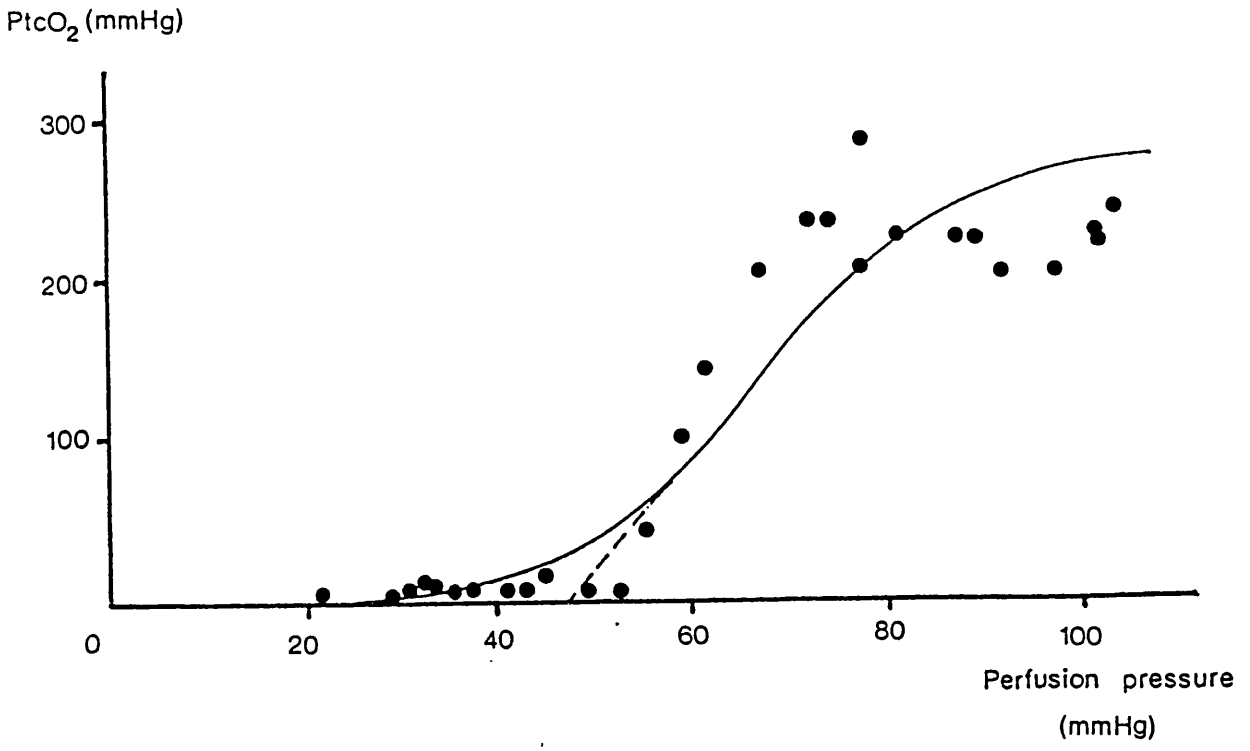


Figure 16 :
Plot of the $PtcO_2$ values observed during ILP against the coincident perfusion pressure measured in the dorsalis pedis artery (patient 7).

As perfusion pressure increases, a critical level is reached above which the $PtcO_2$ rises steeply. Back-extrapolation of the straight-line portion of the sigmoid curve produces an estimate of this critical perfusion pressure (in this case, 47mmHg). At very high perfusion pressures, the $PtcO_2$ curve forms a plateau as it approaches the PaO_2 of the perfusate.

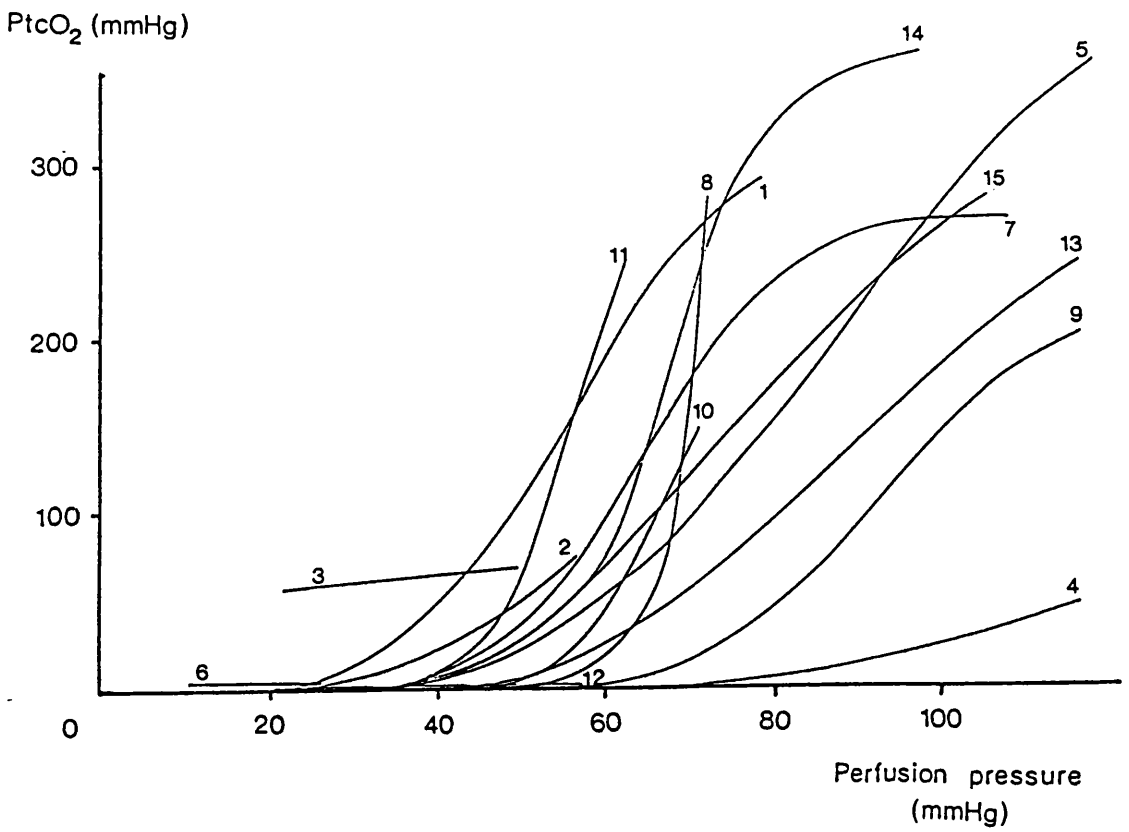


Figure 17 :
Plot of the $PtcO_2$ measured on the isolated limb against the coincident perfusion pressure measured in the dorsalis pedis artery.

The line of "best-fit" for each patient is shown.

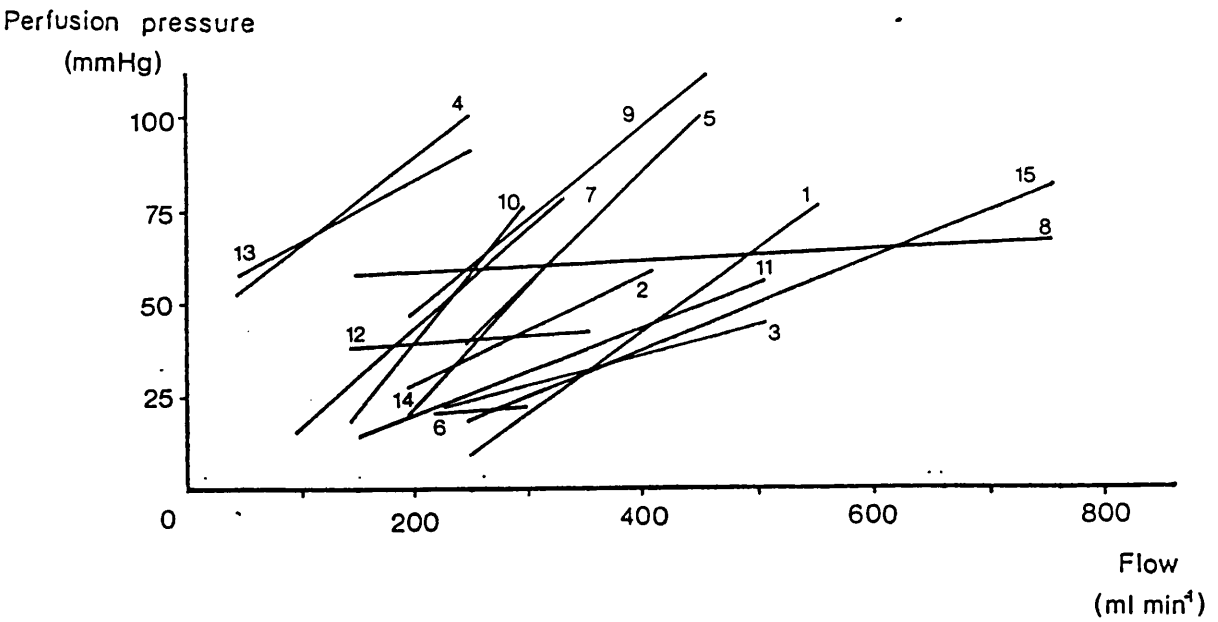


Figure 18 :
Graph of the perfusion pressure (dorsalis pedis artery) generated by varying flow rates in 15 patients.

The "best-fit" straight line for each patient was derived by linear regression.

than the mean systemic pressure in every case, the difference ranging from 6.4 mmHg to 54.7 mmHg with a mean of 26.7 mmHg (SD = 16.2).

The dorsalis pedis artery pressure was then plotted against flow rate for each case and the best fit straight lines were derived by regression. These are shown in Figure 18. The flow rate required to generate the critical "take-off" pressure in the limb was then calculated from these lines. This was highly variable, ranging from 33 ml min^{-1} to 456 ml min^{-1} with a mean of 279.6 ml min^{-1} (SD = 120.9).

3.3.4 Discussion

As one would expect, the surgical isolation of the limb results in effective cessation of perfusion of the tissues of the limb. This is reflected in the rapid decrease in the $PtcO_2$ to zero, which was observed in all but one case in this study. On restoration of inflow to the limb from the extracorporeal circuit, adjustments in the flow rate produced changes in the perfusion pressure but only resulted in successful perfusion of the skin (indicated by a rising $PtcO_2$) when the pressure exceeded a critical level which was different in each case. This can be regarded as the critical closing pressure of the cutaneous arteriolar network. Figure 17 shows that above this level, the $PtcO_2$ measurement rose steeply and tended to form a plateau as it approached the level of the PaO_2. Over the range of flow rates used in this study, the

relation of pressure to flow was more linear (Fig. 18). The sigmoid profile of the $PtcO_2$/pressure curve and the linear profile of the pressure/flow curve are corroborated by the studies of Beran and colleagues[427] who assessed the effect of lowering blood pressure on flow and $PtcO_2$ by applying occlusive plethysmography to arms.

In two cases in this study (cases 6 & 12), the $PtcO_2$ remained low throughout the isolated perfusion. In both of these cases, the perfusion pressure was relatively low (max. 26 and 54 mmHg respectively) and it is probable that the take-off pressure was simply not reached in these cases. In case 3, the $PtcO_2$ remained within normal limits throughout the procedure. This patient had undergone a first isolated limb perfusion at the iliac level one year earlier. It is possible that, by the time of his second ILP, a mature collateral circulation around the pelvis and hip had developed which was not effectively abolished by the surgical isolation procedure. This might account for the persistence of normal tissue oxygenation even after clamping the iliac vessels.

As with all physiological variables, one would expect the value of the critical take-off pressure to fall within a "normal" range. However, this parameter may also be influenced by chemical factors, such as the pH of the circulating blood, circulating vasoactive substances, blood oxygen tension, and temperature, and by physical factors, such as the compliance of the vasculature and the degree of coexistent atherosclerosis. All of these factors contribute to the peripheral resistance and may

account for some of the variability in the critical pressure. The flow rate associated with this pressure in each case was also highly variable.

From this study, it would therefore appear that regulation of the isolated perfusion circuit to produce predetermined flow rates or pressures is an unreliable way of ensuring adequate perfusion of the tissues in all cases. Only by selecting very high flow rates could one be sure of having exceeded the closing pressure of the cutaneous and subcutaneous arterioles, and this would in many cases result in excessively high pressures or in excessive loss of fluid from the reservoir.

In their studies of tissue perfusion during ILP in dogs and humans[416,443,444], Fontijne and his colleagues demonstrated the inadequacy of predetermined flow rates as criteria of successful tissue perfusion. They also determined that tissue oxygenation in the isolated limb could only be maintained at adequate levels if the perfusion pressure was not allowed to drop to more than 15 mmHg below the mean systemic arterial pressure. Interestingly, this applied both to muscle oxygenation in the dogs and to transcutaneous oxygen tension in humans. However, if the perfusion management in ILP is based on maintaining a specified difference between systemic pressure and perfusion pressure, there is still a risk that a decrease in the systemic blood pressure might result in inadequate cutaneous perfusion in the isolated limb by forcing the perfusion pressure below the critical level. In the present study, the difference between the critical perfusion pressure and the mean systemic pressure

during the period of isolated perfusion ranged from 6.4 mmHg to 54.7 mmHg in the 12 evaluable patients. Control of the perfusion pressure at a level of 15 mmHg below the mean systemic pressure would have resulted in failure to exceed the critical perfusion pressure in a further 3 cases. Therefore this method is dependent on the maintenance of normotensive systemic circulation.

The $PtcO_2$ monitor allows the perfusion of the skin to be continuously assessed non-invasively. By applying this method, it should be possible to gradually increase the flow rate in the isolated circuit in every case until a rapidly rising $PtcO_2$ level is observed, indicating that the critical perfusion pressure has been exceeded. From that point on, control of the perfusion circuit would require adjustment of the flow rate in order to maintain these elevated $PtcO_2$ levels during the circulation of the cytotoxic agent, while protecting the stability of the circuit. The effect of this method would be to ensure maximal exposure of the tissues (the skin in particular) to the cytotoxic agent.

3.4 Control of Perfusion by $PtcO_2$ Monitoring

3.4.1 Introduction

Monitoring of the $PtcO_2$ offers the possibility of controlling the perfusion parameters of the isolated circuit in such a way as to ensure adequate perfusion of the skin. If this can be maintained throughout the period of circulation of the cytotoxic drug, the potential benefits of this approach will be not only the protection of the tissues from ischaemic injury but also maximum exposure of the skin to the drug and to the raised temperature of the perfusate. This study was initiated in order to assess the feasibility of this suggested perfusion method.

3.4.2 Patients and Methods

During a period of six months, a modified isolated perfusion technique was adopted for the first time in this centre. The flow rate through the pump, which until then had been adjusted to maintain the stability of the fluid distribution in the isolated circuit, was regulated to ensure effective cutaneous perfusion as evidenced by restoration of an elevated $PtcO_2$ following the ischaemic period of cannulation of the vessels. Maintenance of a stable circuit, while desirable, was regarded as secondary to the fulfilment of this criterion. A potentially greater loss of fluid volume from the reservoir than

previously allowed was deemed acceptable. However, a drop in the fluid level sufficient to risk air embolism by vortex formation at the arterial outlet of the reservoir (reservoir volume < 200 ml), especially at the higher flow rates associated with iliac perfusion, was deemed excessive and an indication to reduce the flow rate. The volume and composition of the priming fluid were those routinely used in this centre (2:1 mixture of Ringer's lactate solution and packed red blood cells containing 3000 units of heparin; total volume = 1100ml).

The surgical technique was unchanged from the standard practice in this centre (see Section 2.1.2).

The systemic arterial pressure was monitored via a 20G teflon radial artery cannula (Viggo-Spectramed, UK). A similar cannula was placed in the dorsalis pedis artery of the perfused limb to monitor the perfusion pressure during iliac ILP. For axillary ILP, the perfusion pressure was measured via the radial artery of the isolated limb. $PtcO_2$ electrodes (E5242, Radiometer, Copenhagen) heated to 43°C were lightly secured to the skin over the proximal half of tibialis anterior (iliac ILP) or the muscle bellies of the wrist flexors (axillary ILP) on both the perfused and the non-perfused sides. These were connected to a continuous display $PtcO_2$ monitor (TCM2, Radiometer, Copenhagen).

Throughout the operation, the mean systemic blood pressure, the mean perfusion pressure in the isolated limb, the flow rate through the pump and the $PtcO_2$ in the isolated limb were all recorded at regular intervals (according to the protocol shown in Figure 14) together

with the temperature of the perfused limb, the volume of perfusate remaining in the reservoir and the $PtcO_2$ on the contralateral limb.

In all, 22 patients were treated during this period. Perfusions were performed at the iliac (16 cases) and axillary (6 cases) levels. Addition of melphalan to the perfusate was delayed until cutaneous perfusion was demonstrated by a rising $PtcO_2$ level or until 20 minutes after the commencement of isolated perfusion, whichever occurred sooner. Perfusion was then continued for 60 minutes before rinsing the limb vasculature with non-recirculated Hartmann's solution as usual.

Two questions were addressed in this study :

[1] can one reliably use this perfusion method to ensure effective delivery of the perfusate to the skin?

[2] can this goal be achieved without jeopardising the safety of the procedure, which is normally safeguarded by maintaining a positive pressure gradient between the systemic and the isolated circulations?

3.4.3 Results

In this study, cannulation of the dorsalis pedis artery was unsuccessful on three occasions and the perfusion pressure could therefore not be recorded directly in these patients. In all of the remaining 19 cases, it was possible to adjust the flow rate in the isolated circuit so that the perfusion pressure reached a level sufficient to cause the $PtcO_2$ to rise from the low values observed during the ischaemic period of the surgical isolation and cannulation. Figure 19 shows the curves of $PtcO_2$ against perfusion pressure for (a) 13 iliac perfusions, and (b) 6 axillary perfusions. As in the earlier observational study (see Section 3.3.3), these curves clearly show a critical take-off pressure above which the $PtcO_2$ rises rapidly from zero. The critical perfusion pressure ranged from 34 to 83 mmHg for iliac perfusions and from 58 to 83 mmHg for axillary perfusions with means of 60.3 mmHg (SD = 13.8) and 64.8 mmHg (SD = 9.2) respectively.

In 18 patients, this pressure was reached within 20 minutes of the commencement of isolated perfusion and therefore before the addition of melphalan to the perfusate. In 4 patients the melphalan was added after 20 minutes despite the fact that the critical perfusion pressure had not yet been reached. In 3 of these, $PtcO_2$ started to rise within five minutes of melphalan administration. In the fourth patient, the critical perfusion pressure of 63 mmHg was only achieved after 55 minutes of perfusion (ie. 35 minutes after melphalan administration) when the flow rate reached 820 ml min^{-1}.

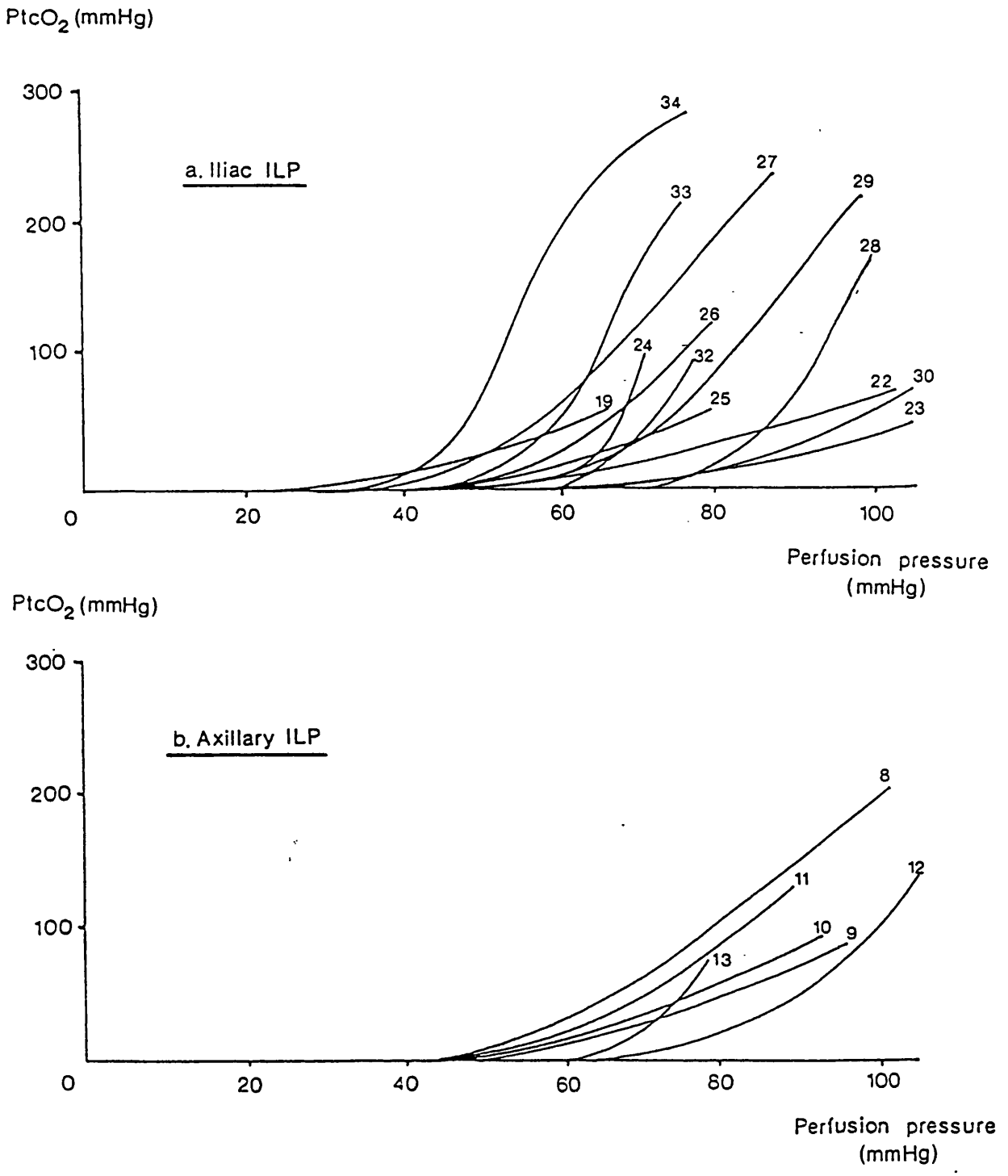
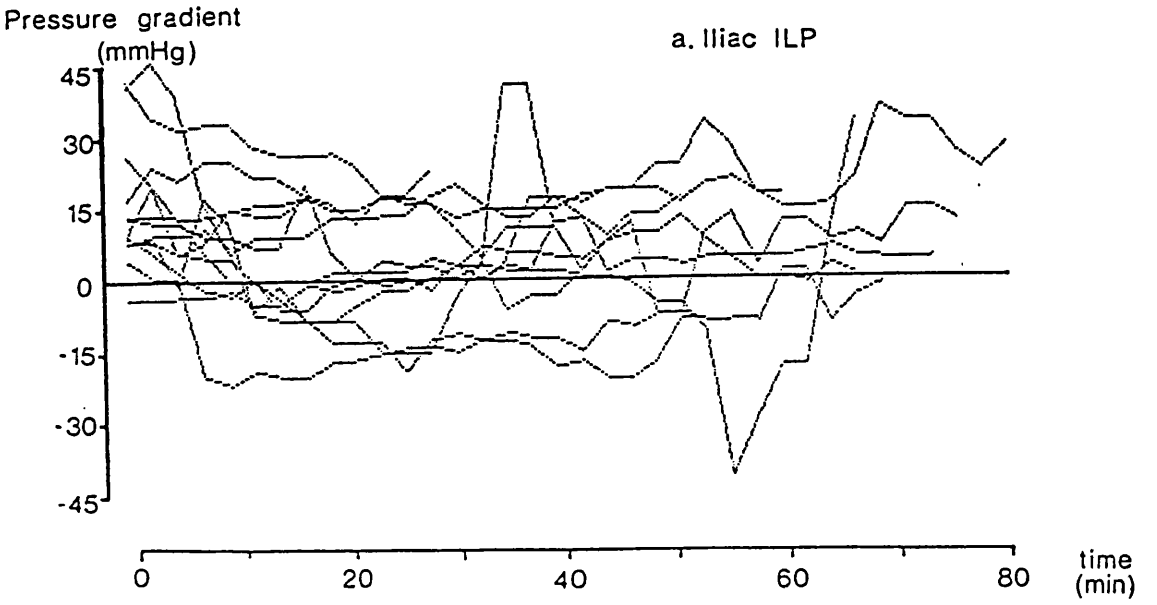


Figure 19 :
Graphs of $PtcO_2$ against perfusion pressure during (a) 13 iliac and (b) 6 axillary ILP's in which the flow rate was controlled to produce and maintain an elevated $PtcO_2$.

Once cutaneous perfusion had been demonstrated by the $PtcO_2$, it was possible to maintain this in most cases with only modest adjustments to the flow rate. In general, a gradual increase in flow rate during the first half of the procedure was matched by a similar decrease during the second half of the procedure, the mean difference between the flow rate at the time of melphalan administration and at the end of the perfusion being -17.5 ml min^{-1} (95% confidence interval: -59, +24) for iliac perfusions (ie. -3%), and -10 ml min^{-1} (95% confidence interval: -73, +53) for axillary perfusions (ie. -4%). In 4 cases, it became necessary to reduce the flow rate to preserve the reservoir volume. This flow reduction was associated with a decrease in $PtcO_2$ which on 3 occasions returned to zero. In the fourth case, the $PtcO_2$, although low, remained higher than during the ischaemic period, indicating that cutaneous perfusion was maintained, albeit at subnormal levels. The mean flow rate in the isolated circuit during the periods of elevated $PtcO_2$ recordings ranged from 286.4 to 820.0 ml min^{-1} in iliac perfusions and from 107.0 to 375.2 ml min^{-1} in axillary perfusions, with means (SD) of 501.6 ml min^{-1}(152.9) and 238.5 ml min^{-1}(101.9) respectively.

The difference between the perfusion pressure during the period of effective cutaneous perfusion and the coincident systemic mean arterial pressure was measured in order to determine the pressure gradient between the systemic and the isolated circulations in each case. This is shown graphically in Figure 20. The mean difference between the



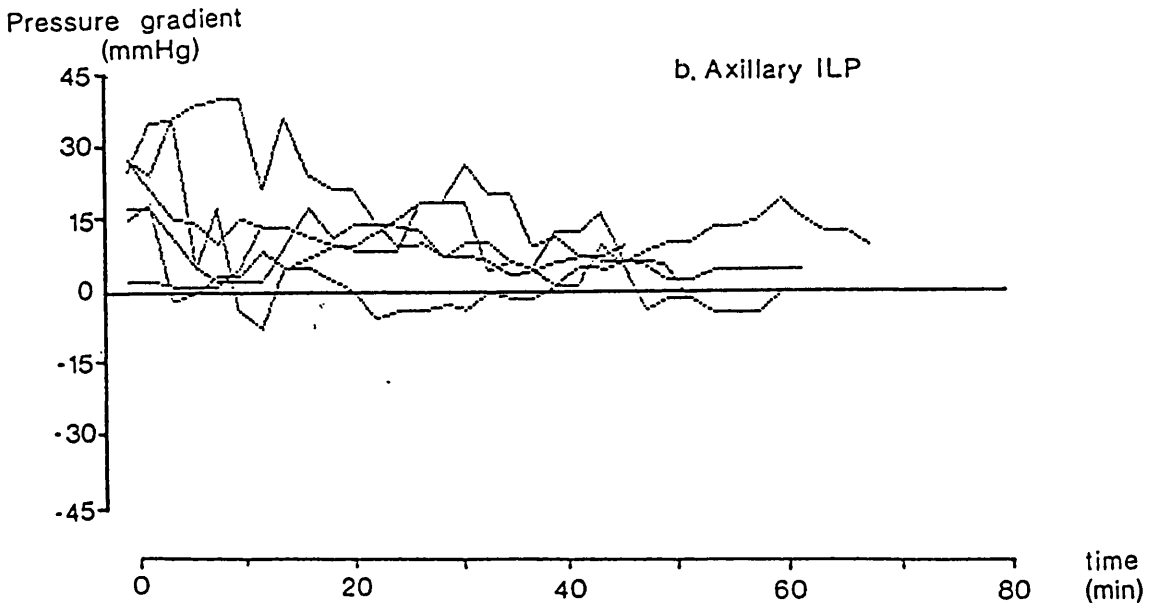


Figure 20 :
Graphs of the pressure gradient between the systemic and isolated circulations during (a) 13 iliac and (b) 6 axillary ILP's in which the flow rate was adjusted to maintain an elevated $PtcO_2$ on the isolated limb.

The gradient was calculated by
$$\frac{\text{mean systemic pressure - mean perfusion pressure}}{}$$
at each of the time points on the data recording sheet (see Fig.14).

systemic and isolated arterial pressures during these periods of perfusion ranged from -10.2 to +21.6 mmHg in iliac perfusions and from +3.1 to +15.6 mmHg in axillary perfusions, with overall means of +7.0 mmHg and +8.8 mmHg respectively (pressure gradient = systemic pressure - isolated perfusion pressure).

3.4.4 Discussion

In 18 of the 22 patients in this study, the perfusion parameters were successfully adjusted to ensure effective perfusion of the skin before the addition of melphalan to the perfusate. This was achieved in all cases by increasing the flow rate in the isolated circuit until the perfusion pressure exceeded the critical perfusion pressure for each patient, as witnessed by a rise in $PtcO_2$ from the very low levels observed during the ischaemic period of isolation and cannulation of the vessels. In a further 3 patients, effective skin perfusion was demonstrated within 5 minutes of the addition of melphalan to the perfusate. Several authors have reported that the pressure in the isolated circuit frequently rises on addition of the melphalan to the perfusate. This is believed to be due to a vasoconstrictor effect of the acid-alcohol diluent resulting in increased peripheral resistance. It is probable that in the three cases in question this produced a further increase in the perfusion pressure sufficient to raise it above the critical level.

In one case, a tall muscular young man (height 1.88m, weight 103 kg, age 26 years), gradual increases in the flow rate only resulted in effective cutaneous perfusion when an exceptionally high flow rate of 820 ml min^{-1} was reached. This was associated with a perfusion pressure of only 63 mmHg. The combination of the patient's physique and highly compliant vessels may account for these exceptional observations.

A comparison of the perfusion data of the patients in this study with those of the previous study (Section 3.3.3, above) shows that the flow rates required to maintain elevated $PtcO_2$ levels during the isolated perfusion were significantly higher than those previously employed in this centre for iliac perfusions (501.6 ml min^{-1} v 337.3 ml min^{-1}; two sample T-test : T=2.91, p=0.0069). Although no axillary perfusions were included in the previous study, a review of the perfusion records of the last six patients undergoing axillary perfusion before the initiation of this study showed that after stabilisation of the circuit the mean flow rate during isolated perfusion of this region ranged from 50.0 to 107.0 ml min^{-1} with a mean of only 79.3 ml min^{-1} (SD = 22.8). The flow rates required to maintain effective cutaneous perfusion during axillary perfusion were therefore also significantly higher than in the previous practice (238.5 ml min^{-1} v 79.3 ml min^{-1}; two sample T-test : T=3.74, p=0.013). These differences are shown graphically in Figure 21.

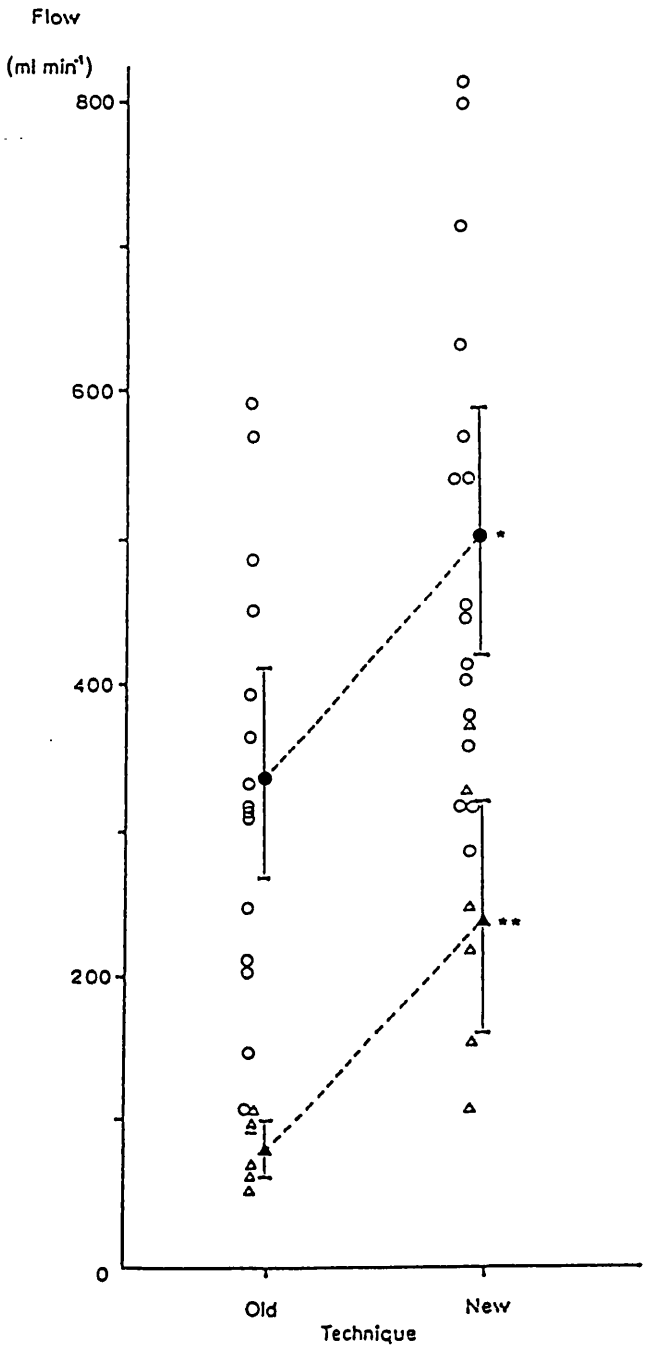


Figure 21 :
Mean flow rate during iliac (●) and axillary (▲) ILP.

"Old" technique : flow rate adjusted to maintain a stable fluid distribution in the extracorporeal circuit.

"New" technique : flow rate adjusted to maintain elevated $PtcO_2$ values in the perfused limb.

Bars indicate 95% confidence intervals around the mean.
*** p = 0.0069 ; ** p = 0.013 (two sample T-test).**

In their studies of ILP controlled to maintain a fixed pressure gradient of +15 mmHg between the systemic and isolated circulations, Fontijne and colleagues also observed that the flow rates required to achieve this gradient varied widely between patients and tended to be significantly higher than in their previous experience (200-1400 ml min^{-1}) [416]. They highlighted the failure of predetermined fixed flow rates to ensure adequate tissue perfusion. The present study, however, confirms that the maintenance of a +15 mmHg pressure gradient is itself an unreliable method of ensuring adequate skin perfusion in both axillary and, especially, iliac perfusion. Indeed, Figure 20 demonstrates that the pressure gradient was less than this for as much as three quarters of the periods of effective cutaneous perfusion (iliac 73.0%; axillary 75.6%). Furthermore, the perfusion pressure actually exceeded the coincident mean systemic pressure, thus reversing the pressure gradient, for up to a quarter of these effective perfusion periods (iliac 28.2%; axillary 13.2%).

Perfusion of the tissues of the isolated limb implies the distribution of perfusate to vascular beds drained of blood during the ischaemic period of isolation and cannulation of the vessels. On commencing isolated perfusion, one can therefore expect a transfer of perfusate volume from the oxygenator reservoir to the limb to fill these vascular beds. The more complete the tissue perfusion, the greater this loss of volume is likely to be. In this study, the loss of perfusate volume in 4 cases was so great that the flow rate (and consequently

the perfusion pressure) had to be reduced to avoid the risk of entrainment of air bubbles into the circulation. This had the predictable, if undesired, effect of reducing the cutaneous perfusion. A simple way of overcoming this problem would be to increase the volume of priming fluid used (eg. from 1100ml to 1500ml). This would have the additional benefit of allowing bolder initial increases in flow rate, so that the critical perfusion pressure could be generated more quickly, even in patients such as the young man described above. While one should be aware of the potential dilutional effect that such a change might have on the melphalan concentration in the perfusate, the dosimetry studies in Chapter 4 suggest that the contribution of the priming fluid to the total volume of distribution of melphalan is much less significant than one might expect. This may therefore not pose a serious problem (see Section 4.3.3).

Since effective cutaneous perfusion cannot be reliably ensured by maintaining a predetermined flow rate or pressure gradient, direct assessment of cutaneous perfusion by $PtcO_2$ monitoring seems a more appropriate method of controlling the perfusion parameters during ILP. The method, however, is associated with higher perfusion pressures than have normally been recommended. This feature, and in particular the reversal of the pressure gradient observed in many cases, may jeopardise the safety of the procedure by increasing the theoretical risk of leakage of cytotoxic agent to the systemic circulation by reversed flow through persistent collateral channels.

3.5 Effect of Pressure Control on the Cutaneous Uptake and Systemic Leakage of Melphalan

3.5.1 Introduction

The theoretical advantages of regulating the perfusion parameters during ILP in the manner described above include

- **maximal exposure** of the skin and subcutaneous tissues to the cytotoxic agent used and to the effects of hyperthermia

and

- **protection of the tissues of the limb** against the effects of ischaemia caused by inadequate perfusion.

The principal potential disadvantage of the method is the **risk of leakage** of perfusate containing high concentrations of cytotoxic drug into the systemic circulation which may result from the reversal of the pressure gradient between the isolated limb and the systemic circulation.

The success of this therapeutic modality is dependent on combining the maximum regional antitumour effect with the minimum systemic side effects and acceptable regional toxicity. Although the efficacy of the treatment can only truly be determined by prolonged follow-up of the treated

patients, it would be helpful to know whether the theoretical benefits and risks of the modifications of the technique described above can be demonstrated in reality.

The aims of the study described in this section were threefold :

[1] to assess the relationship between the **quality of tissue perfusion** during ILP and the **uptake of melphalan** by the tissues;

[2] to assess the **regional toxicity** observed in the perfused limb and compare this with the concentrations of melphalan in the skin and perfusate;

[3] to observe the effect of changes in perfusion pressure on the **leakage of melphalan** into the systemic circulation.

3.5.2 Patients and Methods

During the period of the studies described above (see Sections 3.3 and 3.4), melphalan pharmacokinetics data were collected from 38 patients. All patients gave their informed consent to participation in these studies which were also approved by the local ethical committee.

All patients underwent isolated limb perfusion with melphalan for malignant melanoma, according to the normal practice in Glasgow (see Section 2.1.2). ILP was performed at the iliac level in 27 cases and at the axillary level in 11 cases. Patients undergoing iliac ILP received a dose of 1.75 mg kg^{-1} of melphalan injected as a bolus into the venous compartment of the reservoir. Patients undergoing axillary ILP received 50 or 60 mg of melphalan, depending on body weight and build of the patient. Isolated perfusion was continued for 60 minutes after the addition of melphalan. The circuit was then rinsed with Hartmann's solution before reversing the isolation.

Pharmacokinetics data collection

5 ml samples of perfusate were withdrawn from the arterial tubing of the extracorporeal circuit at 5 minute intervals throughout the hour of isolated perfusion. In 30 cases, a further sample was obtained of the final effluent from the venous cannula at the end of the rinsing period (sample EP). Samples of systemic blood were also withdrawn at 0, 30 and 60 minutes from the start of perfusion with melphalan, and 30 minutes after restoration of the systemic circulation to the limb (ie. approximately 120 minutes after the addition of melphalan to the perfusate). All the samples were collected in lithium heparin tubes and kept in an ice bath until the sample collection was complete. After placing all the

samples in a centrifuge at 2500 rpm for 10 minutes, the plasma from each sample was transferred into a separate tube and stored at -20^{0}C until melphalan assay could be performed by High Performance Liquid Chromatography (HPLC; see below).

Skin samples were also obtained from 21 of the patients undergoing iliac ILP and 10 of the patients undergoing axillary ILP by excising an ellipse of skin (15mm x 5mm) from the medial aspect of the upper thigh or upper arm respectively at the end of the rinsing. The subcutaneous fat was shaved off the deep surface of the samples which were then placed in universal containers and "snap-frozen" by immersion in liquid nitrogen. The frozen tissue was then stored at -20^{0}C until melphalan assay could be performed by HPLC.

Melphalan assay by High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is a technique which harnesses the principles of chromatography - separation of the components of a mixture by exploitation of their different affinities for stationary and mobile phases - without the need to expose the substances analysed to raised temperatures. It is therefore particularly useful for the analysis of heat-labile compounds, such as melphalan. The HPLC column is

pressure-driven rather than gravity-dependent, making the method much less time-consuming than classical chromatographic analysis.

The method used for melphalan assay of all the samples in this study is based on the methods of Furner et al[445], Chang et al[446] and Flora et al[447].

[A] Melphalan assay in blood samples

0.1-1.0ml samples of the thawed plasma in 15ml conical centrifuge tubes are used for analysis. 10ug of dansyl proline (Sigma) is added to each sample to act as an internal standard. Keeping the sample on ice, four volumes of ice-cold acetonitrile (HPLC Grade, BDH) containing 0.1% hydrochloric acid (Analar Grade, BDH) are then added. The tube is immediately vortex-mixed and placed in a centrifuge for 10 minutes at 2000 rpm. The supernatant is transferred to 30ml vials and is evaporated to dryness on a Buchler vortex evaporator, keeping the temperature at 30^0C. The sample is then reconstituted in 500ul of the mobile phase (equal volumes of 0.01 M NaH_2PO_4 and 20% methanol, titrated to pH 3.0 with phosphoric acid, Analar Grade, BDH), centrifuged at 13000 rpm for 5 minutes (MSE micro centrifuge) and transferred to autosampler vials (300ul) for loading into the Altex autosampler (Model 500, Beckman RIIC; Altex injection valve model 210, with 50ul loop).

The sample for analysis is then pumped at 1.5 ml min^{-1} by an Altex solvent programmer (Model 420, Beckman RIIC) and Altex solvent pump (Model 100A, Beckman RIIC) to a 150x4.6mm stainless steel column packed "in-house" with Partisil ODS-2 (10u particle size) and u-Bondapak C18 (Waters) in the pre-column.

Melphalan and dansyl proline were detected using an ultraviolet spectrophotometer (Model 100-10, Hitachi) set at a wavelength of 261 nm. The results were processed using a Milton Roy CI-10 integrator with Sekonics printer/plotter.

[B] Melphalan assay in skin samples

Thawed skin samples are prepared for melphalan assay by weighing, chopping finely with a scalpel, and then homogenising in ice-cold 0.01 M NaH_2PO_4 (pH 3.1) using an Ultra-Turrax homogeniser. 2ml samples of homogenate are then placed in 50ml conical centrifuge tubes and analysed using the same method as that described for plasma samples.

The accuracy of this method was tested by producing standard curves from the analysis of samples of blank plasma spiked with known concentrations of melphalan, ranging from 1-50ug ml^{-1}. The extraction efficiency of melphalan and dansyl proline were 90% and 93%

respectively, the melphalan plot being linear through the tested range (r=0.9967). Inter-assay variability was less than 5%, and the limit of detection of melphalan was 100ng ml^{-1}.

Assessment of regional toxicity

Postoperatively, patients were examined on a daily basis to assess the toxicity in the tissues of the perfused limb. This was graded according to the Wieberdink classification[406] (see Table 8) and the most severe reaction recorded in each case.

