# Isolated Regional Perfusion for Metastases of Malignant Melanoma

## Clinical and Experimental studies

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2013

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*To Ann-Sophie*

"People learn something every day, and a lot of times it's that what they learned the day before was wrong."

William E. ("Bill") Vaughan
*Newspaper Columnist for the Kansas City Star 1946-1977*

# ABSTRACT

## Isolated Regional Perfusion for Metastases of Malignant Melanoma - Clinical and Experimental studies

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**Background** Isolated regional perfusion is a treatment option mainly for localized metastatic disease. The principle idea is to surgically isolate a region of the body and connect the circulation to a heart-lung machine. A high concentration of a chemotherapeutic agent is then delivered to the tumour, while systemic toxicity is avoided. The aim of this thesis was to evaluate clinical outcome of isolated regional perfusion for extremity and liver metastases of malignant melanoma, investigate associated immunological mechanisms, and to explore the potential use of tumour-derived exosomes as future biomarkers.

**Methods** Clinical outcome was analysed by retrospective studies of patient medical records and by data from the national patient registers. Tumour specific T-cells were studied by flow cytometry analyses before and after perfusion. Exosomes were isolated from liver perfusate by ultra-centrifugation and thereafter characterized by electron microscopy, flow cytometry and real-time PCR of the RNA content.

**Results** Between 1984 and 2008, 163 patients with melanoma in-transit metastases underwent isolated limb perfusion (ILP). The overall response rate was 85%, with 65% of the patients having a complete response. Local progression occurred in 63% of the patients after a median time of 16 months. Predictive factors for response were mainly attributed to tumour burden. Thirty-four patients, with uveal melanoma liver metastases, underwent isolated hepatic perfusion (IHP). The overall response rate was 68%. There was a significant median overall survival advantage of 14 months (p=0.029) compared with the longest surviving patients in Sweden during the same time period. Immunological factors were studied in twelve patients after ILP, and the results showed a significant elevation of Melan-A+ CD8+ T-cells after four weeks in 30% of the patients. Exosomes were isolated from the liver perfusate and were shown to be Melan-A positive with a miRNA profile associated with melanoma.

**Conclusion** ILP is a surgical method with a high response rate for the palliative treatment of patients with in-transit metastases of melanoma. IHP is a treatment option with a high response rate, and with a potential survival benefit of more than one year. A small increase in Melan-A specific T-cells is induced after ILP, however the clinical significance needs to be further assessed. Tumour-derived exosomes can be isolated from liver perfusate during IHP. The miRNA characteristics of these exosomes could be a potential source for future biomarkers.

**Keywords**: Malignant Melanoma; Uveal Melanoma; Isolated Limb Perfusion; Isolated Hepatic Perfusion; Immunology; Exosomes

# SAMMANFATTNING PÅ SVENSKA

Isolerad regional perfusion är en kirurgisk behandlingsmetod som gör det möjligt att ge höga doser av cytostatika till en avgränsad del av kroppen med låg systemisk toxicitet. Den drabbade kroppsdelen isoleras från systemcirkulationen genom att dess ven och artär friläggs kirurgiskt. Katetrar förs in i blodkärlen och ansluts till en hjärt-lungmaskin. Höga doser cytostatika tillsätts till hjärt-lungmaskinen och cirkulerar i den isolerade kroppsdelen under 60-90 minuter, därefter återställs den normala blodcirkulationen.

Isolerad regional perfusion är en etablerad metod för behandling av metastaser av malignt melanom lokaliserade till extremiteter, och denna teknik benämns då ILP (isolated limb perfusion). Syftet med delarbete I var att studera långtidsresultat hos 163 patienter som genomgått behandling under åren 1984-2008. Resultaten visade att två av tre patienter erhöll komplett respons och ytterligare en femtedel partiell respons av tumörerna. Majoriteten av patienterna utvecklade lindrig toxicitet, och ingen patient behövde genomgå amputation pga. biverkningar. Efter drygt ett år fick dock två tredjedelar av patienterna åter tillväxt av nya metastaser, och medianöverlevnaden var två och ett halvt år efter behandling. Konklusionen av delarbete I är att ILP är en säker palliativ kirurgisk metod med hög respons, patienterna botas dock inte från sin sjukdom.

Patienter med malignt melanom i ögat utvecklar ofta levermetastaser, och för denna patientgrupp finns ännu ingen etablerad behandling. Syftet med delarbete II var att sammanställa resultat från 34 patienter med denna sjukdom som behandlats med leverperfusion (isolated hepatic perfusion, IHP), samt att jämföra överlevnad med data från det nationella patientregistret. Resultaten visar på en överlevnadsvinst med 14 månader jämfört med de längsta överlevarna i Sverige under samma tidsperiod. Konklusionen är att leverperfusion är en effektiv och säker behandlingsprincip med en potentiell överlevnadsvinst på mer än ett år.

Syftet med delarbete III var att studera om ILP kan leda till en immunologisk aktivering. Tolv patienter som genomgått ILP följdes under 3 månader med blodprov för att bestämma antal och andel av T-, B- och NK-celler samt specifik aktivering av tumörspecifika T-celler. Resultaten visade en signifikant ökning av tumörspecifika T-celler hos 4 av de 12 patienterna, cirka 4 veckor

efter ILP. Andelen av en viss subpopulation av T-celler tycks även utgöra en prognostisk markör för respons, dessa data behöver dock valideras hos ytterligare patienter och en sådan studie är för närvarande pågående.

Delarbete IV syftade till att studera exosomer i samband med IHP. Genom att isolera exosomer direkt från den till hjärt-lungmaskinen kopplade levern, var hypotesen att andelen exosomer med ursprung direkt från levermetastaser kunde öka. Exosomer isolerades med ultracentrifugering av leverperfusat, och karakteriserades sedan med elektronmikroskopi och flödescytometri. Exosomalt RNA extraherades och mikro-RNA profiler associerade med malignt melanom identifierades.

Med en ökad kunskap om resultat och prognostiska faktorer vid perfusionsbehandling kan fortsatt utveckling och förbättrade resultat uppnås. Resultaten från IHP har legat till grund för en nystartad svensk multicenterstudie (SCANDIUM studien) som randomiserar patienter med levermetastaser från ögonmelanom till antingen IHP eller bästa alternativa behandling. Om systemiska immunologiska effekter kan dokumenteras i samband med ILP, kan metoden i framtiden komma att kombineras med andra behandlingsregimer med syfte att förbättra den immunologiska aktiveringen. Slutligen kan studier av tumörexosomer ligga till grund för fortsatta studier med syfte att försöka identifiera specifika exosomer i plasma som en markör för tidig upptäckt av metastaser av ögonmelanom.

# LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I.  **Olofsson R**, Mattsson J, Lindnér P.
    *Long Term Follow-Up of 163 Consecutive Isolated Limb Perfusions for In-Transit Metastases of Malignant Melanoma.*
    Int J Hyperthermia. 2013 Sep;29(6):551-7

II. **Olofsson R**, Cahlin C, All-Ericsson C, Hashimi F, Mattsson J, Rizell M, Lindnér P.
    *Isolated Hepatic Perfusion for Ocular Melanoma Metastasis - Registry Data Suggests a Survival Benefit.*
    Ann Surg Onc. DOI:10.1245/s10434-013-3304-z. In press.

III. **Olofsson R**, Lindberg E, Karlsson-Parra A, Lindnér P, Mattsson J, Andersson B.
    *Melan-A specific CD8+ T lymphocytes after hyperthermic isolated limb perfusion: A pilot study in patients with in-transit metastases of malignant melanoma.*
    Int J Hyperthermia. 2013 May;29(3):234-8

IV. Eldh M[*], **Olofsson R**[*], Lässer C, Svanvik J, Sjöstrand M, Mattsson J, Lindnér P, Choi DS, Gho YS, Lötvall J.
    *MicroRNA in exosomes isolated from the liver circulation in patients with uveal melanoma metastases.*
    Manuscript.
    [*]These authors contributed equally to this work.

# CONTENT

# ABBREVIATIONS

| | |
|---|---|
| AJCC | American Joint Committee on Cancer |
| ALM | Acral lentiginous melanoma |
| APC | Antigen-presenting cell |
| BAC | Best alternative care |
| BMDC | Bone marrow-derived cells |
| CDKN2A | Cyclin-dependent kinase inhibitor 2A |
| CLND | Completion lymph node dissection |
| COMS | The Collaborative Ocular Melanoma Study |
| CR | Complete response |
| CT | Computed tomography |
| CTLA-4 | Cytotoxic T-lymphocyte antigen 4 |
| DFS | Disease-free survival |
| DNA | Deoxyribonucleic acid |
| EGFR | Epidermal growth factor receptor |
| esRNA | Exosomal shuttle RNA |
| HLA | Human leukocyte antigen |
| ICD | International Classification of Diseases |
| ICU | Intensive care unit |
| IHHP | Isolated hypoxic hepatic perfusion |
| IHP | Isolated hepatic perfusion |
| ILI | Isolated limb infusion |
| ILP | Isolated limb perfusion |
| IVC | Inferior vena cava |
| KEGG | Kyoto Encyclopaedia of Genes and Genomes |
| LDH | Lactate dehydrogenase |
| LMM | Lentigo maligna melanoma |
| M-ILP | Melphalan based isolated limb perfusion |
| MAPK | Mitogen-activated protein kinase |
| MHC | Major histocompatibility complex |
| miRNA | Micro RNA |
| MRI | Magnetic resonance imaging |
| mRNA | Messenger RNA |
| MVB | Multivesicular bodies |
| NC | No change |

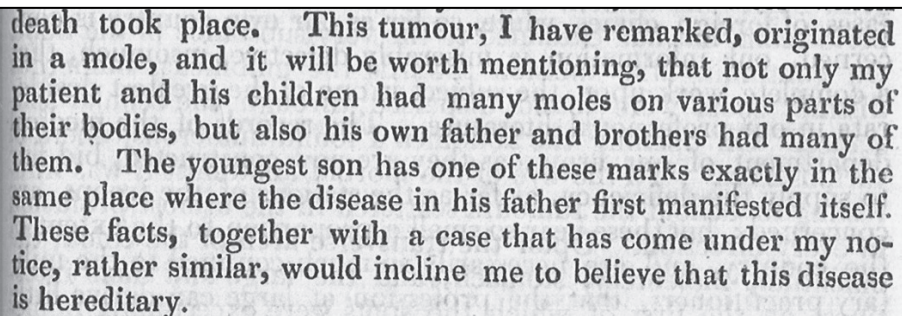| | |
|---|---|
| NM | Nodular melanoma |
| OS | Overall survival |
| PCR | Polymerase chain reaction |
| PD | Progressive disease |
| PET-CT | Positron emission tomography - computed tomography |
| PFS | Progression-free survival |
| PHP | Percutaneous hepatic perfusion |
| PR | Partial response |
| RECIST | Response Evaluation Criteria In Solid Tumours |
| RNA | Ribonucleic acid |
| SD | Stable disease |
| SN | Sentinel node |
| SNB | Sentinel node biopsy |
| SSM | Superficial spreading melanoma |
| TCR | T-cell receptor |
| TM-ILP | TNF-alpha and melphalan based isolated limb perfusion |
| TNF-alpha | Tumour necrosis factor-alpha |
| TTLP | Time to local progression |
| UV | Ultraviolet light |
| UVB | Ultraviolet B light |
| WHO | World Health Organization |

# 1 INTRODUCTION

## 1.1 Malignant melanoma

The first accredited report of melanoma is found in the writings of the Greek physician Hippocrates (460 BC), where he describes "fatal black tumours with metastases" (1). French physician René Laennec (1781-1826) was the first to describe *cancer noire*, the black cancer, as a disease entity. He introduced the term *la mélanose*, derived from the Greek word *mavros* meaning black, during a lecture for the Faculté de Médecine de Paris in 1804 (2).

The first report of melanoma in English literature was in 1820 when William Norris (1792-1877) described a patient with a primary lesion in the abdominal skin originating from a pre-existing mole. He also concluded in this manuscript that melanoma is a hereditary disease (3) (see Figure 1).



**Figure 1.** Quotation from William Norris in the article Case of Fungoid Disease. Edinburgh Medical and Surgical Journal. 1820; 16:562-565.

The oldest evidence of melanoma in humans dates back 2400 years. Nine Incan mummies, from what is now Peru, were examined in the 1960s. These individuals showed melanotic masses in the skin and diffuse metastases to the bones - particularly of the skull and extremities.

The well-known Scottish surgeon John Hunter (1728-1793) is reported to be the first to operate on a metastatic melanoma in 1787. The patient was a 35-year old man with recurrent nodal metastases behind the angle of the lower jaw. Although not knowing precisely what it was, he described it as a

"cancerous fungous excrescence" (4). The excised tumour was preserved in the Hunterian Museum of the Royal College of Surgeons of England. It was not until 1968 that microscopic examination of the specimen revealed it to be an example of metastatic melanoma (5).