Analysis of results

The measured plasma melphalan concentrations were used to generate concentration/time curves for the perfusate from time zero to 60 minutes. The area under the curve (AUC_{0-60}) for each patient was then calculated using the trapezoidal rule. Several modifications were made to this AUC calculation to take account of the influence of the quality of perfusion on the delivery of melphalan to the tissues :

(i) the measured melphalan concentration was doubled for the AUC calculation during periods when the $PtcO_2$ on the perfused limb was elevated above the ischaemic levels recorded during the period of isolation and cannulation (AUC_a)

(ii) the product of the melphalan concentration and the coincident $PtcO_2$ was plotted against time and the AUC of this curve calculated for each case (AUC_b)

(iii) the oxyhaemoglobin dissociation curve for normal blood shows that the oxygen saturation of haemoglobin rises to about 90% at a pO_2 of 70 mmHg. At pO_2 values higher than this, the saturation rises only very little. Several factors are known to affect the position of the dissociation curve on the pO_2 axis, such as pH, temperature and the concentration of 2,3-diphosphoglycerate (2,3-DPG) in erythrocytes. In ILP, the combined effects of the raised temperature and alkaline pH of the perfusate negate that of the low 2,3-DPG content of the priming blood mixture, resulting in an approximately normal position for the oxyhaemoglobin dissociation curve. Therefore, $PtcO_2$ values of 70 mmHg or more can be considered to represent effective arterial perfusion of the skin. In order to avoid inappropriate weighting of the AUC calculations by extremely high $PtcO_2$ values, a perfusion index (PI) was introduced to the AUC calculation :

$$PI = PtcO_2/70 \quad (\text{ if } PtcO_2 < 70 \text{ mmHg })$$

or

$$PI = 1 \quad (\text{ if } PtcO_2 \geq 70 \text{ mmHg })$$

The melphalan concentration at the various time points was then increased by the coincident perfusion index (ie. [MEL] x (1 + PI)) and the AUC of this curve calculated for each case (AUC_C).

The effectiveness of perfusion throughout the period of isolated perfusion (the overall perfusion index, PI_O) could then be expressed as the quotient of AUC_C and AUC_{0-60}.

The potential leakage of melphalan to the systemic circulation during periods of reversal of the pressure gradient between the isolated and systemic circulations was estimated by calculating the AUC of melphalan in the perfusate for these periods only (AUC_{leak}).

The actual systemic exposure to melphalan during ILP and in the period immediately following this was also estimated by generating concentration/time curves for melphalan in the samples of systemic blood and deriving AUC's for time zero to 60 minutes ($_sAUC_{0-60}$) and time zero to 120 minutes ($_sAUC_{0-120}$). A previous study in Glasgow has shown that the systemic blood concentration of melphalan is highest one hour after the end of the isolated perfusion period[448]. This is believed to be due to the rinsing out of residual melphalan from the limb by

the restored systemic circulation. After this time, the systemic melphalan concentration decreases steadily over a period of several hours.

The three stated aims of this study were then addressed as follows :

[1] the skin melphalan concentration was compared to each of the AUC's for melphalan in the perfusate;

[2] the toxicity observed in the perfused limb was compared to the calculated AUC's, the skin melphalan concentration and the PI_o;

[3] the risk of leakage of melphalan to the systemic circulation was assessed by comparing the AUC_{leak} with the $_{sys}AUC_{0-60}$.

3.5.3 Results

The concentrations of melphalan in the perfusate and the systemic circulation for each patient are shown in Appendix B.

Table 19 lists the skin melphalan concentrations together with the four calculated AUC's for melphalan in the perfusate, the overall perfusion index, and the regional toxicity for each patient.

Table 19 :

Pharmacokinetics of Melphalan in Skin and Perfusate during ILP

Patient No	Skin [Mel]	Perfusate AUC_{0-60}	AUC_a	AUC_b (x100)	AUC_c	PI	Toxic Grade#
Iliac							
003	-	842	1684	467	1506	1.79	I
005	-	1437	2875	3138	2698	1.88	II
007	1.07	930	1860	2193	1860	2.00	II
008	2.22	1494	2649	2045	2650	1.77	III
009	1.71	1690	3380	2198	3380	2.00	III
010	1.85	1010	1818	613	1643	1.63	II
011	2.12	1102	1998	1003	1933	1.75	III
012	1.32	1537	1537	10	1552	1.01	II
013	3.38	2448	4896	3702	4538	1.85	II
014	3.66	1397	2795	4300	2795	2.00	II
016	-	1507	3014	3100	3014	2.00	III
*019	6.70	1976	3952	738	3019	1.53	II
*020	4.50	2105	4209	1556	3252	1.54	II
*021	12.02	1891	3782	3434	3445	1.82	II
*022	11.12	2954	5337	1312	5067	1.72	III
*023	7.76	2273	4096	968	3656	1.61	III
*024	3.97	2215	2740	193	2491	1.12	II
*025	1.11	1819	2832	178	2188	1.20	II
*026	2.10	1076	2153	764	1956	1.82	II
*027	5.95	1862	3725	2953	3621	1.94	III
*028	1.85	1127	2253	734	1774	1.57	II
*029	2.81	1661	3321	2036	3311	1.99	III
*030	6.91	2426	4852	1331	4116	1.70	II
*031	0.88	1423	2564	519	2031	1.43	III
*032	-	1845	3282	727	2861	1.55	III
*033	-	3170	6339	4036	6014	1.90	II
*034	-	1580	3159	3604	3159	2.00	III
Axillary							
A03	2.86	1617	1617	0	1617	1.00	III
A04	0.83	1351	1351	0	1351	1.00	IV
A05	0.70	1526	2563	64	1600	1.05	II
A06	3.89	1571	2757	54	1648	1.05	III
A07	1.90	2137	2137	0	2137	1.00	III
*A08	11.51	1855	3495	833	2821	1.52	III
*A09	-	2771	4295	437	3396	1.23	I
*A10	2.78	923	1846	714	1691	1.83	II
*A11	2.89	1280	2137	324	1721	1.34	II
*A12	5.09	1033	2066	1162	1986	1.92	II
*A13	8.44	1034	1526	420	1230	1.19	III

Skin[mel] in ug ml^{-1} AUC's in ug.min ml^{-1}

PI : perfusion index

Wieberdink grades[406] (see Table 8)

* denotes ILP controlled on the basis of $PtcO_2$.

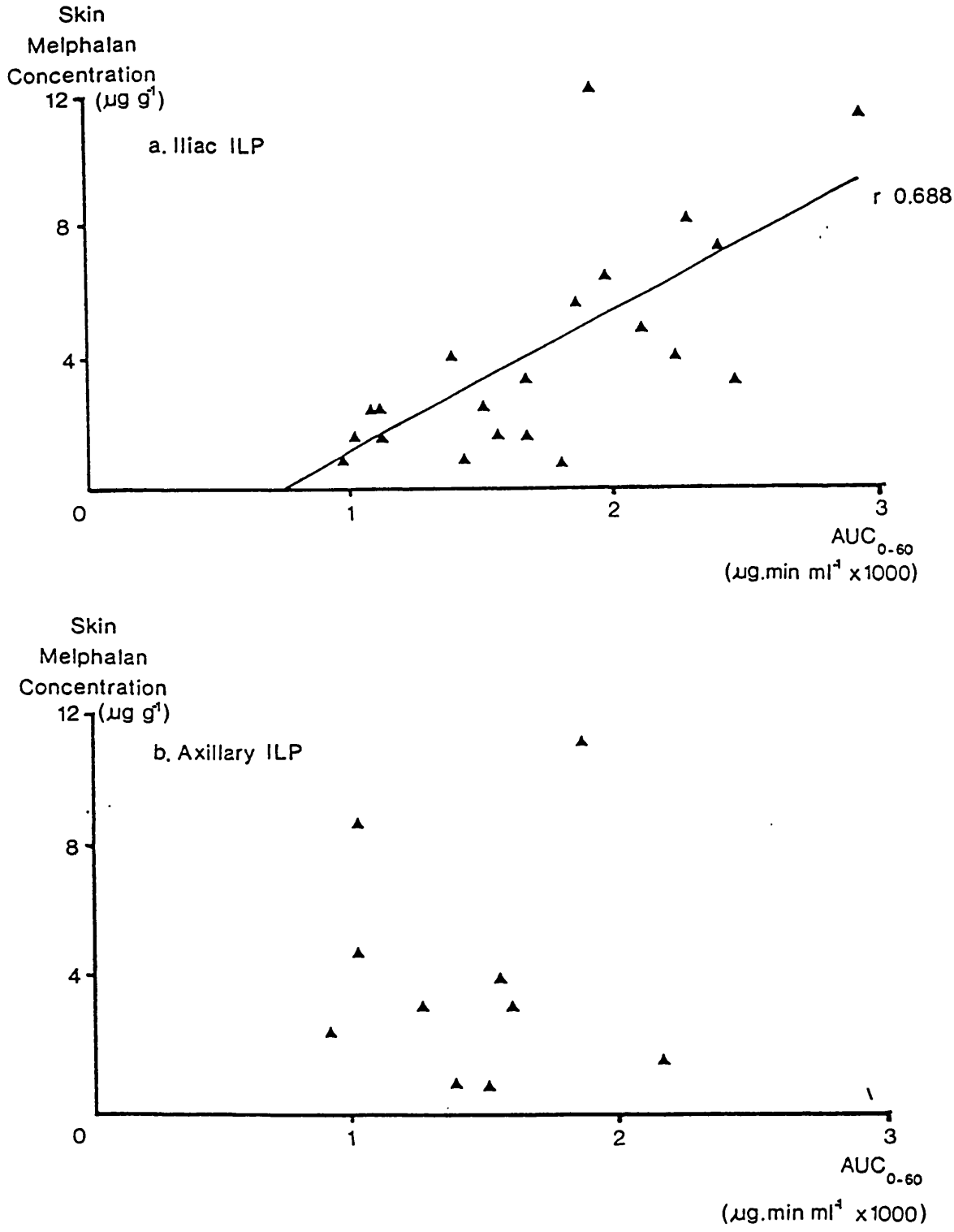


Figure 22 :
Plot of the melphalan concentration in skin biopsies taken at the end of ILP against the AUC_{0-60} of melphalan in the perfusate.

(a) iliac ILP (b) axillary ILP

Although a clear association could be demonstrated between the skin melphalan concentration and the AUC_{0-60} for lower limb ILP (p=0.001), the correlation was rather poor (r=0.688). For upper limb ILP, no association was demonstrated between the skin melphalan concentration and the AUC_{0-60} (r=0.000) (Figure 22). Modification of the AUC calculation to reflect the quality of perfusion resulted in a slight improvement in the correlation for lower limb ILP (AUC_a r=0.726, AUC_b r=0.265, AUC_c r=0.716). These modifications failed to reveal a significant association between the skin melphalan concentration and the modified AUC's for upper limb ILP.

In order to assess the relation between the pharmacokinetic parameters listed in Table 19 and the observed toxicity, the recorded Wieberdink Grades were regrouped into two categories (Grades I-II, Grades III-IV). No significant difference was demonstrated between these two groups when comparing the skin melphalan concentration, AUC_{0-60}, AUC_a, AUC_b, AUC_c, or PI_o (Mann-Whitney U-test). This applied to the overall analysis of the results and to their breakdown according to perfused region.

Table 20 lists the pharmacokinetic data for melphalan in the systemic circulation for the patients in this study. No simple correlation could be demonstrated between the measured systemic exposure to melphalan during the period of isolated perfusion ($_sAUC_{0-60}$) and the estimated leakage

Table 20 :

Pharmacokinetics of Melphalan in Systemic Blood during ILP

Patient No	Systemic sAUC (0-60)	Systemic sAUC (0-120)	Systemic AUC_{leak}	Perfusate# t=60	Perfusate# End	Rinsing Efficiency (%)
Iliac						
003	0.0	86.1	0.0	6.32	-	-
005	11.9	17.6	1077.9	9.97	-	-
007	0.0	9.6	613.8	10.38	-	-
008	2.7	23.7	576.3	12.16	-	-
009	10.2	36.9	1728.0	15.13	-	-
010	5.1	27.3	0.0	8.64	0.94	89.1
011	26.7	56.7	0.0	9.25	1.75	81.1
012	73.7	140.6	0.0	13.19	1.38	89.5
013	37.5	71.7	2021.5	19.44	2.91	85.0
014	0.0	11.1	172.5	11.10	2.82	74.6
016	34.5	150.6	-	9.26	-	-
*019	8.1	31.5	0.0	12.80	2.20	82.8
*020	0.0	16.8	-	11.70	1.28	89.1
*021	0.0	117.6	-	9.03	0.39	95.7
*022	0.0	24.6	1456.5	24.80	4.11	83.4
*023	0.0	84.9	1749.9	-	5.16	-
*024	8.4	30.0	0.0	15.40	5.10	66.9
*025	23.7	46.8	0.0	11.54	1.97	82.9
*026	0.0	3.6	385.3	9.35	1.37	85.3
*027	1.2	38.4	0.0	-	3.21	-
*028	7.5	27.3	569.2	-	3.63	-
*029	39.2	68.6	740.9	9.11	1.19	86.9
*030	0.0	25.5	2445.9	7.90	3.24	59.0
*031	39.2	96.2	-	16.64	1.65	90.1
*032	32.3	49.9	0.0	11.75	0.76	93.5
*033	30.2	56.3	499.9	15.55	2.55	83.6
*034	0.0	0.7	0.0	10.53	1.76	83.3
Axillary						
A03	0.0	0.0	0.0	19.77	-	-
A04	0.0	5.4	0.0	12.11	-	-
A05	0.0	3.3	0.0	13.59	3.47	74.5
A06	0.0	0.0	0.0	14.48	3.68	74.6
A07	0.0	10.8	0.0	17.80	5.38	69.8
*A08	0.0	5.4	94.0	18.80	2.13	88.7
*A09	0.0	3.0	0.0	21.30	3.83	82.0
*A10	0.0	0.0	569.1	12.01	0.80	93.3
*A11	-	-	-	14.76	1.74	88.2
*A12	0.0	0.0	244.5	9.17	0.82	91.1
*A13	0.0	0.0	0.0	11.60	1.42	87.8

Melphalan concentration in samples of final perfusate (t=60 mins) and terminal rinse (End).
* denotes ILP controlled on the basis of $PtcO_2$.
AUC's expressed in ug.min ml^{-1}

(AUC_{leak}) which might have occurred as a result of the reversal of the pressure gradient between the isolated and the systemic circulations. Similarly, there was no correlation between the $_{s}AUC_{0-120}$ and the AUC_{leak}.

3.5.4 Discussion

The cytotoxic action of melphalan takes place at its two chloroethyl groups and is principally mediated within the cell nucleus by the formation of cross-links between strands of DNA[449-451]. It has been suggested that the binding of melphalan to the first strand of DNA takes place rapidly, but that completion of the DNA cross-links by the second chloroethyl group is delayed and reaches maximum levels after 6-12 hours[452]. The intracellular transport of melphalan has been shown experimentally to be dependent on an active carrier mechanism[453-457] but is also counteracted by ready efflux of unbound melphalan from the cell[456].

Since the duration of isolated perfusion with melphalan is limited by surgical considerations, it seems desirable to ensure that the tissues of the limb are exposed to the high melphalan concentrations typically encountered in ILP for as much of this period as possible, in order to saturate the transport mechanism and to counteract the efflux of melphalan from the cell by maintaining a high concentration gradient in the opposite direction. This should result in maximal binding of melphalan molecules to

DNA before the reversal of the concentration gradient between the cytoplasm and the interstitial fluid during the rinsing and restoration of the systemic circulation.

In previous studies, authors have attempted to calculate the tissue uptake of melphalan by measuring the loss of melphalan from the perfusate during ILP and correcting this to take account of loss from other sources such as hydrolysis and uptake by the cellular components of the perfusate[458,459]. This method, however, does not give any indication of the melphalan uptake by specific tissues of the limb but only by the limb as a whole. Three other studies have measured the tissue melphalan concentrations directly after lower limb ILP[460-462]. The first of these, based on only three patients, found higher melphalan concentrations in tumour than in subcutaneous fat[460]. In the other two studies, each based on 12 patients, Scott et al found similar melphalan concentrations in skin and tumour samples taken from the same patients, but were unable to demonstrate any correlation between these and the various pharmacokinetic parameters[461], while Klaase et al, on the other hand, observed a linear relation between tumour melphalan levels and the AUC_{0-60} which was not apparent for skin samples[462].

The skin melphalan concentrations measured in this study show, for the first time, a clear association with the AUC_{0-60} of melphalan in the perfusate during lower limb ILP ($p=0.001$; $r=0.688$). The same association is not apparent for upper limb ILP. Attempts to quantify the quality of cutaneous perfusion during ILP have not

revealed an association between this and the measured skin melphalan concentrations. However, the interpretation of the skin melphalan concentrations must be qualified by several other considerations:

[i] firstly, the HPLC assay measures the concentration of melphalan in the tissue fluid of the sample (ie. interstitial and intracellular). The proportion of the measured melphalan within the interstitial fluid may be partly determined by the efficacy of the rinsing process thus introducing a degree of variability into the technique.

[ii] secondly, the hydrolysis products of melphalan - monohydroxy- and dihydroxy-melphalan - were not measured in this study. Although the dihydroxy- product is believed to have no cytotoxic effect, monohydroxy-melphalan does possess some antitumour activity and may contribute to the overall cytotoxicity of melphalan[463].

[iii] thirdly, only free melphalan is measured by the HPLC assay. The method cannot take into account melphalan molecules already bound to DNA.

The implication of these factors is that the skin melphalan concentration measured by this method can only be regarded as an indirect indicator of the actual exposure of the skin to melphalan.

Alterations of the perfusion technique to ensure effective perfusion of the skin before the addition of melphalan to the perfusate (see Section 3.4) are likely to produce other changes in the perfusion circuit, which in turn might also have some bearing on the uptake of melphalan by the tissues : the more rapid blood flow can be expected to result in a more rapid rise in temperature in the perfused limb; similarly the higher initial flow rates result in a lower reservoir volume into which to add the melphalan and in more rapid mixing of the melphalan in the perfusate. This may therefore alter the pharmacokinetic profile of the drug, including the AUC_{0-60}. Indeed, although the doses of melphalan used in both phases of this study for lower limb ILP were very similar (mean dose : early phase 121.0mg (SD = 21.2), late phase 125.5mg (SD = 19.8)) and the priming fluid identical, the mean AUC_{0-60} and equilibrium concentration of melphalan in the perfusate ($[MEL]_{eq}$) were both significantly higher in the second phase of the study (AUC_{0-60} : 1399 (SEM = 135) v 1963 (SEM = 143) ug.min ml^{-1}, p=0.0083; $[MEL]_{eq}$: 39.60 (SEM = 3.70) v 67.41 (SEM = 7.86) ug ml^{-1}, p=0.0062. Mann Whitney U Test). For upper limb ILP, there was no statistically significant difference in the doses, the AUC_{0-60}, or the $[MEL]_{eq}$ between the two phases of the study. This is perhaps not surprising since although the flow rates used were higher than before (see Section 3.4.4), the relatively larger volume of priming fluid used in upper limb ILP would

result in a longer circulation time for the perfusate and a greater proportion of the fluid remaining in the reservoir.

In fact, the skin melphalan concentrations measured in the second phase of this study were significantly higher overall than those found in the earlier phase (mean (SEM) : 5.47ug ml^{-1} (0.84) v 2.12ug ml^{-1} (0.29); p=0.0045, Mann Whitney U test) (see Figure 23). Two way analysis of variance using a general linear model to test the effect of ILP technique ("old" or "new") and perfused region (upper or lower limb) on the skin melphalan concentration revealed a significant association only with the technique used (F = 10.47, p=0.003) and no significant interaction between these two factors.

The toxicity encountered was similar in both phases of this study and did not show a simple association with any of the pharmacokinetic parameters tabulated. The regional toxicity observed after ILP is thought to be due to a variety of factors: the drug used and its dosage, hyperthermia, and hypoxic injury. These factors are all interrelated, and it is possible that improved oxygenation might protect the tissues against the greater toxicity of higher temperatures and higher tissue concentrations of melphalan. In both phases, the toxic reactions were almost all of Grade II or III severity, distributed in equal proportions between these two bands. It is therefore reassuring to observe that although the

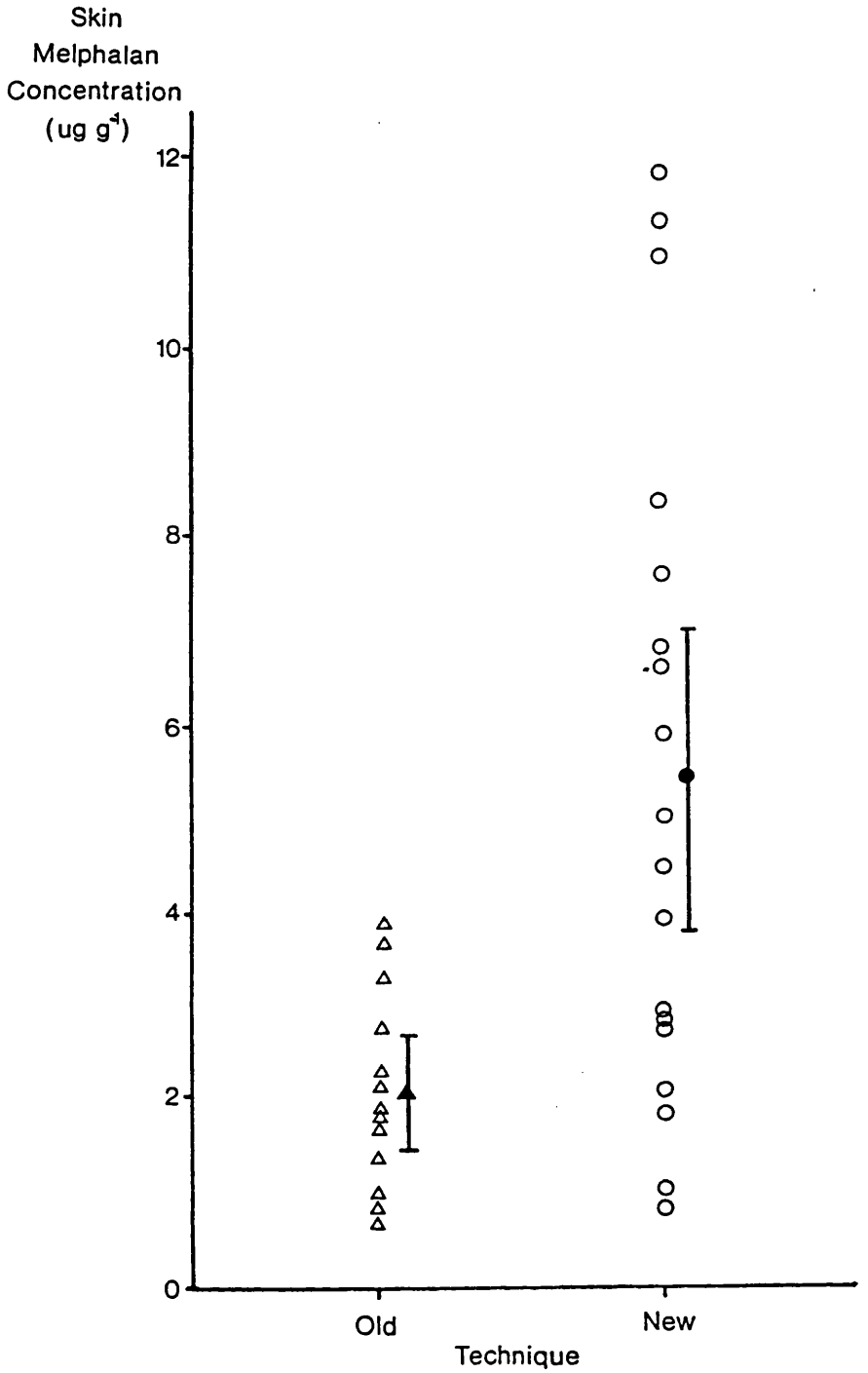


Figure 23 :
Melphalan concentration in skin biopsies taken at the end of ILP separated according to perfusion method employed.

"Old" technique : flow rate adjusted to maintain a stable fluid distribution in the extracorporeal circuit.

"New" technique : flow rate adjusted to maintain elevated $PtcO_2$ values in the perfused limb.

Bars indicate 95% confidence intervals around the mean.
p = 0.0045 (Mann Whiney U test)

technique of perfusion was altered, the toxic effect of the treatment was neither sacrificed nor increased to unacceptable levels.

The measured leakage of melphalan to the systemic circulation was very low during both the hour of ILP and the first hour thereafter. The mean AUC_{0-60} in the perfusate exceeded the mean $_{s}AUC_{0-60}$ and $_{s}AUC_{0-120}$ for lower limb ILP by factors of 116 and 33.65 respectively. In upper limb ILP, no melphalan was detected in the systemic circulation during the period of isolated perfusion and the mean $_{s}AUC_{0-120}$ was less than 0.002 of the mean AUC_{0-60}. There was no correlation between the calculated potential melphalan leak (AUC_{leak}) and the $_{s}AUC_{0-60}$.

The theoretical risk of increased leakage of melphalan to the systemic circulation due to higher perfusion pressures is therefore not supported by this study.

The other principal source of leakage of melphalan to the systemic circulation is the redistribution of melphalan from the perfused limb after the restoration of normal circulation to the limb. This includes the residual melphalan in the intravascular compartment at the end of the rinsing period, the melphalan in the interstitial fluid at that time, and melphalan effluxing from the cytoplasm after ILP. This process is believed to take place over several hours, but the highest systemic levels are reached within the first hour after the return of

normal flow to the limb[448]. Thorough rinsing of the limb vasculature at the end of ILP provides the simplest way of minimising this post-ILP release of melphalan. Samples of the final effluent from the rinsing fluid taken from 30 patients in this study show that this process reduces the melphalan concentration in the intravascular compartment by a mean of 83% for both upper and lower limbs. It may be possible to further reduce this by using a larger volume of rinsing fluid. A side-effect of this step, however, would be the prolongation of the ischaemic period unless a blood containing mixture were used. Furthermore, the delayed peak of the systemic melphalan concentration would suggest that most of this melphalan does not originate from the vascular compartment at the time of reconnection but is more slowly released from the interstitial or intracellular compartments. The low incidence of systemic toxicity encountered in Glasgow to date (see Section 2.1.4) using 2 litres of Hartmann's solution for lower limbs and 1 litre for upper limbs would suggest that this is an effective rinsing method. The modest benefits which might be achieved by changes to this practice should be balanced against the potential disadvantages of prolonged hypoxia and increased duration of anaesthesia.

3.5.5 Conclusion

Contrary to the results of previous smaller series, this study shows a clear correlation between the melphalan concentrations measured in skin biopsies after iliac ILP and the AUC_{0-60} of melphalan in the perfusate. Although no direct association could be shown between the degree of perfusion of the skin ($PtcO_2$) and the skin melphalan levels, alteration of the perfusion method to ensure effective perfusion of the skin especially during the period of highest perfusate melphalan concentrations tended to result in an increased AUC_{0-60} and thus in higher skin melphalan levels than before. The toxicity encountered in this study was similar to that observed previously and was confined to Grades II and III in all but 3 cases, only one of these suffering a more severe reaction (Grade IV). **Higher skin melphalan levels were not associated with more severe toxicity.**

From this study, there is no evidence that perfusion pressures in excess of the mean systemic arterial pressure result in increased leakage of perfusate to the systemic circulation. In this regard, the surgical technique of isolation of the limb circulation is of paramount importance and requires meticulous attention to detail. In Glasgow, the obturator vessels are ligated and the internal iliac artery and vein are both clamped for the duration of ILP in order to minimise cross-pelvic leakage. In upper limb ILP, the technique employed (see Section 2.1.2) resulted in no detectable leakage of melphalan during perfusion. This is in contrast with the experience

of other authors who report 15-40% leakage of melphalan in upper limb ILP performed using the infraclavicular approach[458].

The technique of isolated perfusion can therefore be safely controlled by assessment of the $PtcO_2$ to ensure adequate cutaneous perfusion without jeopardising the isolation of the limb and with reasonable expectation that skin melphalan uptake might be improved.

3.6 Methods to Circumvent the Risk of Systemic Leakage of Melphalan

3.6.1 Introduction

The evidence from the previous section (Section 3.5) suggests that perfusion pressures in excess of the mean systemic arterial pressure need not be associated with increased leakage of melphalan to the systemic circulation. The surgical technique of isolation is naturally of paramount importance in this respect. However there exist situations where a standard surgical technique may prove inadequate. For example, if the vascular anatomy is abnormal or, perhaps more importantly, when the anatomy is distorted due to previous surgery (eg. a previous ILP of the same region), aberrant vessels and intraosseous collateral vascular channels may adopt a greater significance than in the normal situation and may offer an alternative path for leakage of perfusate out of the limb. It would therefore be of interest to explore possible methods of ensuring effective cutaneous perfusion at lower perfusion pressures.

Vasodilatation in the perfused limb should lower the peripheral resistance in the limb. In the ILP technique currently used in Glasgow, the perfusate is oxygenated with 100% oxygen. This results in arterial hyperoxaemia which is known to exert a vasoconstrictor influence on the peripheral vasculature. Alteration of the gas mixture in the oxygenator to reduce the oxygen tension to more physiological levels and to include carbon dioxide, itself

a potent vasodilator, (eg. 95% O_2, 5% CO_2) might therefore provide a way of reducing the peripheral resistance. However, the high pO_2 in the perfusate is not only designed to reverse the acidosis which develops in the limb during the ischaemic period of isolation and to maintain tissue oxygenation thereafter. The originators of the ILP technique suggested at the outset that, in the same way as it was known to enhance the effects of radiotherapy, hyperoxaemia might also increase the cytotoxicity of the "radiomimetic" alkylating agents, such as melphalan[327]. The tumoricidal effect of nitrogen mustard has since been shown, in animal experiments, to be increased by high oxygen tension[340]. Furthermore, tumour angiogenesis is believed to lead to the development of vessels with abnormal morphological characteristics[464]. In particular, they generally seem to lack smooth muscle and adrenergic innervation[465-471]. The effects of hyperoxaemia and hypocapnia on vessels supplying melanoma metastases, even microscopic, may therefore be different from those described above. For these reasons, the author believes that it is appropriate to continue with the present practice.

In most perfusion centres, the pumps incorporated in the extracorporeal circuit generate a constant flow and pressure as opposed to the characteristically pulsatile systemic circulation. Pulsatile flow is believed to maintain peripheral resistance at lower levels than non-pulsatile flow[472]. Furthermore, one can envisage a situation where, although the mean arterial pressure might be lower than the critical closing pressure of a given

vessel, the systolic pressure might be sufficient to allow blood to circulate in that vessel, albeit on an intermittent basis. This is therefore an aspect of perfusion technique which should be explored. Indeed, in recent years, many cardiac surgeons have adopted pulsatile flow in cardiopulmonary by-pass.

A third way of reducing the peripheral resistance would be the addition of vasodilator drugs to the perfusate. These could be added directly to the perfusate during the ILP or administered systemically pre-operatively. However, the effect that any such drug might have on the distribution of melphalan to the tissues of the limb must be considered. Several reports of experimental work with human and rodent tumours have shown that vasoactive drugs can alter tumour blood flow either directly, by their action on the tumour vessels, or indirectly, by an alteration of the tumour:normal tissue perfusion ratio due to their action on normal vessels[468,473-480]. The response in any specific case would depend on the drug, tumour type and host species. This could result in an increased cellular uptake of cytotoxic agent, either by increased exposure to the drug in areas of increased flow or by reduced clearance of the drug in areas of decreased flow[481,482].

The calcium channel blocking drug verapamil, commonly used as a vasodilator or antiarrhythmic agent, is known to increase blood flow through implanted SMT-2A mammary adenocarcinoma in rats[479]. It is also known to potentiate the effect of melphalan against some types of fibrosarcoma and human melanoma xenografts in mice[483,484], but this

effect has been shown to be independent of tumour blood flow and is believed to be due to a direct influence on the cellular transport of melphalan[484]. Indeed, in vitro experiments have shown that verapamil not only increases the efficacy of adriamycin and thiotepa against human bladder cancer cells[485] and of vincristine against human colorectal cancer cells[486], but can also enhance the effects of adriamycin and vincristine against certain anthracycline- or vincristine-resistant cell lines respectively[487-493]. It has been postulated that verapamil interferes with the calcium-mediated drug efflux mechanism of these resistant cell lines.

Before introducing vasoactive agents to the perfusion circuit, it is important to ensure that this manoeuvre will not adversely affect the cytotoxicity of melphalan. The effect of several commonly used, rapidly acting vasodilator drugs on the efficacy of melphalan against human melanoma cells grown in vitro has therefore been studied.

3.6.2 Materials and Methods

Human malignant melanoma cells (B0008) were used for these experiments. This is an established cell line whose biological properties have been characterised[494]. For the experiments in this section, the cells were grown as multicellular tumour spheroids (MTS). These are cellular aggregates which grow by division of the cells in their peripheral layers. Their growth in vitro simulates that

of tumour micrometastases in vivo[495,496]. They can therefore be regarded as an experimental tumour model of intermediate complexity between, on one hand, the standard in vitro monolayer cell culture and, on the other, the in vivo tumour xenografts used in animal experiments[497].

The cells were maintained in culture using Eagle's Minimum Essential Medium (Eagle MEM), to 450ml of which were added foetal calf serum (50ml), 200mM L-glutamine (5ml), penicillin (50,000iu) and streptomycin (50,000iu) (5ml), and amphotericin-B (1.25mg in 5ml) (all solutions from Gibco).

To initiate spheroids, confluent monolayer cultures of B0008 cells were first washed in Earle's balanced salt solution (EBSS; Gibco) and then disaggregated enzymatically with 0.25% trypsin (Gibco) in 5ml EBSS. The trypsin was then neutralised with 5ml of supplemented MEM (see above). The disaggregated cell suspension was then used to initiate MTS growth according to the "agar underlay static method" [498]. This consists in placing 5ml of a high density suspension of the disaggregated cells in supplemented MEM (ie. $1\text{-}2\times10^5$ cells ml^{-1}) into 25ml flasks (Nunclon), base-coated with 5ml of 1.25% Noble agar (Difco) in supplemented MEM. This has the effect of preventing adherence of the cells to the base of the flask without absorbing nutrients from the culture medium. The flasks were then incubated at 37°C in 7% CO_2 at 100% humidity for 6 days, by which time MTS of approximately 200μm diameter had formed and were ready for treatment with melphalan and vasodilators.

The spheroids from the agar base-coated flasks were placed in universal containers (Sterilin), allowed to sediment, washed and resuspended in fresh medium. They were then divided into the number of universal containers required for each experiment and the volume in each container made up to 5ml with MEM. Melphalan and a vasodilator were added to each container in quantities calculated to achieve predetermined concentrations.

Melphalan ("Alkeran", Wellcome Foundation Ltd) was prepared as for intravenous use. The powdered melphalan was prepared in the solvent and diluent provided and then serially diluted in MEM to produce concentrations of 0,10,20 and 40ug ml^{-1}. 5ml aliquots of these solutions were then added to each of the MTS suspensions to give final concentrations of melphalan of 0,5,10 and 20ug ml^{-1}.

The vasodilator drugs used in this study were all selected for their rapid action on the vasculature and consequent suitability for use in the ILP setting. Two of these (verapamil and hydralazine) have been shown experimentally to affect the efficacy of some cytotoxic drugs including melphalan against certain tumours[478,483,484,490]. The others possess a very rapid onset and short duration of action and are commonly used to quickly reduce peripheral vascular resistance in vascular and cardiac surgery.

[i] <u>verapamil</u> ("Cordilox", Abbott Laboratories Ltd) was obtained as the injectable drug (verapamil hydrochloride, 5mg in 2ml). Aliquots of this were added to the

MTS/melphalan suspensions using a micropipette (Gelson Pipetman, Gelson Medical Electronics) to achieve concentrations of 0,2.5 and 5.0ug ml^{-1}.

[ii] hydralazine ("Apresoline", CIBA Laboratories) was obtained as the lyophilised powder (hydralazine hydrochloride, 20mg) and reconstituted in 1ml of sterile water for injection. This was added to the MTS/melphalan suspension using a micropipette to achieve concentrations of 0,2.5,5,10 and 20ug ml^{-1}.

[iii] papaverine was obtained as the injectable drug ("in house" pharmacy preparation of papaverine hydrochloride, 20mg in 10ml). This was added to the MTS/melphalan suspension using a micropipette to produce concentrations of 0,25,50,75 and 100ug ml^{-1}.

[iv] sodium nitroprusside ("Nipride", Roche products Ltd) was reconsituted from the powdered preparation (sodium nitroprusside, 50mg) in 5% dextrose according to the manufacturers' recommendations and added by the same technique as above to the MTS/melphalan suspension to produce concentrations of 0,10^{-4},$5x10^{-4}$ and 10^{-3}ug ml^{-1}. From the moment of preparation of the drug until the end of the experiment, solutions containing sodium nitroprusside were shielded from daylight using metallic foil.

The spheroids were then incubated for 1 hour at 37°C in 7% CO_2 at 100% humidity, shaking the suspensions gently at 15 minute intervals. At the end of the incubation period, the drug-containing medium was aspirated leaving the sedimented spheroids in the conical bottom of the universal containers. These were then washed three times in ice-cold MEM, allowing them to sediment between each wash. Thereafter, the spheroids were resuspended in 3ml of MEM and transferred to 5cm Petri dishes (Flow Laboratories) in a laminar flow cabinet. Under microscopy, single spheroids were picked using a micropipette (aspiration volume 20 ul) with a disposable transparent tip. The spheroids were placed in 0.5ml supplemented MEM in each well of a 24-well tissue culture multidish (Corning 25820), base-coated with 1.25% Noble agar/supplemented MEM (see above). As far as possible, spheroids of similar size (approximate diameter 250um) were selected for this purpose. Each treatment group of spheroids was placed in a different 24-well plate. The spheroids were then incubated at 37°C in 7% CO_2 and 100% humidity, adding 0.5ml supplemented MEM to each well on a weekly basis. Cross-sectional area measurements of each spheroid were made approximately twice weekly for up to 4 weeks starting on the day of treatment using an image analysis system. These area measurements were later converted to volumes using an "in house" computer programme which assumes a spherical configuration of the spheroids. In this way, growth curves could be constructed for each experimental group and the effect of melphalan and the vasodilators quantified by comparison of

the growth delay, which is defined as the time taken for median spheroid volumes to increase by a factor of ten above their initial size[499].

Infected experiments were abandoned and are not reported.

3.6.3 Results

Tables 21-24 show the growth delay calculated for each arm of these experiments using an "in house" computer programme. This is expressed as the median value (95% confidence limits) for all of the spheroids in each arm. Spheroids which failed to regrow and are therefore considered "cured" by the treatment were allocated an arbitrary growth delay of 1000 days. Some spheroids which were not cured by the treatment nevertheless failed to reach 10 times their original volume within the period of the experiment. The growth delay of these spheroids was calculated by extrapolation of the regrowth phase of their growth curve and does not therefore include a 95% confidence interval. In order to compare the effects of the vasodilators on the regrowth of B0008 spheroids, the ratio of the growth delay at each concentration of vasodilator to that at the same concentration of melphalan but without vasodilator is also shown in Tables 21-24.

The growth delay of B0008 spheroids is increased by increasing the concentration of melphalan. No consistent effect of verapamil or hydralazine on this melphalan

Table 21 :

Regrowth Delay of B0008 Melanoma Spheroids after Treatment with Melphalan and Verapamil

VERAPAMIL (ug/ml)	MELPHALAN 0	5	10	20
0	6.23(1.00) [6.11-6.73]	35.50(1.00) @	(-)	(-)
2.50	6.26(1.00) [5.72-7.03]	38.55(1.09) @	(-)	(-)
5	6.67(1.07) [6.39-7.01]	35.36(1.00) @	(-)	(-)
0	8.00(1.00) [7.00-8.99]	(-)	14.61(1.00) [14.29-18.74]	29.10(1.00) @
2.50	7.41(0.93) [6.46-8.09]	(-)	15.94(1.09) [14.15-1000]	27.92(0.96) @
5	6.80(0.85) [6.19-7.97]	(-)	19.94(1.36) [14.70-1000]	21.84(0.75) @
0	6.73(1.00) [6.36-7.11]	58.73(1.0) @	61.11(1.00) @	43.93(1.00) @
2.50	7.38(1.10) [6.60-7.90]	36.02(0.61) @	96.15(1.57) @	70.08(1.60) @
5	6.77(1.01) [6.36-7.16]	65.69(1.12) @	68.93(1.13) @	69.85(1.59) @

Growth Delay is expressed in days. Values are shown as :

Growth Delay (fraction of control plate value)
[95% confidence interval]

@ - confidence interval not quoted as calculation of growth delay required extrapolation beyond the bounds of the data.

Table 22 :

Regrowth Delay of B0008 Melanoma Spheroids after Treatment with Melphalan and Hydralazine

HYDRALAZINE (ug/ml)	MELPHALAN 0	5	10	20
0	6.80(1.00) [6.12-7.12]	13.85(1.00) [12.10-15.00]	14.82(1.00) [14.31-15.67]	41.40(1.00) @
5	6.77(1.00) [6.01-7.57]	9.65(0.70) [9.16-11.19]	17.90(1.21) [15.77-20.62]	27.83(0.67) [27.36-1000]
10	6.23(0.92) [5.99-6.77]	11.46(0.83) [10.39-12.82]	58.28(3.93) @	28.08(0.68) [25.69-1000]
0	6.98(1.00) [6.11-7.85]	15.92(1.00) [15.19-18.90]	19.89(1.00) [13.38-26.18]	34.27(1.00) @
2.50	7.64(1.09) [6.41-8.84]	12.38(0.78) [11.64-13.73]	21.16(1.06) [17.86-1000]	28.05(0.82) [24.85-1000]
20	7.22(1.03) [6.51-9.45]	14.75(0.93) [12.81-17.19]	24.97(1.26) [21.13-1000]	35.62(1.04) @

Growth Delay is expressed in days. Values are shown as :

Growth Delay (fraction of control plate value)
[95% confidence interval]

@ - confidence interval not quoted as calculation of growth delay required extrapolation beyond the bounds of the data.

Table 23 :
Regrowth Delay of B0008 Melanoma Spheroids after Treatment with Melphalan and Papaverine

PAPAVERINE (ug/ml)	MELPHALAN 0	5	10	20
0	17.92(1.00) [16.86-18.86]	28.25(1.00) [23.17-1000]	*(30.45(1.00)	32.65(1.00) @
25	16.74(0.93) [16.27-17.71]	23.02(0.81) [21.14-24.22]	35.21(1.16) @	1000(**) @
50	16.35(0.91) [13.65-17.41]	19.85(0.70) [18.48-22.40]	32.65(1.07) @	91.87(2.81) @
75	15.28(0.85) [11.79-1000]	91.18(3.23) @	1000(**)	1000(**)
100	15.09(0.84) [12.87-1000]	1000(**)	1000(**)	1000(**)

Growth Delay is expressed in days. Values are shown as :

Growth Delay (fraction of control plate value)
[95% confidence interval]

@ - confidence interval not quoted as calculation of growth delay required extrapolation beyond the bounds of the data.

* - plate lost; arbitrary intermediate value selected to allow calculation of growth delay ratio.

** - >50% of spheroids "cured".

Table 24 :
Regrowth Delay of B0008 Melanoma Spheroids after Treatment with Melphalan and Sodium Nitroprusside

SODIUM NITROPRUSSIDE (ug/ml)	MELPHALAN 0	5	10	20
0	14.38(1.00) [11.68-1000]	35.00(1.00) @	(-)	(-)
$5X10^{-4}$	34.77(2.42) @	43.30(1.24) @	1000(**)	1000(**)
10^{-3}	1000(**)	1000(**)	1000(**)	1000(**)
10^{-4}	17.28 [17.03-22.84]	(-)	34.37 @	1000(**)
$5X10^{-4}$	(-)	61.87 @	59.75 @	1000(**)

Growth Delay is expressed in days. Values are shown as :

Growth Delay (fraction of control plate value)
[95% confidence interval]

@ - confidence interval not quoted as calculation of growth delay required extrapolation beyond the bounds of the data.
** - >50% of spheroids "cured".

cytotoxicity was observed and neither drug seemed to produce an independent cytotoxic effect. In the presence of melphalan, papaverine prolonged the growth delay of B0008 spheroids, especially at higher concentrations of both papaverine and melphalan (papaverine > 50 ug ml^{-1}, melphalan > 5ug ml^{-1}). Papaverine alone had no effect on the growth of the spheroids. The experiments using sodium nitroprusside were complicated by the disaggregation of spheroids on several plates. It would appear, however, that even in the absence of melphalan, sodium nitroprusside exerted an independent cytotoxic effect on the B0008 spheroids.

3.6.4 Discussion

As far as possible, identical conditions were maintained for all of these experiments. Spheroids generated by the culture of B0008 cells obtained from several monolayer dishes were pooled prior to treatment in order to avoid the selection of different cell clones for the various arms of the experiments. In spite of this, there was clearly a difference between the growth delay of the spheroids treated with verapamil or hydralazine and those treated with papaverine or sodium nitroprusside. Within each experiment, however, the growth delay of the control spheroids (no melphalan) was very similar with the exception of those treated with high concentrations of nitroprusside (see below).

The results of this pilot study into the effect of vasodilators on the cytotoxicity of melphalan to B0008 cells in vitro suggest that neither verapamil nor hydralazine result in any increase in sensitivity or resistance to the treatment. At the concentrations of melphalan commonly produced in the limb circulation during ILP (10-20ug ml^{-1}), papaverine would appear to increase the cytotoxic effect of melphalan when used at concentrations of 50ug ml^{-1} or more. The growth delay produced by melphalan was increased by the addition of increasing concentrations of sodium nitroprusside. Even in the absence of melphalan, the growth delay of B0008 spheroids was increased by incubation with nitroprusside. In the systemic use of this drug, the ferrous ion of nitroprusside reacts quickly with sulfhydryl-containing compounds in erythrocytes to produce cyanide, which in turn is reduced to thiocyanate in the liver. The absence of this latter step in the "in vitro" preparation may allow a sufficient concentration of cyanide to accumulate in the culture medium during the incubation period to produce a direct cytotoxic effect on the cultured cells. The exclusion of the liver from the isolated circuit of ILP would similarly result in increasing concentrations of cyanide in the perfusate, and this therefore must preclude the use of sodium nitroprusside in ILP.

While verapamil and hydralazine have not been shown to increase the cytotoxicity of melphalan in vitro, the absence of any deleterious effect of these drugs would allow their use in vivo to lower the peripheral resistance

in the isolated limb. The most suitable vasodilator for use in ILP may, however, prove to be papaverine since this combines a very rapid onset and short duration of action with an apparent increase in the cytotoxicity of melphalan in vitro.

3.6.5 Conclusion

In order to reduce the perfusion pressure required to ensure effective cutaneous perfusion during ILP, it may be possible to administer vasodilator drugs which would result in a reduction in peripheral vascular resistance. Clinical studies of the use of verapamil, hydralazine or papaverine for this purpose should now be performed to assess the feasibility of this technique.

Although previous studies have shown that verapamil and hydralazine can potentiate the effects of certain cytotoxic drugs, including melphalan, on various tumour lines in vitro and in vivo, this is not confirmed for melphalan and B0008 human melanoma cells in vitro. Any enhancement of the melphalan effect in ILP would have to be the result of their vasoactive properties. In view of this, papaverine, which may enhance melphalan cytotoxicity independently of its vasoactive properties, may be the most suitable vasodilator by virtue of the ease with which its effects can be controlled - rapid onset and short duration of action.

The addition of vasodilators to the ILP circuit can be expected to produce a greater fall in the volume of perfusate within the reservoir than usual by expanding the intravascular compartment of the limb. The flow rates required to maintain effective perfusion pressures within this "expanded" system may therefore be higher than before, even if the critical perfusion pressure itself is lower. An increase in the volume of the priming fluid in the reservoir may be necessary to counteract these potential difficulties. Although the effect of such a manoeuvre on the melphalan pharmacokinetics should not be ignored, the dosimetry studies described in Section 4.3 suggest that the priming fluid contributes a less significant proportion to the volume of distribution of melphalan in the isolated circuit than hitherto suspected. It should therefore be possible to make adjustments to the priming volume without significantly altering the outcome of the procedure.

4 CHAPTER 4 - A STUDY OF DOSIMETRIC METHODS IN ILP

4.1 Melphalan Dosimetry in ILP - Existing Methods

There is much debate on which melphalan dosage schedule should be applied to isolated limb perfusion. Malignant melanoma has proved relatively resistant to most forms of treatment because unacceptable toxicity to normal tissues is encountered at lower doses of chemotherapeutic agent or radiation than are necessary to produce a clinical response. The great advantage offered by Isolated Limb Perfusion is the ability to exclude vital organs and tissues (eg. liver, bone marrow) from the field of treatment. This allows the surgeon to administer higher, therapeutically effective doses of cytotoxics. For this reason it has generally been accepted that the optimal dose of melphalan is the highest dose tolerated by the normal tissues of the limb[406,411]. There has been a suggestion that more in depth studies should be undertaken in order to assess the effectiveness of lower, less toxic doses[500]. However the uniformity of the toxic reactions observed by the authors of the major studies in this field and the apparent acceptability to patients of this temporary morbidity suggest that this is not necessary. Furthermore, while dose reduction has been made possible in the treatment of many malignancies by fractionation of systemic chemotherapy or radiation therapy, this approach could not be routinely employed in ILP as the repeated exposure and cannulation of the major vessels to the limbs would be associated with major technical

difficulties and with a high risk of severe vascular morbidity. Nevertheless there may be a place for this type of approach in selected cases, particularly in the adjuvant treatment of distal lesions, if a low fraction number can be used[389,411,501].

One limitation of analyses of the results of Isolated Limb Perfusion is the variability in the dosage schedules which exist between, and indeed within, perfusion centres. The main dosage schedules currently employed are based on

[1] total body weight, giving a predetermined dose per kilogram

[2] the volume of the perfused limb, measured by immersion, giving a fixed dose per litre of perfused tissue

[3] the calculated circulating blood volume in the isolated circuit and dose required to achieve a predetermined concentration of cytotoxic agent in this volume.

Historically, most centres have used dosage schedules based on body weight but these have varied between 0.75mg kg^{-1} and 2mg kg^{-1} for femoral and external iliac perfusions, and between 0.5mg kg^{-1} and 1.0mg kg^{-1} for popliteal and axillary perfusions (see Table 25). Many authors recognize that the morphological characteristics

Table 25 :

Weight-based Dosage Formulae for Melphalan in ILP*

Reference(year)	Lower Limb	Upper limb
Stehlin(1963)[146]	iliac/femoral 90-125mg popliteal 50-75mg	50-75mg
Krementz(1963)[409]	1.2mg kg^{-1} **	-
Krementz(1972)[375]	1.0-1.4mg kg^{-1}	0.6-1.0mg kg^{-1}
Sugarbaker(1976)[386]	iliac 1.5mg kg^{-1} popliteal 1.0mg kg^{-1}	1.0mg kg^{-1}
Rosin(1980)[361] (& Skene 1990 [369])	iliac 2.0mg kg^{-1} femoral 1.5mg kg^{-1}	-
Bulman(1980)[382]	1.5mg kg^{-1}	-
Schraffordt Koops (1981)[389],(1992)[501]	1.0-1.5mg kg^{-1}	0.5-0.7mg kg^{-1}
Janoff(1982)[390]	1.0-1.5mg kg^{-1}	0.75-1.0mg kg^{-1}
Rege(1983)[388]	1.5mg kg^{-1} #	-
Briele(1985)[458]	1.0-1.5mg kg^{-1}	-
Ghussen(1984)[377]	1.5mg kg^{-1}	1.0mg kg^{-1}
Minor(1985)[373]	iliac 0.8-1.5mg kg^{-1} @ popliteal 0.75mg kg^{-1}	0.75mg kg^{-1}
Storm(1985)[365]	1.0mg kg^{-1}	-
Krige(1988)[410]	1.0mg kg^{-1}	-
Santinami(1989)[368]	1.5mg kg^{-1}	0.8-1.0mg kg^{-1}

* ranges indicate allowance made for dose modification based on complexion, hair colour and build of the patient.

** ideal body weight.

maximum dose 100mg.

@ recommended dose range 0.75-1.0mg kg^{-1}.

of their patients should have some influence on the exact dose of cytotoxic administered and, indeed, Krementz initially based his dose calculations on ideal body weight[409], reflecting his acknowledgement of this suggestion. Other reports quote doses adjusted (somewhat subjectively) to take account of the build, complexion and hair colour of the patient[146,375,386].

In 1982, Wieberdink et al proposed a dosage schedule based on the volume of the limb to be perfused[406]. This was measured by immersion of the limb in water and estimation of the displaced volume of water. They also graded the regional toxicity according to a five-level system which has now gained acceptance in most ILP centres (see Table 8). The mildest reaction (Grade I) is generally taken to represent a less than optimal cytotoxic effect while Grades IV and V are associated with very severe reactions resulting in objective and subjective impairment of limb function (see Figure 5). Wieberdink found that a fixed dosage of melphalan of 10mg per litre of perfused tissue produced predictable and acceptable toxicity in the limb - Grades II or III in 97% of patients. In subsequent studies, he was able to adjust the dosage schedule for upper limb ILP where, due to the relatively larger contribution of the volume of perfusate in the pump reservoir to the total volume of the perfusion circuit, the dose administered could be raised to 13mg per litre of tissue in order to achieve similar concentrations of drug without significantly increasing the observed toxicity[459]. The predictability and tight control of toxicity produced by this dosage schedule has

since been confirmed by other authors[458] and this system is now widely employed in Europe as the method of choice. Indeed, this was adopted as the standard dosage regime in the protocol of the WHO/EORTC randomized trial of adjuvant limb perfusion with melphalan for melanoma[394].

In common with most studies of Isolated limb Perfusion, Wieberdink's technique was described and is used without particular reference to the cytotoxic drug concentrations achieved in the perfusate. Successive pharmacokinetic studies have revealed that there remains a substantial variation in the melphalan concentrations produced[448,458,459]. In an attempt to base the melphalan dosage on pharmacokinetic principles, a new method was devised whereby the total blood volume in the perfusion circuit could be estimated by measurement of the haematocrit in blood samples taken from the patient's systemic circulation, the priming mixture in the pump, and the mixed perfusion fluid. Melphalan could then be administered to achieve a predetermined concentration within this volume[502]. The authors claimed a close correlation between the expected and observed melphalan concentrations in the perfusate.

All three of the dosage methods described above are currently used by the various perfusion centres. The published results of isolated limb perfusion for melanoma show a wide variation in both the response rate of unexcised in-transit disease and the survival of patients with stage I melanoma[388,411]. In order to achieve more reproducible results, it seems desirable to standardise as many aspects of the treatment as possible.

This study was therefore designed to assess the three dosage schedules described so that the most appropriate method could be recommended.

4.2 Limb Volume Studies

4.2.1 Introduction

The method for determining the volume of the limb to be perfused was described by Wieberdink[406]. He recommended lowering the limb into a cylindrical reservoir (30cm or 15cm in diameter, for legs or arms respectively) until the proximal limit of the region to be perfused was level with the rim of the reservoir. The reservoir was then filled with water. Upon withdrawal of the limb from the reservoir, the drop in the water level could be measured and multiplied by the cross-sectional area of the reservoir to give the volume of the immersed limb. The transparent walls of the reservoir were calibrated for this purpose. In perfusion at the iliac level, a correction factor of 10% was added to the measured volume to allow for that part of the upper thigh and buttock which is included in the perfused region but cannot be immersed in this way. All other limb perfusion regions can be totally immersed and do not therefore require the measured volume to be corrected in any way.

During an initial period of evaluation of this method in Glasgow, several practical problems were encountered by the author. The actual manoeuvre of immersing a leg into such a narrow container can present major difficulties, especially to those patients with limited mobility such as the elderly, arthritic, or those with an impaired sense of balance. Wieberdink suggested that patients should be raised and lowered on a mechanical platform while

weightbearing on one leg, thus leaving the other leg free to hang inside the reservoir. This, however, requires the use of expensive hoists. In addition, the patient does not weightbear with the immersed leg and a pelvic tilt may therefore result, producing an error in the volume estimation. Furthermore, pressure of the rim of the reservoir on the patient's perineum can be uncomfortable, and patients can be embarassed by the close attention given to this part of their anatomy during the procedure. Finally, the exact determination of the water level in a container of this diameter, even if it is absolutely transparent, can be associated with a substantial error. Since the limb volume estimation requires two such measurements, an error of only 1mm could result in a volume error of approximately 140ml.

Measures were taken to overcome several of these difficulties. Rather than use a mechanical platform, patients were asked to climb two small steps and then to raise their foot into the reservoir while supporting their weight on their arms with the help of two parallel bars. A small platform of variable height was placed within the reservoir so that they could then weightbear fully on both legs. In order to facilitate the movement of the leg into and out of the reservoir, a cylinder of greater diameter (37.4cm) was used. To improve the accuracy of the water level recordings, a piece of transparent plastic tubing was connected to the reservoir so that the bottom of the meniscus could be clearly seen in the tubing. All measurements were then made from this tubing.

The remainder of the difficulties associated with the limb immersion technique (i.e. pelvic tilt, perineal discomfort, patient embarassment) stem from the need to immerse the leg up to the level of the perineum. A method of measuring the leg volume without the need for total immersion would therefore overcome these problems. An attempt has been made to derive a formula for calculating the total leg volume by studying its correlation with partial leg volume measurement and other anthropometric measurements.

4.2.2 Patients and Methods

The subjects for this study were volunteer junior doctors or medical students. In total, 34 subjects were studied (18 males, 16 females). Sex, weight (WT) and height (HT) were recorded. Body surface area (SA) was derived from these measurements according to a published nomogram[503]. Leg volume was then measured in two ways:

[i] a horizontal line was drawn on the thigh one third of the distance down between the upper border of the greater trochanter of the femur and the head of the fibula. The leg was then immersed to this level with the subject weightbearing on both legs and the drop in the water level on subsequent withdrawal of the leg from the reservoir was recorded as the **Partial Limb Volume (PLV)**.

[ii] the height of the platform in the reservoir was adjusted so that, on reimmersion of the leg, the subject's perineum rested on the rim of the reservoir and the volume of the leg could be measured as in Wieberdink's description[406]. This was recorded as the Total Limb Volume (TLV).

The relation between TLV and the other variables recorded was then tested by single and multiple regression analysis in order to derive the most accurate predictive formula for obtaining an estimate of TLV using less awkward techniques than direct measurement.

4.2.3 Results

All the recorded observations are tabulated in Appendix C. There were 18 male subjects and 16 female subjects. The mean height was 1.723m (range 1.58-1.92m) and the mean weight was 63.5kg (range 49-81kg). The mean body surface area derived from the nomogram was 1.767m^2 (range 1.51-2.05m^2). The mean PLV measured by immersion was 8.499 litres (range 6.27-11.77 litres) and the mean TLV was 10.936 litres (range 8.47-15.40 litres).

TLV was plotted against each of the other four variables in turn. The closest correlation was found with PLV (r=0.847), and this plot is shown in Figure 24.

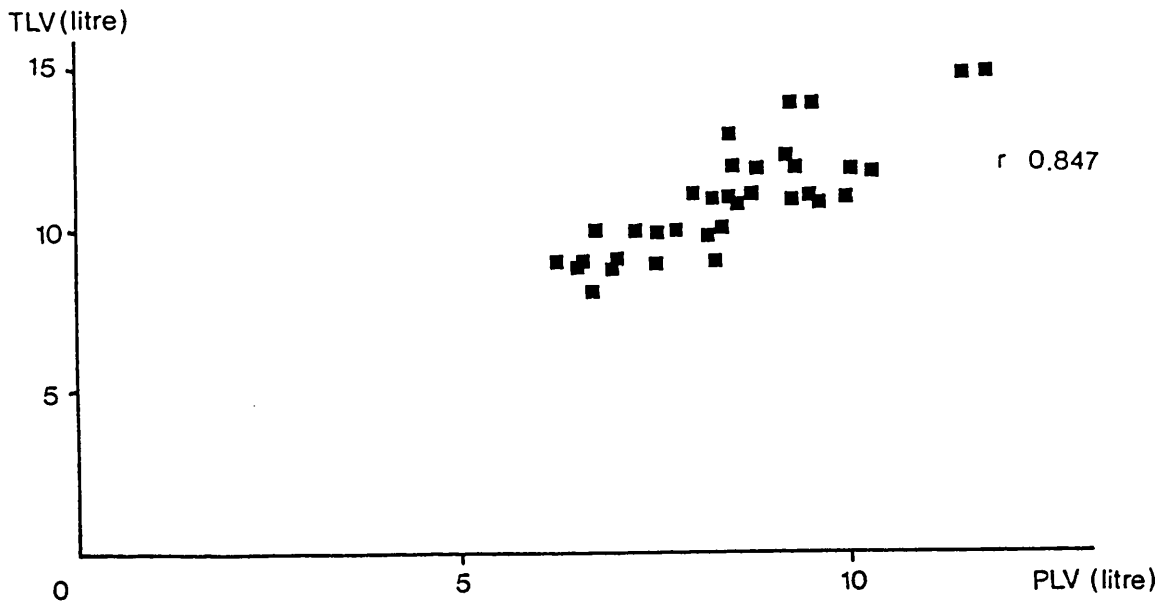


Figure 24 :
Plot of the Partial(PLV) and Total(TLV) limb volumes measured by immersion in 34 volunteer subjects.

Table 26 :

Regression of Total Limb Volume(TLV) on Partial Limb Volume(PLV), Height(HT), Weight(WT), Surface Area(SA) and Sex

Predictor	Coefficient					K	R^2adj	F
	PLV	HT	WT	SA	Sex			
PLV	1.10 (0.00)					1.58	70.8	81.0
PLV,Sex	1.15 (0.00)				0.69 (0.04)	0.88	73.9	47.8
HT		7.27 (0.04)				-1.59	9.9	4.6
HT,Sex		15.73 (0.00)			2.03 (0.01)	-17.12	25.6	6.7
WT			0.16 (0.00)			0.75	51.7	36.4
WT,Sex			0.20 (0.00)		1.57 (0.00)	-2.78	68.0	36.0
SA				7.85 (0.00)		-2.93	38.3	21.5
SA,Sex				13.25 (0.00)	2.45 (0.00)	-13.64	69.4	38.4
PLV,HT	1.13 (0.00)	-0.83 (0.71)				2.80	70.0	39.5
PLV,HT, Sex	1.04 (0.00)	4.85 (0.10)			1.21 (0.01)	-6.82	75.5	34.8
PLV,WT	1.22 (0.00)		-0.02 (0.63)			2.00	70.1	39.7
PLV,WT, Sex	0.85 (0.01)		0.06 (0.28)		0.97 (0.02)	-0.55	74.1	32.5
PLV,SA	1.17 (0.00)			-0.87 (0.65)		2.51	70.1	39.6
PLV,SA, Sex	0.73 (0.00)			5.67 (0.04)	1.47 (0.00)	-5.99	76.5	36.8

Numbers in brackets are p-values for the regression coefficients.

Table 26 shows the results of the regression of TLV on each of the predictors, singly and in combination. Sex was introduced as a binary variable (male = 0, female = 1). In all of these calculations, SA was never used in combination with HT and/or WT, since SA is itself calculated from these measurements. Regression of SA on HT and WT using the data obtained from the subjects of this study yielded the following formula :

$$SA = 0.781xHT + 0.0113xWT - 0.297$$

The correlation between the SA observed and the SA calculated from this formula produced a correlation coefficient of 0.999 and, as a result, HT and WT were not used in combination either during any calculations of TLV.

The R^2 values listed in Table 26 show that the inclusion of sex improved the closeness of the correlation between TLV and all the other variables, taken singly and in combination. The products of the predictor variables and the sex code for each subject were therefore introduced into the analysis and stepwise regression of TLV on all the predictor variables (HT, WT, SA, PLV, HTxSex, WTxSex, SAxSex, and PLVxSex) was performed. By this method, the best model for predicting TLV was given by the equation

$$TLV = 0.67xPLV + 5.1xSA + 0.162xPLVxSex - 4.462$$

$$(F=36.89;\ p=0.000;\ R^2_{adj}=78.67)$$

This was associated with a mean 95% prediction interval of ±15.65% (SD=2.10) over the range of observations in this study. The close correlation between the TLV values calculated by this equation and those actually measured in the same subjects is demonstrated in Figure 25.

The following formulae were therefore adopted for the calculation of TLV :

Male subjects $TLV = 0.67xPLV + 5.1xSA - 4.462$

Female subjects $TLV = 0.832xPLV + 5.1xSA - 4.462$

In the context of Isolated Limb Perfusion at the iliac level, Wieberdink had recommended that a correction factor of 10% be added to the TLV to take account of that part of the iliac region which cannot be immersed[406]. The iliac perfusion volume (IPV) can therefore be calculated by the corrected formulae :

$$IPV_{male} = 0.74xPLV + 5.6xSA - 4.91$$

and

$$IPV_{female} = 0.92xPLV + 5.6xSA - 4.91$$

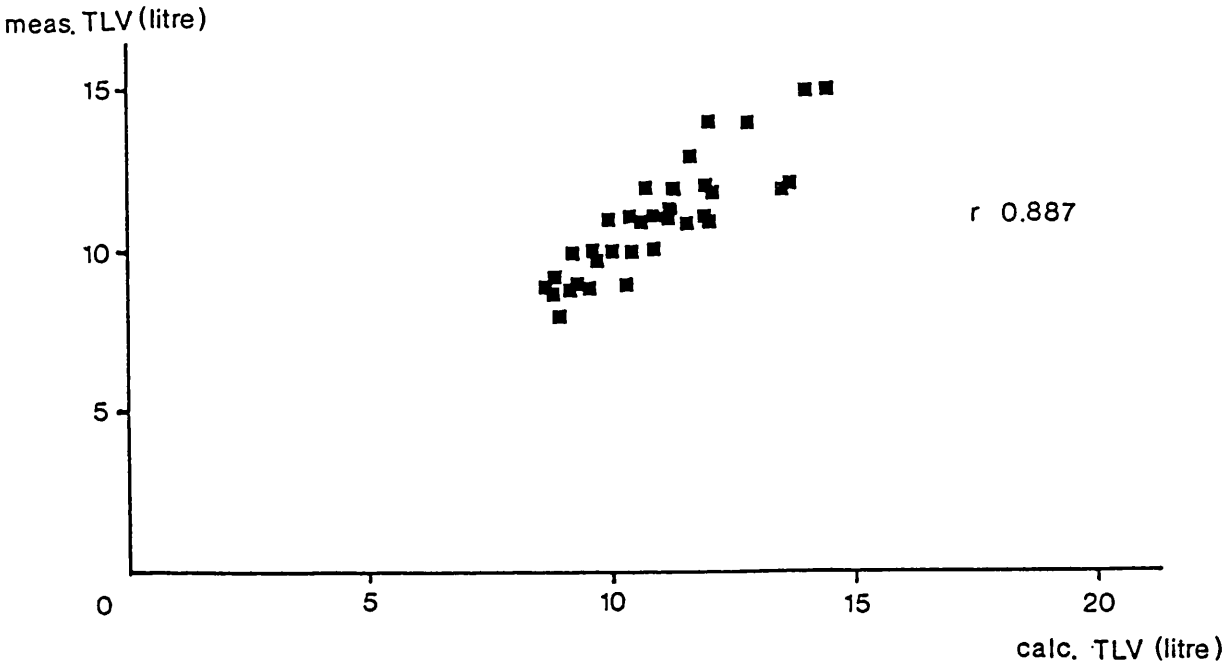


Figure 25 :
Plot of the measured TLV against the TLV calculated from the equation
TLV = 0.67PLV + 5.1SA + 0.162PLV.Sex - 4.462

(Sex = 0 for male, 1 for female)

4.3 A Comparison of Dosimetric Methods in ILP

4.3.1 Introduction

In Glasgow, the normal practice is to administer a dose of melphalan based on the body weight of the patient. A phase I study of melphalan pharmacokinetics in mildly hyperthermic Isolated Limb Perfusion performed in this centre has demonstrated that this dose can be raised to 1.75mg kg^{-1} without increasing the tissue toxicity to unacceptable levels[448]. Excessive toxicity has been reported when using very high doses of melphalan, and as a result a maximum dose limit of 150mg is applied.

A comparative study was undertaken to assess which of the dosimetric methods listed above (Section 4.1) would most consistently lead to the administration of the highest doses. The actual dose administered was compared to the doses which would have been prescribed according to the other methods. The reliability of the calculation of the theoretical volume of distribution of melphalan was also assessed by studying the pharmacokinetic profile of melphalan in the perfusate.

4.3.2 Patients and Methods

All the patients in this study underwent Isolated Limb Perfusion according to the techniques described in Section 2.1.2. The appropriate dose of melphalan for each patient was determined using each of the three dosage schedules as follows:

[1] Body weight method

All patients were weighed using the same scales. For iliac perfusion, melphalan was administered in a dose of 1.75mg kg^{-1} (maximum 150mg) injected as a bolus into the venous compartment of the pump reservoir. The dose administered in popliteal perfusions was a fraction of this iliac dose proportional to the apparent fraction of the iliac region isolated below the tourniquet. For axillary perfusion, doses of 50mg or 60mg were given, the exact dose being selected according to the age and morphology of the patient.

[2] Limb volume method (Wieberdink)

Limb volumes were measured by immersion. For patients undergoing iliac perfusion, the same technique was employed as described in Section 4.2.2 to measure the Partial Limb Volume (PLV). Height and weight were also recorded so that body surface area could be derived from

the same nomogram as was used in Section 4.2.2. The Iliac Perfusion Volume (IPV) was then calculated using the following formulae :

$$IPV_{male} = 0.74 \times PLV + 5.6 \times SA - 4.91$$

$$IPVfemale = 0.92 \times PLV + 5.6 \times SA - 4.91$$

The melphalan dose according to this method was then calculated at 10mg l^{-1}.

For axillary and popliteal perfusions, the perfused region is totally immersible and was therefore measured directly. The melphalan doses were then calculated using 13mg l^{-1} for axillary perfusions and 10mg l^{-1} for popliteal perfusions.

[3] Haematocrit method (Lejeune)

During the isolation procedure, 5ml samples of blood were withdrawn from the patients' systemic circulation and from the priming fluid in the pump reservoir immediately before cannulation of the vessels. A third sample was taken from the pump reservoir 10-15 minutes after commencing the isolated perfusion to allow sufficient time for thorough mixing of the priming fluid and the residual blood in the limb at the time of isolation. The haematocrits of all three samples were then measured.

Since the volume of priming fluid was known, it was then possible to calculate the residual volume of blood within the limb (termed ELBV - the Exchangeable Limb Blood Volume - by Lejeune[502]) according to the following formula :

$$ELBV = \frac{(HCT_p - HCT_m)}{(HCT_s - HCT_m)} \times VOL_p$$

where VOL_p is the volume of the priming fluid, and HCT_p, HCT_m, and HCT_s are the haematocrits of the Priming fluid, Mixed perfusion fluid and Systemic blood of the patient respectively.

The total blood volume (TBV) within the isolated circuit (limb + extracorporeal cicuit) is then represented by the sum of the priming volume (VOL_p) and ELBV. The composition of the priming fluid for Isolated Limb Perfusion is such that high dilution of the red blood cells occurs. The difference between the haematocrits of the prime and of the mixed perfusion fluid will therefore yield a negative number. Since only the magnitude of the difference is significant in the calculation of ELBV, the terms can be reversed in the formula. Thus,

$$TBV = VOL_p + ELBV$$

$$= VOL_p + \frac{(HCT_m - HCT_p)}{(HCT_s - HCT_m)} \times VOL_p$$

$$= VOL_p \times 1 + \frac{(HCT_m - HCT_p)}{(HCT_s - HCT_m)}$$

$$= VOL_p \times \frac{(HCT_s - HCT_m)}{(HCTs - HCTm)} + \frac{(HCT_m - HCT_p)}{(HCTs - HCTm)}$$

$$= VOL_p \times \frac{HCT_s - HCT_p}{HCT_s - HCT_m}$$

Since we use a standard volume of 1100ml of priming fluid, we can simplify this to :

$$TBV = 1100 \times \frac{HCT_s - HCT_p}{HCT_s - HCT_m}$$

If HCT_s is equal to either of the other two haematocrits (and, logically, HCT_s should be equal to both or neither of them), then this formula becomes inapplicable.

The dose of melphalan for each patient was then determined according to Lejeune's protocol which prescribes 40ug ml^{-1} of TBV for lower limb perfusion and 20ug ml^{-1} of TBV for upper limb perfusion.

Comparison of dosage methods

The dose of melphalan administered to each patient (1.75mg kg^{-1}) was then compared with the doses prescribed by each of the other two methods using matched pairs tests.

In order to test the reliability of the method for determining the volume of distribution of melphalan based on three haematocrits, the pharmacokinetic profile of melphalan in the perfusate was measured for each patient. Samples of blood (5ml) were withdrawn from the arterial side of the extracorporeal circuit at 5 minute intervals throughout the 60 minutes of isolated perfusion with melphalan and were put into lithium heparin containers. The samples were placed on ice and were then processed immediately after the end of isolated perfusion. Samples were centrifuged at 2500rpm for 10 minutes. The plasma was then separated from each sample and stored at -20°C until melphalan assay by HPLC could be performed. The method for the assay was that described in Section 3.5.2 (above). The melphalan concentrations in the perfusate were plotted on a logarithmic scale against time. The equilibrium concentration of melphalan in the perfusate was then calculated by regression of the beta-phase of the concentration/time curve (ie. the 20-60 minute portion of the curve) and extrapolation of this line back to time zero minutes. This value, the measured equilibrium concentration of melphalan ($meas[MEL]_{eq}$), was then compared with the equilibrium concentration which would be

expected ($exp[MEL]_{eq}$) from the dose given if the method of calculating the volume of distribution of the drug were accurate.

4.3.3 Results

During a 2 year period, 73 patients were entered into this study. 51 of these underwent Isolated Limb Perfusion at the iliac level and 5 at the popliteal level, while the other 17 were treated at the axillary level. Owing to the large number of time-consuming observations and blood samplings, the data collection was not complete for every patient. Nevertheless, 86% of lower limb volumes and 76% of upper limb volumes were measured; the three haematocrit measurements were obtained from all except four patients (94% lower limbs, 94% upper limbs); pharmacokinetic profiles were obtained for 80% of lower limb perfusions and 65% of upper limb perfusions.

The measurements of height, weight and body surface area (n=73) are listed in Appendix D, together with the measured limb volume (n=59) and the calculated total iliac perfusion volumes where appropriate (n=44). The age of the patients varied considerably (range 16-78years) and, accordingly, the observations of height, weight and limb volume also showed a wide range of values. The sets of haematocrit measurements obtained from each ILP (n=69) are listed in Appendix E together with the corresponding calculated total blood volume. The variability in the haematocrit of the priming fluid (mean 24.96 ; SD 2.7) is

explained by the slightly different proportions of crystalloid and red cell concentrate in the fluid in each case. The volume of the prime was kept constant at 1100ml.

The dose of melphalan given to each patient and the doses which would have been given, had either of the other two dosage schedules been employed, are listed in Table 27. These are also displayed graphically in Figure 26, with lines joining each of the three doses for individual patients. For iliac perfusions, the mean dose (SD) was 121.41mg (23.68) by body weight, 126.16mg (23.67) by limb tissue volume and 83.83mg (24.91) by total blood volume. The mean doses (SD) prescribed by each of the three methods for axillary perfusions were 54.71mg (5.14), 43.85mg (6.14) and 28.25mg (11.54), and for popliteal perfusions 62.0mg (13.04), 58.5mg (3.54), and 55.6mg (9.07) respectively. These results were analysed by comparing the three combinations of matched pairs of doses using Wilcoxon's signed-rank test (Table 28). This revealed that the dosage schedules based on body weight and limb volume led to the administration of very significantly higher doses of melphalan for both iliac (+38.0mg and +42.5mg respectively) and axillary (+28.75mg and +16.0mg respectively) perfusions than did the method based on TBV estimation, even allowing for multiple testing. These differences can be seen very clearly in Figure 26. The popliteal perfusions were too few in number to allow a meaningful comparison in their own right. Taken in combination with the axillary perfusions (which are associated with similar administered doses of

Table 27 :

Melphalan Doses Prescribed for ILP by Three Dosimetric Schedules

Patient No	Schedule LTV	WT	TBV	Patient No	Schedule LTV	WT	TBV
Iliac				Iliac(cont)			
001	159*	140	99	042	-	107	88
002	137	163*	98	043	161*	137	70
003	99	100	88	044	90	96	66
004	95	84	51	045	111	107	50
005	151*	166*	107	046	99	89	70
006	114	103	70	047	134	117	132
007	115	107	88	048	119	103	59
008	-	151*	81	049	104	112	94
009	-	137	101	050	146	158*	61
010	93	85	53	051	-	110	79
011	116	105	88	052	122	130	-
012	116	112	88	053	104	112	-
013	144	133	71				
014	134	119	147	Axillary			
016	155*	133	88	A01	40	60	70
017	-	126	57	A02	51	60	25
019	127	117	79	A03	40	60	22
020	-	105	52	A04	-	50	25
021	166*	145	82	A05	-	60	24
022	97	100	50	A06	39	60	25
023	111	98	69	A07	-	60	25
024	170*	180*	101	A08	44	50	-
025	139	144	-	A09	38	50	22
026	97	105	69	A10	46	50	31
027	157*	151*	73	A11	38	50	24
028	111	119	121	A12	56	60	26
029	151*	151*	95	A13	49	50	24
030	129	119	132	A14	51	60	28
031	169*	156*	99	A15	38	50	33
032	138	119	70	A16	-	50	22
033	112	109	66	A17	40	50	26
034	121	128	73				
035	143	135	66	Popliteal			
036	105	91	95	P01	-	50	50
037	154*	163*	167*	P02	61	70	55
038	104	95	77	P03	56	60	66
039	94	91	73	P04	-	50	44
040	138	126	88	P05	-	80	63
041	-	103	83				

Dosimetry schedules based on limb tissue volume(LTV), weight(WT), and total blood volume(TBV).

* denotes prescribed doses which exceed the maximum administered dose of 150mg.

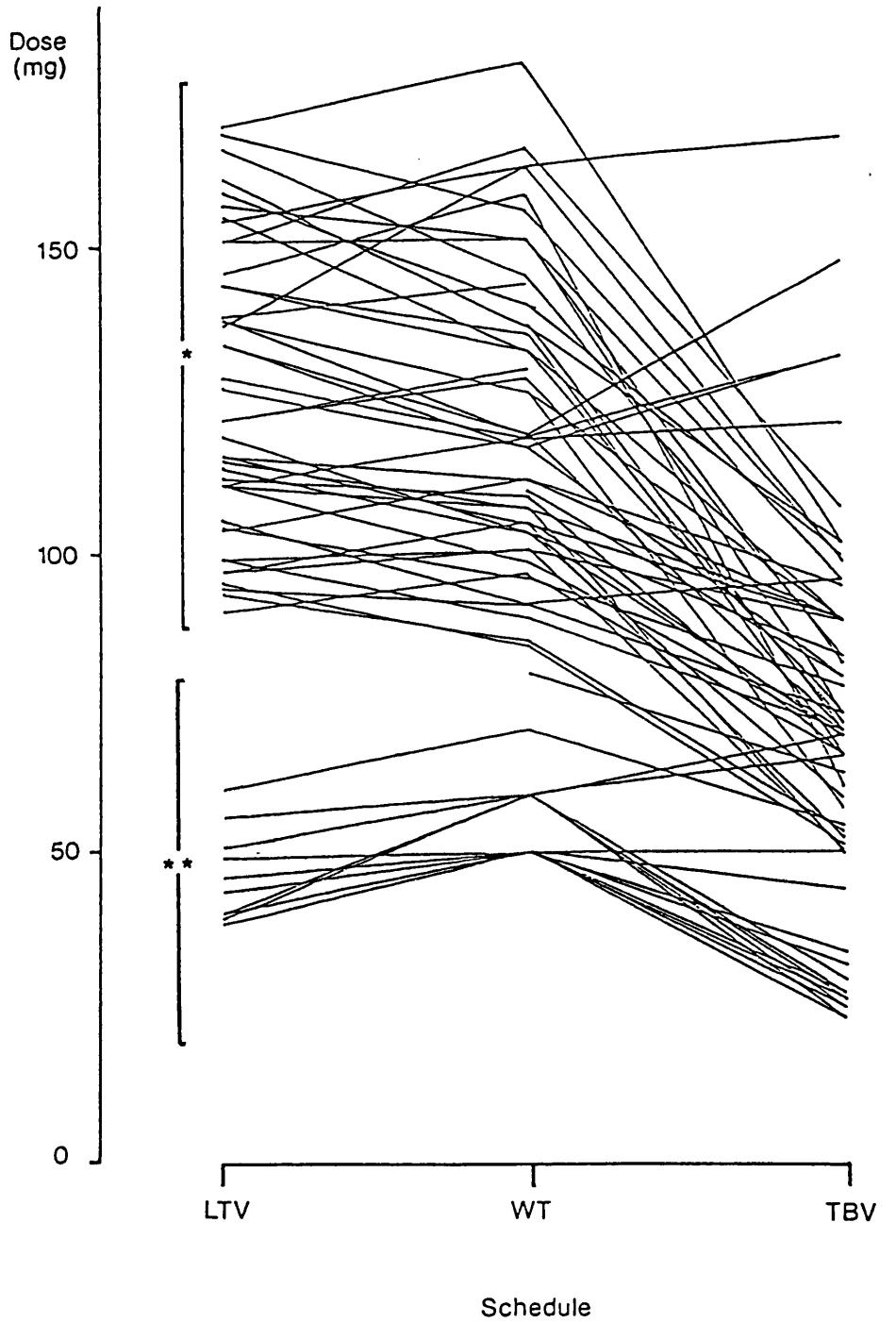


Figure 26 :
Doses of melphalan calculated according to three dosimetric schedules for 73 patients undergoing ILP.

Dosimetric schedules based on limb tissue volume(LTV), weight(WT), and total blood volume(TBV).

(* iliac ILP; ** axillary & popliteal ILP)

Table 28 :

Comparison of Melphalan Doses
Calculated by Three Dosimetric Schedules[#]

Region	WT v LTV	WT v TBV	LTV v TBV
Iliac	-5.0 (-9.0,-1.0) n=44 p=0.007	+38.0 (29.5,45.5) n=48 p<0.001	+42.5 (33.5,52.0) n=41 p<0.001
Iliac (clinical)*	-4.5 (-7.5,-1.0) n=44 p=0.001	+36.5 (28.5,43.5) n=48 p<0.001	+40.0 (32.0,49.5) n=41 p<0.001
Axillary	+10.5 (6.5,15.0) n=13 p=0.002	+28.75 (23.5,32.0) n=16 p=0.001	+16.0 (9.5,22.0) n=12 p=0.034
Axillary and Popliteal	+10.5 (6.5,14.5) n=15 p=0.001	+24.5 (15.5,30.0) n=21 p<0.001	+14.5 (3.0,20.5) n=14 p=0.026

[#] using Wilcoxon's signed rank test for matched pairs

Data shown as :
median difference(mg)
(95% confidence interval)
no. of patients
p-value

WT=Weight LTV=limb tissue volume
TBV=total blood volume
* maximum permissible dose = 150mg.

melphalan), they confirm the relative insufficiency of the dose calculated by TBV estimation. Calculation of the iliac perfusion dose by body weight resulted in the administration of a slightly lesser amount of melphalan (-5.0mg) than the dosage schedule based on limb volume.

In the clinical situation, an upper limit of 150mg is applied to the dose of melphalan (see doses marked with an asterisk in Table 27). Analysis of these "clinical" doses by the same method (Wilcoxon's signed rank test) produced similar results (Table 28), although the difference between weight-based doses and volume-based doses was slightly less (-4.50mg).

Pharmacokinetics data were obtained from 56 ILPs and the melphalan concentrations in the perfusate are listed in Appendix B. By linear regression of the logarithmic plot of the melphalan concentrations between time = 20 minutes and time = 60 minutes, a drug disappearance curve was drawn for each ILP. The half-life of the beta phase of the curve could therefore be calculated from the gradient, and the projected equilibrium concentration of melphalan ($meas[MEL]_{eq}$) was obtained by back-extrapolation of the regression line to time = 0 minutes. The equation of the line was of the form:

$$\log[MEL] = a + b.t$$

where t = time, a = the intercept on the log[MEL] axis (ie. the equilibrium concentration of melphalan, on the logarithmic scale), and b = the gradient of the line.

The half-life of melphalan in the perfusate could therefore be expressed as the time at which

$$\log[MEL] = \log([MEL]_{eq}/2).$$

From the above equation,

$$t = \frac{\log[MEL] - a}{b}$$

So,

$$t_{1/2} = \frac{\log([MEL]_{eq}/2) - \log[MEL]_{eq}}{b}$$

$$= \frac{\log \frac{[MEL]_{eq}/2}{[MEL]_{eq}}}{b}$$

$$= \frac{\log 1/2}{b}$$

The mean equilibrium concentration of melphalan in the perfusate was 46.81ug ml^{-1} (SD=24.69) and its mean half-life was 37.05 minutes (SD=16.31). Table 29 shows these figures separated according to the region perfused.

Table 29 :
Melphalan Pharmacokinetics in ILP

Region	Equilibrium concentration ($[MEL]_{eq}$)	Half-life ($t_{1/2}$)
Iliac (n=40)	48.49(4.32)	34.12(1.66)
Popliteal (n=5)	37.02(3.26)	41.14(5.61)
Axillary (n=11)	45.32(5.91)	45.84(8.75)
Overall (n=56)	46.81(3.30)	37.05(2.18)

$[MEL]_{eq}$ expressed in ug ml^{-1}

$t_{1/2}$ expressed in minutes

All values given as mean(SEM).

Table 30 lists the measured equilibrium concentrations of melphalan (meas$[MEL]_{eq}$) derived from the pharmacokinetic profiles of 56 ILPs. The table also includes the dose of melphalan administered in each case together with the estimated total blood volume in the isolated circuit (TBV), and the equilibrium concentration which one might predict from these two values (exp$[MEL]_{eq}$). The last column in the table contains estimates of the volume of distribution of the drug derived from the administered dose and the measured equilibrium concentration.