## 1.1.1 Cutaneous malignant melanoma

The incidence of cutaneous malignant melanoma has been rising for several decades in many fair-skinned populations, especially in the Scandinavian countries (6). Between 1970 and 2010, the incidence of melanoma in Sweden increased from 7.1 to 32.0/100.000 for men and from 8.4 to 26.6/100.000 for women (7, 8). During the 1990s there was some evidence that the incidence was levelling off, these results were in part attributed to different programs for primary prevention (9). However, recent data for the last decade show a yearly increase in incidence of over 4%, which now makes malignant melanoma responsible for about 5% of all cancers in Sweden.

### Risk factors

The principal environmental risk factor for cutaneous melanoma is ultraviolet (UV) radiation, where intermittent sun exposure and severe sunburns, especially during childhood, and use of tanning beds have been associated with an increased risk of cutaneous melanoma (10). The exact biological mechanisms involved in this process are not completely known (11). However, ultraviolet B radiation (UVB) has a direct mutagenic effect on DNA by a photochemical reaction between UVB and pyrimidine bases resulting in a distorted DNA helix affecting transcription and DNA replication (12).

The risk for developing melanoma has been directly correlated to the total number of melanocytic nevi on the body (13). Melanocytic nevi are benign accumulations of melanocytes, and they may be acquired or congenital. Several mechanisms have been suggested to explain the association between nevi and melanoma; patients with many nevi have more total melanocytes at risk for malignant transformation, it may indicate a greater genetic risk or it might be an indicator of increased sun exposure (14). Common acquired nevi typically appear after 6 to 12 months of age, they increase in frequency up to the third and fourth decades, and then slowly start to disappear (14). Both environmental and genetic factors play a role in determining the number of nevi that will develop. The frequency and amount of intermittent sun

exposure influence both the number and size of nevi; especially blistering sunburns during childhood (15).

Approximately 5-10% of melanomas develop in individuals who have one or more first-degree relatives with melanoma, and patients with a history of melanoma in a first-degree relative have approximately twice the risk (16). Most familial clusters occur by chance or are due to family members having the same host characteristics and lifestyles; only a small percentage of patients have an inherited mutation in a melanoma susceptibility gene (17). An example of such a gene is the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene; it encodes two proteins controlling cell proliferation, $p16^{INK4a}$ and $p14^{ARF}$. For a person with a CDKN2A mutation, the lifetime risk for developing melanoma is between 60% and 90% (18).

## Subtypes

Although more than 95% of all melanomas are found in the skin, melanoma is not exclusively a skin cancer. Sites of primary melanoma also include ocular (19), mucosal (20), gastrointestinal (21), genitourinary (22) and the leptomeninges (23).

Cutaneous melanomas are classified into four major clinical subtypes. Superficial spreading melanoma (SSM) is the most common form, approximately 60% (24) and is related to severe sunburns in childhood and intermittent sun exposure in adult life. The growth of a SSM begins with a horizontal growth phase, appearing as a slowly evolving macule, often with multiple colours and pale areas of regression (25). This is in contrast to nodular melanomas (NM), which often show an aggressive vertical growth phase with a short or absent horizontal growth. NM accounts for approximately 15-20% of melanomas (24), and most often occurs on the trunk and limbs of patients in their fifth or sixth decade of life, with males being more commonly affected than females (26). Lentigo maligna melanoma (LMM) is typically found in sun-exposed regions, often in the face, and correlates to long-term sun exposure and increasing age. It accounts for about 5-15% of melanoma (24, 27). Acral lentiginous melanoma (ALM) accounts for about 1-5% of the cases and is not associated with sun-exposure. It is found on the palms, the soles and at subungual sites (24, 28). There also exist other more rare forms of melanoma, e.g. desmoplastic and amelanotic melanomas (29).

## Prognostic factors

In 1970 Alexander Breslow published a retrospective analyses of 98 patients where he stated that tumour thickness measured from the top of the granular cell layer of the epidermis to the deepest point of invasion was a risk factor (30). This was later confirmed in a larger series where he reported that the incidence of metastatic disease is directly proportional to the tumour thickness. Since then, tumour thickness is the most powerful prognostic factor, with a 10-year survival rate of 93% for melanoma less than 1 mm compared to 42% for tumours larger than 6 mm (31).

The first prognostic factor was Clark's level of invasion (32), which describes a five-stage scale of invasion depth. However, in the current seventh edition of the American Joint Committee on Cancer (AJCC) tumour staging manual, the mitotic rate has replaced the Clark level. The Clark level was no longer significant in multivariate analyses when mitotic rate was included (33).

In 1953 Allen and Spitz established ulceration of the primary tumour as another important prognostic factor. Ulceration is defined as the absence of an intact epidermis overlying a major portion of the primary melanoma (34).

In the early 1990s, Morton and colleagues developed the concept of sentinel node biopsy (SNB) and selective lymphadenectomy (35, 36). SNB is based on the hypothesis that lymph draining from a tumour site passes to a sentinel node (SN) before the passage to other regional nodes. If the SN is without tumour cells, tumour cells are unlikely to be present in the other regional nodes. SNB provides important independent prognostic information; however there is no clear evidence that removal of these nodes, followed by completion lymph node dissection (CLND) if SN is positive, improves survival (37, 38).

## Staging

Melanoma is staged according to the TNM staging system developed by AJCC (31). The TNM system is based on the extent of the primary tumour (T), presence of lymph node metastases (N), and the presence of distant metastasis (M). Stage I and II includes patients without evidence of lymph node or distant metastases. Stage I is defined as a primary tumour thickness

less or equal to 1 mm and stage II as a tumour thickness more than 1 mm (see Table 1).

**Table 1.** AJCC classification of primary melanoma (Stage I-II) (31)

| T Classification | Thickness (mm) | Ulceration/Mitoses |
|---|---|---|
| T1 | ≤1.0 | a: without ulceration and mitosis <1mm$^2$ |
| | | b: with ulceration and mitosis ≥1mm$^2$ |
| T2 | 1.01-2.0 | a: without ulceration |
| | | b: with ulceration |
| T3 | 2.01-4.0 | a: without ulceration |
| | | b: with ulceration |
| T4 | >4.0 | a: without ulceration |
| | | b: with ulceration |

Stage III is defined as lymphatic dissemination. Nodal involvement is defined as N1 to N3 by the number of nodes, and is subcategorized as Na and Nb depending on whether there are microscopically (micro-metastases) or clinically detectable metastases (macro-metastases) (31). Approximately 5-10% of patients with high-risk melanoma will develop in-transit metastases (39) - a form of tumour spread within intradermal and subcutaneous lymphatic channels between the primary site and the regional lymph nodes (40). The presence of satellite or in-transit metastases is defined as N2c when nodal metastases are absent and N3 when there is concomitant nodal disease (31) (see Table 2). For staging purposes, the current recommendation is that patients with T1b-T4 tumours undergo SNB to determine lymph node status.

**Table 2.** AJCC classification of lymph node metastases (Stage III) (31)

| N Classification | No. of metastatic nodes | Micro/Macrometastases |
|---|---|---|
| N1 | 1 node | a: micrometastasis |
| | | b: macrometastasis |
| N2 | 2-3 nodes | a: micrometastasis |
| | | b: macrometastasis |
| | | c: in-transit metastases/satellites without metastatic nodes |
| N3 | 4 or more nodes, matted nodes, or N2c with metastatic nodes | |

**Figure 2.** In-transit metastases of malignant melanoma on the lower limb. (A) Pigmented lesions in a light skinned patient. (B) Amelanotic lesions in a dark skinned patient. Photo courtesy of Dr Jan Mattsson, Department of Surgery, Sahlgrenska University Hospital, Sweden.

Stage IV is defined as distant metastases with the most common localisation being skin, soft tissues, lung, liver, brain, bone and the gastrointestinal tract. A significant difference in survival has lead to a sub categorization of the M category. M1a for distant metastasis in skin/subcutaneous tissue and for distant lymph node metastases, M1b for lung metastasis and M1c for metastases at any other site (M1c). In addition, the presence of an elevated lactate dehydrogenase (LDH) level, irrespective of metastatic site, places the patient in the M1c category (see Table 3) (31). Median survival has been

reported as 16, 14 and 9 months for the M1a, M1b and M1c categories respectively (41).

**Table 3.** AJCC classification of distant metastases (Stage IV) (31)

| M Classification | Site | Serum LDH |
|---|---|---|
| M1a | Distant skin, subcutaneous or nodal metastasis | Normal |
| M1b | Lung metastasis | Normal |
| M1c | All other metastasis | Normal |
| | Any distant metastasis | Elevated |

**Table 4.** Staging of melanoma according to AJCC (31)

| Pathologic staging | T | N | M |
|---|---|---|---|
| 0 | Tis | N0 | M0 |
| IA | T1a | N0 | M0 |
| IB | T1b | N0 | M0 |
| | T2a | N0 | M0 |
| IIA | T2b | N0 | M0 |
| | T3a | N0 | M0 |
| IIB | T3b | N0 | M0 |
| | T4a | N0 | M0 |
| IIC | T4b | N0 | M0 |
| IIIA | T1-4a | N1a | M0 |
| | T1-4a | N2a | M0 |
| IIIB | T1-4b | N1a | M0 |
| | T1-4b | N2a | M0 |
| | T1-4a | N1b | M0 |
| | T1-4a | N2b | M0 |
| | T1-4a | N2c | M0 |
| IIIC | T1-4b | N1b | M0 |
| | T1-4b | N2b | M0 |
| | T1-4b | N2c | M0 |
| | Any T | N3 | M0 |
| IV | Any T | Any N | M1 |

# 1.1.2 Uveal malignant melanoma

Uveal melanoma is a tumour in the eye arising from melanocytes in the uveal tract. It is most common in the choroidea (90%), but can also be found in the ciliary body (7%) and in the iris (3%) (42). Choroidal and ciliary body melanomas are together named posterior uveal melanomas, whereas iris melanomas are named anterior uveal melanomas. Anterior melanomas have a more benign clinical course compared with posterior melanomas (43).

The uveal melanoma incidence is highest in Caucasian populations. In Europe, the incidence shows a gradient from north-to-south, decreasing from over 8-9 per million in Scandinavia to less than 2 per million in the southern European countries (44, 45). The incidence is increasing with age, and the median age at the time of diagnose is about 60 years. There are no major differences in incidence between genders (46).
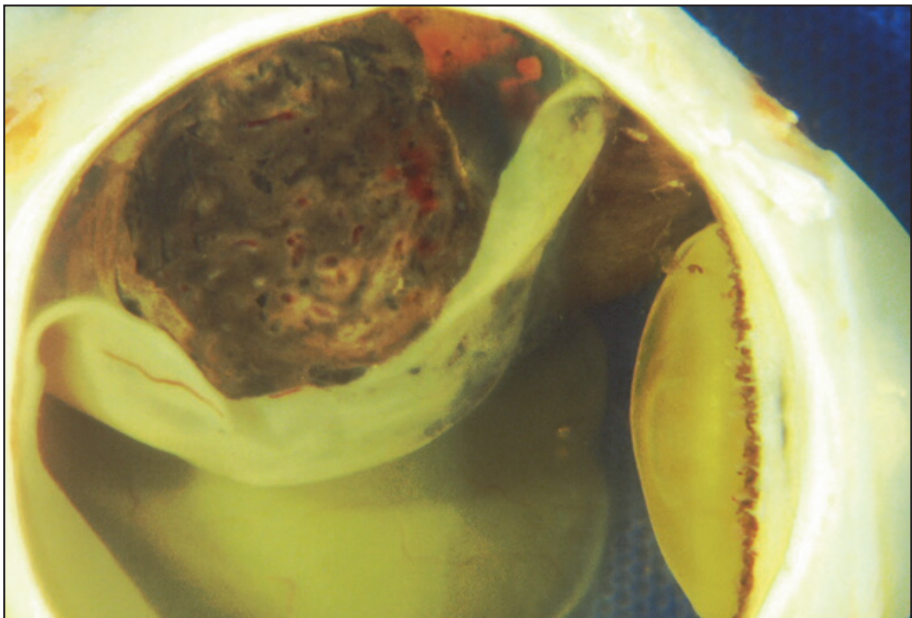


**Figure 3.** Large choroidal melanoma pushing the retina. Photo courtesy of Professor Stefan Seregard, St Erik Eye Hospital, Stockholm, Sweden.

## Risk factors

Several host factors have been associated with an increased risk for uveal melanoma. Ocular melanocytosis is a predisposing factor, with a lifetime risk

estimated to be about 0.3% (47). The prevalence of choroidal nevi in Caucasian populations is between 5% and 8%, but the risk of malignant transformation is very low, about 0.01% (48). A meta-analysis have shown that light eye colour, fair skin colour, and the inability to tan are independent risk factors (49).