The measured $[MEL]_{eq}$ correlated very poorly with the expected $[MEL]_{eq}$ (iliac ILP: r=0.212; axillary + popliteal ILP: r=0.392; overall r=0.257). This is shown graphically in Figure 27. Furthermore, in almost all cases the measured value was significantly lower than the expected value as can be seen from the fact that most of the points on the graph lie below the line of identity (meas$[MEL]_{eq}$=exp$[MEL]_{eq}$). The typical difference between the two values can be quantified using Wilcoxon's signed-rank test. This reveals a median difference of 15.89$\underline{u}$g ml^{-1} (95% C.I. = +7.8, +22.6; p=0.001) for iliac perfusions. Greater predictive accuracy was achieved with axillary or popliteal perfusions, the median difference in this group being only 5.59$\underline{u}$g ml^{-1} (95% C.I. = -4.9, +11.9; p=0.148). A similar comparison of the TBV estimated from the three haematocrit measurements and the calculated volume of distribution of melphalan at equilibrium shows a marked underestimation of the volume by the former method, resulting in a median difference of 942.3ml for iliac perfusion (95% C.I. = +574, +1292; p=0.000) and 264.2ml

Table 30 :

Melphalan Pharmacokinetics and Estimated Volume of Distribution in 56 ILPs

Patient	Dose	TBV	$[MEL]_{eq}$ meas.	$[MEL]_{eq}$ obs.	Vol. of Distr.
Iliac					
003	100	2200	45.45	35.41	2824
005	150	2671	56.16	38.16	3931
006	103	1760	58.52	28.77	3580
007	107	2200	48.64	28.21	3793
008	150	2017	74.37	41.13	3647
009	137	2514	54.49	49.91	2745
010	85	1320	64.39	26.79	3173
011	105	2200	47.73	30.43	3451
012	112	2200	50.91	41.27	2714
013	133	1787	74.43	70.60	1884
014	119	3667	32.45	40.06	2971
016	133	2200	60.45	35.41	3756
019	117	1980	59.09	62.81	1863
020	105	1300	80.77	56.85	1847
021	145	2043	70.97	85.51	1696
022	100	1238	80.78	109.19	916
023	98	1729	56.68	66.79	1467
024	150	2514	59.67	44.27	3388
025	144	-	-	45.38	3173
026	105	1728	60.76	31.05	3382
027	150	1833	81.83	55.66	2695
028	119	3025	39.34	30.94	3846
029	150	2383	62.95	75.44	1988
030	119	3300	36.06	119.40	997
031	150	2475	60.61	36.15	4149
032	119	1760	67.61	67.43	1765
033	109	1650	66.06	137.32	794
034	128	1833	69.83	54.38	2354
039	91	1833	49.65	27.03	3367
040	126	2200	57.27	47.87	2632
041	103	2063	49.93	24.96	4127
042	107	2200	48.64	22.90	4672
043	137	1760	77.84	52.62	2604
044	96	1650	58.18	19.89	4827
045	107	1238	86.43	36.74	2912
046	89	1760	50.57	24.24	3671
047	117	3300	35.45	32.74	3574
048	103	1467	70.21	25.47	4044
049	112	2338	47.90	24.77	4522
050	150	1513	99.14	35.77	4193

(cont)

Table 30(cont):
Melphalan Pharmacokinetics and Estimated Volume of Distribution in 56 ILPs

Patient	Dose	TBV	$[MEL]_{eq}$ meas.	$[MEL]_{eq}$ obs.	Vol. of Distr.
Axillary					
A03	60	1100	54.55	46.11	1301
A04	50	1269	39.40	44.17	1132
A05	60	1179	50.89	45.23	1327
A06	60	1247	48.12	52.98	1133
A07	60	1257	47.73	70.20	855
A08	50	-	-	57.00	877
A09	50	1100	45.45	80.99	1301
A10	50	1571	31.83	17.79	2811
A11	50	1184	42.23	36.14	1384
A12	60	1283	46.77	23.10	2597
A13	50	1179	42.41	24.79	2017
Popliteal					
P01	50	1257	39.78	34.00	1471
P02	70	1375	50.91	39.68	1764
P03	60	1650	36.36	31.41	1910
P04	50	1100	45.45	48.59	1029
P05	80	1571	50.92	31.41	2547

TBV : total blood volume
$[MEL]_{eq}$: equilibrium concentration of melphalan.

Dose expressed in mg.
TBV and Volume of Distribution expressed in ml.
$[MEL]_{eq}$ expressed in ug ml^{-1}

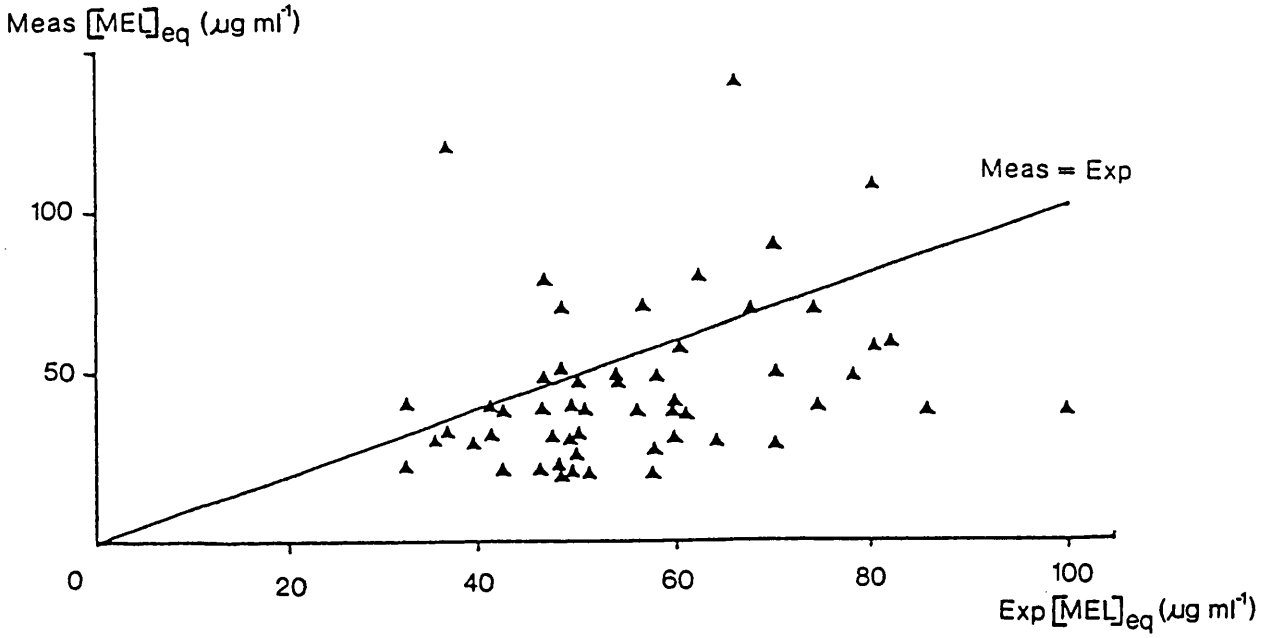


Figure 27 :
The equilibrium concentration of melphalan measured in samples of perfusate (meas$[MEL]_{eq}$) plotted against that predicted from the dose administered and the estimated total blood volume into which it is distributed (exp$[MEL]_{eq}$).

Most of the points lie below the line of identity reflecting an underestimation of the volume of distribution by the TBV method.

for axillary and popliteal prfusion (95% C.I. = +43, +622ml; p=0.016). These median differences amounted to as much as 31.5% and 16.1% of the mean volumes of distribution of the two groups respectively.

4.4 Discussion

Measurement of the volume of a limb by immersion seems an attractive proposition since it allows the clinician to apply a numerical dosage formula where in the past he relied on a subjective impression of the patient's morphology when adjusting the weight-based melphalan dose. However, the practice of immersing a whole leg is awkward and can at times be painful or even dangerous. The study described in Section 4.2 confirms that partial immersion of a leg allows accurate calculation of the total leg volume while avoiding the risks of imbalance, pain and embarassment. The 95% prediction intervals for the formula derived enclose a substantial range, and the dose calculation may therefore be associated with an error of up to 16mg. However, there have been no studies so far to determine the accuracy and reproducibility of the limb volumetry protocol. Inherent in the method are several steps which each contribute, to a greater or lesser degree, to a potential error : lack of stability of the non-weightbearing limb, perineal discomfort resulting in an unbalanced posture, visual estimation of the two water levels, an arbitrary 10% correction factor to include the upper thigh and buttock in the total volume. In this study, attempts have been made to reduce these errors by using a narrow piece of transparent tubing to gauge the water level and by allowing the subjects to weightbear on both legs. In this way, reproducibility at least is more probable.

Iliac ILP

In his original paper describing a dosage schedule based on limb tissue volume, Wieberdink[406] found that when the melphalan doses administered were expressed in mg kg^{-1} body weight, this resulted in a mean dosage of 1.58mg kg^{-1}. Other authors have subsequently adopted his method, and have reported dosages equivalent to 1.77mg kg^{-1} body weight[504]. In the first part of this study (Section 4.2), the limb volume measurements would theoretically have yielded a mean dose equivalent to 1.90mg kg^{-1} ($\pm$ 0.03, SEM) and, indeed, in the clinical study described in this chapter (Section 4.3.2), the mean dose calculated by the limb volume technique would have been equivalent to 1.83mg kg^{-1} ($\pm$ 0.02, SEM). Therefore this modified technique of limb volume measurement would appear to produce similar results to that of Wieberdink, with calculated doses in a similar (though, if anything, slightly higher) range.

The main reason for seeking new dosimetric methods is the need to standardise treatment. The majority of perfusion centres in Europe currently employ the dosage schedule based on limb tissue volume. In Section 4.3.3, it has been shown that the doses calculated by body weight for iliac ILP are only marginally lower than those based on limb volume. Previous reports have suggested that calculations based on body weight can result in wide variations of melphalan dose expressed per tissue volume in the limb[406] and that this can produce variable, unpredictable and sometimes unacceptable toxicity.

However, in most studies, the doses have not been strictly defined. They are, rather, selected from a range of dosage schedules (see Table 25) according to the build, complexion, hair colour and age of the patient, red-haired and fair-skinned individuals being thought to be more susceptible to the toxic effects of melphalan. In this centre, the calculated dose of melphalan is not adjusted except to limit the maximum dose to 150mg. All patients undergoing iliac perfusion are given 1.75mg kg^{-1} body weight. In this study, the dose administered for iliac perfusion was equivalent to 9.63 $\pm$ 0.13mg l^{-1} (mean $\pm$ SEM) of perfused tissue, which is only slightly lower than the recommended 10mg l^{-1}, suggesting that a large part of the previously observed variability of dosage and toxicity may have resulted from subjective assessment of the patients' morphological characteristics and from technical variations such as inconsistent temperature regulation during ILP. The toxic reactions encountered after iliac ILP in this study were largely acceptable with no reactions exceeding Grade III and only 4 falling into Grade I (Table 31).

Of the three dosimetric methods assessed, that based on estimation of the total blood volume in the perfusion circuit (TBV) consistently prescribed the lowest dose. Calculation of the TBV led to inaccurate prediction of the equilibrium concentration of melphalan, and in fact the estimated volume of distribution of the drug at equilibrium was, on average, as much as 31.5% higher than the calculated TBV for iliac perfusions. Clearly, it would appear that TBV does not represent the volume of

Table 31 :

Toxicity Observed in Normal Tissues of 73 Limbs after ILP with Melphalan

Region	Grade of Reaction*				
	I	II	III	IV	V
Iliac n=51	4 (8)	33 (65)	14 (27)	-	-
Polpiteal n=5	-	4 (80)	1 (20)	-	-
Axillary n=17	1 (6)	8 (47)	6 (35)	2 (12)	-
Overall n=73	5 (7)	45 (62)	21 (29)	2 (3)	-

* Toxic reaction graded according to Wieberdink[406] (see Table 8).

Numbers in brackets are percentages.

distribution of the drug. The much larger volume calculated from the measured equilibrium concentration and the administered dose is based on the beta phase of the drug disappearance curve from the perfusate. During this phase, the decrease in melphalan concentration is believed to be due to hydrolysis of the melphalan in the perfusate and to uptake of the drug by the oxygenator and the plastic components of the circuit[448,458]. The alpha phase of the curve represents the changes in melphalan concentration caused by mixing of the drug in the perfusate and distribution of the drug to the cellular components of the perfusate and to the interstitial fluid of the limb. Most of the cellular uptake of the drug in the limb is also believed to take place during this phase, and intracellular transport of melphalan in the later part of the perfusion is probably matched by efflux of intact melphalan back into the interstitial fluid. Therefore the volume upon which to base calculations of equilibrium concentrations of melphalan should more appropriately include TBV, the interstitial fluid of the limb and a proportion of the intracellular fluid. The estimated contribution of the patients' peripheral blood to the total blood volume in the perfusion circuit in this study is slightly greater than, but of similar magnitude to, that measured by Lejeune[502] (mean $\pm$ SEM: 952 $\pm$ 90ml and 768 $\pm$ 72ml respectively). Yet this study has identified an apparent further significant contribution of 921 $\pm$ 182ml (mean $\pm$ SEM; p=0.000, Wilcoxon's signed-rank test) to the total volume of distribution of the drug. This is a variable volume which represents the extra

compartments into which the melphalan is distributed (see above). No significant correlation has been observed between this volume and the limb tissue volume (r=-0.22) or the estimated TBV (r=-0.37). The method of dose calculation based on TBV is therefore unreliable.

Popliteal ILP

In Isolated Limb Perfusion at the popliteal level, we calculate the total iliac dose of melphalan (1.75mg kg^{-1}) and then estimate the proportion of the iliac region which is included below the tourniquet. An equivalent proportion of the total dose of melphalan is then administered. In this study this has resulted in a mean popliteal dose of 0.93mg kg^{-1} (SD 0.05). Due to the small number of popliteal perfusions (n=5), statistical analysis of differences between dosage schedules is of very limited value. The dosage schedule employed consistently prescribed a higher dose than either of the other two schedules (WT dose = LTV dose + 6.5mg and TBV dose + 6.0mg). For further analysis of the popliteal perfusions, the data from these perfusions have been combined with those from the axillary perfusions (indicated at the appropriate passages) since the doses prescribed by all three techniques tend to fall into similar ranges for both of these regions.

Axillary ILP

It is generally held that there is a tendency to underestimate the appropriate dose for axillary ILP. After initially recommending the same dosage schedule of

10mg l^{-1} for both upper and lower limbs, Wieberdink and his colleagues acknowledged this underestimation by increasing their recommended dose for upper limb perfusions to 13mg l^{-1} [459,505]. The doses recommended in the literature according to body weight range from 0.5mg kg^{-1} to 1.0mg kg^{-1} (see Table 25). Our past experience has led us to apply a maximum dose of 60mg in order to avoid the excessive toxicity frequently caused by higher doses. In fact, at present only two doses of melphalan are employed in Glasgow, 50 or 60mg, selected according to body weight and our impression of the build of the patient. In this study, this has resulted in a mean body weight dose of 0.75mg kg^{-1} (SD 0.01) and higher administered doses than the systems based on limb volume (median difference = +10.5mg) or TBV estimation (median difference = +28.75mg). Expressed as melphalan per litre of perfused tissue, <u>all</u> the administered doses in this study exceeded the prescribed 13mg l^{-1}, and the mean value of 16.35mg l^{-1} was significantly higher than this ($p=0.002$). Excessive toxicity (Grade IV) was observed in only two patients. This is in direct contrast to Wieberdink's claim that doses in excess of 13mg l^{-1} <u>invariably</u> resulted in reactions greater than Grade III. However, his experience was based on only three cases receiving 13-17mg l^{-1} ($n=2$) or 19mg l^{-1}($n=1$). Three patients in this study received doses in the range 19-20mg l^{-1} and all experienced Grade III reactions. While Grade IV toxicity is undesirable, modification of the dosage does not seem to be necessary at present. It is likely that, among the other factors contributing to the observed

toxicity, temperature control to avoid excessive hyperthermia can also limit the reaction to an acceptable level. Inspection of the intraoperative records of the patients in this group has revealed that the two patients who displayed a Grade IV reaction were also those in whom the limb temperatures were the highest (both higher than 38.8^{o}C). In this study, 53% of patients suffered toxic reactions less severe than Grade III. Reduction of the dose of melphalan would presumably have resulted in an even greater proportion of patients falling into Grades I and II. It has been suggested that Grade I indicates relative underdosage of melphalan[406,504]. In terms of potential disease-control, this is undesirable.

The TBV estimation produced a closer approximation of the calculated volume of distribution of melphalan in axillary than in iliac perfusions, the median difference being 200.5ml (13.7% of the mean volume of distribution). The data from popliteal perfusions yielded a degree of accuracy intermediate between these two groups, the median difference in this setting being 301.5ml (17.3% of the mean volume of distribution). Accordingly, the predicted equilibrium concentration of melphalan in the perfusate was more accurate in axillary and popliteal perfusions than in the iliac group.

The observation that the TBV more closely approximated to the volume of distribution of melphalan in perfusion of regions of smaller volumes would tend to support the hypothesis that interstitial fluid plays a large part in the calculation of volume of distribution of the drug. Adipose tissue, a relatively avascular tissue, is not

evenly distributed over the body and occupies a relatively greater proportion of lower limb volume than upper limb volume, especially in women. Even within the lower limb, there is a disproportionate accumulation of adipose tissue in the thigh relative to the calf. By contrast, muscle is a very vascular tissue and although it does constitute the main bulk of the thigh, it occupies a lesser proportion of this part of the body than of the calf or arm. Therefore we could expect the volume of blood per litre of tissue to be lower in the thigh than in the calf or arm and to form a lesser part of the total compartment of distribution of melphalan during Isolated Limb Perfusion.

4.5 Conclusions

The main aim of any dosimetric method for ILP is to allow administration of the highest dose of cytotoxic drug consistent with acceptable morbidity. In order to standardise the treatment, the method must ideally be easily reproducible.

Each method of calculating the dose of melphalan for Isolated Limb Perfusion has its own merits. A schedule based on body weight is simple to implement, and is easily reproducible. In ILP of the iliac region, the toxicity produced by doses of 1.75mg kg^{-1} can be confined to Grades II and III in 92% of patients, and in this series never exceeded Grade III. The doses calculated on the basis of limb tissue volume (10mg l^{-1}) are generally slightly higher than these, but in this study the difference was only of the order of 5.0mg (4%). The toxicity reported using this schedule is similar to that observed when melphalan is administered using 1.75mg kg^{-1} body weight. The stated aim of the dosage schedule based on estimation of the total blood volume in the perfusion circuit is to generate predictable concentrations of cytotoxic in the perfusate. While this may seem an attractive approach and while it may offer the same possibilities of gradually increasing the dose of melphalan to the maximum tolerable level as the other two methods, this study has shown it to be unreliable. The melphalan concentrations achieved in the perfusate in this large series of patients correlated very poorly with the predicted concentrations, contrary to Lejeune's experience[502]. However, although

his initial report contained details of 31 cases, his claim that the equilibrium concentration of melphalan was highly predictable was based on only 12 patients of whom 8 had undergone iliac ILP and 4 had undergone axillary ILP. Furthermore, the equilibrium concentrations produced in the present study by doses based on body weight were higher than his recommended targets of 40ug ml^{-1} for lower limbs and, especially, 20ug ml^{-1} for upper limbs. One can therefore conclude that, even when the method achieves higher accuracy (eg. when perfusing regions of smaller volume), this schedule results in less than optimal dosage of melphalan.

In perfusion of the axillary or popliteal region as practised in Glasgow, the dose calculation includes an element of subjective assessment of the patient's build. This clearly is not easy to standardise. The separation of doses for axillary perfusion into only two categories, while reducing the variability of the dosage, also reduces the specificity of the dose in each case. It may be more appropriate to use limb volume as the determinant factor in dose calculation for these regions. This study has shown that melphalan administered in a dose equivalent to 16mg l^{-1} is associated with toxicity reactions of Grade III or less in 88% of axillary perfusions. The currently recommended dose of 13 mg l^{-1} may therefore not be optimal.

It is concluded that for Isolated Limb Perfusion of the iliac region a dose based on body weight is the most appropriate by virtue of its simplicity, the high doses

which it prescribes, and the well controlled toxicity which it produces. Further Phase I studies of slightly higher doses than the current 1.75mg kg^{-1} might allow the total dose administered to be raised, in line with the calculated dose of 1.83mg kg^{-1} associated with the limb volume dose in this study. Limb volumetry by immersion is, at best, an awkward technique. It can be uncomfortable for the patient and may even at times be dangerous. The alternative method of partial volumetry is simpler, less dangerous, and less uncomfortable; it does not require the use of an expensive mechanical hoist. The accuracy and reproducibility of these methods has not been proven and may be suspect.

Despite the use of a higher dose in every case than the recommended 13mg l^{-1} for perfusion of the axillary region (equivalent to a mean of 16mg l^{-1} of perfused tissue), the toxicity encountered in this study was within acceptable limits. Further Phase I studies of dosage for perfusion of the axillary region based on limb volume are therefore also advocated.

5 CHAPTER 5 - TARGETING THERAPY TO CANCER

5.1 Targeting Methods - Monoclonal Antibodies

The treatment of most cancers is limited by the deleterious effect of the treatment on the normal tissues of the host and in particular on organs such as the liver or bone marrow. ILP offers a method of reducing the insult to these tissues and thus of applying more aggressive therapeutic measures to the tumour. The principal limiting factor in ILP for malignant melanoma, however, remains the toxicity observed in the normal tissues of the limb.

For long, researchers have sought ways of increasing the specificity of anticancer agents in order to enhance the therapeutic index (relative uptake in tumour compared to normal tissues). One approach has been the use of toxic substrates to reactions known to take place within the malignant cells, for example the use of radioactive iodine in the treatment of thyroid cancer. In the treatment of melanoma, the chemical pathway of melanin synthesis offers a possible target for toxic substrates. Indeed, it had originally been hoped that melphalan, because of its chemical structural similarity with phenylalanine, might act as a false precursor in melanin synthesis[506,507]. More recently, "false substrates" converted to active metabolites by the enzyme tyrosinase have been under investigation, in particular 4-hydroxyanisole[283]. The incorporation of aminothiol compounds into newly-synthesized melanin resulting from their reaction

with orthoquinones produced during melanogenesis offers an alternative route of attack in the melanogenic pathway. Substances such as thiourea, 2-thiouracil, 5-iodo-2-thiouracil and methimazole have all been shown to be incorporated into melanin in vivo and in vitro[508-513]. The substitution of radioactive isotopes of iodine or sulphur in these compounds may provide a way of targeting radiotherapy to melanin-synthesizing cells.

In the last two decades, the development of techniques allowing the production of monoclonal antibodies to specific antigens has given rise to great optimism that targeted therapy of cancer might soon be possible.

Monoclonal antibodies

In 1975, Kohler and Milstein described an elegant method for producing pure antibodies of predetermined specifity[514]. They immunised mice repeatedly with a defined antigen and later removed the spleen from which they harvested proliferating B lymphocytes. These B cells were then "immortalised" by fusion with mouse myeloma cells. The resultant hybrid, maintained in tissue culture, was able to secrete large amounts of antibody to the original antigen.

Early evidence for the existence of tumour-associated antigens was obtained by Pressman[515] who demonstrated that radiolabelled antibody from the serum of a rabbit immunised against rat sarcoma accumulated at the tumour sites when injected into rats bearing osteogenic sarcomas.

Since then, several tumour-associated antigens have been identified on human tumours including carcinomas of the colon, breast, lung, ovary, uterine cervix, renal cell and bladder, as well as lymphomas, leukaemias, melanoma and bone and soft tissue sarcomas[516]. Although they were initially believed to be tumour-specific, it has become clear that many of these antigens are proteins not normally present on most adult cells but produced by the foetus. These oncofoetal proteins include carcinoembryonic antigen (CEA), alpha foetoprotein (AFP) and human chorionic gonadotrophin (HCG). Other human tumour-associated antigens may well be distinct from foetal antigens but seem to be only preferentially or inappropriately expressed on malignant cells[517,518]. They are therefore not exclusive to tumour and may be expressed in small amounts by normal tissues.

Monoclonal antibodies raised against these tumour-associated antigens offer the potential for targeting treatment to malignant cells. There are abundant reports of the successful localisation of tumour deposits by monoclonal antibodies raised against antigens expressed by malignant melanoma as well as carcinomas of the colon, breast and ovary[519-523]. Although the therapeutic value of monoclonal antibodies has until now been limited, their potential applications fall into two main categories :

[1] they may be used in the unmodified state to activate the host's natural effector mechanisms against tumour cells. Phagocytosis, antibody-dependent cellular cytotoxicity, and complement fixation are all activated by the constant region (Fc) of cell-bound antibody[524]. There have been several reports of this type of treatment associated with variable responses[525-528]. Certain patients seem to mount an immune response to the monoclonal antibody itself and, in particular, to the antigen-binding portion (idiotype) of the antibody. It has been suggested that these anti-idiotype antibodies may act as mirror images of the targeted tumour-associated antigen to stimulate host immunity against the tumour[529].

[2] the more likely therapeutic application of monoclonal antibodies would appear to be as targeting vectors, allowing selective delivery of anticancer agents to tumour sites. The principal agents which have been conjugated to monoclonal antibodies are radionuclides, cytotoxic drugs and toxins. To date, most of the experience of this type of approach has been with radionuclides due to the ease with which the distribution of the radiolabelled antibody can be assessed by scintigraphy. The success of such an approach to cancer therapy is dependent on several factors:

- the selective delivery of the agent to tumour cells (this will in turn depend on the degree of specificity of the tumour-associated antigen)

- the concentration of antigenic sites on the target cell, and the ability of the cell, if necessary, to internalise the agent conjugated to the antibody in sufficient quantities for cell kill

- the retention of the antibody's antigen-binding specificity and of the cytotoxic agent's activity after conjugation.

Many drugs in fact lose much of their activity when bound to proteins[530], possibly by stearic hindrance or by alteration of the uptake mechanism. One way of counteracting this problem is to couple the drugs to carrier molecules (eg. human serum albumin) which can be heavily substituted with drug and to conjugate these molecules to the antibody. This "amplification" technique can allow as much as a tenfold increase in the amount of drug carried by the antibody[531]. The specificity of these conjugates may be improved as a result, since only those cells with high numbers of antigenic sites (ie. tumour cells) will receive enough drug for irreversible damage to occur. Although most reported studies have used single antibody preparations, tumours are thought to express a variety of tumour-associated surface antigens and it is possible that "cocktails" of antibodies will allow a higher degree of antibody binding to tumour cells[518,524]. It is also important to ensure that, while the active agent may have to be released once the target cell has

been reached, dissociation of the conjugate does not occur prematurely with release of the agent into the systemic circulation.

One difficulty with the antibody targeting method is poor tumour localisation, particularly by larger conjugates including drug-carrier-antibody complexes. This is thought to be due to their inability to permeate through the capillary walls and to their rapid uptake by the reticulo-endothelial system[529]. This latter mechanism carries the added disadvantage that the cytotoxic agent accumulates in normal tissue, further reducing the therapeutic index. For this reason, attempts have been made to produce smaller conjugates by using antibody fragments (Fab or $F(ab')_2$ fragments). By virtue of their smaller size, these fragments are able to pass more rapidly out of the capillaries into the interstitial fluid. They are also readily excreted into the urine, thereby shortening their plasma half-life[524]. Hopes that these fragments might allow more effective irradiation of tumour deposits by improving the penetration of the conjugates into tumour parenchyma have unfortunately not been realised[524].

Murine antibodies are known to elicit a strong antimouse protein immune response which reduces their circulating half-life. Modern techniques of antibody engineering have allowed chimerisation and humanisation of monoclonal antibodies by incorporating respectively the variable region or antigen-binding portion of the murine antibody into a human antibody structure[532]. This also increases the antibody's ability to activate the host's natural

immune mechanisms, the murine Fc being a rather weak activator of human effector functions[524]. Several antibodies to human tumours have now been humanised[533] and at least one has already been associated with a clinically significant response[534].

Targeted cytotoxic agent

Of the anticancer agents used for targeted therapy to date, cytotoxic drugs have the advantage that their modes of action and side effects are already well recognised. However, their usefulness in targeted therapy is severely hindered by factors such as loss of activity due to covalent linkage and the need for high intracellular concentrations in order to achieve cell kill. The cytocidal dose of most active drugs is in excess of one million molecules per sensitive cell. In contrast, single molecules of plant toxins (eg. ricin) are believed to be capable of killing cells[535]. Conjugates of these toxins with anti-tumour monoclonal antibodies, also known as **immunotoxins**, may therefore be more cytotoxic than drug-antibody conjugates but also require a much greater degree of antibody specificity to avoid non-target tissue damage.

Various radionuclides have been conjugated to monoclonal antibodies, mainly for diagnostic purposes but also for therapeutic studies. The most commonly used radionuclides have been isotopes of iodine (I^{123}, I^{125}, I^{131}) due to the ease with which they can be substituted into the

antibody molecule. Other commonly used radionuclides include ^{111}Indium, ^{99}Technetium and, increasingly, ^{90}Yttrium. The chemistry of linking these isotopes to antibodies is more complex than that of iodine. For therapeutic use, however, ^{131}Iodine presents problems of its own. In vivo deiodination of the antibody can occur readily, resulting in a non-specific systemic distribution of the isotope. The use of ^{90}Yttrium-labelled antibodies is similarly limited by instability of the label when diethylene triamine pentacetic acid (DTPA) is used as the chelating agent[536]. Attempts to circumvent this problem by using much stronger chelating agents have been hindered by the immune responses generated in vivo by these chelates[537]. Furthermore, the radiation characteristics of many of these isotopes are not ideal for therapeutic use : for example, the beta emissions from ^{131}I are principally dissipated about 1mm distant from the source of the emission and those of ^{90}Y are dissipated 6mm from the source[538]. The irradiated nuclei would therefore be those not of the targeted cells but of cells at these respective distances from the antibody. The most effective use of these isotopes would therefore be against tumour deposits large enough to allow "cross-fire" irradiation of targeted cells by isotope bound to other similar cells nearby[538]. A side-effect of this approach would, however, be the irradiation of non-target cells in the vicinity and thus a loss of specificity of treatment. Other isotopes, such as ^{211}Astatine and ^{67}Copper (short range alpha- and beta- emitters respectively), may provide a better method for targeting micrometastases and

single-cell disease[538,539], and these are currently being investigated.

5.2 ILP using an Anti-melanoma Monoclonal Antibody

5.2.1 Introduction

Several melanoma-associated tumour antigens have been identified. The best known of these are the so-called "high molecular weight" melanoma-associated antigen (HMW-MAA), a chondroitin sulphate proteoglycan with a molecular weight of approximately 240,000 which is expressed by up to 90% of melanoma lesions[540], and the glycoprotein "p97" with a molecular weight of 97,000 and a chemical structure similar to that of transferrin[541]. The latter antigen is found in very high concentrations (400,000 molecules/cell) on more than 50% of melanomas, but is also found in naevi, foetal colon and, at lower concentrations, in most normal adult tissues[517,541]. Monoclonal antibodies have been produced against both of these surface antigens. While antigen p97 is reported to be present on 67% of clinically detectable melanoma deposits, the HMW-MAA is expressed by 80% of such lesions[542]. Overall, more than 90% of melanomas express one or both of these antigens[542]. Much experience has been gained in the use of these antibodies for the detection of metastatic melanoma by immunoscintigraphy[540,542-544] and some reports include details of patients receiving therapeutic doses of radiation by this method[542].

In Glasgow, immunoscintigraphy using a radiolabelled murine anti-HMW-MAA antibody (XMMME-001) has been found to have similar sensitivity and specificity to computerised

axial tomography for the detection of melanoma metastases[545]. In the same study, some patients were found to have tumour uptakes which would have resulted in cell kill had an alpha- or beta-emitting isotope been used. Hepatic and splenic uptake would, however, have proved to be a limiting factor to this therapeutic approach.

A clinical study of the potential for targeting melanoma deposits within a limb by introducing radiolabelled monoclonal antibodies into the Isolated Limb Perfusion circuit has therefore been performed.

5.2.2 Patients and methods

After receiving the approval of the local Ethical Committe and of the Administration of Radioactive Substances Advisory Committee (ARSAC) for this study, informed consent was obtained from 6 patients undergoing iliac ILP to add to the perfusate a diagnostic quantity (50-80 MBq) of ^{111}In-labelled antibody XMMME-001 (Xoma Corporation). All six patients were being treated for locoregionally recurrent melanoma and had clinically detectable tumour deposits on the lower leg or foot at the time of ILP.

Antibody labelling technique:

The antibody was provided already conjugated to DTPA cyclic anhydride at a concentration of 1mg ml^{-1} in 10mM Hepes buffer, 0.15M sodium chloride (pH 7). 0.2ml of this

solution were added to a mixture of 0.2ml sodium citrate buffer (pH 5) and 70-100 MBq in 0.2ml of ^{111}In (Amersham International plc). This was then incubated at 4°C for 2 hours before addition of 1ml of 20% human serum albumin (HSA) solution and passage through a 0.22um filter. The labelling efficiency was tested by thin layer chromatography (TLC) of an aliquot of the final solution in each case. This was found to be above 97% in all cases.

The standard iliac ILP technique described in Section 2.1.2 was employed in all cases.

[1] Patients 1-3:

In the first three cases, 200ug of ^{111}In-labelled antibody was added to the perfusate 5 minutes after the injection of melphalan into the ILP circuit. In order to maximise the "first pass" effect, the antibody was injected directly into the arterial cannula. Gamma emissions from the isotope were continuously monitored during the ILP using a scintillation probe (Nuclear Enterprises) positioned over the site of melanoma deposits and a second similar probe positioned over the muscle bulk of the thigh. Both probes were connected to a scaler and chart recorder (MS310, J&P).

[2] Patients 4-6:

In this group of patients, the melphalan and ^{111}In-labelled antibody were added simultaneously to the perfusate and the distribution of the radiolabel within the limb was then imaged using a mobile gamma camera (LEM, Siemens) and computer system (Map 2000, Link Systems). One minute images were collected at 64^2 resolution throughout the period of perfusion. The imaging data were later analysed off-line by drawing regions of interest (ROIs) around the sites of tumour deposits and of adjacent normal tissue and generating activity-time curves for these regions, normalised for the difference in area of each ROI. At the end of the procedure, tissue samples were obtained from two patients in this group (patients 4 & 5) so that the uptake of activity in skin, fat and tumour tissue could be measured. These were then assayed in an automatic gamma counter (Cobra, Packard) and the activity in each sample recorded as counts per minute per gram of tissue.

In all cases, perfusate samples were drawn from the venous limb of the perfusion circuit at 5 minute intervals throughout the perfusion period in order to count the activity in the perfusate. After rinsing the isolated circuit in the usual manner at the end of the ILP, the extracorporeal apparatus was emptied into a designated main drain and rinsed with water. The oxygenator and vascular tubing were then monitored for residual

radioactivity before being returned to the radioactive waste store.

5.2.3 Results

[1] Patients 1-3:

In all cases, the probe situated over the site of clinically detectable tumour recorded increasing levels of activity throughout the period of perfusion. In contrast, the thigh probe, after showing an initial rapid increase in activity during the first few minutes, then recorded a much higher but steadily diminishing activity throughout the remainder of the perfusion period. Fig. 28 shows the recordings obtained from one of the patients in this group.

[2] Patients 4-6:

The images obtained from each of these patients at the end of the isolated perfusion are shown in Fig. 29. In the case of patient 4, no significant concentration of radioactivity can be seen apart from the area of the calf muscles. In cases 5 and 6, accumulation of activity is clearly visible at sites corresponding to deposits of metastatic melanoma. Tumour:normal tissue uptake ratios at the end of the procedure as measured in the selected ROIs were 3.5:1 and 2.5:1 respectively for these two patients. The gamma emission counts measured in the tissue samples confirmed the absence of selective uptake

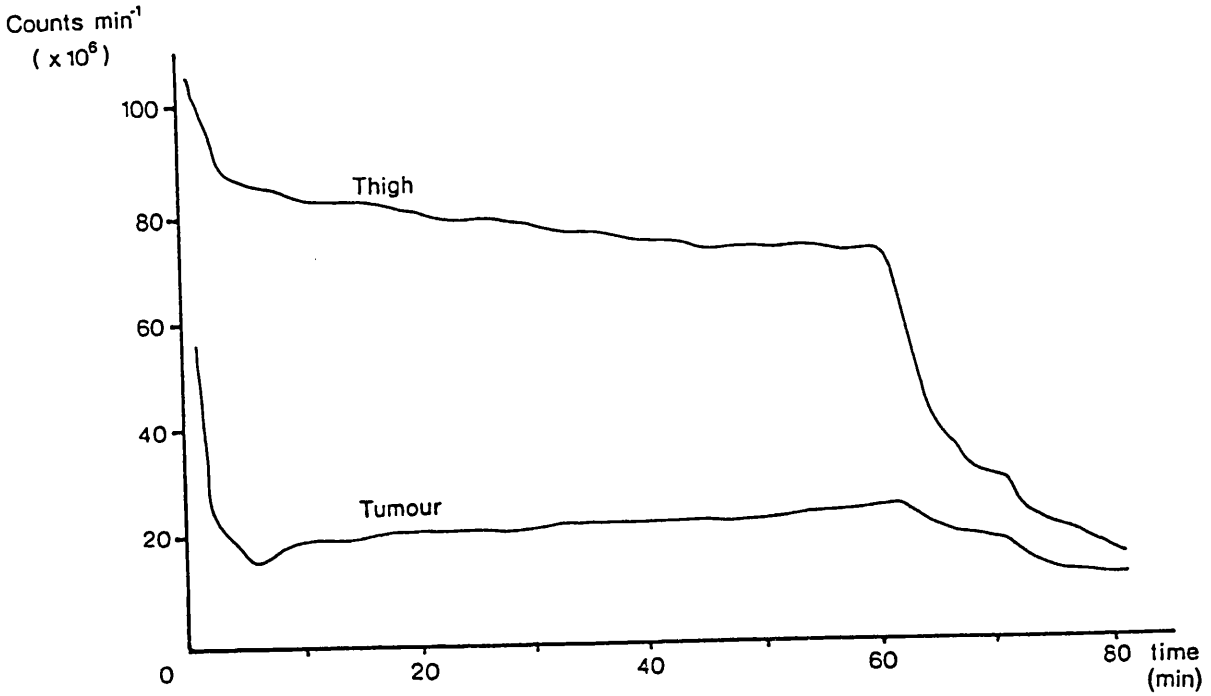
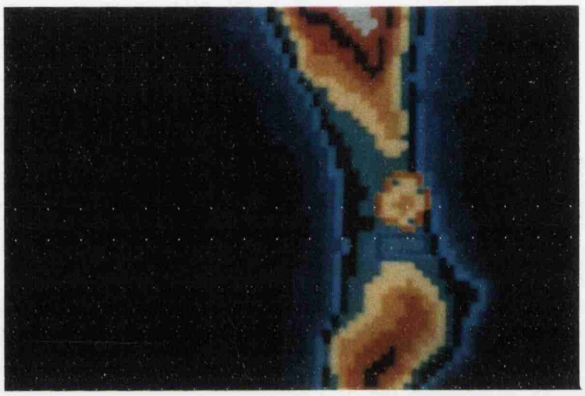


Figure 28 :
Recordings obtained from scintillation probes positioned over the thigh muscles and tumour deposits of patients undergoing iliac ILP with 111-indium-labelled anti-melanoma monoclonal antibody (XMMME-001).

4.

5.



6.

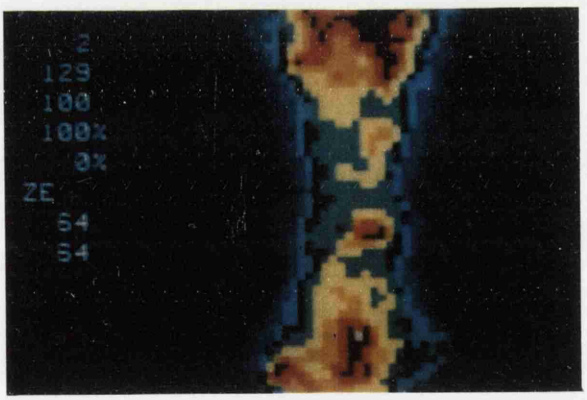


Figure 29 :
Gamma camera images obtained from 3 patients at the end of iliac ILP with 111-indium-labelled anti-melanoma monoclonal antibody (XMMME-001).

of activity by tumour in patient 4 and a tumour:normal tissue uptake ratio of approximately 3.1:1 in patient 5 (Table 32).

The activity measured in serial samples of perfusate taken during ILP is shown in Fig 30. This bears a similar profile to the curve of melphalan concentration found in perfusate during ILP, with apparent extraction of approximately one third of the activity over the perfusion period. Radiochromatography of the final sample of perfusate revealed that more than 95% of the radioactivity was still associated with a species having the same molecular weight as the original antibody. There was therefore no evidence of significant deconjugation of the radiolabel or aggregation of the antibody during the procedure. The leakage of radiolabelled antibody from the isolated limb to the systemic circulation during ILP was minimal, the activity in samples of systemic blood reaching only 2.7% of that in the perfusate at the end of ILP.

Post-operative monitoring of the extracorporeal apparatus showed no residual activity after rinsing, indicating no measurable adsorption of radioactivity onto the components of the circuit.

The radiation dose rate at the surgeon's operating position was 2uSv/hr and at the surface of the oxygenator reservoir, where the highest recording was obtained, reached only 25uSv/hr.

Table 32 :

Activity Measured in Skin, Fat and Tumour Samples Taken at the End of ILP with 111-In-labelled Anti-melanoma Monoclonal Antibody XMMME-001

Patient	Skin	Fat	Tumour
4	5.33 (0.025)	2.72 (0.013)	3.37 (0.016)
5	5.49 (0.026)	3.94 (0.018)	14.70 (0.070)

Values are expressed as counts x 1000 min^{-1} g^{-1}.

Numbers in brackets show the uptake of activity per gram of tissue expressed as a percentage of the total activity administered to the limb (total = activity added to the perfusate - activity recovered in the rinse).

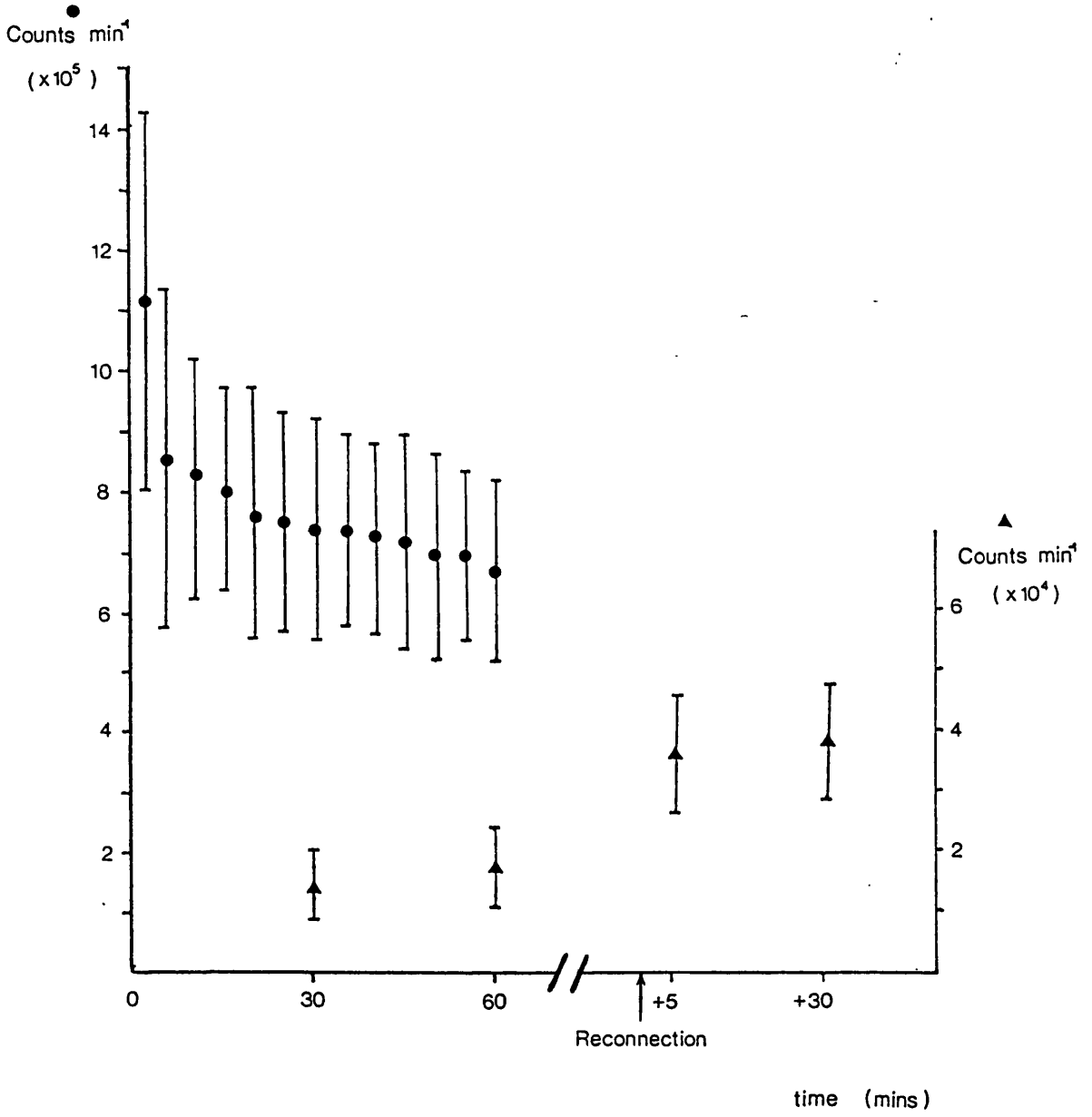


Figure 30 :
Curve of activity measured in serial samples of perfusate (●) and systemic blood (▲) taken during 6 iliac ILPs with 111-indium-labelled anti-melanoma monoclonal antibody XMMME-001.