Exposure to solar UV radiation as a risk factor in the development of uveal melanoma is still an unanswered question (50). However, it has been shown that artificial UV radiation from welding and use of sunlamps increases the risk for posterior melanoma (51).

## Symptoms and clinical presentation

The clinical presentation of uveal melanoma largely depends on the size and the location of the lesion. Patients may be diagnosed after developing symptoms of various visual disturbances or, if asymptomatic, during a routine eye examination. The majority of patients with uveal melanoma are symptomatic with about 30% being asymptomatic. The most common symptoms are blurred vision, visual field defect, irritation and pain (46).

Choroidal melanoma usually presents as a subretinal mass that can cause secondary retinal detachment with consequent visual loss. The colour of the tumour can vary from typically brown pigmentation to being amelanotic. Ciliary body melanoma can cause lens displacement with consequent refractive and accommodation disturbances. The condition can be asymptomatic for long periods before causing any clinical manifestation (52).

Iris melanomas usually manifest themselves as the growth of a previously noted iris lesion, as a new-pigmented lesion noticed by the patient or being discovered during a routine eye examination (53).

## Subtypes

Uveal melanoma has been subclassified into two major molecularly defined prognosis groups: one group with low metastatic risk associated with gains of the short arm of chromosome 6, and a second group characterized by aggressive tumours with high metastatic risk, exhibiting loss of chromosome 3 and gain of 8q (54). Expression profiling of mRNA has also identified two distinct tumour classes with better prognosis (Class 1) and worse prognosis (Class 2) according to Onken et al (55).

## Local treatment

Local treatment of uveal melanoma used to consist of enucleation, but currently, posterior uveal melanoma is primarily treated with plaque radiation therapy. Other options include particle beam radiotherapy, trans-pupillary thermotherapy, laser photocoagulation, gamma knife stereotactic radiosurgery or local surgical resection (56). The Collaborative Ocular Melanoma Study (COMS) trial did not find any difference in mortality rates between patients managed by brachytherapy or enucleation, but showed that brachytherapy had the advantage of preserved vision (57). For locally advanced tumours, especially with extra-ocular involvement, enucleation is still the mainstay therapy (58). However, the improvements in local therapy have not improved survival rates (59), and metastatic disease is the leading cause of death (60).

## Distant metastases

At the time of initial diagnosis, about 2-4% of all patients have metastatic disease (60, 61), but patients with uveal melanoma have a high risk of developing metastatic disease. In the COMS trial, the cumulative 5 and 10-year rates were 25% and 34% respectively (62). Also late metastases are frequent, with approximately 50% of the patients ultimately developing metastases (60).

The liver is the most common site for metastases (89%) and these metastases are generally refractory to systemic treatment. The median survival for patients with liver metastases is about 6 months (62). Patients with metastases outside the liver, or with the liver not being the first site, have a better survival (63). For patients with liver metastases, regardless of treatment, the mortality rate is approximately 90% at 2 years with only about 1% of the patients surviving more than 5 years (62).

# 1.2   Isolated regional perfusion

When metastases are located in a defined region, e.g. in-transit metastasis in the limbs or isolated liver metastases, one treatment option is isolated regional perfusion. The principle idea behind this technique is to surgically isolate a region of the body and then deliver a high concentration of a chemotherapeutic agent to the tumour, while avoiding systemic toxicity. The basis stems from the early experiments in the 1950s, by Klopp and

colleagues, where they showed an improved effect of chemotherapeutics when infused into the tumour-supplying artery together with compression of the venous return (64).
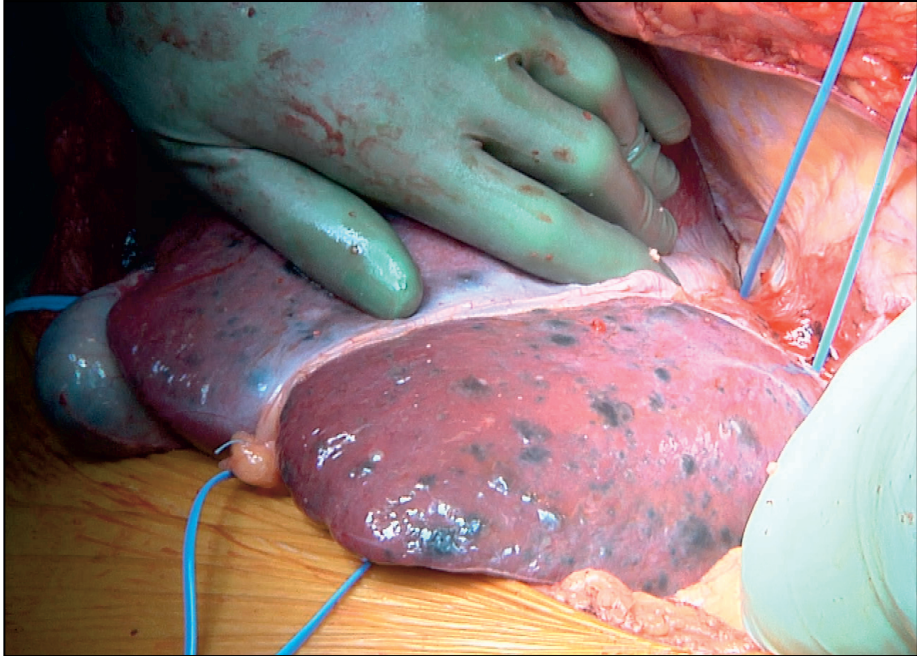


**Figure 4.** Multiple liver metastases from uveal malignant melanoma. Photo courtesy of Dr Jan Mattsson, Department of Surgery, Sahlgrenska University Hospital, Sweden.