Values shown are means with 95% confidence bars.

No detectable anti-mouse immune response was observed in the serum of any of these patients one month after ILP.

5.2.4 Discussion

The counts recorded by the thigh probe in the first three patients reflect the activity-time curve of the perfusate samples, indicating that this probe measured activity in the large blood pool of the thigh musculature. The recordings of the probe placed over the tumour sites differed substantially from this, suggesting that selective uptake of the radiolabelled monoclonal antibody by the tumour was taking place. For this reason, the protocol was altered for the next three patients to allow direct imaging of the tumour-bearing portion of the limb. In two cases, selective uptake of radioactivity at the tumour sites was confirmed. Late scans of these two patients revealed that the tumour:normal tissue ratios observed immediately after ILP were maintained for up to 72 hours. However they also revealed increased uptake of the radiolabel in the central organs (liver and spleen) after 24 hours.

The radiation dose received by attendant staff during the procedure was well within permissible limits. In the therapeutic situation, the use of alpha- or beta-emitters would result in an even lower external dose rate.

These observations confirm that monoclonal antibody targeting of melanoma deposits within a limb is possible, even within the limited time scale of ILP. The exclusion

of the central organs from exposure to the highest concentrations of antibody during ILP together with the ability to flush the limb vasculature before reconnecting it to the systemic circulation reduce the potential uptake of antibody by non-target organs, in particular by the liver. In this study, the highest activity measured in samples of systemic blood was less than 3% of the lowest activity measured in the perfusate during the isolated perfusion. The markedly increased hepatic and splenic uptake of radiolabel observed after 24 hours in patients 5 and 6 must have resulted from deconjugation of the radiolabel from the antibody, fragmentation of the antibody, or detachment of the antibody from its binding site on the target antigen. In the therapeutic situation, it may be possible to avoid serious toxic complications of this redistribution by using $F(ab')_2$ fragments of the antibody to minimise hepatic uptake or by using a radioisotope with a very short half-life (eg. ^{212}Bismuth: $t_{1/2}$ = 1 hour; ^{211}Astatine: $t_{1/2}$ = 7 hours), so that physical decay can occur before the isotope is released to the systemic circulation. If these conditions were met, the limits on the radiotherapy of melanoma deposits by this method would be imposed by the radiation tolerance of the limb rather than the sensitivity of the liver or bone marrow. If, as has been suggested, the highest dose of radiation tolerable by a limb is of the order of 30Gy [546], the tumour:normal tissue ratios recorded in patients 5 and 6 would have allowed the administration of as much as 90Gy (or its dose-rate corrected radiobiological equivalent[547]) to the tumour.

The therapeutic ratio achievable by ILP may be further improved by increasing the duration of ILP or by the use of two mutually avid components[542], such as streptavidin and biotin[548,549]. A monoclonal antibody-streptavidin complex could be administered systemically and, after allowing the optimum time for antibody localisation of the tumour deposits, this could be followed by ILP with radiolabelled biotin so that very rapid accumulation of isotope at the target site in the limb could be achieved. Successful and rapid detection of metastases of carcinoembryonic antigen-positive tumours has already been reported using this type of strategy in the systemic circulation[550]. The benefits of this approach could be further exploited by the use of an isotope with a very short half-life (eg. ^{212}Bi, ^{211}At) in order to allow the maximum cytotoxic effect to be achieved before redistribution of the radiolabelled antibody.

In one case (patient 4), no localisation of tumour by the radiolabelled monoclonal antibody was observed. This problem could result from the use of a single antibody if the melanoma did not express the target antigen. However, in this instance the patient's tumour had already been successfully imaged scintigraphically using the same radiolabelled antibody administered systemically[545]. Furthermore, when the same patient underwent a second ILP three months later (patient 6), the tumour deposits were successfully demonstrated. It is probable that the physiological characteristics of the first ILP resulted in inadequate perfusion of the tumour-bearing portion of

the limb so that the antibody was not sufficiently exposed to the antigen for significant uptake to occur (see section 5.3).

The absence of a detectable immune response to the murine antibody in these patients (including one patient exposed to the antibody on a total of three occasions) is of significant interest. Although 200ug of protein was injected in each case, the majority of this dose was recovered in the perfusate and rinsing fluid at the end of ILP. The systemic exposure of the patient to the foreign protein was therefore minimal. Repeated administration of murine antibodies may therefore be possible by ILP without the limitations imposed on their systemic use by the strong anti-mouse immune response which they usually elicit[532]. Radiation dose fractionation would then allow delivery of a greater total dose to the tumour, although the number of fractions would itself be limited to two or three by the technical difficulties associated with repeated surgical access to the regional vessels.

5.3 Clinical and Scintigraphic Evidence of the Value of $PtcO_2$

5.3.1 Introduction

In order to treat melanoma of the limbs by ILP, it is important that effective concentrations of cytotoxic agent reach the tissues most likely to harbour clinical and/or subclinical tumour deposits - ie. the skin and subcutaneous tissues. It is suggested in Chapter 3 that $PtcO_2$ monitoring is a simple and effective way of ensuring an adequate perfusion of these tissues during ILP. However, the value of this method can only be proven by demonstrating a clinical benefit to the patient, and this requires prolonged follow-up, especially for those patients with no clinical evidence of disease at the time of treatment. In the presence of visible or palpable tumour deposits, tumour response to the treatment may give an indication of the effectiveness of the perfusion, although other factors such as temperature, dose, and sensitivity of the individual patient's tumour to the cytotoxic agent may all contribute to the eventual outcome.

One patient has now undergone ILP four times in Glasgow, twice by the external iliac approach. An identical procedure was used on both occasions with regard to the surgical technique, the dose of melphalan and the preparation of the priming fluid. Furthermore, on both of these occasions, ^{111}In-labelled anti-HMW-MAA antibody was added to the perfusate as part of the study described in

Section 5.2. The results of these two ILPs were very different and offer some indication of the value of $PtcO_2$ monitoring during ILP.

5.3.2 Patient and methods

The patient was a woman of 64 years who had presented ten years previously with a nodular melanoma (Breslow thickness 2.40mm) of the right lower leg. This had been treated by wide excision of the primary lesion with a 5cm lateral clearance and application of a split thickness skin graft. Nine years later, the tumour recurred locally in the form of a nodular tumour deposit within the area of grafted skin. Excision of this nodule was followed three months later by the appearance of several other similar nodules. There was no clinical evidence of central spread of the disease. Scintigraphy using ^{111}In-labelled anti-HMW-MAA murine monoclonal antibody (XMMME-001) highlighted an area of uptake of activity in the region of the tumour deposits. In addition, there appeared to be increased uptake of radioactivity in the left shoulder and mediastinum, although these were not confirmed by CAT scanning[545]. The tumour showed no response to systemic combination chemotherapy (BELD).

First ILP

ILP was performed using the external iliac approach according to the standard method described in Section 2.1.2. Satisfactory isolation was demonstrated using fluorescein before adding 100mg of melphalan (equivalent to 1.75mg kg^{-1}) to the perfusate. A bolus of 200ug of ^{111}In-labelled monoclonal antibody XMMME-001 was simultaneously injected into the arterial cannula and the mixture was then allowed to circulate for 1 hour during which the flow rate was regulated to maintain a stable distribution of perfusate within the compartments of the isolated circuit. The limb was then rinsed with 2 litres of balanced electrolyte solution and the isolation procedure was reversed. Throughout the period of isolated perfusion, the tumour-bearing limb was monitored using a mobile gamma camera as described in Section 5.2 (above).

Second ILP

A similar surgical technique was employed for this ILP as for the first. After confirmation of satisfactory isolation, melphalan (100mg) and ^{111}In-labelled antibody (200ug) were added to the circuit. On this occasion, the perfusion parameters were controlled to ensure effective perfusion of the skin, as demonstrated by an increase in the $PtcO_2$ level from the ischaemic values observed during the period of isolation and cannulation (see Section 3.4.2). Otherwise, the protocol was identical to that of the patient's first ILP.

5.3.3 Results

Figure 31 shows the appearance of the patient's leg at the time of her first ILP. A cluster of 4 melanoma nodules is visible in the upper half of the grafted area. A fifth nodule lies at the lower border of the skin graft. A palpable subcutaneous deposit lying 2cm posterior to this is not visible from the angle of this photograph.

Figure 32 shows the blood flow rate, perfusion pressure and $PtcO_2$ recorded during both procedures. In the first ILP, low flow rates (mean 208ml min^{-1}) resulted in a mean perfusion pressure of only 22.4mmHg. This in turn led to the failure of the $PtcO_2$ to rise from the ischaemic values observed after application of the vascular clamps and tourniquet. In contrast, during the second ILP, much higher flow rates (mean 382.6ml min^{-1}) were employed to generate a mean perfusion pressure of 39.8mmHg. This resulted in effective perfusion of the skin, as evidenced by the rise in the $PtcO_2$.

The gamma camera images obtained at the end of both perfusions are shown in Figure 33 alongside photographs of the patient's leg taken one month after each operation. After the first ILP, no focus of increased radioactivity could be seen apart from the highly vascular muscle group of the calf. The clinical photogaph shows no change in the appearance of the melanoma deposits on the leg one month later. After the second ILP, increased activity was clearly visible in two distinct areas, corresponding to the cluster of nodules in the upper portion of the graft and the two nodules at the lower posterior edge of the

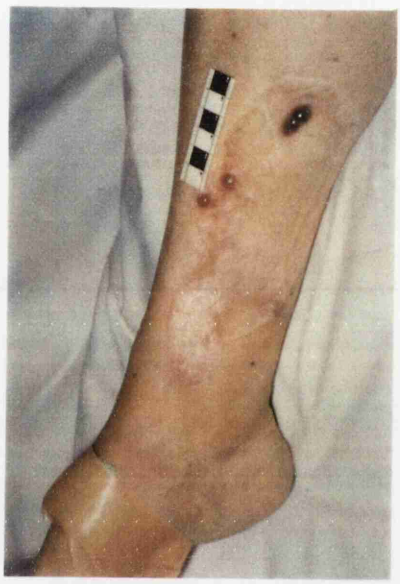


Figure 31 :
Photograph of patient's leg before the first of two isolated perfusions with melphalan and radiolabelled anti-melanoma monoclonal antibody.

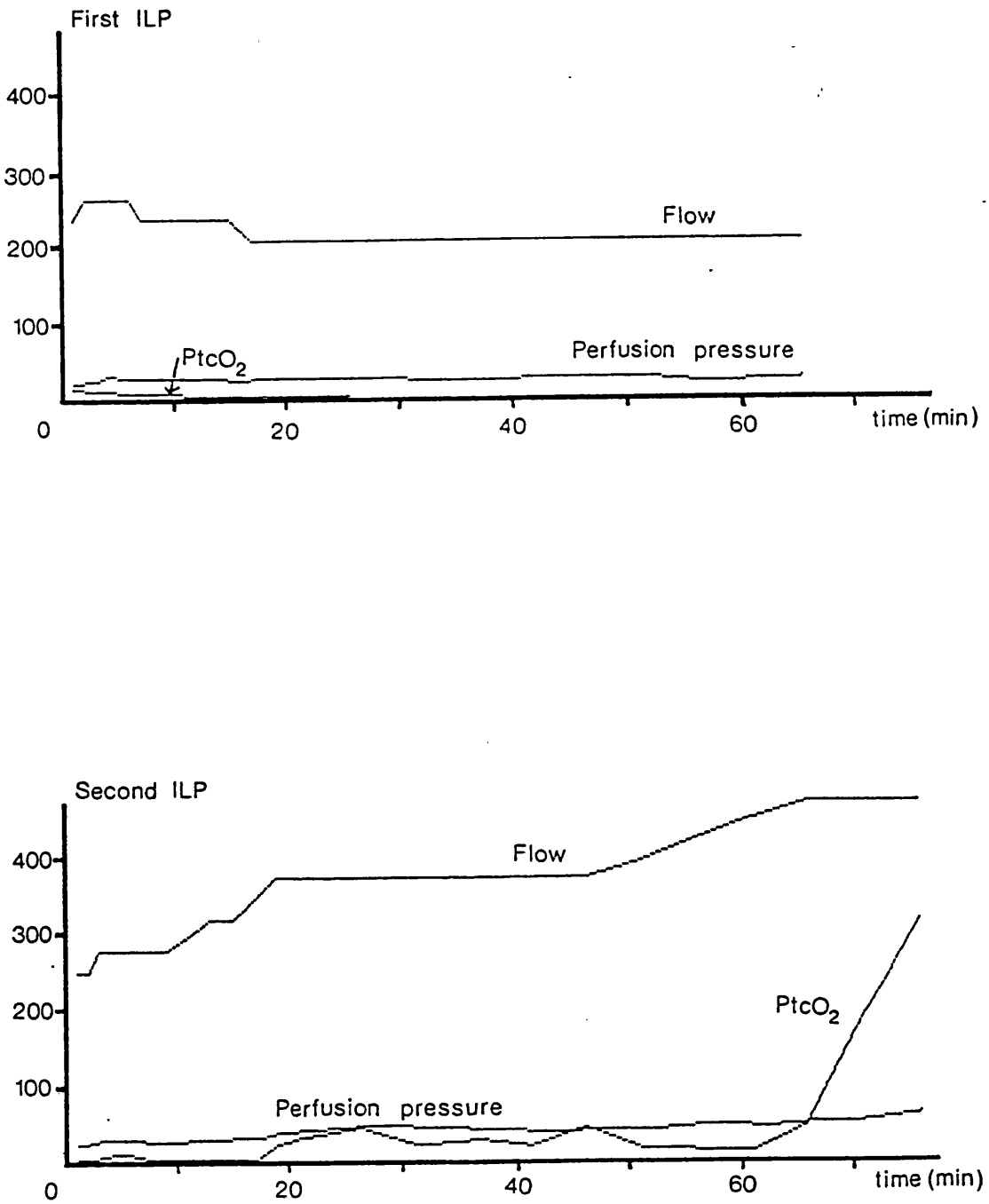
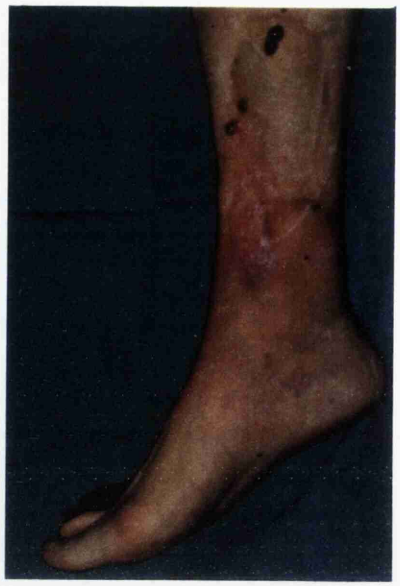
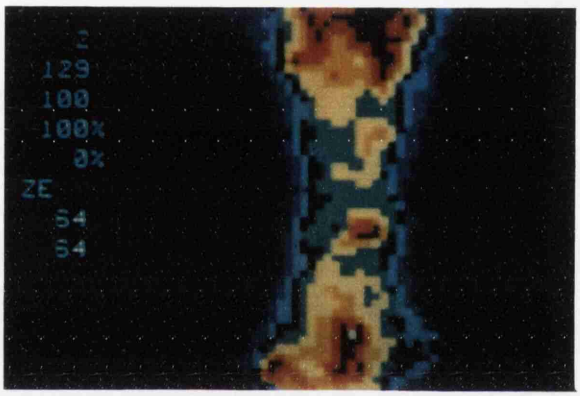


Figure 32 :
Graphs showing the variations in perfusion pressure and $PtcO_2$ in the same patient during two isolated perfusions with melphalan and radiolabelled anti-melanoma monoclonal antibody.

First ILP



Second ILP



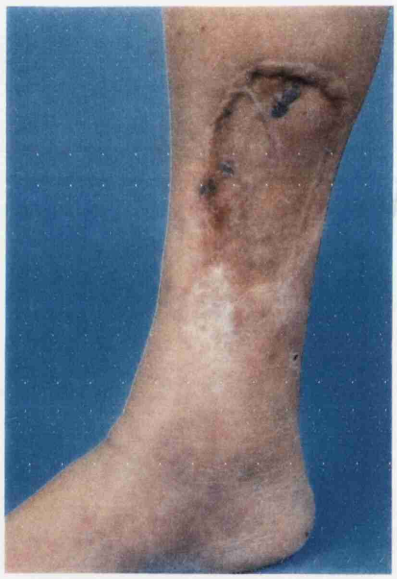


Figure 33 :
Gamma camera images obtained from the same patient at the end of two isolated perfusions with melphalan and radiolabelled anti-melanoma monoclonal antibody. Each image is accompanied by a photograph of the patient's leg taken one month later.

At the end of the first ILP, there is no evidence of uptake of activity at the tumour sites and the tumour shows no response to melphalan one month later. After the second ILP, areas of increased uptake of activity can be seen corresponding to the sites of tumour deposits. In the photograph taken one month later, the tumour deposits have become smaller, flat and dull in response to the treatment.

graft. One month later, all the visible tumour deposits had become flattened and dull and were starting to ulcerate.

5.3.4 Discussion

By acting as her own control, this lady offers a unique opportunity to assess the potential clinical value of modifying the perfusion technique in the manner suggested in Section 3.4.2. The results above provide evidence that the absence of a rise in the $PtcO_2$ is indicative of a **failure to perfuse the skin and subcutaneous tissues adequately.** In this patient's first ILP, this resulted in failure of the monoclonal antibody to locate its target antigen on the cutaneous and subcutaneous melanoma deposits. This was associated with **no detectable response of the tumour** to melphalan. In the second ILP, **effective skin perfusion,** indicated by the higher $PtcO_2$ levels, was confirmed by the successful detection of the melanoma deposits by the antibody and produced an obvious **clinical response of the tumour** to melphalan.

One of the fundamental requirements of any drug treatment is that the drug should be able to reach its desired site of action in effective concentrations. Successful treatment of malignant melanoma by ILP is therefore dependent on effective perfusion of the skin and subcutaneous tissues (including lymphatics) with melphalan-rich perfusate. $PtcO_2$ monitoring offers a

simple and non-invasive method of ensuring that this aim is achieved.

5.4 Animal Experiments of Melanoma Targeting with Monoclonal

5.4.1 Introduction

While the clinical study described in Section 5.2 indicates that monoclonal antibody targeting of melanoma deposits on a limb by Isolated Limb Perfusion is possible, further studies will be required to establish its true therapeutic potential. The development of a suitable animal model of ILP would therefore be a valuable addition to the existing clinical application. Most of the early experimental studies, which concentrated on the mechanical aspects of ILP and the normal tissue tolerance to high dose chemotherapy and to vascular isolation, were performed on dogs[328,329,551-553]. However the lack of naturally occurring or transplantable tumours in dogs make it an unsuitable model for studying the response of tumour to treatment. Subsequent reports have described perfusion of the rat hind limb bearing transplanted Walker 256 carcinosarcomas[554,555].

In order to study the uptake of monoclonal antibody XMMME-001 by human melanoma, experimental studies were performed using athymic nude rats bearing human melanoma xenografts. These studies were performed with the approval of the Home Office under the Animals (Scientific Procedures) Act 1986 (Project Licence PPL 60/00905).

5.4.2 Materials and methods

Barrier-reared 4-8 week old PVG-rnu athymic nude rats (Harlan Olac Ltd), housed in isolation from other animals and maintained on irradiated diet and sterile water, were used in all experiments. The animals were caged in groups of three on autoclaved bedding. One week after delivery from the suppliers, all animals were innoculated with 0.5-1.0 x 10^6 human melanoma cells (B0008) by subcutaneous injection on one hindlimb. Tumours were then allowed to grow at the site of injection until they reached a diameter of 5-7mm. The rats were then treated in three groups, divided according to the route of administration of ^{111}In-labelled monoclonal antibody XMMME-001 : Group A received an intravenous injection into a lateral tail vein; Group B was treated by intraarterial injection into the femoral artery of the tumour-bearimg hind limb; Group C underwent isolated perfusion of the tumour-bearing limb at the common femoral level.

Preparation and injection of B0008 melanoma cells

B0008 human melanoma cells were obtained by disaggregation of confluent monolayer cultures using the method described in Section 3.6.2. The disaggregated cells were then suspended in normal saline at densities of 2.5-5.0 x 10^6 cells ml^{-1}. Each rat received a subcutaneous injection of 0.2ml of the B0008 cell suspension on the lower thigh of the left hind limb.

Preparation of monoclonal antibody

Monoclonal antibody XMMME-001 was prepared using the ^{111}In-labelling technique described in Section 5.2.2. The resultant solution (containing 200ug of protein and 100-120MBq in 1.6ml) was divided into 0.2ml aliquots for administration to each rat.

Techniques of antibody administration

The rats were anaesthetised by intraperitoneal injection of a mixture of fluanisone 10mg ml^{-1}/fentanyl citrate 0.315mg ml^{-1} ("Hypnorm", Janssen) and midazolam 2mg ml^{-1} ("Hypnovel", Roche) (1 part Hypnorm, 1 part Hypnovel, 2 parts water for injection) in a dose of 2.0ml kg^{-1}.

Group A : 0.2ml of the labelled antibody preparation were injected into one lateral tail vein using a 27G stainless steel needle.

Group B : through a 1.0-1.5cm groin crease incision, the femoral vessels of the tumour-bearing hind limb were exposed using microsurgical techniques. 0.2ml of the labelled antibody preparation were then injected slowly (ie. over 5-10 seconds) into the common femoral artery using a 27G or 30G needle. After removing the needle from the artery, the skin was then sutured using 5/0 plain catgut.

Group C : through a 2.0-2.5cm groin crease incision, the common femoral vessels of the tumour-bearing hind limb were exposed using microsurgical techniques. These were

then ligated proximally, and the ligatures were used to exert gentle traction on the vessels. A microarteriotomy and microvenotomy were then created so that a 2F gauge teflon cannula could be inserted into each vessel. The cannulae were introduced for a distance of 4-5mm and secured to the vessels with nylon ligatures above and below the point of insertion. A 3mm rubber sling was then passed deep to the vessels proximal to the cannula insertion sites and brought out through separate stabwounds at either end of the main incision. The sling was passed round the root of the limb and secured in position as a tourniquet. The wound was then sutured using 5/0 plain catgut sutures and the cannulae were further secured by suturing them to the wound edges and to the anterior abdominal wall with nylon sutures. The labelled antibody solution (1.6ml containing 200ug of protein) was then added to a syringe containing warm normal saline ($T=32^{o}C$) to a final volume of 30ml. The syringe was placed in a constant rate adjustable infusion pump (MS 2000, Graseby Medical) and connected to the arterial cannula. The venous cannula was connected to an emptied IV fluid administration bag which was laid beside the anaesthetised rat. An arterial flow rate of 0.5ml min^{-1} was employed. Venous effluent was allowed to drain by gravity into the empty bag.

Imaging of rats

Gamma camera images of the rats in groups A and B were obtained at hourly intervals after injection of the antibody for the first four hours and 24 hours after injection. Rats in group C were imaged continuously during the antibody infusion.

Strict aseptic technique was observed when performing all the procedures described above.

Any rat showing evidence of deviation from normal health other than the growth of a single tumour at the site of injection was euthanised. All other rats were euthanised within 24 hours of the experiments (groups A and B) or at the end of the perfusion (group C).

5.4.3 Results

A total of 60 rats were innoculated with human melanoma cells. In 46 cases, visible tumour growth at the site of injection was recorded. Tumours first became visible (2-3mm) between 3-4 weeks after innoculation and reached a diameter of 5-7mm after 8 weeks. 33 rats with tumours survived long enough to be included in one of the three experimental groups (group A : 12; group B : 15; group C : 6). 27 rats died or were euthanised before this. Post-mortem examination of these animals revealed no macroscopic evidence of tumour growth in 14 cases and solitary tumours on the left hind limb in 13 cases. There

was no evidence of metastatic tumour deposits in the liver or lungs of any of these animals. The rats included in the experiments all weighed between 185g and 285g.

Group A : Lateral tail vein injection was attempted in 12 cases. In all cases, extravasation of the antibody solution at the injection site occurred. 3 rats died under anaesthesia within 5 minutes of the intravenous injection and were therefore not scanned. Successful localisation of the tumour was detected in 5 of the remaining 9 rats. In all cases, this was evident on the first scan (taken at 1 hour in 4 cases and at 2 hours in 1 case). Increasing uptake was apparent with successive scans. The brightest spot on all scans was the injection site on the tail of the animal. Accumulation of isotope was also recorded in the liver and heart in all scans. With increasing time after injection of the labelled antibody, distribution of the isotope became more generalised, although the localised tumours remained evident. An example of the scans taken from one rat in this group is shown in Figure 34. Only three rats survived more than four hours after the injection and were scanned at 24 hours. None of these showed evidence of isotope uptake at the tumour site at any time. Of the other six rats, four died before recovering from the first anaesthetic and only two required a further injection of the anaesthetic mixture to immobilize them during the scan at 4 hours.

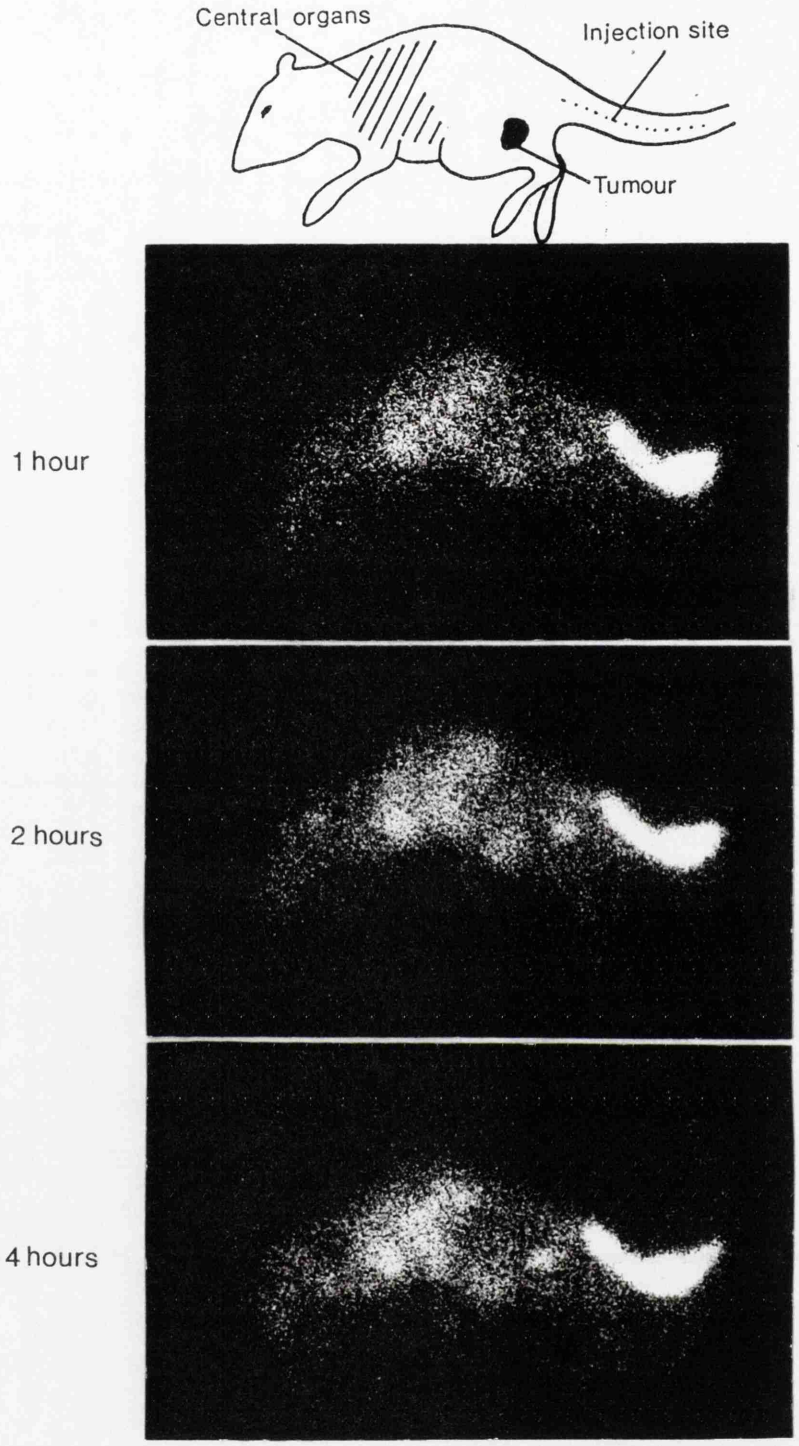


Figure 34 :
Gamma camera immages taken (a) 1 hour, (b) 2 hours and (c) 4 hours after injection of 0.2ml of 111-indium-labelled monoclonal antibody XMMME-001 into the lateral tail vein of a rat bearing a subcutaneous melanoma (B0008) xenograft on the left hind limb.

An area of increasing uptake of activity is evident at the site of the tumour on successive scans.

Group B : Intraarterial injection was attempted in 15 cases and was successfully achieved in 7 cases. The failures were due to traumatic division of the artery by the cutting edge of the needle in 3 cases, avulsion of the artery in 1 case, and intramural injection and/or extravasation of the majority of the injected volume in 4 cases. After experiencing three traumatic divisions of the femoral artery using 27G needles, the author used 30G needles for all subsequent injections. Three of the rats successfully injected died within 5 minutes of the injection and were not scanned. The other four rats were scanned hourly for three hours without showing any evidence of uptake of isotope at the tumour site. Only one rat survived beyond this time. A scan taken after 24 hours failed to reveal any accumulation of isotope at the tumour site. Three of the rats displayed a "bright spot" over the upper thigh but alteration of the position of the rat from the lateral decubitus position to the supine position confirmed that this was due to extravasated antibody solution at the injection site. All four rats had evidence of ischaemia of the left hind limb (cyanosis, cool temperature, loss of function).

Group C : Cannulation of the femoral vessels with 2F gauge cannulae was attempted in 6 rats. This proved to be technically extremely difficult. The femoral vein was successfuly cannulated in 5 cases but both vessels could be cannulated in only 2 rats. In these two cases, perfusion of the limb with the antibody solution was

initiated but had to be aborted within 10 minutes due to failure of the venous drainage, extravasation of the solution, and visible swelling of the limb in both cases.

The rats whose intraarterial injections failed or in whom cannulation of the femoral vessels was unsuccessful were all euthanised.

5.4.4 Discussion

Immunodeficient animals, such as athymic nude rats and mice, provide useful models for the study of human malignancies due to their ability to support the growth of human tumours without mounting a cell-mediated immune response to the foreign tissue. It is generally held that the biological characteristics of these human tumour xenografts largely reflect those of the parent tumour. In this study it was posssible to initiate xenografts of the human melanoma cell line B0008 in 77% of innoculated animals. Autopsies performed on those rats which died or were euthanised prematurely showed no evidence of metastatic disease. The loss of metastasising potential is a recognised feature of many human tumour xenografts including melanomas[556]. One particular advantage of this property is the opportunity to study "in vivo" the response of a single tumour to treatment in an otherwise healthy host.

The successful localisation of tumour by the labelled antibody in 5 of the 9 rats in group A suggests that the B0008 cell line may prove a useful model for exploring the potential of antibody-targeted therapy of human melanoma in vivo and in vitro.

Previous descriptions of the isolated perfusion of the rat hind limb have referred to normally immunocompetent rats. Nude rats and mice grow to a much smaller size than their normal counterparts and this is reflected in the low body weight of the animals in this study (mean : 223g; range : 185-285g). The technical difficulties encountered in this study with injection and cannulation of the vessels could all be attributed to the small size of the animals. Whereas previous authors have been able to cannulate the femoral vessels of normal adult rats (Sprague Dawley) weighing 400-550g using 3F or 4F cannulae[555], the smaller 2F cannulae used in this study proved too large for the vessels of the nude rat. Although the femoral vein was cannulated successfully in 5/6 cases, the vessel wall was visibly stretched over the cannula in 4 of these, and it is not surprising that the smaller, thicker-walled corresponding artery could not accommodate a cannula of this calibre. Similarly, the trauma inflicted on the arterial wall by the 27G and even 30G needles used in group B demonstrates the difficulty of using such a small animal model. The finding that all rats given an intraarterial injection then developed an ischaemic limb is in marked contrast to the experience of other authors who found that ligation of the femoral vessels of adult Sprague Dawley rats was completely

innocuous[555]. The significantly worse outcome in this experiment may have resulted from a more extensive propagation of thrombus from the arterial injection site abolishing inflow of blood to the limb via collateral channels, although a direct effect of the antibody solution on the vessels of the limb is also possible. The ischaemia observed in these limbs is the probable explanation of the failure of the labelled antibody to accumulate significantly at the site of the tumour.

Although they were housed in isolators and maintained on sterilized food, water and bedding, and despite the use of strict aseptic technique for all procedures, nearly half of all the rats innoculated in this study (27/60) died prematurely or were euthanised when they showed signs of illness (weight loss, anorexia, inactivity). Furthermore, 6 of the 19 rats successfully injected in groups A and B died within a few minutes of the onset of anaesthesia and another 10 only survived a few hours longer. The susceptibility of athymic animals to bacterial, viral and fungal infections is a major problem in experimental work[557], especially when, as in this study, the animals must be maintained for long periods to allow tumours to grow to a suitable size. In addition, their smaller than normal size and their relative absence of hair renders them more sensitive to heat loss than normally coated, larger rats.

In order to overcome the technical difficulties encountered in this study, a larger animal would be required. Normally developed, non-mutant but surgically thymectomised rats or rabbits may prove to be a more suitable model for ILP of human tumour-bearing limbs.

5.4.5 Conclusions

The human melanoma cell line B0008 can be used to establish xenografts successfully in athymic nude rats. Anti-melanoma monoclonal antibody XMMME-001 administered by intravenous injection localised the B0008 xenograft in more than 50% of cases in this study. The B0008 cell line therefore appears to be an effective model for in vivo and in vitro studies of the uptake of antibody by melanoma.

The athymic nude mutant rat, being much smaller than its normal counterpart, presents very severe technical difficulties which make it unsuitable as an animal model of ILP. In order to study the effects of ILP on experimental human tumours, a larger immunosuppressed (surgically thymectomised) animal may prove to be a better model.

PART C

CONCLUSIONS

Isolated Limb Perfusion has now become a well-established form of therapy for malignant melanoma in Glasgow. The results presented in Chapter II indicate that the technique is safe and is associated with similar objective response rates to those observed in the largest centres in the world. In the treatment of Stage I melanoma of the limbs, a non-randomized case-control study shows an apparent benefit from Adjuvant ILP in Glasgow of 20% (survival) and 15% (disease-free survival) at three years' follow-up.

The studies described in Part B were performed in an attempt to standardise some of the variable aspects of the ILP technique and to investigate a potential method of increasing the specificity of this treatment using monoclonal antibodies as targeting vectors. The following conclusions can be drawn from these studies :

Chapter 3 :

- current ILP methods do not reliably ensure effective perfusion of the skin with cytotoxic drug. This can be achieved by adjusting the perfusion parameters to generate perfusion pressures in excess of a critical value in each case. The effectiveness of this method can be confirmed simply and non-invasively by peroperative transcutaneous oxygen tension ($PtcO_2$) monitoring.

- higher skin melphalan concentrations than previously measured have been achieved using this method without a concomitant increase in regional toxicity or leakage of melphalan to the systemic circulation.
- the vasodilators verapamil, hydralazine and papaverine have no adverse effect on the cytotoxicity of melphalan to melanoma cells "in vitro"; papaverine may exert a potentiating effect on melphalan cytotoxicity. All three drugs could therefore be added to the ILP circuit in order to lower the critical perfusion pressure required for effective cutaneous perfusion. This should now be investigated in a clinical study.

Chapter 4 :

- the melphalan dosage schedules based on body weight and limb volume prescribe very similar doses of melphalan for individual patients and are associated with similar regional toxicity.
- the dosage schedule based on total blood volume estimation consistently prescribes lower doses than both of these schedules.
- the method of total limb volume measurement by immersion is awkward but can be simplified by the application of a formula for its calculation based on a partial measurement.
- the dosage schedule based on body weight remains the most appropriate method at present by virtue of its simplicity and the high doses which it prescribes.

Chapter 5 :

- targeting of melanoma by monoclonal antibodies is possible within the ILP protocol. Tumour : normal tissue uptake ratios of up to 3.5:1 (sufficient to allow the administration of effective radiation doses) can be achieved by this method.
- an "in vivo" experimental model of human melanoma has been established by producing human melanoma xenografts in athymic nude mutant rats.
- the human melanoma B0008 cell line is recognised in xenografts by monoclonal antibody XMMME-001 to the high molecular weight melanoma-associated antigen.
- attempts to develop an "in vivo" model of ILP in nude rats have been limited by the small size of this animal compared to its non-mutant counterparts.

A standard method of controlling the ILP circuit can therefore be recommended which should maximise the exposure of the tissues to melphalan by ensuring effective cutaneous pefusion with high concentrations of the drug. The value of this method is illustrated by the improved clinical response achieved in one patient during a second ILP (Section 5.3).

Further studies into ways of limiting the non-target tissue uptake of anti-melanoma monoclonal antibody are suggested so that the true therapeutic potential of this approach can be evaluated.

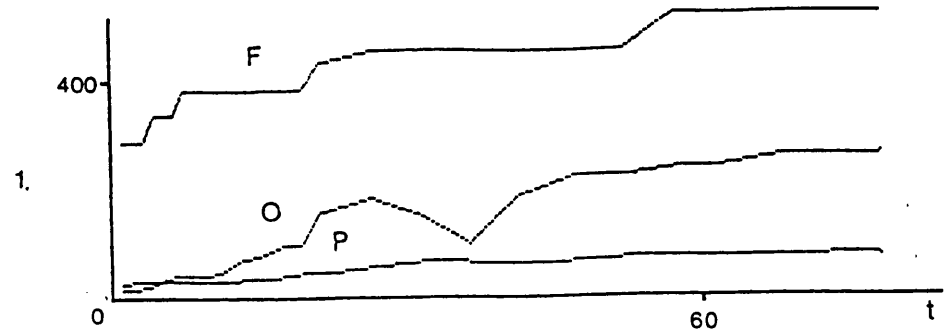
APPENDIX A

Blood pressure (P), blood flow (F), and transcutaneous oxygen tension (O) recordings from 15 patients undergoing iliac ILP (Section 3.3.3).

Units :

blood pressure and transcutaneous oxygen tension - mmHg

blood flow - ml min^{-1}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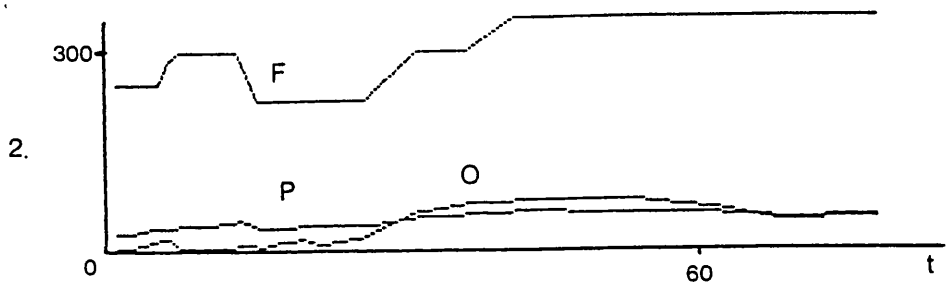
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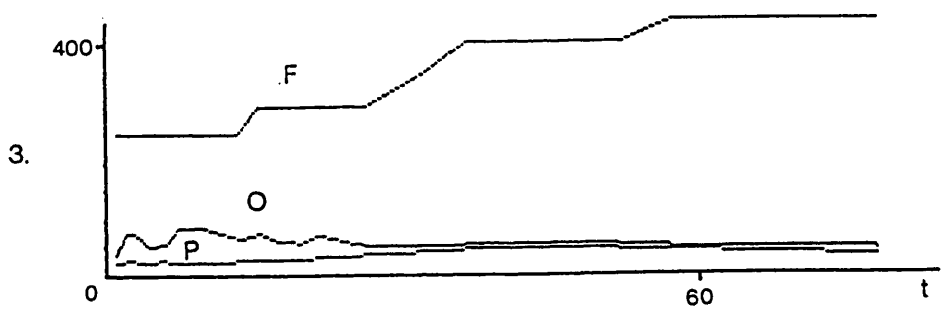
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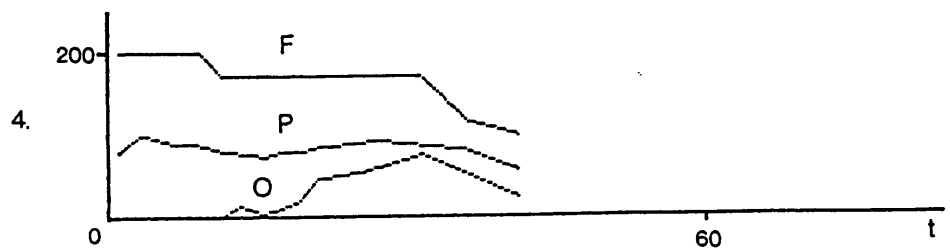
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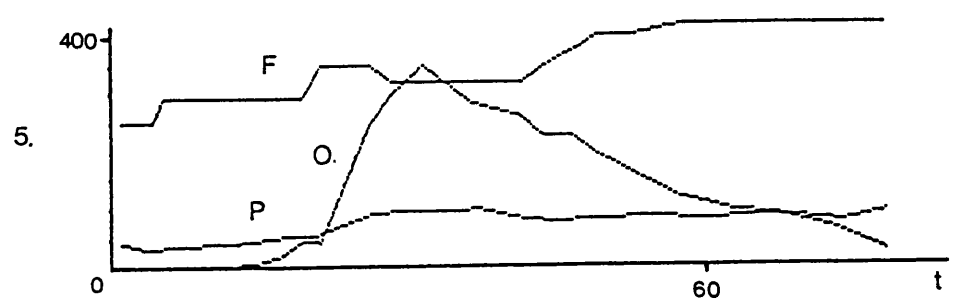
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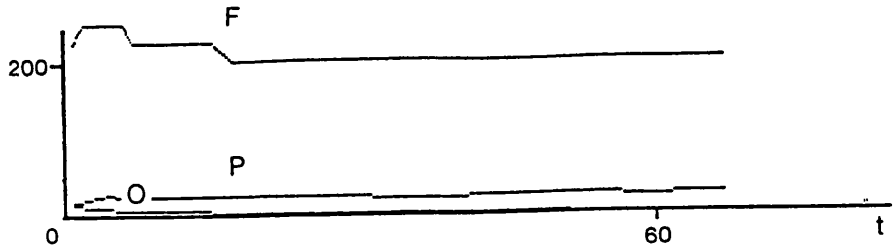
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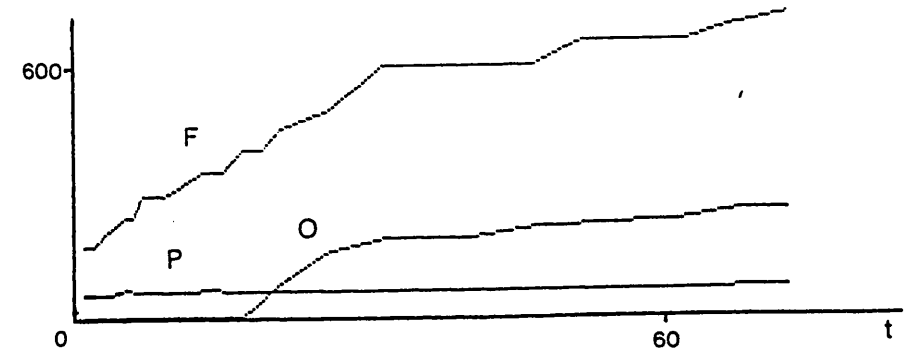
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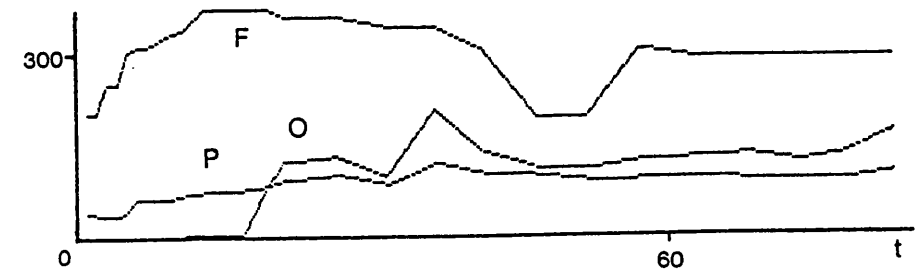
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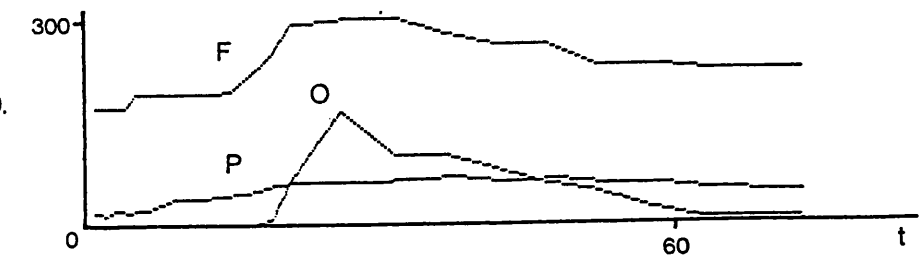
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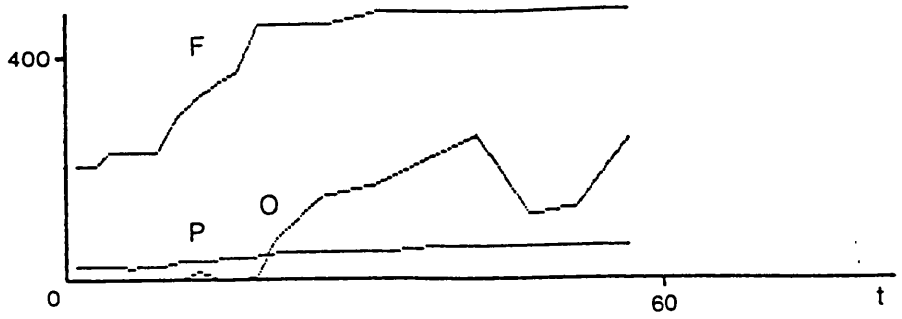
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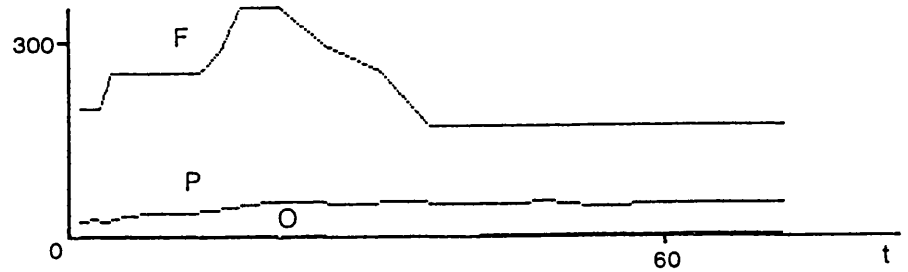
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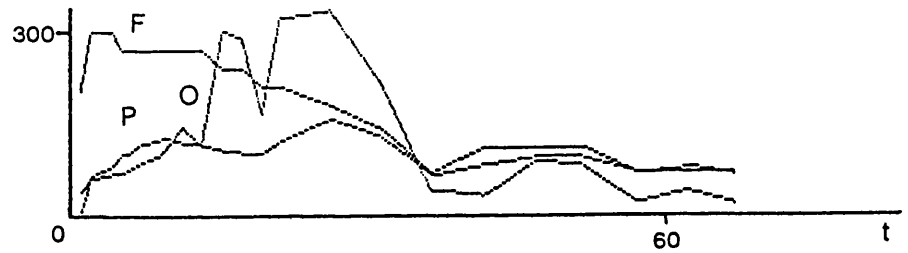
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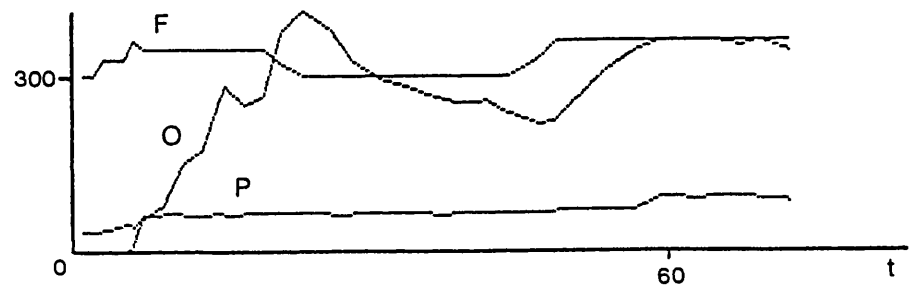
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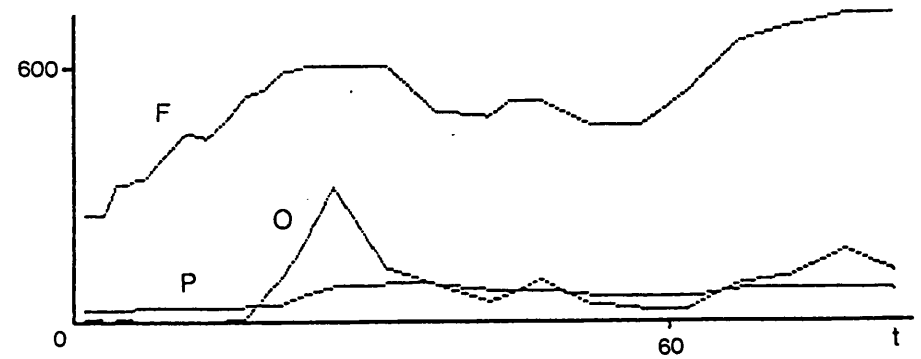
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APPENDIX B

Melphalan Pharmacokinetics

Patient:	003	005	006	007	008	009	010
Study:							
Chapter 3	*	*		*	*	*	*
Chapter 4	*	*	*	*	*	*	*
Sample(mins)							
1	1.11	8.87	2.28	.18	47.94	.00	38.00
5	36.14	65.69	36.90	25.30	67.52	68.51	40.31
10	19.72	44.55	28.06	30.05	47.73	49.10	28.34
15	16.96	37.38	23.74	22.16	36.53	41.74	23.07
20	17.77	29.52	19.36	22.05	26.63	31.53	20.08
25	19.46	20.54	17.57	17.87	25.02	32.28	16.30
30	15.17	16.33	14.04	13.08	19.29	25.26	13.55
35	10.33	17.16	11.54	12.95	18.53	20.53	13.07
40	8.07	10.09	12.61	11.18	16.14	17.99	11.93
45	-	19.52	11.30	9.49	13.17	16.39	11.75
50	7.21	11.30	10.74	7.26	10.42	13.52	10.28
55	6.75	10.42	8.81	9.40	11.62	13.62	8.93
60	6.32	9.97	7.33	10.38	12.16	15.15	8.64
EP	-	-	1.95	-	-	-	.94
S-0	.00	.00	.00	.00	.00	.00	.00
S-30	.00	.00	.00	.00	.00	.42	.12
S-60	.00	.30	.00	.00	.18	.00	.10
S-90	2.87	.19	.00	.32	.52	.47	.64
$[MEL]_{eq}$	33.65	38.16	28.77	28.21	41.13	49.91	26.79
$t_{1/2}$	23.19	29.78	31.92	32.33	29.66	29.72	35.62

Patient:	011	012	013	014	016	019	020
Study:							
Chapter 3	*	*	*	*	*	*	*
Chapter 4	*	*	*	*	*	*	*
Sample(mins)							
1	56.27	2.68	89.81	7.38	3.48	59.71	85.69
5	41.10	53.63	95.27	57.13	106.46	96.25	112.80
10	29.86	54.62	75.19	42.31	40.64	59.61	66.80
15	29.00	37.79	61.69	35.25	27.88	40.26	41.80
20	20.85	29.56	51.49	27.70	25.10	38.24	33.67
25	20.54	24.71	40.15	23.11	19.66	38.70	30.68
30	14.94	22.14	32.83	19.20	16.49	23.67	33.90
35	12.92	19.02	30.76	16.81	15.00	22.86	20.80
40	13.07	17.49	25.39	15.69	12.87	20.12	18.60
45	-	14.68	24.40	13.34	11.77	16.68	21.39
50	-	13.32	21.20	11.69	10.45	16.90	17.85
55	-	13.79	21.53	11.71	10.44	15.50	16.80
60	9.25	13.19	19.44	11.10	9.26	12.80	11.70
EP	1.75	1.38	2.91	2.82	-	2.20	1.28
S-0	.00	.00	.00	.00	.00	.00	.00
S-30	.50	1.84	1.01	.00	.00	.26	.00
S-60	.60	1.23	.48	.00	.23	.02	.00
S-90	.40	1.00	.66	.37	1.57	.76	.56
$[MEL]_{eq}$	30.43	41.27	70.60	40.06	35.41	62.81	56.85
$t_{1/2}$	33.26	33.26	30.25	30.04	29.54	25.64	28.59

Melphalan Pharmacokinetics(cont)

Patient:	021	022	023	024	025	026	027
Study:							
Chapter 3	*	*	*	*	*	*	*
Chapter 4	*	*	*	*	*	*	*
Sample(mins)							
1	26.98	129.00	-	316.10	195.68	60.59	95.15
5	69.23	114.00	85.11	130.50	97.62	43.24	101.80
10	66.69	56.70	97.00	71.50	58.01	26.83	101.62
15	56.71	73.20	61.06	49.10	41.10	24.12	32.08
20	45.62	67.90	50.21	34.80	29.43	23.76	31.50
25	37.71	63.80	33.20	25.30	26.08	19.49	29.90
30	24.06	42.70	38.00	26.89	21.20	14.76	23.67
35	19.30	53.90	26.00	23.00	21.76	13.90	-
40	15.56	24.20	24.90	19.80	18.21	11.06	21.62
45	14.08	25.70	27.40	18.50	16.17	12.00	15.23
50	12.90	30.70	23.40	19.60	15.61	10.89	-
55	11.80	25.50	-	16.40	12.88	10.55	-
60	9.03	24.80	-	15.40	11.54	9.35	-
EP	.39	4.11	5.16	5.10	1.97	1.35	3.21
S-0	.00	.00	.00	.00	.00	.00	.00
S-30	.00	.00	.21	.20	.79	.00	.00
S-60	.00	.00	.16	.16	.00	.00	.08
S-90	3.92	.82	2.83	.56	.77	.12	1.16
$[MEL]_{eq}$	85.51	109.19	66.79	44.27	45.38	31.05	55.66
$t_{1/2}$	18.04	25.42	31.32	38.45	30.72	32.86	25.86

Patient:	028	029	030	031	032	033	034
Study:							
Chapter 3	*	*	*	*	*	*	*
Chapter 4	*	*	*	*	*	*	*
Sample(mins)							
1	83.00	11.59	375.75	99.38	71.98	280.76	121.76
5	48.43	52.48	77.89	50.62	81.49	157.21	61.62
10	38.78	52.24	71.14	37.22	58.56	99.98	46.38
15	33.02	43.46	61.46	28.85	48.80	67.97	37.32
20	22.94	31.16	51.07	24.25	37.89	59.91	36.29
25	-	27.05	55.95	20.95	35.08	49.50	24.94
30	19.30	34.15	35.06	30.51	29.11	45.20	-
35	17.45	30.90	29.92	25.11	18.05	42.11	19.55
40	16.45	18.39	26.90	16.07	14.98	41.08	17.62
45	15.65	15.82	23.40	15.02	14.00	32.05	16.94
50	-	11.59	25.90	14.27	13.03	14.72	15.06
55	-	-	22.59	13.35	12.07	16.92	12.68
60	-	9.11	7.90	16.64	11.75	15.55	10.53
EP	3.63	1.19	3.24	1.65	.76	2.55	1.76
S-0	.00	.00	.00	.00	.00	.00	.00
S-30	.16	1.18	.00	.98	.52	.90	.00
S-60	.18	.25	.00	.65	1.11	.21	.00
S-90	.48	.73	.85	.64	.65	.66	.29
$[MEL]_{eq}$	30.94	75.44	119.40	36.15	67.43	137.32	54.38
$t_{1/2}$	44.33	20.03	18.84	42.52	21.49	18.73	25.75

Melphalan Pharmacokinetics(cont)

Patient:	039	040	041	042	043	044
Study:						
Chapter 3						
Chapter 4	*	*	*	*	*	*
Sample(mins)						
1	87.20	85.25	11.50	83.95	112.75	64.50
5	38.30	70.55	30.95	47.85	69.45	32.90
10	29.25	42.00	28.55	40.25	58.65	24.25
15	28.80	40.90	17.55	22.95	47.60	18.40
20	19.95	30.55	17.75	18.40	40.90	15.80
25	18.65	32.55	15.15	16.20	33.75	14.50
30	16.90	-	13.85	15.40	30.55	12.10
35	-	-	-	-	-	-
40	13.85	21.00	11.50	13.85	25.55	10.80
45	-	-	-	-	-	-
50	12.35	18.20	8.05	12.55	22.75	8.45
55	-	-	-	-	-	-
60	10.85	15.15	8.70	10.60	20.15	9.80
EP	-	-	-	-	-	-
S-0	.00	.00	.91	.00	.00	-
S-30	1.91	.00	.44	.44	.18	.00
S-60	2.12	.00	.44	.31	.31	.00
S-90	2.34	1.39	.39	.74	1.91	4.15
$[MEL]_{eq}$	27.03	47.87	24.96	22.90	52.62	19.89
$t_{1/2}$	44.47	35.79	35.50	54.93	41.46	48.01

Patient:	045	046	047	048	049	050
Study:						
Chapter 3						
Chapter 4	*	*	*	*	*	*
Sample(mins)						
1	1.55	72.70	140.66	105.60	112.75	114.48
5	68.82	47.85	47.62	41.15	48.49	-
10	38.53	32.05	39.62	32.25	40.48	45.46
15	26.20	25.55	28.80	27.05	24.69	33.56
20	25.99	20.60	24.69	20.80	21.01	27.07
25	21.66	17.55	23.61	18.85	17.55	22.96
30	18.63	14.50	20.58	17.35	16.68	20.14
35	-	-	-	-	-	-
40	17.11	14.10	18.41	14.95	15.16	14.09
45	-	-	-	-	-	-
50	13.44	11.70	16.25	13.65	13.65	14.09
55	-	-	-	-	-	-
60	-	11.90	-	12.80	12.35	13.00
EP	-	-	-	-	-	-
S-0	.00	.61	.00	.00	.00	.00
S-30	1.13	.65	.57	.00	.13	.00
S-60	1.17	.61	1.26	.00	.00	.00
S-90	1.09	.83	1.82	.39	.96	.48
$[MEL]_{eq}$	36.74	24.24	32.74	25.47	24.77	35.77
$t_{1/2}$	34.40	52.35	48.63	57.01	58.00	37.40

Melphalan Pharmacokinetics(cont)

Patient:	039	040	041	042	043	044
Study:						
Chapter 3						
Chapter 4	*	*	*	*	*	*
Sample(mins)						
1	87.20	85.25	11.50	83.95	112.75	64.50
5	38.30	70.55	30.95	47.85	69.45	32.90
10	29.25	42.00	28.55	40.25	58.65	24.25
15	28.80	40.90	17.55	22.95	47.60	18.40
20	19.95	30.55	17.75	18.40	40.90	15.80
25	18.65	32.55	15.15	16.20	33.75	14.50
30	16.90	-	13.85	15.40	30.55	12.10
35	-	-	-	-	-	-
40	13.85	21.00	11.50	13.85	25.55	10.80
45	-	-	-	-	-	-
50	12.35	18.20	8.05	12.55	22.75	8.45
55	-	-	-	-	-	-
60	10.85	15.15	8.70	10.60	20.15	9.80
EP	-	-	-	-	-	-
S-0	.00	.00	.91	.00	.00	-
S-30	1.91	.00	.44	.44	.18	.00
S-60	2.12	.00	.44	.31	.31	.00
S-90	2.34	1.39	.39	.74	1.91	4.15
$[MEL]_{eq}$	27.03	47.87	24.96	22.90	52.62	19.89
$t_{1/2}$	44.47	35.79	35.50	54.93	41.46	48.01

Patient:	045	046	047	048	049	050
Study:						
Chapter 3						
Chapter 4	*	*	*	*	*	*
Sample(mins)						
1	1.55	72.70	140.66	105.60	112.75	114.48
5	68.82	47.85	47.62	41.15	48.49	-
10	38.53	32.05	39.62	32.25	40.48	45.46
15	26.20	25.55	28.80	27.05	24.69	33.56
20	25.99	20.60	24.69	20.80	21.01	27.07
25	21.66	17.55	23.61	18.85	17.55	22.96
30	18.63	14.50	20.58	17.35	16.68	20.14
35	-	-	-	-	-	-
40	17.11	14.10	18.41	14.95	15.16	14.09
45	-	-	-	-	-	-
50	13.44	11.70	16.25	13.65	13.65	14.09
55	-	-	-	-	-	-
60	-	11.90	-	12.80	12.35	13.00
EP	-	-	-	-	-	-
S-0	.00	.61	.00	.00	.00	.00
S-30	1.13	.65	.57	.00	.13	.00
S-60	1.17	.61	1.26	.00	.00	.00
S-90	1.09	.83	1.82	.39	.96	.48
$[MEL]_{eq}$	36.74	24.24	32.74	25.47	24.77	35.77
$t_{1/2}$	34.40	52.35	48.63	57.01	58.00	37.40

Melphalan Pharmacokinetics(cont)

Patient:	A03	A04	A05	A06	A07	A08
Study:						
Chapter 3	*	*	*	*	*	*
Chapter 4	*	*	*	*	*	*
Sample(mins)						
1	20.90	3.90	2.44	.00	68.46	12.70
5	26.60	38.41	53.34	38.83	65.26	43.00
10	37.26	44.92	41.34	38.24	53.65	42.90
15	36.95	29.29	32.10	36.98	48.10	48.50
20	35.28	29.24	29.34	33.91	50.90	47.08
25	32.57	31.06	26.81	30.67	35.70	29.90
30	28.86	18.56	23.20	27.06	38.30	22.70
35	26.66	17.48	24.34	24.10	27.90	32.80
40	22.56	15.30	21.58	23.14	29.00	24.50
45	23.17	13.78	16.79	21.32	23.80	24.10
50	20.85	13.23	15.53	17.69	23.70	21.60
55	19.79	12.79	14.13	14.96	22.16	24.50
60	19.77	12.11	13.59	14.48	17.80	18.80
EP	-	-	3.47	3.68	5.38	2.13
S-0	.00	.00	.00	.00	.00	.00
S-30	.00	.00	.00	.00	.00	.00
S-60	.00	.00	.00	.00	.00	.00
S-90	.00	.18	.11	.00	.36	.18
$[MEL]_{eq}$	46.11	44.17	45.23	52.98	70.20	57.00
$t_{1/2}$	45.20	29.28	33.67	31.79	30.78	37.53

Patient:	A09	A10	A11	A12	A13
Study:					
Chapter 3	*	*	*	*	*
Chapter 4	*	*	*	*	*
Sample(mins)					
1	172.00	2.01	.00	62.15	40.50
5	117.00	18.62	36.83	48.89	-
10	73.50	21.23	30.29	28.28	26.23
15	59.20	19.42	26.00	21.05	21.57
20	51.90	17.85	24.96	17.39	19.30
25	46.90	16.22	29.40	16.61	16.55
30	41.00	14.71	-	13.64	14.85
35	35.90	11.62	16.71	13.56	18.37
40	35.40	13.95	-	10.39	12.52
45	30.20	-	15.44	11.77	15.26
50	29.80	15.61	15.67	10.37	14.28
55	22.90	14.62	14.18	10.07	8.94
60	21.30	12.01	14.76	9.17	11.60
EP	3.83	.80	1.74	.82	1.42
S-0	.00	.00	.00	.00	.00
S-30	.00	.00	.00	.00	.00
S-60	.00	.00	.00	.00	.00
S-90	.10	.00	.17	.00	.00
$[MEL]_{eq}$	80.99	17.79	36.14	23.10	24.79
$t_{1/2}$	31.49	130.88	39.66	43.75	50.26

Melphalan Pharmacokinetics(cont)

Patient:	P01	P02	P03	P04	P05
Study:					
Chapter 3					
Chapter 4	*	*	*	*	*
Sample(mins)					
1	.00	.00	3.32	2.41	-
5	31.62	44.90	44.64	42.80	57.52
10	28.51	35.30	35.29	41.70	32.24
15	22.40	31.48	25.56	37.16	25.62
20	22.76	28.38	29.84	36.55	22.06
25	20.72	22.93	21.59	29.72	19.89
30	18.02	19.24	18.62	27.00	17.60
35	13.91	18.04	23.13	26.00	16.48
40	15.87	16.53	20.30	25.50	17.19
45	13.70	14.92	19.19	18.34	13.65
50	12.78	12.73	18.75	23.18	12.21
55	10.29	12.54	-	23.00	11.18
60	9.53	11.35	-	13.90	11.06
EP	1.63	1.49	.79	1.51	1.31
S-0	.00	.00	.00	.00	.00
S-30	.00	.00	.00	.00	.00
S-60	.00	.00	.14	.00	2.57
S-90	.02	-	.15	1.52	.26
$[MEL]_{eq}$	34.00	39.68	31.41	48.59	31.41
$t_{1/2}$	33.08	32.02	62.85	39.30	38.45

Patients are numbered according to the region perfused:
O.. - iliac, **A**.. - axillary, **P**.. - popliteal.
Sample EP is taken from the final rinsing effluent.
Samples prefixed S- are taken from the systemic blood.

All concentrations are expressed in ug ml^{-1}.
$[MEL]_{eq}$: equilibrium concentration of melphalan
$t_{1/2}$: Half-life of melphalan in the perfusate (mins).

APPENDIX C

Limb Volume Measurements - Controls

Sex	Height	Weight	Surface Area*	PLV	TLV
M	1.85	75	2.000	9.24	12.32
M	1.68	69	1.790	9.46	10.56
M	1.81	66	1.860	8.36	10.34
M	1.80	67	1.865	10.12	11.44
M	1.84	72	1.950	9.57	10.89
M	1.79	81	2.050	11.77	14.52
M	1.80	62	1.810	8.25	10.45
M	1.70	61	1.720	7.04	9.35
M	1.75	70	1.860	9.13	10.67
M	1.82	58	1.780	7.48	8.69
M	1.81	65	1.850	8.03	10.67
M	1.77	63	1.800	7.37	10.34
M	1.82	63	1.835	8.58	10.67
M	1.66	65	1.730	8.47	10.56
M	1.74	70	1.850	8.80	11.55
M	1.70	67	1.790	8.36	8.91
M	1.76	60	1.755	7.48	9.79
M	1.92	68	1.975	9.35	13.75
F	1.63	64	1.700	8.47	11.88
F	1.62	50	1.535	6.71	8.47
F	1.62	52	1.560	7.04	9.35
F	1.70	64	1.750	8.58	13.09
F	1.63	52	1.565	6.27	9.02
F	1.71	58	1.695	8.69	11.11
F	1.68	72	1.820	11.44	15.40
F	1.70	67	1.785	9.57	14.08
F	1.65	60	1.670	8.25	10.56
F	1.61	54	1.575	7.70	9.68
F	1.74	76	1.910	10.23	11.99
F	1.67	65	1.740	9.35	11.88
F	1.79	71	1.900	9.90	12.34
F	1.60	54	1.570	6.71	9.90
F	1.63	49	1.530	6.60	8.80
F	1.58	50	1.510	6.60	8.80

Units:
height : m, weight : kg, surface area : m^2, volumes : l.

* Surface area derived from nomogram[503].
PLV - partial limb volume
TLV - total limb volume

APPENDIX D

Limb Volume Measurements - Patients

Patient	Sex	Height	Weight	Surface Area*	PLV	IPV
Iliac						
001	F	1.70	80	1.920	10.89	15.86
002	M	1.78	93	2.110	9.13	13.66
003	M	1.71	57	1.680	7.26	9.87
004	F	1.51	48	1.430	6.93	9.47
005	M	1.79	95	2.140	10.89	15.13
006	F	1.61	59	1.695	7.37	11.36
007	F	1.66	61	1.630	7.92	11.50
008	F	1.64	86	1.920	-	-
009	F	1.60	78	1.820	-	-
010	F	1.63	49	1.535	6.05	9.25
011	F	1.35	60	1.675	7.70	11.55
012	F	1.63	64	1.700	7.59	11.59
013	F	1.56	76	1.760	10.23	14.36
014	F	1.66	68	1.770	9.14	13.40
016	F	1.71	76	1.890	10.89	15.48
017	F	1.59	72	1.745	-	-
019	F	1.66	67	1.755	8.47	12.71
020	F	1.56	60	1.605	-	-
021	F	1.71	83	1.960	11.44	16.59
022	F	1.45	57	1.485	6.82	9.68
023	F	1.71	56	1.670	7.26	11.12
024	M	1.88	103	2.310	12.10	16.98
025	F	1.67	82	1.910	8.80	13.88
026	M	1.66	60	1.680	7.04	9.71
027	F	1.58	86	1.865	11.00	15.65
028	M	1.67	68	1.770	8.25	11.11
029	M	1.78	86	2.040	11.66	15.14
030	F	1.58	68	1.705	9.02	12.94

Units:
height : m, weight : kg, surface area : m^2, volumes : l.

* Surface area derived from nomogram[503].
PLV - partial limb volume
IPV - iliac perfusion volume

Limb Volume Measurements - Patients(cont)

Patient	Sex	Height	Weight	Surface Area*	PLV	IPV
Iliac(cont)						
031	F	1.67	89	1.980	11.66	16.91
032	F	1.59	68	1.710	9.90	13.77
033	F	1.63	62	1.675	7.26	11.15
034	M	1.72	73	1.870	8.80	12.07
035	F	1.68	77	1.870	9.46	14.27
036	F	1.52	52	1.485	7.70	10.49
037	M	1.81	93	2.140	11.22	15.38
038	F	1.56	54	1.540	7.26	10.39
039	F	1.54	52	1.500	6.38	9.36
040	F	1.65	72	1.800	9.35	13.77
041	M	1.76	59	1.745	-	-
042	F	1.63	61	1.665	-	-
043	F	1.64	78	1.850	11.55	16.08
044	F	1.56	55	1.555	5.60	8.95
045	F	1.61	61	1.650	7.37	11.11
046	F	1.63	51	1.560	6.60	9.90
047	F	1.67	67	1.765	9.18	13.42
048	F	1.67	59	1.680	8.03	11.89
049	M	1.75	64	1.800	7.04	10.38
050	M	1.75	90	2.080	10.56	14.55
051	M	1.73	63	1.770	-	-
052	F	1.62	74	1.790	11.35	12.20
053	F	1.55	64	1.630	10.10	10.44

Units:
height : m, weight : kg, surface area : m^2, volumes : l.

* Surface area derived from nomogram[503].
PLV - partial limb volume
IPV - iliac perfusion volume

Limb Volume Measurements - Patients(cont)

Patient	Sex	Height	Weight	Surface Area*	Perfusion Volume
Axillary					
A01	M	1.66	65	1.730	3.09
A02	F	1.58	96	1.960	3.91
A03	F	1.59	66	1.690	3.09
A04	M	1.67	56	1.640	-
A05	M	1.78	78	1.975	-
A06	M	1.79	78	1.985	3.03
A07	F	1.74	74	1.900	-
A08	F	1.54	76	1.740	3.36
A09	F	1.61	68	1.725	2.96
A10	F	1.63	67	1.730	3.50
A11	F	1.61	58	1.620	2.96
A12	M	1.88	100	2.275	4.32
A13	M	1.76	79	1.960	3.77
A14	M	1.64	80	1.865	3.91
A15	F	1.57	65	1.665	2.96
A16	M	1.70	74	1.860	-
A17	F	1.65	68	1.760	3.09
Popliteal					
P01	F	1.52	53	1.495	-
P02	F	1.62	74	1.790	6.05
P03	F	1.55	64	1.630	5.55
P04	F	1.54	43	1.390	-
P05	F	1.56	89	1.875	-

Units:
height : m, weight : kg, surface area : m^2, volume

* Surface area derived from nomogram[503].

APPENDIX E

TBV Calculations in 69 ILPs

Patient	Hct* s	Hct* p	Hct* m	TBV#
001	34	25	30	2475
002	39	19	30	2444
003	35	25	30	2200
004	36	22	24	1283
005	42	25	35	2671
006	39	23	29	1760
007	34	28	31	2200
008	38	27	32	2017
009	38	22	31	2514
010	31	25	26	1320
011	38	26	32	2200
012	38	28	33	2200
013	39	26	31	1788
014	34	24	31	3667
016	34	26	30	2200
017	35	22	25	1430
019	35	17	25	1980
020	34	21	23	1300
021	34	21	27	2043
022	37	28	29	1238
023	38	27	31	1729
024	40	24	33	2514
026	36	25	29	1729
027	38	23	29	1833
028	35	24	31	3025
029	38	25	32	2383
030	33	24	30	3300
031	36	27	32	2475
032	35	27	30	1760
033	37	22	27	1650
034	39	24	30	1833
035	37	25	29	1650
036	36	23	30	2383
037	43	24	38	4180
038	35	28	31	1925
039	37	27	31	1833
040	37	27	32	2200
041	42	27	34	2063
042	34	26	30	2200
043	33	25	28	1760
044	37	25	29	1650
045	34	25	26	1238
046	33	25	28	1760
047	32	29	31	3300
048	31	27	28	1467
049	40	23	32	2338
050	36	25	28	1513
051	40	31	35	1980
Axillary				
A01	45	26	39	3483
A02	37	28	29	1238
A03	32	27	27	1100
A04	35	20	22	1269
A05	41	26	27	1179
A06	37	20	22	1247
A07	32	24	25	1257
A09	35	25	25	1100
A10	36	26	29	1571
A11	37	23	24	1185
A12	42	28	30	1283
A13	36	21	22	1179
A14	39	29	31	1375
A15	32	29	30	1650
A16	34	26	26	1100
A17	35	29	30	1320
Popliteal				
P01	31	23	24	1257
P02	40	25	28	1375
P03	33	24	27	1650
P04	32	24	24	1100
P05	36	26	29	1571

* Haematocrits :
s = systemic, p = priming fluid, m = mixed perfusate

\# TBV = calculated total blood volume.

REFERENCES

1. Rawles ME. - Origin of melanosomes and their role in the development of colour patterns in vertebrates. Physiol Rev 1948;28:383-408.

2. Birbeck MSC - Electron microscopy of melanocytes : the fine structure of hair bulb premelanosomes. Ann NY Acad Sci 1963;100:540-547.

3. Seiji M, Iwashita S - Intracellular localisation of tyrosinase and site of melanin formation in melanocytes. J Invest Dermatol 1965;45:305-314.

4. Wellings SR, Siegel BV - Electron microscopic studies on the subcellular origin and ultrastructure of melanin granules in mammalian melanomas. Ann NY Acda Sci 1963;100:548-568.

5. Jimbow K, Takahashi M, Sato S, Kukita A - Ultrastructural and cytochemical studies of melanogenesis in melanocytes of normal human hair matrix. J Electron Micros (Tokyo) 1971;20:87.

6. Rappaport H, Nakai T, Swift H - The fine structure of normal and neoplastic melanocytes in the Syrian hamster with particular reference to carcinogen-induced melanotic tumours. J Cell Biol 1963;16:171-186.

7. Moyer FH - Genetic effects on melanosome fine structure and ontogeny in normal and malignant cells. Ann NY Acad Sci 1963;100:584-606.

8. Demopoulos HB, Yuen TGH - Fine structure of melanosomes in S-91 mouse melanomas. Am J Pathol 1967;50:559-569.

9. Novikoff AB, Albala A, Biempica L - Ultrastructure and cytochemical observations on B-16 and Harding Passey mouse melanomas - the origin of premelanosomes and compound melanosomes. J Histochem Cytochem 1968;16:299-319.

10. Maul GG - Golgi-melanosome relationship in human melanoma in vitro. J Ultrastruct Res 1969;26:163-176.

11. Maul GG, Brumbaugh JA - On the possible function of coated vesicles in melanogenesis of the regenerating fowl feather. J Cell Biol 1971;48:41-48.

12. Masson P - Pigment cells in man. in Minor RW, Gordon M eds. The Biology of Melanomas, Vol IV. New York Academy of Science, 1948; 15.

13. Fitzpatrick TB, Szabo G - The melanocyte : cytology and cytochemistry. J Invest Dermatol 1959;32:197-209.

14. Pathak MA, Fitzpatrick TB - The role of natural photoprotective agents in human skin. in Pathak MA, Harber LC, Seiji M, Kukita A eds. Sunlight and Man. University of Tokyo Press, 1974; 725-750.

15. MacKie RM, Freudenberger T, Aitchison TC - Personal risk-factor chart for cutaneous melanoma. Lancet 1989(ii):487-490.

16. Amer MH, Al Sarraf M, Vaitkevicius VK - Clinical presentation, natural history and prognostic factors in advanced malignant melanoma. Surg Gynecol Obstet 1979;149:687-692.

17. MacKie RM - The pathogenesis of cutaneous malignant melanoma. Br Med J 1983;287:1568-1569.

18. Swerdlow AJ - International trends in cutaneous melanoma. Ann NY Acad Sci 1990;609:235-251.

19. Jensen OM, Bolander AM - Trends in malignant melanoma of the skin. World Health Statistics Quarterly, WHO 1980;33:2-26.

20. Green A - Incidence and reporting of cutaneous malignant melanoma in Queensland. Australas J Dermatol 1982;23:105-109.

21. Greene A, Siskind V - Geographical distribution of cutaneous malignant melanoma in Queensland. Med J Austr 1983;1:407-410.

22. Malek E, Eklund G - The changing incidence of malignant melanoma of the skin in Sweden 1959-1968. Scand J Plast Reconst Surg 1978;12:19-27.

23. Magnus K - Habits of sun exposure and risks of melanoma. An analysis of incidence rates in Norway 1955-1977 by cohort, sex, age and primary tumour site. Cancer 1981;48:2329-2335.

24. Magnus K - The nordic profile of skin cancer incidence. A comparative epidemiological study of the three main types of skin cancer.Int J Cancer 1991;47:12-19.

25. Schreiber MM, Bozzo PD, Moon TE - Malignant melanoma in Southern Arizona. Increasing incidence and sunlight as an etiologic factor. Arch Dermatol 1981;117:6-11.

26. Pathak DR, Samet JM, Howard CA, Key CR - Malignant melanoma of the skin in New Mexico 1969-1977. Cancer 1982;50:1440-1446.

27. MacKie Rm, Hunter JAA - Cutaneous malignant melanoma in Scotland. Br J Cancer 1982;46:75-80.

28. MacKie RM, Smyth JF, Soutar DS, et al - Malignant melanoma in Scotland 1979-1983. Lancet 1985(ii):859-862.

29. Hunter JAA, MacKie RM, Smyth JF, Soutar DS, McLaren K, Kemp IW - Malignant melanoma and the Scottish Melanoma Group. Health Bulletin 1986;4:302-308.

30. MacKie RM, Hunter JAA, Aitchison TC, et al - Cutaneous malignant melanoma, Scotland 1979-1989. Lancet 1992;339:971-975.

31. Office of Population Censuses and Surveys. Cancer Statistics : survival, England and Wales. London HMSO 1982, series MB1, No 9.

32. Magnus K - Incidence of malignant melanoma of the skin in Norway 1955-1970 : variations in time and space and solar radiation. Cancer 1973;32:1275-1286.

33. Elwood JM, Lee JAH - Recent data on the epidemiology of malignant melanoma. in Clark WH, Goldman LI, Mastrangelo MJ eds. Human Malignant Melanoma. New York : Grune and Stratton, 1979; 261-272.

34. MacKie RM - The role of sunlight in the aetiology of cutaneous malignant melanoma. Clin Exp Dermatol 1981;6:407-410.

35. Lee JAH - Melanoma and exposure to sunlight. Epidemiol Rev 1982;4:110-136.

36. Briele HA, Das Gupta TK - Natural history of cutaneous malignant melanoma. World J Surg 1979;3:255-270.

37. Nicholls EM - Development and elimination of pigmented moles, and the anatomical distribution of primary malignant melanoma. Cancer 1973;32:191-195.

38. Pack GT, Lenson N, Gerber DM - Regional distribution of moles and melanomas. Arch Surg 1952;65:862-870.

39. Creagan ET, Fraumeni JF Jr - Cancer mortality among American Indians 1950-1967. J Natl Cancer Inst 1972;49:959-967.

40. Jung HD, Kolzsch J - Zur Epidemiologie von pracancerosen und bosartigen Tumoren der Haut. V. Melanosis circumscripta praecancerosa und malignes Melanom. Hautartz 1968;19:350-356.

41. McGovern VJ - Aetiology of melanoma : classification and histologic reports. Spontaneous regression : frozen section diagnosis. in Milton GW, ed. Malignant Melanoma of the Skin and Mucous Membranes. Edinburgh : Churchill Livingstone, 1977; 1-25.

42. Elder DE, Greene MH, Bondi EE, Clark WH - Acquired melanocytic nevi and melanoma : the dysplastic nevus syndrome. in Ackerman AB ed. Pathology of Malignant Melanoma. New York : Masson, 1981; 185-215.

43. McNeer G, Das Gupta TK - Prognosis in malignant melanoma. Surg 1964;56:512-518.

44. Cameron JRF - Melanoma of the skin - clinical account of 209 cases. J R Coll Surg Edinb 1968;13:233-254.

45. Cochran AJ - Malignant melanoma: a review of 10 years' experience in Glasgow, Scotland. Cancer 1969;23:1190-1199.

46. Clark WH, Mastrangelo MJ, Ainsworth AM, Berd D, Bellet RE, Bernardino EA - Current concepts of the biology of human cutaneous malignant melanoma. Adv Cancer Res 1977;24:267-338.

47. Frichot BC, Lynch HT, Giurgis HA, Harris RE, Lynch JF - New cutaneous phenotype in familial malignant melanoma. Lancet 1977(i):864-865.

48. Clark WH, Reimer PR, Greene MH, Ainsworth AM, Mastrangelo MJ - Origin of familial malignant melanoma from heritable melanocytic lesionms : "The B-K mole syndrome". Arch Dermatol 1978;114:732-738.

49. Greene MH, Clark WH, Tucker MA, et al - Precursor naevi in cutaneous malignant melanoma : a proposed nomenclature. Lancet 1980(ii):1024.

50. Elder DE, Goldman LI, Goldman SC, Greene MH, Clark WH - Dysplatic naevus syndrome : a phenotypic association of sporadic cutaneous melanoma. Cancer 1980;46:1787-1794.

51. Kraemer KH, Greene MH, Tarone R, Elder DE, Clark WH, Guerry D - Dysplastic naevi and cutaneous malignant melanoma risk. Lancet 1983(ii):1076-1077.

52. Greene MH, Clark WH, Tucker MA, Kraemer KH, Elder DE, Fraser MC - The high risk of malignant melanoma in melanoma prone families with dysplastic naevi. Ann Int Med 1985;102:458-465.

53. Swerdlow AJ, Green A - Melanocytic naevi and melanoma : an epidemiological perspective. Br J Dermatol 1987;117:137-146.

54. Ackerman AB, Mihara I - Dysplasia, dysplastic melanocytes, dysplastic naevi, the dysplastic naevus syndrome, and the relationship between dysplastic naevi and malignant melanomas. Human Pathol 1985;16:87-91.

55. Reed WB, Becker SW, Becker SW Jr, Nickel WR - Giant pigmented nevi, melanoma and leptomeningeal melanocytosis. Arch Dermatol 1965;91:100-119.

56. Mark GJ, Mihm MC, Liteplo MG, Reed RJ, Clark WH - Congenital melanocytic nevi of the small and garment type. Human Pathol 1973;4:395-418.

57. MacKie RM - Melanocytic Naevi. in Rook A, Wilkinson DS, Ebling FJG, Champion RH, Butor JL, eds. Textbook of Dermatology, 4th edition, Vol.1. London : Blackwell Sci Publ, 1986; 179-190.

58. MacKie RM - Risk factors, diagnosis, and detection of melanoma. Curr Opin Oncol 1991;3:360-363.

59. Swerdlow AJ, English J, MacKie RM, et al - Benign melanocytic naevi as a risk factor for malignant melanoma. Br Med J 1986;292:1555-1559.

60. Goldman RJ, Kopf AW, Bart RS, Levenstein M - Does a history of melanoma correlate with the clinical presentation of dysplastic nevi? J Dermatol Surg Oncol 1990;16:538-542.

61. Goldgar DE, Cannon-Albright LA, Meyer LJ, Piepkorn MW, Zone JJ, Skolnick MH - Inheritance of nevus number and size in melanoma and dysplastic nevus syndrome kindreds. J Natl Cancer Inst 1991;83:1726-1733.

62. Norris W - A case of fungoid disease. Edinb Med Surg J 1820;16:562-565.

63. Greene MH, Fraumeni JF - The hereditary variant of malignant melanoma. in Clark WH, Goldman LI, Mastrangelo MJ, eds. Human Malignant Melanoma. New York : Grune and Stratton, 1979; 139-166.

64. Wallace DC, Beardmore GL, Exton LA - Familial malignant melanoma. Arch Surg 1973;177:15-20.

65. Anderson DE - Clinical characteristics of the genetic variety of cutaneous melanoma in man. Cancer 1971;28:721-725.

66. Weber FP, Schwarz E, Hellenschmied R - Spontaneous innoculation of melanotic sarcoma from mother to foetus. Br Med J 1930;1:537-539.

67. George PA, Fortner JG, Pack GT - Melanoma with pregnancy: a report of 115 cases. Cancer 1960;13:854-859.

68. Fountain JW, Karayiorgou M, Graw SL, et al - Chromosome 9p involvement in melanoma. Am J Hum Genet 1991;49(suppl):45.

69. Fountain JW, Karayiorgou M, Taruscio D, et al - Genetic and physical map of the interferon region on chromosome 9p. Genomics 1992;14:105-112.

70. Olopade OI, Bohlander SP, Pomykala H, et al - Mapping of the shortest region of overlap of deletions of the short arm of chromosome 9 associated with human neoplasia. Genomics 1992;14:437-443.

71. Nancarrow DJ, Mann GJ, Holland EA, et al - Confirmation of chromosome 9p linkage in familial melanoma. Am J Hum Genet 1993;53:936-942.

72. Cowan JM, Halaban R, Francke U - Cytogenetic analysis of melanocytes from premalignant naevi and melanomas. J Natl Cancer Inst 1988;80:1159-1164.

73. Murty VV, Bouchard B, Mathew S, Vijayasaradhi S, Houghton AN - Assignment of the human TYRP (brown) locus to chromosome region 9p23 by non-radioactive in situ hybridisation. Genomics 1992;13:227-229.

74. Klepp O, Magnus K - Some environmental and bodily characteristics of melanoma patients. A case-control study. Int J Cancer 1979;23:482-486.