The technique of isolated limb perfusion (ILP) was developed in the late 1950s, when Creech and Krementz (65) at the Charity Hospital in New Orleans, pioneered the technique. The first patient, a 76-year-old man with extensive melanoma in-transit metastases who refused amputation, was treated in 1957. A normothermic ILP was performed through the femoral artery and vein (Figure 5). The leg was isolated with an Esmarch tourniquet proximal to the cannulated vessels and the catheters were connected to an oxygenated perfusion circuit. Melphalan was injected in 4 doses at 5-minute intervals, for a total perfusion time of 23 minutes. The patient had a remarkable complete response (CR) to the treatment and died of unrelated causes 16 years later (66).
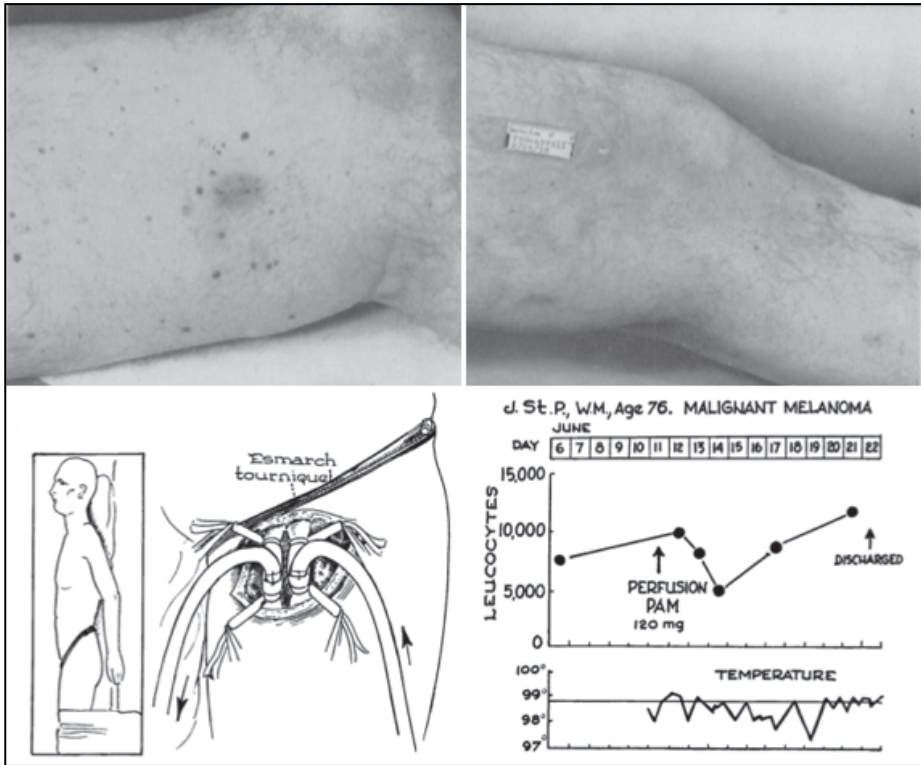
**Figure 5.** Drawings from the original paper by Creech and colleagues describing the techniques and results of the first patient treated with isolated limb perfusion (65). Reprinted with permission from Wolters Kluwer Health.

Using the same rationale as for isolated limb perfusion, isolated hepatic perfusion (IHP) is a treatment modality that can expose hepatic metastases to a high local concentration of a chemotherapeutic agent with minimal systemic exposure. Ryan et al originally designed an experimental model of IHP in a canine model (65), and later Robert Ausman developed the technique in a porcine model (67). In 1960, Ausman reported the outcome of the first five patients treated with IHP using nitrogen mustard for 60 minutes (68) (Figure 6).

Since then, IHP has been clinically evaluated in several studies, mainly for liver metastases derived from colorectal cancer, melanoma, and neuroendocrine tumours, but also for primary hepatic malignancies (69-71).
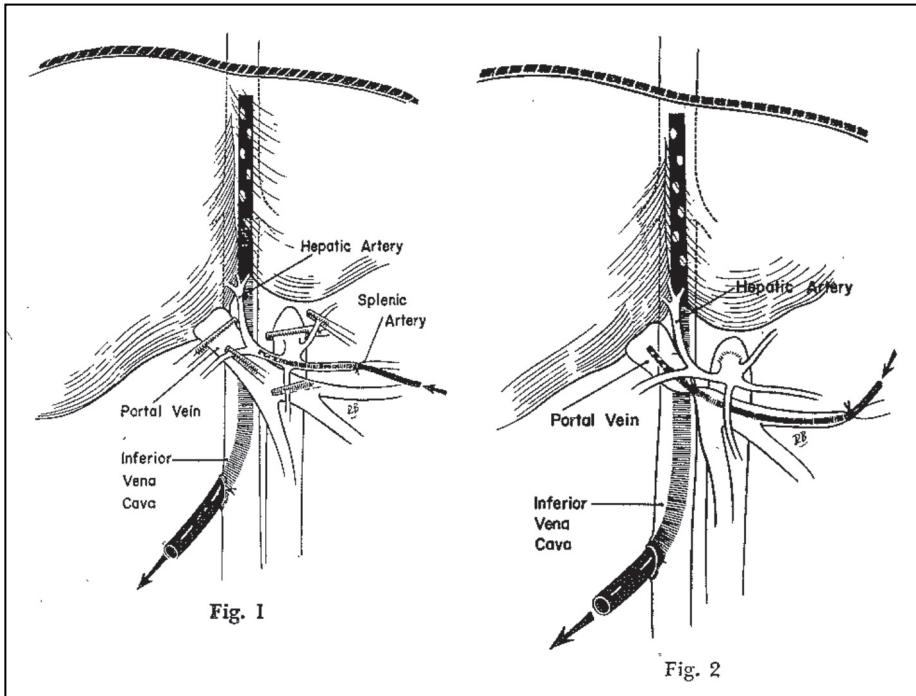
**Figure 6.** Drawings from the original paper by Ausman and Aust describing the techniques for isolated hepatic perfusion, both through the hepatic artery (left) and the portal vein (right) (67). Reprinted with permission from Elsevier.

## 1.2.1 Melphalan

Melphalan (L-phenylalanine mustard) is a phenylalanine derivate of nitrogen mustard. It is an alkylating agent, which cross-links DNA by binding at the N7 position of guanine, resulting in interference with mitosis and cell division. Bergel and Stock first synthesized melphalan in 1953; three years later the first report describing growth cessation of implanted melanoma in mice was published (72). However, melphalan is ineffective as a systemic treatment for melanoma as the maximally tolerated dose is lower than the effective dose (73). But due to a favourable local toxicity profile, melphalan has been used as the standard chemotherapeutic agent in both the IHP and ILP setting.

An early pharmacokinetic study using melphalan in an ILP setting showed that high peak perfusate concentrations were achieved (6-115 mg/ml) and that these levels could be about 20 to 100 times higher than the peak levels

achieved with the usual intravenous doses of melphalan (74). In another study, different melphalan concentrations were tested using isolated limb infusion in a nude rat model with melanoma xenografts. Notably, a CR or a PR was always achieved when the concentration of melphalan in the perfusate was above 15 mg/mL. There was a plateau in the treatment response, starting at approximately 25 mg/mL; above this level no additional tumour response was seen (up to 400 mg/mL) (75).