75. Beitner H, Ringborg U, Wennersten G, Lagerlof B - Further evidence for increased light sensitivity in patients with malignant melanoma. Br J Dermatol 1981;104:289-294.

76. Shapiro MP, Keen P, Cohen L, Murray JF - Skin cancer in the South African Bantu. Br J Cancer 1953;7:45-57.

77. Lewis MG - Malignant melanoma in Uganda. Br J Cancer 1967;21:483-495.

78. Davies JNP, Tank R, Meyer R, Thurston P - Cancer of the integumentary tissues in Ugandan Africans - the basis for prevention. J Natl Cancer Inst 1968;41:31-51.

79. Tuyns AJ - Les cancers cutanes en Afrique et dans le monde. Med Afr Noire 1971;18:471-481.

80. Kaplan I, Youngleson J - Malignant melanoma in the South African Bantu. Br J Plastic Surg 1972;25:65-68.

81. Camain R, Tuyns AJ, Sarrat H, Quenum C, Faye I - Cutaneous cancer in Dakar. J Natl Cancer Inst 1972;48:33-49.

82. Krementz ET, Sutherland CM, Carter RD, Ryan RF - Malignant melanoma in the american black. Ann Surg 1976;183:533-542.

83. Crombie IK - Racial differences in melanoma incidence. Br J Cancer 1979;40:185-193.

84. Houghton A, Flannery J, Viola MV - Malignant melanoma in Connecticut and Denmark. Int J Cancer 1980;25:95-104.

85. Lane Brown MM, Sharp CAB, MacMillan DS, McGovern VJ - Genetic predisposition to melanoma and other skin cancers in Australia. Med J Austr 1971;1:852-853.

86. Clemmesen J - Statistical studies in the aetiology of malignant neoplasms. I. Review and results. Acta Pathol Microbiol Scand 1965;Suppl.174:190.

87. Crombie IK - Distribution of malignant melanoma on the body surface. Br J Cancer 1981;43:842-849.

88. MacKie RM, Aitchison TC - Severe sunburn and subsequent risk of primary cutaneous malignant melanoma in Scotland. Br J Cancer 1982;46:955-960.

89. Lee JAH, Strickland D - Malignant melanoma : social status and outdoor work. Br J Cancer 1980;41:757-763.

90. Giannelli F - Xeroderma pigmentosum. in Symington T, Carter RL, eds. Scientific Foundation in Oncology; London, William Heinemann Medical Books Ltd, 1976; 476-489.

91. Cleaver JE - Defective repair replication of DNA in xeroderma pigmentosum. Nature 1968;218:652-656.

92. Lindley D - CFC's cause part of global ozone decline. Nature 1988;332:293.

93. MacKie RM, Rycroft MJ - Health and the ozone layer. Br Med J 1988;297:369-370.

94. Swerdlow AJ, English JSC, MacKie RM, et al - Fluorescent lights, ultraviolet lamps and risk of cutaneous melanoma. Br Med J 1988;297:647-653.

95. Retsas S - Sunbeds and melanoma (letter). Br Med J 1983;286:892.

96. Walter SD, Marrett LD, From L, Hertzman C, Shannon HS, Roy P - The association of cutaneous malignant melanoma with the use of sunbeds and sunlamps. Am J Epidemiol 1990;131:232-243.

97. Veronesi U, Cascinelli N, Bufalino R - Evaluation of the risk of multiple primaries in malignant cutaneous melanoma. Tumori 1979;62:127-130.

98. Vaisman I, Bellet RE, Mastrangelo MJ, Lustbader E - Additional primary malignancies in patients with cutaneous melanoma. in Clark WH, Goldman LI, Mastrangelo MJ, eds, Human Malignant Melanoma, New York, Grune and Stratton, 1979; 243-260.

99. Greene MH, Hoover RN, Fraumeni JF - Subsequent cancer in patients with chronic lymphatic leukaemia : a possible immunologic mechanism, J Natl Cancer Inst 1978;61:337-340.

100. Hoxter EO, Mandel JS, Murray SS, Schuman LM, Goltz RW - Incidence of skin carcinoma after renal transplantation. Arch Dermatol 1977;113:436-438.

101. Greene MH, Young TI, Clark WH - Malignant melanoma in renal transplant recipients. Lancet 1981(i); 1196-1199.

102. Kripke ML, Fisher MS - Immunological parameters of ultraviolet carcinogenesis. J Natl Cancer Inst 1976;57:211-215.

103. Hersey P, Bradley M, Hasic E, Haran G, Edwards A, McCarthy WH - Immunological effects of solarium exposure. Lancet 1983(i):545-548.

104. Shiu MH, Schottenfield D, MacLean B, Fortner JG - Adverse effect of pregnancy on melanoma. A reappraisal. Cancer 1976;37:181-187.

105. Fisher RI, Neifeld JP, Lippman ME - Oestrogen receptors in human malignant melanoma. Lancet 1976(ii):337-338.

106. Neifeld JP, Lippman M - Steroid hormone receptors and melanoma. J Invest Dermatol 1980;74:379-381.

107. Chaudhuri PK, Walker MJ, Briele HA, Beattie CW, Das Gupta TK - Incidence of oestrogen receptor in benign nevi and human malignant melanoma. JAMA 1980;244:791-793.

108. Slinglufff CL, Reintgen DS, Vollmer RT, Seigler HF - Malignant melanoma arising during pregnancy. Ann Surg 1990;211:552-559.

109. Everson TC, Cole WH - Spontaneous regression of cancer. Philadelphia, WB Saunders, 1966; 164-220.

110. Bodurtha AJ - Spontaneous regression of malignant melanoma. in Clark WH, Goldman LI, Mastrangelo MJ, eds, Human Malignant Melanoma,New York, Grune and Stratton. 1979; 227-241.

111. Little JH - Partial regression in primary cutaneous malignant melanoma. Pathology 1971;3:62.

112. Nathanson L - Spontaneous regression of malignant melanoma. A review of the literature on incidence, clinical features and possible mechanisms. NCI Monograph 1976;44:67-76.

113. Shimkin MB, Boldrey EB, Kelly lh, Bierman HR, Ortega P, Naffziger HC - Effect of surgical hypophysectomy in a man with malignant melanoma. J Clin Endocrinol Metab 1952;12:439-453.

114. Cade S - Malignant melanoma. Br Med J 1957;1:119-124.

115. Smith JR, Stehlin JS - Spontaneous regression of primary malignant melanomas with regional metastases. Cancer 1965;18:1399-1415.

116. Stephenson HE, Delmez JA, Renden DI, et al - Host immunity and spontaneous regression of cancer evaluated by computertised data reduction study. Surg Gynecol Obstet 1971;133:649-655.

117. Cole WH - Spontaneous regression of cancer : the metabolic triumph of the host? Ann NY Acad Sci 1974;230:111-141.

118. Bodurtha AJ, Berkelhammer J, Kim YH, Laucius JF, Mastrangelo MJ - A clinical, histological and immunological study of a case of metastatic malignant melanoma undergoing spontaneous regression. Cancer 1976;37:735-742.

119. Mastrangelo MJ, Kim YH, Bornstein RS, et al - Clinical and histological correlation of melanoma regression after intralesional BCG therapy : a case report. J Natl Cancer Inst 1974;52:19-24.

120. Jonk A, Kroon BBR, Rumke P, Mooi WJ, Hart AAM, van Dongen JA - Lymph node metastasis from melanoma with an unknown primary site. Br J Surg 1990;77:665-668.

121. The EJ, Eibergen R, Lamberts HB, et al - Immune phagocytosis in vivo of human malignant melanoma cells. Acta Med Scand 1972;192:141-144.

122. Das Gupta T, Bowden L, Berg JW - Malignant melanoma of unknown primary origin. Surg Gynecol Obstet 1963;117:341-345.

123. Smith JL, Stehlin JS - Spontaneous regression of primary malignant melanomas with regional metastases. Cancer 1965;18:1399-1415.

124. Lopez R, Holyoke ED, Moore RH, Karakousis CP - Malignant melanoma with unknown primary site. J Surg Oncol 1982;19:151-154.

125. Reintgen DS, McCarty KS, Woodard B, Cox E, Seigler HF - Metastatic malignant melanoma with an unknown primary. Surg Gynecol Obstet 1983;156:335-340.

126. Baab GH, McBride CM - Malignant melanoma. The patient with an unknown site of primary origin. Arch Surg 1975;110:896-900.

127. Giuliano AE, Moseley HS, Morton DL - Clinical aspects of unknown primary melanoma. Ann Surg 1980;191:98-104.

128. Chang P, Knapper WH - Metastatic melanoma of unknown primary. Cancer 1982;49:1106-1111.

129. Milton GW, Shaw HM, McCarthy WH - Occult primary malignant melanoma : factors influencing survival. Br J Surg 1977;64:805-808.

130. Pack GT, Miller TR - Metastatic melanoma with indeterminate primary site. JAMA 1961:176:139-140.

131. Ridolfi RL, Rosen PP, Thaler H - Nevus cell aggregates associated with lymph nodes : estimated frequency and clinical significance. Cancer 1977;39:164-171.

132. Ewing - cited in Ariel IM : Biologic regression with disappearance of malignant melanoma. in Ariel IM ed. Malignant melanoma; New York, Appleton Century Crofts 1981; 119-128.

133. Shendy BV, Fort L(III), Benjamin SP - Malignant melanoma primary in lymph node. Am J Surg Pathol 1987;11:140-146.

134. Ariel IM - Biologic regression with disappearance of malignant melanoma. in Ariel IM ed. Malignant Melanoma; New York, Appleton Century Crofts 1981; 119-128.

135. Clark WH - A classification of malignant melanoma in man correlated with histogenesis and biologic behaviour. in Mothena W, Hu F eds. Advances in Biology of the Skin - the Pigmentary System, Vol 8, 1st edition; London, Pergamon press, 1967: 621-649.

136. Arrington JH, Reed RJ, Ichinose H, Krementz ET - Plantar lentiginous melanoma : a distinctive variant of human cutaneous malignant melanoma. Am J Surg Pathol 1977;1:131-143.

137. Holmes EC, Clark WH, Morton DL, Eilber FR, Bochow AJ - Regional lymph node metastases and the level of invasion of primary melanomas. Cancer 1976;37:199-202.

138. Clark WH, Ainsworth AM, Bernardino EA, Yang CH, Mihm MC, Reed RJ - The developmental biology of primary human malignant melanoma. Semin Oncol 1975;2:83-103.

139. Mihm MC, Fitzpatrick TB, Lane Brown MM, Raker JW, Malt RA, Kaiser JS - Early detection of primary cutaneous malignant melanoma. A color atlas. N Engl J Med 1973;289:989-996.

140. Clark WH, Folberg R, Ainsworth AM - Tumour progression in primary human cutaneous malignant melanoma. in Clark WH, Goldman LI, Mastrangelo MJ eds. Human malignant melanoma; Grune and Stratton, 1979; 15-23.

141. Hutchinson J - On tissue dotage. Arch Surg 1892;3:315-322.

142. Hutchinson J - Lentigo melanosis : a further report. Arch Surg 1894;5:253-256.

143. Rippey JJ, Rippey E - Malignant melanoma with adjacent Hutchinson's melanotic freckle in black Africans. Pathology 1977;9:105-109.

144. Stehlin JS, Smith JL, Jing B-S, Sherrin D - Melanoma of the extremities complicated by in transit metastases. Surg Gynecol Obstet 1966;122:2-14.

145. McCarthy JG, Haagensen CD, Hertel FP - The role of groin dissection in the management of melanoma of the lower extremity. Ann Surg 1974;179:156-159.

146. Stehlin JS, Clark RL, Vickers WE, Monges A - Perfusion for malignant melanoma of the extremities. Am J Surg 1963;105:607-614.

147. Milton GW, Shaw HM, Farago GM, McCarthy WH - Tumour thickness and the site and time of first recurrence in cutaneous malignant melanoma (Stage I). Br J Surg 1980;67:543-546.

148. Roses DF, Harris MN, Rigel D, Carrey Z, Friedmann R, Kopf AW - Local and in transit metastases following definitive excision for primary cutaneous malignant melanoma. Ann Surg 1983;198:65-69.

149. McNeer G, Das Gupta T - Prognosis in malignant melanoma. Surg 1964;56:512-518.

150. McBride CM, Clark RL - Experience with L-phenylalanine mustard dihydrochloride in isolation perfusion of extremities for malignant melanoma. Cancer 1971;28:1293-1296.

151. Ketcham AS, Christopherson WO - A staging system for malignant melanoma. World J Surg 1979;3:271-278.

152. Fortner JG, Das Gupta T, McNeer G - Primary malignant melanoma on the trunk. Ann Surg 1965;161:161-169.

153. Goldsmith HS, Shah JP, Kim DH - Prognostic significance of lymph node dissection in the treatment of malignant melanoma. Cancer 1970;26:606-609.

154. Shah JP, Goldsmith HS - Prognosis of malignant melanoma in relation to clinical presentation. Am J Surg 1972;123:286-288.

155. Gutterman JV, Mavligit G, Gottlieb JA, et al - Chemoimmunotherapy of disseminated malignant melanoma with dimethyl triazeno imidazole carboxamide and bacillus Calmette-Guerin. N Engl J Med 1974;291:592-597.

156. Mastrangelo MJ, Bellet RE, Berd R - Prognostic factors. in Clark WH, Goldman LI, Mastrangelo MJ, eds. Human Malignant Melanoma; New York, Grune and Stratton, 1979: 273-282.

157. Balch CM, Soong S, Shaw HM, Milton GW - An analysis of prognostic factors in 4000 patients with cutaneous melanoma. in Balch CM, Milton GW, eds. Cutaneous melanoma. Clinical management and treatment results worldwide; Philadelphia, JP Lippincott, 1985: 321-352.

158. Heise H, Krementz ET - Survival experience of patients with malignant melanoma of the skin 1950-1957. NCI Monograph 1961;6:69-84.

159. Bodenham DC - Basic principles of surgery - malignant melanoma. in McCarthy WH ed. Melanoma and skin cancer; Sydney, VCN Blight, Government Printer, 1972; 375-383.

160. Day CL, Mihm MC, Sober AJ, et al - Prognostic factors for melanoma patients with lesions 0.76-1.69mm in thickness : an appraisal of "thin" level IV lesions. Ann Surg 1982;195:30-34.

161. Cascinelli N, Vaglini M, Bufalino R, Morabito A - BANS : a cutaneous region with no prognostic significance in patients with melanoma. Cancer 1986;57:441-444.

162. Mehnert JH, Heard JL - Staging of malignant melanomas by depth of invasion, Am J Surg 1965;110:168-176.

163. Clark WH, From L, Bernardino EA, Mihm MC - The histogenesis and biologic behaviour of primary human malignant melanomas of the skin. Cancer Res 1969;29:705-727.

164.Breslow A - Thickness, cross-sectional area and depth of invasion in the prognosis of cutaneous melanoma. Ann Surg 1970;172:902-908.

165. Wanebo HJ, Fortner JG, Woodruff J, MacLean B, Binkowski E - Selection of the optimum surgical treatment of Stage I melanoma by depth of microinvasion : use of the combined microstage technique (Clark-Breslow). Ann Surg 1975;182:302-315.

166. Balch CM, Murad TM, Soong S, Ingalls AL, Halpern NB, Maddox WA - A multifactorial analysis of melanoma : prognostic histopathological features comparing Clark's and Breslow's staging methods. Ann Surg 1978;188:732-742.

167. Eldh J, Boeryd B, Peterson LE - Prognostic factors in cutaneous malignant melanoma in Stage I : a clinical, morphological and multivariate analysis. Scand J Plast Reconstr Surg 1978:12:243-255.

168. Schmoeckel C - Histological features of prognostic significance in cutaneous malignant melanoma. in MacKie RM ed. Malignant Melanoma , Pigment Cell Vol 6; Basel, Karger, 1983; 22-35.

169. Schmoeckel C, Bockelbrink A, Bockelbrink H, Braun-Falco O - Low- and high-risk malignant melanoma. II. Multivariate analyses for a prognostic classification. Eur J Cancer Clin Oncol 1983;19:237-243.

170. Lehman JA, Cross FS, Richey DWG - A clinical study of forty-nine patients with malignant melanoma. Cancer 1966;19:611-619.

171. Jones WM, Williams WJ, Roberts MM, Davies K - Malignant melanoma of the skin : prognostic value of clinical features and the role of treatment in 111 cases. Br J Cancer 1968;22:437-451.

172. Perzik SL, Baum RK - Individualization in the management of melanoma : a review of 164 consecutive cases. Amer Surg 1969;35:177-180.

173. McLeod GR - Factors influencing prognosis in malignant melanoma. in McCarthy WH ed, Melanoma and skin cancer; Sydney, VCN Blight, Government Primter, 1972; 367-373.

174. Cochran AJ - Histology and prognosis in malignant melanoma. J Pathol 1969;97:459-468.

175. Balch CM, Wilkerson JA, Murad TM, Soong S, Ingalls AL, Maddox WA - The prognostic significance of ulceration of cutaneous melanoma. Cancer 1980;45:3012-3017.

176. Day CL, Mihm MC, Lew RA, et al - Prognostic factors for patients with clinical Stage I melanoma of intermediate thickness (1.51-3.99mm). A conceptual model for tumor growth and metastasis. Ann Surg 1982;195:35-43.

177. Balch CM, Soong SJ, Murad TM, Ingalls AL, Maddox WA - A multifactorial analysis of melanoma. III. Prognostic factors in melanoma patients with lumph node metastases (Stage II). Ann Surg 1981;193:377-388.

178. Callery C, Cochran AJ, Roe DJ, et al - Factors prognostic for survival in patients with malignant melanoma spread to the regional lymph nodes. Ann Surg 1982;196:69-75.

179. Ghussen F, Kruger I, Groth W - The value of current staging systems for melanoma of the extremities. Cancer 1990;66:396-401.

180. Fortner JG, Woodruff J, Schottenfeld D, MacLean B - Biostatistical basis of elective node dissection for malignant melanoma. Ann Surg 1977;186:101-103.

181. Balch CM, Soong SJ, Milton GW, et al - A comparison of prognostic factors and surgical results in 1786 patients with localized (Stage I) melanoma treated in Alabama, USA, and New South Wales, Australia. Ann Surg 1982;196:677-684.

182. Veronesi U, Adamus J, Bandiera DC, et al - Inefficacy of immediate node dissection in Stage I melanoma of the limbs. N Engl J Med 1977;297:627-630.

183. Das Gupta TK - Current status of surgical treatment of melanoma. Semin in Oncol 1988;15:566-568.

184. Milton GW, Shaw HM, McCarthy WH, Pearson L, Balch CM, Soong SJ - Prophylactic lymph node dissection in clinical Stage I cutaneous malignant melanoma : results of surgical treatment in 1319 patients. Br J Surg 1982;69:108-111.

185. Reintgen DS, Cox EB, McCarty KS, Vollmer RT, Seigler HF - Efficacy of elective lymph node dissection in patients with intermediate thickness primary melanoma. Ann Surg 1983;198:379-385.

186. Morton DL - Current management of malignant melanoma (ed). Ann Surg 1990;212:123-124.

187. Karakousis CP, Temple DF, Moore R, Ambrus JL - Prognostic parameters in recurrent malignant melanoma. Cancer 1983;52:575-579.

188. Balch CM, Shaw H, Soong S, Milton GW - Changing trends in the clinical and pathological features of melanoma. in Balch CM, Milton GW eds. Cutaneous melanoma. Clinical management and treatment results worldwide. Philadelphia, JP Lippincott, 1985; 313-319.

189. Doherty VR, MacKie RM - Experience of a public education programme on early detection of cutaneous malignant melanoma. Br Med J 1988;297:388-391.

190. Davis NC, McLeod GR, Beardmore GL, Little JH, Quinn RL, Holt J - Primary cutaneous melanoma : a report from the Queensland melanoma project. in Ariel IM ed. Malignant Melanoma. New York, Appleton Century Crofts, 1981; Pp 281-287.

191. Sampson-Handley W - The pathology of melanotic growths in relation to their operative treatment (The Hunterian Lectures). Lancet 1907(1):996-1003.

192. Pringle JH - A method of operation in cases of melanotic tumours of the skin. Edinb Med J 1908;23:496-499.

193. Petersen NC, Bodenham DC, Lloyd OC - Malignant melanoma of the skin. A study of the origin, development, aetiology, spread, treatment and prognosis. Part II. Br J Plast Surg 1962;15:97-116.

194. Das Gupta TK - Results of treatment of 269 patients with primary cutaneous melanoma : a five year prospective study. Ann Surg 1977;186:201-209.

195. Balch CM, Murad TM, Soong SJ, Ingalls AL, Richards PC, Maddox WA - Tumour thickness as a guide to surgical management of clinical Stage I melanoma patients. Cancer 1979;43:883-888.

196. Cascinelli N, van der Esch EP, Breslow A, Morabito A, Bufalino R - Stage I melanoma of the skin : the problem of resection margins. Eur J Cancer 1980;16:1079-1085.

197. Day CL, Mihm MC, Sober AJ, Fitzpatrick TB, Malt RA - Narrower margins for clinical Stage I malignant melanoma. N Engl J Med 1982;306:479-482.

198. Urist MM, Balch CM, Soong SJ, Shaw HM, Milton GW, Maddox WA - The influence of surgical margin and prognostic factors predicting the risk of local recurrence in 3445 patients with primary cutaneous melanoma. Cancer 1985;55:1398-1402.

199. Welvaart K, Hermans J, Zwaveling A, Ruiter DJ - Prognosis and surgical treatment of patients with Stage I melanomas of the skin : a retrospective analysis of 211 patients. J Surg Oncol 1986;31:79-86.

200. Griffiths RW, Briggs JC - Incidence of locally metastatic ("recurrent") cutaneous malignant melanoma following conventional wide margin excisional surgery for invasive clinical Stage I tumours : importance of maximal primary tumour thickness. Br J Surg 1986;73:349-353.

201. Elder DE, Guerry D (IV), Heiberger RM, et al - Optimal resection margins for cutaneous malignant melanoma. Plast Reconstr Surg 1983;71:66-72.

202. Epstein E, Bragg K, Linden G - Biopsy and prognosis of malignant melanoma. JAMA 1969;208:1369-1371.

203. Veronesi I, Cascinelli N, Adamus J, et al - Thin Stage I primary cutaneous malignant melanoma. Comparison of excision with margins of 1 or 3 cm. New Engl J Med 1988;318:1159-1162.

204. Brandt SE, Welvaart K, Hermans J - Is long-term follow-up justified after excision of a thin melanoma ($\leq$1.5mm)? : a retrospective analysis of 206 patients. J Surg Oncol 1990;43:157-160.

205. Kelly JW, Sagebiel RW, Calderon W, Murillo L, Dakin RL, Blois MS - The frequency of local recurrence and microsatellites as a guide to reexcision margins for cutaneous malignant melanoma. Ann Surg 1984;200:759-763.

206. Balch CM - Excising melanomas : how wide is enough? And how to reconstruct? (ed). J Surg Oncol 1990;44:135-137.

207. Papachristou DN, Fortner JG - Melanoma arising under the nail. J Surg Oncol 1982;21:219-222.

208. Olsen G - The malignant melanoma of the skin. New theories based on a study of 500 cases. Acta Chir Scand 1966;suppl.365:1-222.

209. Kenady DE, Brown BW, McBride CM - Excision of underlying fascia with a primary malignant melanoma : effect on recurrence and survival rates. Surg 1982;92:615-618.

210. Balch CM - The role of elective lymph node dissection in melanoma : rationale, results and controversies. J Clin Oncol 1988;6:163-172.

211. Cascinelli N, Preda F, Vaglini M, et al - Metastatic spread of Stage I melanoma of the skin. Tumori 1983;69:449-454.

212. Pack GT, Scharnagel IM, Morfit M - The principle of excision and dissection in continuity for primary and metastatic melanoma of the skin. Surg 1945;17:849-866.

213. McCarthy WH, Shaw HM, Milton GW - Efficacy of elective lymph node dissection in 2347 patients with Stage I malignant melanoma. Surg Gynecol Obstet 1985;161:575-580.

214. Karakousis CP, Emrich LJ, Driscoll DL, Rao U - Survival after groin dissection for malignant melanoma. Surg 1991;109:119-126.

215. Morton DL, Wen DR, Wong JH, et al - Technical details of intraoperative lymphatic mapping for early stage melanoma. Arch Surg 1992;127:392-399.

216. Sim FH, Taylor WF, Ivins JC, Pritchard DJ, Soule EH - A prospective randomised study of the efficacy of routine elective lymphadenectomy in management of malignant melanoma : preliminary results. Cancer 1978;41:948-956.

217. Veronesi U, Adamus J, Bandiera DC, et al - Delayed regional lymph node dissection in Stage I melanoma of the skin of the lower extremities. Cancer 1982;49:2420-2430.

218. Sim FH, Taylor WF, Pritchard DJ, Soule EH - Lymphadenectomy in the management of Stage I malignant melanoma : a prospective randomised study. Mayo Clin Proc 1986;61:697-705.

219. Cochran AJ, Wen DR, Morton DL - Management of the regional lymph nodes in patients with cutaneous malignant melanoma. World J Surg 1992;16:214-221.

220. Morton DL, Wen DR, Foshag LJ, Essner R, Cochran AJ - Intraoperative lymphatic mapping and selective cervical lymphadenectomy for early-stage melanoma of the head and neck. J Clin Oncol 1993;11:1751-1756.

221. Wanebo HJ, Binns R - Surgical treatment of recurrent and metastatic malignant melanoma. in Nathanson L ed. Contemporary Issues in Clinical Oncology, Vol 6 : Management of Advanced Melanoma. New York, Churchill Livingstone, 1986; 185-194.

222. Price WE, Du Val MK - Regional lymph node dissection and malignant melanoma. Arch Surg 1963;87:747-750.

223. Nicolle FV, Mathews WH, Palmer JD - Treatment of malignant melanoma of the skin. Arch Surg 1966;93:209-214.

224. Conrad FG - Treatment of malignant melanoma. Arch Surg 1972;104:587-593.

225. Rosin RD - The treatment of malignant melanoma. Eur J Surg Oncol 1985;11:111-115.

226. Balch CM, Urist MM, Maddox WA, Milton GW, McCarthy WH - Management of regional metastatic melanoma. in Balch CM, Milton GW eds. Cutaneous melanoma. Clinical management and treatment results worldwide. Philadelphia, JP Lippincott, 1985; 93-130.

227. Bowsher WG, Taylor BA, Hughes LE - Morbidity, mortality and local recurrence following regional node dissection for melanoma. Br J Surg 1986;73:906-908.

228. Dasmahapatra KS, Karakousis CP - Therapeutic groin dissection in malignant melanoma. Surg Gynecol Obstet 1983;156:21-24.

229. Hueston JT - Integumentectomy for malignant melanoma of the limbs. Aust NZ J Surg 1970;40:114.

230. Jones RF, Dickinson WE - Total integumentectomy of the leg for multiple "in-transit" metastases of melanoma. Am J Surg 1972;123:588-590.

231. Bowers RF - Eleven years experience with quarterectomy for malignant melanoma. Arch Surg 1960;81:752-756.

232. McPeak CJ, McNeer GP, Whiteley HW, Booher RJ - Amputation for melanoma of the extremity. Surg 1963;54:426-431.

233. Turnbull A, Shah J, Fortner J - Recurrent melanoma of an extremity treated by major amputation. Arch Surg 1973;106:496-498.

234. Cox KR - Survival after amputation for recurrent melanoma. Surg Gynecol Obstet 1974;134:720-722.

235. Patel JK, Didolkar MD, Pickren JW, Moore RH - Metastatic pattern of malignant melanoma. A study of 216 autopsy cases. Am J Surg 1978;135:807-810.

236. McCormack PM, Bains MS, Beattie EJ Jr, Martini N - Pulmonary resection in metastatic carcinoma. Chest 1978;73:163-166.

237. Feun LG, Gutterman J, Burgess MA, et al - The natural history of resectable metastatic melanoma (Stage IV A melanoma). Cancer 1982;50:1656-1663.

238. Wong JH, Skinner KA, Kim KA, Foshag LJ, Morton DL - The role of surgery in the treatment of non-regionally recurrent melanoma. Surgery 1993;113:389-394.

239. Amer MH, Al Sarraf M, Baker LH, Vaitkevicius VK - Malignant melanoma and central nervous system metastases : incidence, diagnosis, treatment and survival. Cancer 1978;42:660-668.

240. Hafstrom L, Jonsson P-E, Stromblad L-G - Intracranial metastases of malignant melanoma treated by surgery. Cancer 1980;46:2088-2090.

241. Katz HR - The relative effectiveness of radiation therapy, corticosteroids, and surgery in the management of melanoma metastatic to the central nervous system. Int J Radiat Oncol Biol Phys 1981;7:897-906.

242. Balch CM, Soong SJ, Murad TM, Smith JW, Maddox WA - A multifactorial analysis of melanoma. IV. Prognostic factors in 200 melanoma patients with distant metastases (Stage III). J Clin Oncol 1983;1:126-134.

243. Stewart WR, Gilberman RH, Harrelson JM, Seigler HF - Skeletal metastases of melanoma. J Bone Joint Surg 1978;60(A):645-649.

244. Bellet RE, Mastrangelo MJ, Berd D, Lustbader E - Chemotherapy of metastatic malignant melanoma. in Clark WH, Goldman LI, Mastrangelo MJ eds. Human Malignant Melanoma. New York, Grune and Stratton, 1979; 325-354.

245. Geeraerts L, Nathanson L - Non-investigational cytotoxic agents. in Nathanson L ed. Contemporary Issues in Clinical Oncology, Vol 6 : Management of Advanced Melanoma. New York, Churchill Livingstone, 1986; 1-31.

246. Legha SS - Current therapy for malignant melanoma. Semin Oncol 1989;16(suppl.1):34-44.

247. Thatcher N - Recombinant Interleukin-2 and other types of treatment of advanced malignant melanoma. Curr Opin Oncol 1991;3:364-376.

248. Currie VE, Wong PP, Krakoff IH, Young CW - Phase I trial of vindesine in patients with advanced cancer. Cancer Treat Rep 1978;62:1333-1336.

249. Retsas S, Newtan KA, Westbury G - Vindesine as a single agent in the treatment of advanced malignant melanoma. Cancer Chemother Pharmacol 1979;2:257-260.

250. Retsas S, Peat I, Ashford R, et al - Updated results of vindesine as a single agent in the therapy of advanced malignant melanoma. Cancer Treat Rev 1980;7(suppl):87-90.

251. Carmichael J, Atkinson RJ, Calman KC, MacKie RM, Naysmith AM, Smyth JF - A multicentre Phase II trial of vindesine in malignant melanoma. Eur J Cancer Clin Oncol 1982;18:1293-1295.

252. Quagliana JM, Stephens RL, Baker LH, Costanzi JJ - Vindesine in patients with metastatic malignant melanoma : a Southwest Oncology Group Study. J Clin Oncol 1984;4:316-319.

253. Coates AS, Durant JR - Chemotherapy for metastatic melanoma. in Balch CM, Milton GW eds. Cutaneous Melanoma. Clinical Management and Treatment Results Worldwide. Philadelphia, JP Lippincott, 1985; 275-282.

254. Ariel IM, Pack GT - Treatment of disseminated melanoma with phenylalanine mustard (melphalan) and autogenous bone marrow transplants. Surg 1962;51:583-591.

255. McElwain TJ, Hedley DW, Burton G, et al - Marrow autotransplantation accelerates haematological recovery in patients with malignant melanoma treated with high dose melphalan. Br J Cancer 1979;40:72-80.

256. Cornbleet MA, McElwain TJ, Kumar PJ, et al - Treatment of advanced malignant melanoma with high dose melphalan and autologous bone marrow transplantation. Br J Cancer 1983;48:329-334.

257. Lazarus HM, Herzig RH, Wolff SN et al - Treatment of metastatic malignant melanoma with intensive melphalan and autologous bone marrow transplantation. Cancer Treat Rep 1985;69:473-477.

258. Thatcher N, Lind M, Morgenstern G, et al - High-dose, double alkylating agent chemotherapy with DTIC, melphalan or ifosfamide and marrow rescue for metastatic malignant melanoma. Cancer 1989;63:1296-1302.

259. Lakhani S, Selby P, Bliss JM, Perren TJ, Gore ME, McElwain TJ - Chemotherapy for malignant melanoma : combinations and high doses produce more response without survival benefit. Br J Cancer 1990;61:330-334.

260. Glover D, Glick J, Weiler C, et al - High dose cisplatin (DDP) and WR-2721 (WR) in metastatic melanoma. Proc Am Soc Clin Oncol 1987;5:574-578. cited in McClay EF, Mastrangelo MJ - Systemic chemotherapy for metastatic melanoma. Semin Oncol 1988;15:569-577.

261. McClay EF, Mastrangelo MJ - Systemic chemotherapy for metastatic melanoma. Semin Oncol 1988;15:569-577.

262. Del Prete SA, Maurer LH, O'Donnell J, Forcier RS, Lemarbre P - Combination chemotherapy with cisplatin, carmustine, dacarbazine and tamoxifen in metastatic melanoma. Cancer Treat Rep 1984;68:1403-1405.

263. McClay EF, Mastrangelo MJ, Bellet RE, et al - Combination chemotherapy and hormonal therapy in the treatment of malignant melanoma. Cancer Treat Rep 1987;71:465-469.

264. McClay EF, Mastrangelo MJ, Sprandio JD, Bellet RE, Berd D - The importance of tamoxifen to a cisplatin-containing regimen in the treatment of metastatic melanoma. Cancer 1989;63:1292-1295.

265. McClay ET, Mastrangelo MJ, Berd D, Bellet RE - Effective combination of chemo/hormonal therapy for malignant melanoma : experience with three consecutive trials. Int J Cancer 1992;50:553-556.

266. Reintgen D, Saba H - Chemotherapy for Stage 4 melanoma : a three-year experience with cisplatin, DTIC, BCNU, and tamoxifen. Semin Surg Oncol 1993;9:251-255.

267. Fierro MT, Bertero M, Novelli M, Appino A, Doveil GC, Coconna S - Therapy for metastatic melanoma : effective combination of dacarbazine, carmustine, cisplatin and tamoxifen. Melanoma Res 1993;3:127-131.

268. Crowell EB Jr, Higa GM - The chemohormonal therapy of metastatic melanoma : possible benefits of tamoxifen. W V Med J 1993;89:233-235.

269. Cohen SM, Ohnuma T, Ambinder EP, Holland JF - Lomustine, bleomycin and cisplatin in patients with metastatic malignant melanoma. Cancer Treat Rep 1986;70:688-689.

270. Young DW, Lever RS, English JSC, MacKie RM - The use of BELD combination chemotherapy (bleomycin, vindesine, CCNU, DTIC) in advanced malignant melanoma. Cancer 1985;55:1879-1881.

271. Stables GI, Doherty VR, MacKie RM - Nine years' experience of BELD combination chemotherapy (Bleomycin, vindesine, CCNU and DTIC) for metastatic melanoma. Br J Dermatol 1992;127:505-508.

272. Gundersen S - Dacarbazine, vindesine, and cisplatin combination chemotherapy in advanced malignant melanoma : a Phase II study. Cancer Treat Rep 1987;71:997-999.

273. Carmo-Pereira J, Costa FO, Henriques E - Combination cytotoxic chemotherapy with procarbazine, vincristine and lomustine (POC) in disseminated malignant melanoma : 8 years follow up. Cancer Treat Rep 1984;68:1211-1214.

274. Shelly W, Quirt I, Bodurtha A, et al - Lomustine, vincristine and procarbazine in the treatment of metastatic malignant melanoma. Cancer Treat Rep 1985;69:941-944.

275. Chawla SP, Legha SS, Benjamin RS - Detrorubicin - an active anthracycline in untreated metastatic melanoma. J Clin Oncol 1985;11:1529-1534.

276. Einzig A, Trump DL, Sasloff J - Phase II pilot study of taxol in patients with malignant melanoma. Proc Am Soc Clin Oncol 1988;7:249.

277. Legha SS, Ring S, Papadopoulos N, Raber M, Benjamin RS - A phase II trial of taxol in metastatic melanoma. Cancer 1990;65:2478-2481.

278. Bellet RE, Catalano RB, Mastrangelo MJ, Berd D - Positive phase II trial of dibromodulcitol in patients with metastatic melanoma refractory to DTIC and a nitrosourea. Cancer Treat Rep 1978;62:2095-2099.

279. Simmonds MA, Lipton A, Harvey HA, Ellison N, White DS - Phase II study of mitolactol in metastatic malignant melanoma. Cancer Treat Rep 1985;69:65-67.

280. Jacquillat C, Khayat D, Banzett P, et al - Chemotherapy by fotemustine in cerebral metastases of disseminated malignant melanoma. Cancer Chemother Pharmacol 1990;25:263-266.

281. Aamdal S, Radford J, Thatcher N, D'Incalci M, Gerard B - Phase II trial of the sequential administration of DTIC and fotemustine in advanced malignant melanoma. Proc Am Assoc Cancer Res 1990;31:200.

282. Nolte H, Gjedde SB, Lindegaard-Madsen E, Bergh J, Blomquist E, Mouridsen HT - Phase II study of tauromustine in disseminated malignant melanoma. Eur J Cancer Clin Oncol 1989;25:655-657.

283. Smyth JF, Gundersen S, Renard J, Pinedo HM - Randomised phase II trial of TCNU versus mitozolamide in malignant melanoma. Eur J Cancer Clin Oncol 1989;25:755-757.

284. Pezzuto JM, Shieh H-L, Shaughnessy E, Beattie CW - Approaches for drug development in treatment of advanced melanoma. Semin Oncol 1988;15:578-588.

285. Carrel S, Theilkaes L - Evidence for a tumour-associated antigen in malignant melanoma. Nature 1973;242:609-610.

286. Viza D, Phillips J - Identification of an antigen associated with malignant melanoma. Int J Cancer 1975;16:312-317.

287. Hollinshead AC - Analysis of soluble melanoma cell membrane antigens in metastatic cells of various organs and further studies of antigens present in primary melanoma. Cancer 1975;36:1282-1288.

288. Morton DL, Malmgren RA, Holmes EC, Ketcham AS - Demonstration of antibodies against human malignant melanoma by immunofluorescence. Surg 1968;64:233-240.

289. Romsdahl MM, Cox IS - Human malignant melanoma antibodies demonstrated by immunofluorescence. Arch Surg 1970;100:491-497.

290. Hellstrom I, Hellstrom KE, Sjogren HO, Warner GA - Demonstration of cell-mediated immunity to human neoplasms of various histologic types. Int J Cancer 1971;7:1-16.

291. Holmes EC, Roth JA, Morton DL - Delayed cutaneous hypersensitivity reaction to melanoma antigens. Surg 1975;78:160-164.

292. Mastrangelo MJ, Bellet RE, Berd D - Immunology and immunotherapy of human cutaneous malignant melanoma. in Clark WH, Goldman LI, Mastrangelo MJ eds. Human Malignant Melanoma. New York, Grune and Stratton, 1979; 355-416.

293. Morton DL, Eilber FR, Joseph WL, Wood WC, Trahan E, Ketcham AS - Immunological factors in human sarcomas and melanomas : a rational basis for immunotherapy. Ann Surg 1970;172:740-749.

294. Nathanson L - Regression of intradermal melanoma after intralesional injection of Mycobacterium bovis strain BCG. Cancer Chemother Rep 1972;56:659-665.

295. Mastrangelo MJ, Sulit HL, Prehn LM, Bornstein RS, Yarbro JW, Prehn RT - Intralesional BCG in the treatment of metastatic malignant melanoma. Cancer 1976;37:684-692.

296. Morton DL, Foshag LJ, Nizze JA, et al - Active specific immunotherapy in malignant melanoma. Semin Surg Oncol 1989;5:420-425.

297. Mastrangelo MJ, Schultz S, Kane M, Berd D - Newer immunologic approaches to the treatment of patients with melanoma. Semin Oncol 1988;15:589-594.

298. McIlmurray MB, Embleton MJ, Reeves WG, Langman MJS, Deane M - Controlled trial of active immunotherapy in management of Stage IIB malignant melanoma. Br Med J 1977;1:540-542.

299. Hedley DW, McElwain TJ, Curria GA - Specific active immunotherapy does not prolong survival in surgically treated patients with Stage IIB malignant melanoma and may promote early recurrence. Br J Cancer 1978;37:491-496.

300. Rosenberg SA, Lotze MT, Yang JC, et al - Experience with the use of high dose interleukin-2 in the treatment of 652 cancer patients. Ann Surg 1989;210:474-483.

301. Thatcher N, Dazzi H, Johnson RJ, et al - Recombinant interleukin-2 (rIL-2) given intrasplenically and intravenously for advanced malignant melanoma. A phase I and II study. Br J Cancer 1989;60:770-774.

302. Dutcher JP, Creekmore S, Weiss GR, et al - A phase II study of interleukin-2 and lymphokine-activated killer cells in patients with metastatic malignant melanoma. J Clin Oncol 1989;7:477-485.

303. West WH - Clinical application of continuous infusion of recombinant interleukin-2. Eur J Cancer Clin Oncol 1989;25(suppl.3):11-15.

304. Parkinson DR, Abrams JS, Wiernik PH, et al - Interleukin-2 therapy in patients with metastatic malignant melanoma : a phase II study. J Clin Oncol 1990;8:1650-1656.

305. Habeshaw T, Wheldon TE - Malignant melanoma : the role of radiotherapy. in MacKie RM ed. Clinics in Oncology : Melanoma. London, Saunders publishers, 1984: 571-595.

306. Wheldon TE - Optimal fractionation for the radiotherapy of tumour cells possessing wide-shouldered survival curves. Br J Radiol 1979;52:417-418.

307. Habermalz HJ, Fischer JJ - Radiation therapy of malignant melanoma : experience with high individual treatment doses. Cancer 1976;38:2258-2262.

308. Hornsey S - The relationship between total dose, number of fractions and fraction size in the response of malignant melanoma in patients. Br J Radiol 1978;51:905-909.

309. Strauss AJ, Dritschilo A, Nathanson L, Piro AJ - Radiation therapy of malignant melanomas : an evaluation of clinically used fractionation schemes. Cancer 1981;47:1262-1266.

310. Oberley LW, Buettner GR - Role of superoxide dismutase in cancer : a review. Cancer Res 1979;39:1141-1149.

311. Katz HR - The results of different fractionation schemes in the palliative irradiation of metastatic melanoma. Int J Radiat Oncol Biol Phys 1981;7:907-911.

312. Hilaris BS, Raben M, Calabrese AS, Phillips RF, Henschke UK - Value of radiation therapy for distant metastases from malignant melanoma. Cancer 1963;16:765-773.

313. Wagstaff J, Thatcher N, Rankin E, Crowther D - Tamoxifen in the treatment of metastatic malignant melanoma. Cancer Treat Rep 1982;66:1771.

314. Beecher R, Kloke O, Hoffken K, et al - Phase II study of high dose medroxyprogesterone acetate in advanced malignant melanoma. Br J Cancer 1989;59:948.

315. McClay EF, Albright KD, Jones JA, Christen RD, Howell SB - Tamoxifen modulation of cisplatin sensitivity in human malignant melanoma cells. Cancer Res 1993;53:1571-1576.

316. Karakousis CP, Didolkar MS, Lopez R, Baffi R, Moore R, Holyoke ED - Chemoimmunotherapy (DTIC and Corynebacterium parvum) as adjuvant treatment in malignant melanoma. Cancer Treat Rep 1979;63:1739-1743.

317. Hill GJ (II), Moss SE, Golomb FM, et al - DTIC and combination therapy for melanoma : III. DTIC (NSC-45388) surgical adjuvant study COG protocol 7040. Cancer 1981;47:2556-2562.

318. Jacquillat C, Banzet P, Maral J - Clinical trials of chemotherapy and chemoimmunotherapy in primary malignant melanoma. Rec Results Cancer Res 1982;80:254-258.