In the earlier days of regional perfusion treatment, melphalan was administered based on body weight, but this approach did not take into account the distribution of body mass. Consequently, dosing based on limb volume has become the preferred approach with a typical target level of 10–13 mg/L limb volume, depending on upper or lower extremity perfusion.

Factors influencing the concentration of melphalan in the perfusion circuit depend on the combination of limb volume, drug redistribution, drug metabolism, tumour uptake and leakage out of the perfusion circuit. After a bolus injection of melphalan, the concentration curve has a biphasic appearance with a half-life of about 10 minutes for the initial phase and about 25 minutes for the second phase (76). The early and relatively rapid disappearance of melphalan is interpreted as being caused by binding of the drug to proteins and cellular components, whereas the latter portion of the curve represents the hydrolysis of melphalan in plasma (77). The hydrolysis is more rapid at elevated temperatures with a half-life ranging from about 5.5 hours at 20°C to about 1 hour at 39-40°C (77).

## 1.2.2   Hyperthermia

The first application of hyperthermia for regional cancer control dates back to 1898 when the Swedish gynaecologist Frans Westermark (1853-1941) treated cervical cancer by running hot water through an intracavitary spiral tube. He noted excellent clinical response in the seven patients treated (78).

In 1967 Cavaliere reported the effects of ILP using only hyperthermia in 22 patients with recurrent extremity tumours. The duration of hyperthermia (>40°C) ranged from 50 minutes to almost 7 hours. Six patients died in the postoperative period with 12 of the 22 patients being alive without evidence of disease at 3 to 28 months of follow-up (79). This finding is also supported by experiments using a melanoma rat model, where ILP without

chemotherapeutics showed a marked tumour response when comparing the use of normothermic perfusion (37°C) with hyperthermic perfusion (41.5°C) (80).

In 1969, Stehlin combined extreme hyperthermia (46.1°C) together with ILP to potentiate the effect of melphalan (81). It has later been demonstrated that hyperthermia mediates an increased uptake of chemotherapeutics by tumour cells through changes in tumour blood flow and cellular membrane permeability (82). During ILP, an increase in temperature from 37°C to 39.5°C doubles the concentration of cisplatin in tumours while at the same time decreasing the concentration in surrounding healthy tissue (83). Hyperthermia also acts synergistically with melphalan leading to an increased toxicity in human melanoma cell lines (84).

Moreover, hyperthermia causes a selective destruction of tumour vasculature (85), where capillary endothelial cells of tumours seems to be more sensitive to hyperthermia than are endothelial cells of normal tissues (86).

Tissue temperatures of 41.5 degrees or more generates a high response rate, but also increases local toxicity (87). As a compromise between response rate and toxicity, the current standard is to use tissue temperatures of mild hyperthermia (39-40°C).

## 1.2.3   TNF-alpha

In 1988 the addition of TNF-alpha (Tumour necrosis factor-alpha) to standard melphalan based ILP (TM-ILP) was introduced by Lejeune and Lienard. The initial discovery of TNF was made when mice were injected with BCG and endotoxin, resulting in the release of a factor that induced necrosis in a murine sarcoma model (88). The use of systemic TNF-alpha resulted in severe side effects similar to those seen in septic shock, which limited its utility, but by using TNF-alpha in an isolated regional perfusion setting, the systemic toxicity could be abrogated. There are at least two different effects of TNF-alpha in the setting of perfusion; one causes a direct toxic effect on tumour cells (89), the other causes a specific destruction of tumour vasculature (90).

The initial reports generated great interest in TM-ILPs and prompted many single-institution, non-randomized trials (91). Only one prospective

randomized trial between M-ILP and TM-ILP have been presented. This trial included 103 patients and found no evidence of improved response (92). The trial has been criticized on the basis of the early time point for assessing response (3 months), for the lower response rates observed when compared with other trials and that the issue of bulky tumours was not addressed (93). In Europe, the result of this trial has largely been ignored, and the current indications for TM-ILP mainly include re-perfusion and perfusion of bulky melanoma. TNF-alpha is currently not available in the US due to patent and licensing rights (94).

The addition of TNF-alpha to melphalan in IHP for uveal melanoma metastases has been reported in smaller case series. One trial reporting on 22 patients found a significantly longer median duration of response for melphalan together with TNF-alpha (14 versus 6 months). There was more toxicity with the addition of TNF-alpha; however, most of the toxicity was of a transient nature and in most circumstances not clinically significant (95).

## 1.2.4   Technical development

### Isolated limb infusion (ILI)

In 1996 John Thompson at the Sydney Melanoma Unit reported the technique of isolated limb infusion (ILI) as a low-morbidity, technically simpler alternative to ILP. Instead of achieving vascular access to the affected limb by surgical exposure, arterial and venous catheters are placed by interventional radiology via the contralateral groin. Once the correct placement of the catheters is confirmed by x-ray, a pneumatic or Esmarch tourniquet is placed at the proximal aspect of the affected limb in order to isolate it. Melphalan is then infused manually during 20-30 minutes via a syringe and high-flow, three-way stopcock (96).

No randomized comparisons between ILI and ILP have been reported. Observationally, there appear to be differences in both outcome and complication rates; whether these differences are significant is yet to be assessed. In one recent non-randomized trial with 215 patients, 134 patients underwent ILI and 81 patients ILP. The CR rates were 30% and 44% for ILI and ILP respectively. The difference in the number of patients experiencing local progressive disease was even greater, 44% vs. 11%, for ILI and ILP respectively.  In a multicentre trial of ILI including 162 patients, a CR rate of

31% was reported (97), which can be compared to the systematic review of Moreno-Ramirez reporting a CR rate of 58% (91). Despite these major differences in efficacy, the use of ILI is increasing worldwide, mainly with the argument that the procedure is simpler and more cost-effective (98).

## Isolated hypoxic hepatic perfusion (IHHP)

IHP is a major and complex surgical intervention. Since the first report by Ausman, there have been many developments in surgical technique that have decreased morbidity and improved response rates. One of the differences between studies has been whether to either shunt the portal vein, include it in the perfusion or to clamp it.