319. Beretta G - Trial 6 : randomized study of prolonged chemotherapy, immunotherapy, and chemoimmunotherapy as an adjuvant to surgery for Stage I and Stage II melanoma : a progress report. Rec Results Cancer Res 1982;80:259-263.

320. Veronesi U, Adamus J, Aubert C, et al - A randomized trial of adjuvant chemotherapy and immunotherapy in cutaneous melanoma. N Engl J Med 1982;307:913-916.

321. Knost JA, Reynolds V, Greco FA, Oldham RK - Adjuvant chemoimmunotherapy Stage I/II malignant melanoma. J Surg Oncol 1982;19:165-170.

322. Balch CM, Murray D, Presant C, Bartolucci AA (and the South Eastern Cancer Study Group) - Ineffectiveness of adjuvant chemotherapy using DTIC and cyclophosphamide in patients with resectable metastatic melanoma. Surg 1984;95:454-459.

323. Creagan ET, Cupps RE, Ivins JC, et al - Adjuvant radiation therapy for regional nodal metastases from malignant melanoma : a randomized prospective study. Cancer 1978;42:2206-2210.

324. Klopp CT, Alford TC, Bateman J, Berry GN, Winship T - Fractionated intra-arterial cancer chemotherapy with methyl bis-amine hydrochloride; a preliminary report. Ann Surg 1950;132:811-832.

325. Bierman HR, Shimkin MB, Byron RL, Miller ER - The effects of intraarterial administration of nitrogen mustard. in Maisin J ed. Fifth International Cancer Congress, Paris 1950;186-187[abst].

326. Donovan TJ - The uses of plastic tubes in the reparative surgery of battle injuries to arteries with and without intraarterial heparin administration. Ann Surg 1949;130:1024-1043.

327. Creech O Jr, Krementz ET, Ryan RF, Winblad JN - Chemotherapy of cancer : regional perfusion utilizing an extracorporeal circuit. Ann Surg 1958;148:616-632.

328. Ryan RF, Krementz ET, Creech O Jr, Winblad JN, Chamblee W, Cheek H - Selected perfusion of isolated viscera with chemotherapeutic agents using an extracorporeal circuit. Surg Forum 1958;8:158-161.

329. Ryan RF, Winblad JN, Hottinger GC, Creech O Jr - Effects of various perfusates in isolated vascular beds using an extracorporeal circuit. Surg Forum 1958;9:194-196.

330. Hales DR, Hall BE, Franco J, Jerner RW, Willett FM, Feichtmeier TV - p-di(2 chloroethyl)amino-L-phenylalanine in the treatment of metastatic melanoma. Proc Am Assoc Cancer Res 1957;2:210.

331. Creech O Jr, Krementz ET, Ryan RF, Reemtsma K, Winblad JN - experience with isolation perfusion technics in the treatment of cancer. Ann Surg 1959;149:627-640.

332. Creech O Jr, Krementz ET - Cancer chemotherapy by perfusion. Adv Cancer Res 1961;6:111-147.

333. Stehlin JS, Clark RL, Dewey WC - Continuous monitoring of leakage during regional perfusion. Arch Surg 1961;83:943-949.

334. Westbury G - Regional chemotherapy in malignant disease. Ann Roy Coll Surg Eng 1963;32:358-379.

335. Alexander P, Stacey KA - Comparison of the changes produced by ionising radiations and by the alkylating agents : evidence for a similar mechanism at the molecular level. Ann NY Acad Sci 1958;68:1225-1237.

336. Gray LH, Conger AD, Ebert M, Hornsey S, Scott OCA - The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. Br J Radiol 1953;26:638-648.

337. Churchill-Davidson I, Sanger C, Thomlinson RH - High pressure oxygen and radiotherapy. Lancet 1955(i):1091-1095.

338. Gray LH - Oxygenation in radiotherapy. I. Radiobiological considerations. Br J Radiol 1957;30:403-406.

339. Churchill-Davidson I, Sanger C, Thomlinson RH - Oxygenation in radiotherapy. II. Clinical applications. Br J Radiol 1957;30:406-422.

340. Krementz ET, Knudson L - The effect of increased oxygen tension on the tumoricidal effect of nitrogen mustard. Surg 1961;50:266-273.

341. Busch W - Uber den Einfluss welchen heftigere Erysipeln zuweilen auf organisierte Neubildungen ausuben. Verhandl Natur Preuss Rhein Westphal 1866;23:28-30.

342. Bruns P - Die Heitwirkung des Erysipels auf Geschwulste. Beitr Klin Chir 1887;3:443-466.

343. Coley WB - The treatment of malignant tumours by repeated inoculation of erysipelas - with a report of ten original cases. Am J Med Sci 1893;105:487-511.

344. Westermark H - Uber die Behandlung ulcerierender Cervix Carcinoma mittels konstanter Warme. Centralb f Gyn 1898;222:1335-1339.

345. Percy JF - Heat in the treatment of carcinomas of the uterus. Surg Gynecol Obstet 1916;22:77-79.

346. Nauts HC, Fowler GA, Bogatko FH - A review of the influence of bacterial infection and bacterial products (Coley's toxin) in malignant tumours in men. Acta Med Scand 1953;suppl.276:1-103.

347. Rohdenburg GL, Prime F - Effect of combined radiation and heat on neoplasms. Arch Surg 1921;2:116-129.

348. Johnson HL - The action of short radiowaves on tissues - III. A comparison of the thermal sensitivities of transplantable tumours in vivo and in vitro. Am J Cancer 1940;38:533-550.

349. Burger FJ, Fuhrman FA - Evidence of injury by heat in mammalian tissue. Am J Physiol 1964;206:1057-1061.

350. Burger FJ, Fuhrman FA - Evidence of injury to tissues after hyperthermia. Am J Physiol 1964;206:1062-1064.

351. Cavaliere R, Ciocatto EC, Giovanella BC, et al - Selective heat sensitivity of cancer cells. Biochemical and clinical studies. Cancer 1967;20:1351-1381.

352. Stehlin JS - Hyperthermic perfusion with chemotherapy for cancers of the extremities. Surg Gynecol Obstet 1969;129:305-308.

353. Stehlin JS Jr, Giovanella BC, de Ipolyi PD, Muenz LR, Anderson RF - Results of hyperthermic perfusion for melanoma of the extremities. Surg Gynecol Obstet 1975;140:339-348.

354. Krementz ET, Carter RD, Sutherland CM, Campbell M - The use of regional chemotherapy in the management of malignant melanoma. World J Surg 1979;3:289-304.

355. Martijn H, Koops HS, Milton GW, et al - Comparison of two methods of treating primary malignant melanomas Clark IV and V, thickness 1.5mm and greater, localised on the extremities. Cancer 1986;57:1923-1930.

356. Stehlin JS Jr - Perfusion for melanoma of the extremities : $6^1/_2$ years experience with 221 cases. Proc Natl Cancer Conf 1964;5:525-531.

357. Rochlin DB, Smart CR - Treatment of malignant melanoma by regional perfusion. Cancer 1965;18:1544-1550.

358. Irvine WT, Luck RJ - Review of regional limb perfusion with melphalan for malignant melanoma. Br Med J 1966;1:770-774.

359. Au FC, Goldman LI - Isolation perfusion in limb melanoma : a critical assessment and literature review. in Clark WH, Goldman LI, Mastrangelo MJ eds. Human Malignant Melanoma. New York, Grune and Stratton, 1979; 295-308.

360. Hansson JA, Simert G, Vang J - The effect of regional perfusion treatment on recurrent melanoma of the extremities. Acta Chir Scand 1977;143:33-39.

361. Rosin RD, Westbury G - Isolated limb perfusion for malignant melanoma. The Practitioner 1980;224:1031-1036.

362. Lejeune FJ, Deloof T, Ewalenko P, et al - Objective regression of unexcised melanoma in-transit metastases after hyperthermic isolation perfusion of the limbs with melphalan. Rec Results Cancer Res 1983;86:268-276.

363. Jonsson PE, Hafstrom L, Hugander A - Results of regional hyperthermic perfusion for primary and recurrent melanoma of the extremities. Rec Results Cancer Res 1983;86:277-282.

364. Vaglini M, Andreola S, Attili A, et al - Hyperthermic antiblastic perfusion in the treatment of cancer of the extremities. Tumori 1985;71:355-359.

365. Storm FK, Morton DL - Value of therapeutic hyperthermic limb perfusion in advanced recurrent melanoma of the lower extremity. Am J Surg 1985;150:32-35.

366. Cavaliere R, Calabro A, di Filippo F, Carlini S, Giannarelli D - Prognostic parameters in limb recurrent melanoma treated with hyperthermic antiblastic perfusion [abst]. 3rd ICRCT, 1987;G7:163.

367. Kroon BBR, van Geel AN, Benckhuijsen C, Wieberdink J - Normothermic isolation perfusion with melphalan for advanced melanoma of the limbs. Anticancer Res 1987;7:441-442.

368. Santinami M, Belli F, Cascinelli N, Rovini D, Vaglini M - Seven years experience with hyperthermic perfusion in extracorporeal circulation for melanoma of the extremities. J Surg Oncol 1989;42:201-208.

369. Skene AI, Bulman AS, Williams TR, Meirion Thomas J, Westbury G - Hyperthermic isolated perfusion with melphalan in the treatment of advanced malignant melanoma of the lower limb. Br J Surg 1990;77:765-767.

370. Hayward JL, Carbone PP, Heuson J-C, Kumaoka S, Segaloff A, Rubens RD - Assessment of response to therapy in advanced breast cancer. Cancer 1977;39:1289-1294.

371. World Health Organization - Handbook for reporting results of cancer. Reprint in : Neoplasma 1980;27:607-619.

372. Watson JV - What does "response" in cancer chemotherapy really mean ? Br Med J 1981;283:34-37.

373. Minor DR, Allen RE, Alberts D, Peng Y-M, Tardelli G, Hutchinson J - A clinical and pharmacokinetic study of isolated limb perfusion with heat and melphalan for melanoma. Cancer 1985;55:2638-2644.

374. Martijn H, Oldhoff J, Schraffordt Koops H - Regional perfusion in the treatment of patients with a locally metastasized malignant melanoma of the limbs. Eur J Cancer 1981;17:471-476.

375. Krementz ET, Ryan RF - Chemotherapy of melanoma of the extremities by perfusion : fourteen years clinical experience. Ann Surg 1972;175:900-917.

376. Lejeune FJ, Lienard D, El Douaihy M, Vadoud Seyedi J, Ewalenko P - Results of 206 isolated limb perfusions for malignant melanoma. Eur J Surg Oncol 1989;15:510-519.

377. Ghussen F, Nagel K, Groth W, Muller JM, Stutzer H - A prospective randomized study of regional extremity perfusion in patients with malignant melanoma. Ann Surg 1984;200:764-768.

378. Ghussen F, Kruger I, Groth W, Stutzer H - The role of regional hyperthermic cytostatic perfusion in the treatment of extremity melanoma. Cancer 1988;61:654-659.

379. Ghussen F, Kruger I, Smalley RV, Groth W - Hyperthermic perfusion with chemotherapy for melanoma of the extremities. World J Surg 1989;13:598-602.

380. Shingleton WW, Seigler HF, Stocks LH, Downs RW - Management of recurrent melanoma of the extremity. Cancer 1975;35:574-579.

381. Stehlin JS, Giovanella BC, de Ipolyi PD, Anderson RF - Eleven years experience with hyperthermic perfusion for melanoma of the extremities. World J Surg 1979;3:305-307.

382. Bulman AS, Jamieson CW - Isolated limb perfusion with melphalan in the treatment of malignant melanoma. Br J Surg 1980;67:660-662.

383. Krementz ET - Regional perfusion. Current sophistication, what next ? The Lucy Wortham James lecture. Cancer 1986;57:416-432.

384. Hartley JW, Fletcher WS - Improved survival of patients with Stage II melanoma of the extremity using hyperthermic isolation perfusion with L-phenylalanine mustard. J Surg Oncol 1987;36:170-174.

385. Karakousis CP, Choe KJ, Holyoke ED - Biologic behaviour and treatment of in-transit metastasis of melanoma. Surg Gynecol Obstet 1980;150:29-32.

386. Sugarbaker EV, McBride CM - Survival and regional disease control after isolation perfusion for invasive Stage I melanoma of the extremities. Cancer 1976;37:188-198.

387. Wagner DE - A retrospective study of regional perfusion for melanoma. Arcg Surg 1976;111:410-413.

388. Rege VB, Leone LA, Soderberg CH, et al - Hyperthermic adjuvant perfusion chemotherapy for Stage I malignant melanoma of the extremity with literature review. Cancer 1983;52:2033-2039.

389. Schraffordt Koops H, Beekhuis H, Oldhoff J, Oosterhuis JW, van der Ploeg E, Vermey A - Local recurrence and survival in patients with (Clark level IV/V and over 1.5mm thickness) Stage I malignant melanoma of the extremities after regional perfusion. Cancer 1981;48:1952-1957.

390. Janoff KA, Moseson D, Nohlgren J, Davenport C, Richards C, Fletcher WS - The treatment of Stage I melanoma of the extremities with regional hyperthermic isolation perfusion. Ann Surg 1982;196:316-321.

391. Franklin HR, Schraffordt Koops H, Oldhoff J, et al - To perfuse or not to perfuse ? A retrospective comparative study to evaluate the effect of adjuvant isolated regional perfusion in patients with Stage I extremity melanoma with a thickness of 1.5mm or greater. J Clin Oncol 1988;6:701-708.

392. Edwards MJ, Soong SJ, Boddie AW, Balch CM, McBride CM - Isolated limb perfusion for localised melanoma of the extremity. Arch Surg 1990;125:317-321.

393. Ghussen F - reply to letter from Monson JRT, Donohue JH. World J Surg 1990;14:845-846.

394. EORTC Newsletter. Letter 110. Haemmerli G ed. April 1983:7.

395. Cox KR - Survival after regional perfusion for limb melanoma. Aust NZ J Surg 1975;45:32-36.

396. Golomb FM - Perfusion of melanoma : 133 isolated perfusions in 114 patients. Panminerva Med 1976;18:8-10.

397. Aigner K, Hild P, Breithaupt H, et al - Isolated extremity perfusion with DTIC. An experimental and clinical study. Anticancer Res 1983;3:87-94.

398. Aigner K, Hild P, Henneking K, Paul E, Hundeiker M - Regional perfusion with cis-platinum and dacarbazine. Rec Results Cancer Res 1983;86:239-245.

399. Aigner K, Jungbluth A, Link KH, et al - Die isolierte hypertherme Extremitaten Perfusion mit Vindesin, Dacarbazine und Cis-Platin bei der Behandlung maligner Melanoma. Onkologie 1984;7:348-353.

400. Shiu MK, Knapper WH, Fortner JG, et al - Regional isolated limb perfusion of melanoma in-transit metastases using mechlorethamine (nitrogen mustard). J Clin Oncol 1986;4:1819-1826.

401. Vaglini M, Belli F, Marolda R, Prada A, Santinami M, Cascinelli N - Hyperthermic antiblastic perfusion with DTIC in Stage IIIA-IIIAB melanoma of the extremities. Eur J Surg Oncol 1987;13:127-129.

402. Pfefferkorn RO, Didolkar MS - Regional perfusion for melanoma of the extremities. J Extracorp Technol 1982;14:475-479.

403. Lienard D, Lejeune FJ, Ewalenko P - In transit metastases of malignant melanoma treated by high dose rTNF-alpha in combination with Interferon-gamma and melphalan in isolation perfusion. World J Surg 1992;16:234-240.

404. Vaglini M, Belli F, Ammatuna M, et al - Treatment of primary or relapsing limb cancer by isolated perfusion with high-dose alpha-tumour necrosis factor, gamma-interferon, and melphalan. Cancer 1994;73:483-492.

405. Schraffordt Koops H - Prevention of neural and muscular lesions during hyperthermic perfusion. Surg Gynecol Obstet 1972;135:401-403.

406. Wieberdink J, Benckhuijsen C, Braat RP, van Slooten EA, Olthuis GAA - Dosimetry in isolation perfusion of the limb by assessment of perfused tissue volume and grading of toxic tissue reactions. Eur J Cancer Clin Oncol 1982;18:905-910.

407. Schraffordt Koops H, Oldhoff J, van der Ploeg E, Vermey A, Eibergen R, Beekhuis H - Some aspects of the treatment of primary malignant melanoma of the extremities by isolated regional perfusion. Cancer 1977;39:27-33.

408. Peto R, Pike MC, Armitage P, et al - Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. Analysis and examples. Br J Cancer 1977;35:1-39.

409. Krementz ET, Kokame GM - Current status of regional chemotherapy by perfusion. Am J Surg 1963;105:598-606.

410. Krige JEJ, King HS, Strover RM - Prophylactic hyperthermic limb perfusion in Stage I melanoma. Eur J Surg Oncol 1988;14:321-326.

411. Kroon BBR - Regional isolation perfusion in melanoma of the limbs; accomplishments, unsolved problems, future. Eur J Surg Oncol 1988;14:101-110.

412. Klein ES, Ben-Ari GY - Isolation perfusion with cisplatin for malignant melanoma of the limbs. Cancer 1987;59:1068-1071.

413. Ganong WF - Cardiovascular homeostasis in health and disease. in Ganong WF. Review of Medical Physiology, 11th edition. Los Altos, California; Lange Medical Publications, 1983; 506-519.

414. Rodbard S - Physics of blood flow. in Abrahams DI ed. Blood Vessels and Lymphatics, London Academic Press 1962; Chapter 2, Section 2.

415. Wieberdink J - Physiological considerations regarding isolation perfusion of the extremities (with special reference to regional chemotherapy of cancer). Netherlands Cancer Institute monograph; Amsterdam, the Netherlands, 1978.

416. Fontijne WPJ, Mook PH, Schraffordt Koops H, Oldhoff J, Wildevuur CRH - Improved tissue perfusion during pressure regulated hyperthermic regional isolated perfusion. A clinical study. Cancer 1985;55:1455-1461.

417. Lubbers DW - Theoretical basis of the transcutaneous blood measurements. Crit Care Med 1981;9:721-733.

418. Grossmann U, Huber J, Fricke R, et al - A new model for simulating oxygen pressure field of skin. in Kovach AGB, Dova Kessler M, Silver JA eds. Oxygen Transport to Tissue. Budapest, Pergamon-Press, 1981; 319-320.

419. Clark LC - Monitor and control of blood and tissue oxygen tensions. Trans Am Soc Artf Intern Organs 1956;2:41-48.

420. Evans NTS, Naylor PFD - The dynamics of changes in dermal oxygen tension. Resp Physiol 1966;2:61-72.

421. Evans NTS, Naylor PFD - The systemic oxygen supply to the surface of human skin. Resp Physiol 1967;3:21-37.

422. Eberhardt P, Mindt W, Jann F, Hammacher K - Continuous PO_2 monitoring in the neonate by skin electrodes. Med Biol Eng Comput 1975;13:436-442.

423. Huch A, Huch R - The development of the transcutaneous PO_2 technique into a clinical tool. Birth Defects 1979;15:5-12.

424. Huch A, Huch R - Technical, physiological and chemical aspects of transcutaneous PO_2 measurements. in Taylor DEM, Whamond J eds. Noninvasive Clinical Measurement. London, Pitman, 1977; 171-185.

425. Tremper KK, Waxman K, Shoemaker WC - Effects of hypoxia and shock on transcutaneous PO_2 values in dogs. Crit Care Med 1979;7:526-531.

426. Tremper KK, Shoemaker WC - Transcutaneous oxygen monitorng of critically ill adults, with and without low flow shock. Crit Care Med 1981;9:706-709.
427. Beran AV, Tolle CD, Huxtable RF - Cutaneous blood flow and its relationship to transcutaneous $O_2\backslash CO_2$ measurements. Crit Care Med 1981;9:736-741.

428. Swift AC - Transcutaneous blood gas monitoring : part I. ITCM 1991;12:15-19.

429. Rithalia SVS - Transcutaneous blood gas and subcutaneous tissue pH monitoring. ITCM 1989;10:200-211.

430. Rooth G, Hedstrand ULF, Tyden H, Ogren C - The validity of the transcutaneous oxygen tension method in adults. Crit Care Med 1976;4:162-165.

431. Tremper KK, Waxman K, Bowman R, Shoemaker WC - Continuous transcutaneous oxygen monitoring during respiratory failure, cardiac decompensation, cardiac arrest and CPR : transcutaneous oxygen monitoring during arrest and CPR. Crit Care Med 1980;8:377-381.

432. McCollum PT, Spence VA, Walker WF - Oxygen inhalation induced changes in the skin as measured by transcutaneous oxymetry. Br J Surg 1986;73:882-885.

433. Swift AC - Transcutaneous blood gas monitoring : part II. ITCM 1991;12:24-27.

434. Severinghaus JW - Transcutaneous blood gas analysis. Resp Care 1982;27:152-159.

435. Linge K, Roberts DH, Dowd GSE - Indirect measurement of skin blood flow and transcutaneous oxygen tension in patients with peripheral vascular disease. Clin Phys Physiol Meas 1987;8:293-302.

436. Keller HP, Klave P, Hockerts T, Lubbers DW - Transcutaneous PO_2 measurements on skin transplants. Birth Defects 1979;15:511-516.

437. Dowd GSE, Linge K, Bentley G - Measurement of transcutaneous oxygen pressure in normal and ischaemic skin. J Bone Joint Surg 1983;65:79-83.

438. Byrne P, Provan JL, Ameli FM, Jones DP - The use of transcutaneous oxygen tension measurements in the diagnosis of peripheral vascular insufficiency. Ann Surg 1984;200:159-165.

439. Ohgi S, Ito K, Mori T - Quantitative evaluation of the skin circulation in ischaemic legs by transcutaneous measurement of oxygen tension. Angiology 1981;32:833-839.

440. Ito K, Ohgi S, Mori T, Urbanyi B, Schlosser V - Determination of amputation level in ischaemic legs by means of transcutaneous oxygen pressure measurement. Int Surg 1984;69:59-61.

441. Dowd GSE - Predicting stump healing following amputations for peripheral vascular disease using the transcutaneous oxygen monitor. Ann R Coll Surg Engl 1987;69:31-35.

442. Evans NTS, Naylor PFD - The oxygen tension gradient across human epidermis. Resp Physiol 1967;3:38-42.

443. Fontijne WPJ, de Vries J, Mook PH, et al - Improved tissue perfusion during pressure-regulated hyperthermic regional isolated perfusion in dogs. J Surg Oncol 1984;26:69-76.

444. Fontijne WPJ, Mook PH, Elstrodt JM, Schraffordt Koops H, Oldhoff J, Wildevuur CRH - Isolated hindlimb perfusion in dogs : the effect of perfusion pressures on the oxygen supply (p_tO_2 histogram) to the skeletal muscle. Surgery 1985;97:278-284.

445. Furner RL, Brown RK, Duncan G - Pharmacokinetics of the absorption, distribution and elimination of melphalan in the dog. Cancer Treat Rep 1977;61:1637-1696.

446. Chang SY, Alberts DS, Melnick LR, Walson PD, Salmon SE - High pressure liquid chromatographic analysis of melphalan in plasma. J Pharm Sci 1978;67:679-682.

447. Flora KP, Smith SL, Cradock JC - Application of a simple high-performance liquid chromatographic method for the determination of melphalan in the presence of its hydrolysis products. J Chromatogr 1979;177:91-97.

448. Scott RN, Kerr DJ, Blackie R, et al - The pharmacokinetic advantages of isolated limb perfusion with melphalan for malignant melanoma. Br J Cancer 1992;66:159-166.

449. Ross WE, Ewig RAG, Kohn KW - Differences between melphalan and nitrogen mustard in the formation and removal of DNA cross-links. Cancer Res 1978;38:1502-1506.

450. Zwelling LA, Michaels S, Schwartz H, Dobson PP, Kohn KW - DNA cross-linking as an indicator of sensitivity and resistance of mouse L1210 leukaemia to cis-diaminedichloroplatinum (II) and L-phenylalanine mustard. Cancer Res 1981;41:640-649.

451. Hansson J, Lewensohn R, Ringborg U, Nilsson B - Formation and removal of DNA cross-links induced by melphalan and nitrogen mustard in relation to drug-induced cytotoxicity in human melanoma cells. Cancer Res 1987;47:2631-2637.

452. Kohn KW - Molecular mechanisms of cross-linking by alkylating agents and platinum complexes. in Sartorelli CA, Lazlo JS, Bertino JR eds. Molecular Actions and Targets for Cancer Chemotherapeutic Agents. New York Academic Press 1981; 3-16.

453. Goldenberg GJ, Lee M, Lam H-Y P, Begleiter A - Evidence for carrier-mediated transport of melphalan by L5178Y lymphoblasts in vitro. Cancer Res 1977;37:755-760.

454. Vistica DT, Toal JN, Rabinovitz M - Amino acid-conferred protection against melphalan : characterization of melphalan transport and correlation of uptake with cytotoxicity in L1210 murine leukaemia cells. Biochem Pharmacol 1978;27:2865-2870.

455. Begleiter A, Lam H-Y P, Grover J, Froese E, Goldenberg GJ - Evidence for active transport of melphalan by two amino acid carriers in L5178Y lymphoblasts in vitro. Cancer Res 1979;39:353-359.

456. Redwood WR, Colvin M - Transport of melphalan by sensitive and resistant L1210 cells. Cancer Res 1980;40:1144-1149.

457. Begleiter A, Froese EK, Goldenberg GJ - A comparison of melphalan transport in human breast cancer cells and lymphocytes in vitro. Cancer Lett 1980;10:243-251.

458. Briele HA, Djuric M, Jung DT, Mortell T, Patel MK, Das Gupta TK - Pharmacokinetics of melphalan in clinical isolation perfusion of the extremities. Cancer Res 1985;45:1885-1889.

459. Benckhuijsen C, Kroon BBR, van Geel AN, Wieberdink J - Regional perfusion treatment with melphalan for melanoma in a limb : an evaluation of drug kinetics. Eur J Surg Oncol 1988;14:157-163.

460. Stotter AT, Robinson B, Clutterbuck R, et al - A comparison of melphalan levels achieved in isolated limb perfusion and systemic high dose therapy. J Exp Clin Cancer Res 1987;6:161-165.

461. Scott RN, Blackie R, Kerr DJ, et al - Melphalan concentration and distribution in the tissues of tumour-bearing limbs treated by isolated limb perfusion. Eur J Cancer 1992;28A:1811-1813.

462. Klaase JM, Kroon BBR, Beijnen JH, van Slooten G, van Dongen JA - Melphalan tissue concentrations in patients treated with regional isolated perfusion for melanoma of the lower limb. in Regional Isolated Perfusion for Melanoma of the Extremities. Klaase JM (thesis); Netherlands Cancer Institute, Amsterdam, the Netherlands, 1993; 151-155.

463. Goodman GE, Chang SY, Alberts DS - The antitumour activity of melphalan and its hydrolysis products. Proc Am Assoc Cancer Res (suppl. to Cancer Res) 1980;21:301.

464. Warren B - The vascular morphology of tumours. in Peterson HI ed. Tumour blood circulation : angiogenesis, vascular morphology and blood flow of experimental and human tumours. Florida 1979, CRC Press; 1-47.

465. Lindgreen AGH - The vascular supply of tumours with special reference to the capillary angioarchitecture. Acta Pathol Microbiol Scand 1945;22:493-520.

466. Natadze TG - Regulation of blood circulation in malignant tumours. Probl Oncol 1959;5:14-23.

467. Kligerman MM, Henel DK - Some aspects of the microcirculation of a transplantable experimental tumour. Radiol 1961;76:810-817.

468. Kruuv JA, Inch WR, McCredie JA - Blood flow and oxygenation of tumours in mice. II. Effects of vasodilator drugs. Cancer 1967; 20:60-65.

469. Krylova NV - Characteristics of microcirculation in experimental tumours. Bibl Anatom 1969;10:301-303.

470. Papadimitriou JW, Woods AE - Structural and functional characteristics of the microcirculation in neoplasms. J Path 1975;116:65-72.

471. Krylova NV - Microcirculatory mechanisms in experimental tumours. Bibl Anatom 1977;15:285-287.

472. Ganong WF - Dynamics of blood and lymph flow. in Ganong WF. Review of Medical Physiology, 11th edition; Los Altos, California, Lange Medical Publications, 1983; 462-476.

473. Algire GH, Legallais FY - Vascular reactions of normal and malignant tissues in vivo. IV. The effect of peripheral hypotension on transplanted tumors. J Natl Cancer Inst 1951;12:399-421.

474. Cater DB, Grigson CMB, Watkinson DA - Changes of oxygen tension in tumours induced by vasoconstrictor and vasodilator drugs. Acta Radiol 1962;58:401-408.

475. Jirtle R, Clifton KH, Rankin JHG - Effects of several vasoactive drugs on the vascular resistance of MT-W9B tumors in W/Fu rats. Cancer Res 1978;38:2385-2390.

476. Debreczeni LA, Farsang C, Takacs L - Effect of phenoxybenzamine, propranolol, phenylephrine and isoproterenol on the circulation of rats bearing Guerin carcinoma. Acta Phys Acad Sci Hungar 1980;56:341-365.

477. Mattson J, Lilja J, Peterson HI - Influence of vasoactive drugs on local tumor blood flow. Eur J Cancer Clin Oncol 1982;18:677-684.

478. Voorhees WD (II), Babbs CF - Hydralazine-enhanced selective heating of transmissible venereal tumor implants in dogs. Eur J Cancer Clin Oncol 1982;19:1027-1033.

479. Kaelin WG, Shrivastav S, Shand DG, Jirtle RL - The effect of verapamil on malignant tissue blood flow in SMT-2A tumor-bearing rats. Cancer Res 1982;42:3944-3949.

480. Chan RC, Babbs CF, Vetter RJ, Lamar CH - Abnormal response of tumor vasculature to vasoactive drugs. J Natl Cancer Inst 1984;72:145-150.

481. Nugent LJ, Jain RK - Extravascular diffusion in normal and neoplastic tissue. Cancer Res 1984;44:238-244.

482. Stratford IJ, Adams GE, Godden J, Nolan J, Howells N, Timpson N - Potentiation of the antitumour effect of melphalan by the vasoactive agent, hydralazine. Br J Cancer 1988;58:122-127.

483. Robinson BA, Clutterbuck RD, Millar JL, McElwain TJ - Verapamil potentiation of melphalan cytotoxicity and cellular uptake in murine fibrosarcomas and bone marrow. Br J Cancer 1985;52:813-822.

484. Robinson BA, Clutterbuck RD, Millar JL, McElwain TJ - Effects of verapamil and alcohol on blood flow, melphalan uptake and cytotoxicity in murine fibrosarcomas and human melanoma xenografts. Br J Cancer 1986;53:607-614.

485. Simpson WG, Tseng MT, Anderson KC, Harty JI - Verapamil enhancement of chemotherapeutic efficiency in human bladder cancer cells. J Urol 1984;132:574-576.

486. Ince P. Appleton DR, Finney KJ, Sunter JP, Watson AJ - Verapamil increases the sensitivity of primary human colorectal carcinoma tissue to vincristine. Br J Cancer 1986;53:137-139.

487. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y - Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res 1981;41:1967-1972.

488. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y - Increased accumulation of vincristine and adriamycin in drug-resistant P388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors. Cancer Res 1982;42:4730-4733.

489. Rogan AM, Hamilton TC, Young RC, Klecker RW Jr, Ozols RF - Reversal of adriamycin resistance by verapamil in human ovarian cancer. Science 1984;224:994-996.

490. Kessel D, Wilberding C - Mode of action of calcium antagonists which alter anthracycline resistance. Biochem Pharmacol 1984;33:1157-1160.

491. Twentyman PR, Fox NE, Bleehen NM - Drug resistance in human lung cancer cell lines : cross resistance studies and effects of the calcium transport blocker, verapamil. Int J Radiat Oncol Biol Phys 1986;12:1355-1358.

492. Merry S, Fetherston CA, Kaye SB, Freshney RI, Plumb JA - Resistance of human glioma to adriamycin in vitro : the role of membrane transport and its circumvention with verapamil. Br J Cancer 1986;53:129-135.

493. Chatterjee M, Robson CN, Harris AL - Reversal of multidrug resistance by verapamil and modulation by alpha$_1$-acid glycoprotein in wild-type and multidrug resistant chinese hamster ovary cell lines. Cancer Res 1990;50:2818-2822.

494. Creasey AA, Smith HS, Hackett AJ, Fukuyama K, Epstein WL, Madin SH - Biological properties of human melanoma cells in culture. In Vitro 1979;15:342-350.

495. West GW, Weichselbaum R, Little JB - Limited penetration of methotrexate into human osteosarcoma spheroids as a proposed model for solid tumour resistance to adjuvant chemotherapy. Cancer Res 1980;40:3665-3668.

496. Sutherland R, Carlsson J, Durand R, Yuhas J - Spheroids in cancer research. Cancer Res 1981;41:2980-2984.

497. Sutherland RM - Cell and environment interaction in tumor microregions : the muticell spheroid model. Science 1988;240:177-184.

498. Yuhas JM, Li AP, Martinez AO, Ladman AJ - A simplified method for production and growth of multicellular tumor spheroids. Cancer Res 1977:37:3639-3643.

499. Twentyman PR - Growth delay in small EMT6 spheroids induced by cytotoxic drugs and its modification by misonidazole pretreatment under hypoxic conditions. Br J Cancer 1982;45:565-570.

500. Mikhail RA, Boddie AW Jr, Ames FC, Zimmerman SO, McBride CM - Effect of variation of drug dosage on disease control and regional toxicity in prophylactic perfusion for Stage I extremity melanoma. J Surg Oncol 1984;27:215-218.

501. Schraffordt Koops H, Kroon BBR, Oldhoff J, Hoekstra HJ - Controversies concerning adjuvant regional isolated perfusion for Stage I melanoma of the extremities. World J Surg 1992;16:241-245.

502. Lejeune FJ, Ghanem GE - A simple and accurate new method for cytostatics dosimetry in isolation perfusion of the limbs based on exchangeable blood volume determination. Cancer Res 1987;47:639-643.

503. Boothby WM, Sandiford RB - Nomogram for determining body surface area of children and adults. Boston Med Surg J 1921;185:337 (reproduced by ICI Pharmaceuticals).

504. van Os J, Schraffordt Koops H, Oldhoff J - Dosimetry of cytostatics in hyperthermic regional isolated perfusion. Cancer 1985;55:698-701.

505. Wieberdink J, Benckhuijsen C, van der Esch EP, et al - Pharmacokinetics of melphalan. Annual Report of the Netherlands Cancer Institute 1983; Section VIII;4:174.

506. Luck JM - Action of p-{di(2-chloroethyl)}-amino-L-phenylalanine on Harding-Passey mouse melanoma. Science 1956;123:984-985.

507. Holland JF, Regelson W - Studies of phenylalanine nitrogen mustard (CB 3025) in metastatic malignant melanoma of man. Ann NY Acad Sci 1958;68:1122-1125.

508. Whittaker JR - Biosynthesis of a thiouracil pheomelanin in embryonic pigment cells exposed to thiouracil. J Biol Chem 1971;246:6217-6226.

509. Dencker L, Larsson B, Olander K, Ullberg S, Yokota M - False precursors of melanin as selective melanoma seekers. Br J Cancer 1979;39:449-452.

510. Dencker L, Larsson B, Olander K, Ullberg S, Yokota M - Incorporation of thiouracil and some related compounds into growing melanin. Acta Pharmacol Toxicol 1981;49:141-149.

511. Dencker L, Larsson B, Olander K, Ullberg S - A new melanoma seeker for possible clinical use : selective accumulation of radiolabelled thiouracil. Br J Cancer 1982;45:95-104.

512. Olander K, Larsson B, Dencker L - Thioamides as false melanin precursors : studies in murine melanomas. Acta Pharmacol Toxicol 1983;52:135-142.

513. Franken NAP, van Langevelde A, Pauwels EKJ, Journee de Korver JG, Bakker CNM, Oosterhuis JA - Radio-iodine-labelled 5-iodo-2-thiouracil : a potential radiopharmaceutical for establishing the viability of ocular melanoma after radiation therapy. Nucl Med Comm 1986;7:797-809.

514. Kohler G, Milstein C - Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975;256:495-497.

515. Pressman D - Radiolabelled antibodies. Ann NY Acad Sci 1957;69:644-650.

516. Ghose T, Blair AH - Antibody-linked cytotoxic agents in the treatment of cancer : current status and future prospects. J Natl Cancer Inst 1978;61:657-676.

517. Carrasquillo JA, Krohn KA, Beaumier P, et al - Diagnosis and therapy for solid tumours with radiolabelled antibodies and immune fragments. Cancer Treat Rep 1984;68:317-328.

518. Baldwin RW, Byers VS - Monoclonal antibodies in cancer treatment. Lancet 1986(i):603-605.

519. Epenetos AA, Britton KE, Mather S, et al - Targeting of iodine-123-labeled tumour-associated monoclonal antibodies to ovarian, breast, and gastrointestinal tumours. Lancet 1982(ii):999-1004.

520. Farrands PA, Perkins AC, Pimm MV, et al - Radioimmunodetection of human colorectal cancers by an anti-tumour monoclonal antibody. Lancet 1982(ii):397-400.

521. Smedley MM, Finan P, Lennox ES, et al - Localisation of metastatic carcinoma by radiolabeled monoclonal antibody. Br J Cancer 1983;47:253-259.

522. Larson SM, Brown JP, Wright PW, Carrasquillo JA, Hellstrom I, Hellstrom KE - Imaging of melanoma with I-131-labelled monoclonal antibodies. J Nucl Med 1983;24:123-129.

523. Mach J-P, Chatal J-F, Lumbroso J-D, et al - Tumor localization in patients by radiolabeled monoclonal antibodies against colon carcinoma. Cancer Res 1983;43:5593-5600.

524. Russell SJ, Llewelyn MB, Hawkins RE - Monoclonal antibodies in medicine : principles of antibody therapy. Br Med J 1992;305:1424-1429.

525. Herlyn DM, Steplewski Z, Herlyn MF, Koprowski H - Inhibition of growth of colorectal carcinoma in nude mice by monoclonal antibody. Cancer Res 1980;40:717-721.

526. Ritz J, Pesando JM, Sallan SE, et al - Serotherapy of acute lymhoblastic leukemia with monoclonal antibody. Blood 1981;58:141-152.

527. Koprowski H, Herlyn D, Lubeck M, de Freitas E, Sears HF - Human anti-idiotype antibodies in cancer patients : is the modulation of the immune response beneficial for the patient ? Proc Natl Acad Sci (USA) 1984;81:216-219.

528. Meeker TC, Lowder J, Maloney DG, et al - A clinical trial of anti-idiotype therapy for B-cell malignancy. Blood 1985;65:1349-1363.

529. Embleton MJ - Drug-targeting by monoclonal antibodies. Br J Cancer 1987;55:227-231.

530. Embleton MJ - Targeting of anti-cancer therapeutic agents by monoclonal antibodies. Biochem Soc Trans 1986;14:393-395.

531. Garnett MC, Embleton MS, Jacobs E, Baldwin RW - Preparation and properties of a drug-carrier-antibody conjugate showing selective antibody-directed cytotoxicity in vitro. Int J Cancer 1983;31:661-670.

532. Hawkins RE, Llewelyn MB, Russell SJ - Monoclonal antibodies in medicine : adapting antibodies for clinical use. Br Med J 1992;305:1348-1352.

533. Russell SJ, Llewelyn MB, Hawkins RE - The human antibody library : entering the next phage. Br Med J 1992;304:585-586.

534. Hale G, Dyer MJS, Clark MR, et al - Remission induction in non-Hodgkin lymphoma with reshaped human monoclonal antibody CAMPATH-1H. Lancet 1988(ii):1394-1399.

535. Eiklid K, Olsnes S, Pihl A - Entry of lethal doses of abrin, ricin and modeccin into the cytosol of HeLa cells. Exp Cell Res 1980;126:321-326.

536. Rowlinson-Busza G, Epenetos AA - Targeted delivery of biologic and other antineoplastic agents. Curr Opin Oncol 1992;4:1142-1148.

537. Kosmas C, Snook D, Gooden CS, et al - Development of humoral immune responses against a macrocyclic chelating agent (DOTA) in cancer patients receiving radioimmunoconjugates for imaging and therapy. Cancer Res 1992:52:904-911.

538. Kemshead JT, Hopkins K - Uses and limitations of monoclonal antibodies (MoAbs) in the treatment of malignant disease : a review. J R Soc Med 1993;86:219-224.

539. Wheldon TE, O'Donoghue JA, Hilditch TE, Barrett A - Strategies for systemic radiotherapy of micrometastases using antibody-targeted ^{131}I. Radiother Oncol 1988;11:133-142.

540. Buraggi GL, Callegaro L, Mariani G, et al - Imaging with ^{131}I-labeled monoclonal antibodies to a high-molecular weight melanoma associated antigen in patients with melanoma : efficacy of whole immunoglobulin and its $F(ab')_2$ fragments. Cancer Res 1985;45:3378-3387.

541. Brown JP, Hewick RM, Hellstrom I, Hellstrom KE, Doolittle RF, Dreyer WJ - Human melanoma-associated antigen p97 is structurally and functionally related to transferrin. Nature 1982;296:171-173.

542. Larson SM - Lymphoma, melanoma, colon cancer : diagnosis and treatment with radiolabeled monoclonal antibodies. Radiology 1987;165:297-304.

543. Lotze MT, Carrasquillo JA, Weinstein JN, et al - Monoclonal antibody imaging of human melanoma. Ann Surg 1986;204:223-235.

544. Kirkwood JM, Neumann RD, Zoghbi SS, et al - Scintigraphic detection of metastatic melanoma using indium-111/DTPA conjugated anti-gp240 antibody (ZME-018). J Clin Oncol 1987;5:1247-1255.

545. Elliott AT, MacKie RM, Murray T, Doherty VR, Adams FG - A comparative study of the relative sensitivity and specificity of radiolabelled monoclonal antibody and computerised tomography in the detection of sites of disease in human malignant melanoma. Br J Cancer 1989;59:600-604.

546. Dobbs J, Barrett A, Ash D - Practical Radiotherapy Planning, 2nd edition; London, Arnold, 1992.

547. O'Donoghue JA, Wheldon TE - Dose-rate effects in biologically targeted radiotherapy. Int J Radiat Biol 1989;56:745-749.

548. Hnatowich DJ, Virzi F, Rusckowski M - Investigations of avidin and biotin for imaging applications. J Nucl Med 1987;28:1294-1302.

549. Hnatowich DJ, Rowlinson G, Rusckowski M, Snook D, Epenetos AA - Tumour localisation studies with streptavidin and biotin. Br J Cancer 1989;59:308.

550. Paganelli G, Magnani P, Zito F, et al - Three-step monoclonal antibody tumour targeting in carcinoembryonic antigen-positive patients. Cancer Res 1991;51:5960-5966.

551. Parkins WM, Ravdin RG, Cogins PF - Exploratory study of vascular occlusion and perfusion techniques for the localization of action of cytotoxic drugs. Surg Forum 1959;9:591-594.

552. Woodhall B, Hall K, Mahaley S, Jackson J - Chemotherapy of brain cancer : experimental and clinical studies in localised hypothermic cerebral perfusion. Ann Surg 1959;150:640-652.

553. Shingleton WW, Reeves JW Jr, Keppel RI, Mahaley S, Taylor HM - Studies on abdominal organ perfusion for cancer chemotherapy. Ann Surg 1960;151:741-749.

554. Turner FW, Tod DM, Francis GJ, Greenhill BJ, Couves CM - The technic for isolation perfusion of the rat hind-limb. Cancer Res 1962;22:49-52.

555. Yates A, Fisher B - Experimental studies in regional perfusion. Arch Surg 1962;85:827-836.

556. Povlsen CO - Heterotransplantation of human malignant melanoma to the mouse mutant nude. Acta Pathol Microbiol Scand 1976[A];84:9-16.

557. Morgan DR - Observations on the breeding and maintenance of athymic nude mice. J Inst Anim Tech 1977;28:83-90.