There have also been efforts trying to more radically change and simplify the procedure. In 2004, van Etten and colleagues reported the first clinical results using the isolated hypoxic hepatic perfusion (IHHP) technique. A veno-venous bypass was not required and a perfusion system without an oxygenator was used. The perfusion was performed for 20-30 minutes under mild hyperthermic conditions using melphalan. Two different perfusion methods were used, both with inflow via the hepatic artery. In the first eight patients, all with colorectal liver metastases, the portal vein was occluded and there was an orthograde outflow via a double-balloon catheter placed in the inferior vena cava (IVC). The leakage rate was more than 50% and no overall response was recorded. In the next 10 patients, the technique was changed to a retrograde outflow via the portal vein while blocking the IVC completely using a triple balloon catheter. The retrograde IHHP technique still had a 35% leakage rate, but resulted in a 20% PR (99). A follow-up study, including eight patients with uveal melanoma metastases, used the retrograde outflow technique and reported an overall response rate of 37.5%, without any CR, and with a median time to progression of 6 months (100).

## Percutaneous hepatic perfusion (PHP)

In the early 1990s, three independent groups developed a novel percutaneous hepatic perfusion system using extracorporeal chemofiltration. The technique combined a conventional hepatic artery infusion with a dual-balloon vena cava catheter collecting the outflow from the liver. The venous outflow was then connected to an extracorporeal venous bypass circuit, including a carbon filter, to recover any of the drug that was not absorbed by the liver (101-103). In 1992, the first phase I dose escalation trial included 15 treatments in 8

patients. Aside from one patient who could not tolerate balloon inflation, each treatment was well tolerated.

In 1994, two follow-up studies including various tumours and either 5-FU or cisplatin were reported. One study included 23 patients (104) and the other one included 11 patients (105), and the results confirmed the feasibility in a total of 75 procedures. Ten years later another phase I dose escalation study using melphalan included 28 patients. The results showed an overall response rate of 30%, and in ten patients with melanoma metastases, the response rate was 50% (106).

This finding lead to the initiation of a phase III study, randomizing 93 patients to either PHP or best alternative care (BAC). The final results have not yet been published, but during a presentation at the ASCO 2012 meeting, some of the results were described. There was a significant increase in the primary endpoint, hepatic progression free survival (245 days vs. 49 days), and also in overall response (34% vs. 2%) (107). Any conclusions concerning overall survival are difficult to make, due to the high proportion of patients crossing over from BAC to PHP.

## 1.2.5  Toxicity

### Systemic toxicity

Using melphalan, a leakage of more than 15% into the systemic circulation may cause toxic effects such as bone marrow depression, gastrointestinal toxicity, hair loss and pruritus (108). These complications are most often manageable, but after the introduction of TNF-alpha in the early nineties, the issue of leakage became more important. A leakage of TNF-alpha into the systemic circulation produces more severe side effects than melphalan alone. At the common dose of 4 mg TNF-alpha, even a small leakage of 1% may result in hypotension of the patient, while a 10% leakage can cause a potentially fatal septic shock like syndrome (109).

### Leakage monitoring

Since strict isolation of the limb or the liver is not always achievable, due to anatomical variations or technical reasons, continuous leakage monitoring becomes important. Stehlin introduced a method in the early 1960s to monitor leakage. The technique used a radioactive tracer administered into

the perfusion circuit and a scintillation probe positioned over the heart (110). Any leak from the perfusion circuit into the systemic circulation was registered by a rise in the radioactive count. The technique has been refined and changed over the years, and different approaches using different radioactive tracers including $^{125/131}$I-labeled albumin (111), $^{99m}$Technetium-labeled albumin (108), or $^{99m}$Technetium-labeled red blood cells (112) have been described. When using radiotracers, the leakage of chemotherapeutics is determined indirectly; however a good correlation with the actual melphalan leakage has been shown (113).

## Local toxicity in ILP

ILP is a palliative treatment with the aim of improving or maintaining a good quality of life. Therefore avoiding or limiting the side effects associated with ILP is imperative for preserving the patient's limb and minimizing long-term morbidity. Within 48 hours of ILP, slight edema, erythema, and discomfort develop in many patients. At about 14 days after ILP, the maximal reaction occurs, most often in the form of edema, erythema and pain. The erythema eventually fades and continues to lighten over a period of 3 to 6 months. Less common local manifestations include drying or blistering of the skin, temporary loss of nails, transient neuralgia (with the feeling of "walking on glass") and inhibition of hair growth. Most of these symptoms appear to subside over time (114).

**Table 5.** Acute regional toxicity grading score, according to Wieberdink et al. (115)

| Grade | Reaction |
|-------|----------|
| I | No reaction |
| II | Slight erythema and/or edema |
| III | Considerable erythema and/or edema with some blistering; slightly disturbed motility permissible |
| IV | Extensive epidermolysis and/or obvious damage to the deep tissues, causing definite functional disturbances; threatened or manifest compartment syndrome |
| V | Reaction that may necessitate amputation |

The Wieberdink grading system of regional toxicity is commonly used to grade the toxic reactions of the normal tissues after ILP. It classifies tissue reaction as mild (grade I or II) and more severe (III or IV). Grade V toxicity is any reaction that may necessitate amputation (see Table 5) (115).

# 1.3 Systemic treatment for melanoma

Conventional systemic chemotherapy for patients with both metastatic cutaneous and uveal melanoma has failed to show any prolonged survival. A response rate of about 20% has been observed using dacarbazine or temozolomide in patients with metastatic cutaneous melanoma; however, the response rates for uveal melanomas are reported to be less than 5% (116, 117).

Different multidrug regimens such as CVD (cisplatin, vinblastine and dacarbazine) and the BOLD regimen (bleomycin, vincristine, lomustine and dacarbazine) have reported response rates of about 30% in cutaneous melanoma metastases. The response rates for uveal melanoma metastases are lower than 10% (118-121).

## 1.3.1 Targeted therapies

In both cutaneous and uveal melanoma, the activation of the RAS/RAF/MEK/ERK pathway, also known as the mitogen-activated protein kinase (MAPK) pathway, is a common event. The MAPK pathway has a mitogenic effect in melanocytes where it activates transcription factors important for cell proliferation. The driving mutations are different between the two types; mutations in BRAF and NRAS are seen in about 50% and 20% of the cutaneous melanomas respectively (122, 123). Uveal melanoma typically lacks BRAF and NRAS mutations but commonly has GNAQ or GNA11 mutations - leading to a downstream activation of MEK (124).

Selective inhibitors of the mutated BRAF kinase have been developed. Both the drugs vemurafenib and dabrafenib have shown a response rate of about 50% in phase III trials including patients with cutaneous melanoma (125, 126). Vemurafenib has also shown improved overall survival compared with dacarbazine, with the latest update showing a median survival of 13.6 months compared to 9.7 months (127).

Trametinib was the first MEK inhibitor to undergo randomized phase III testing in patients with cutaneous BRAF V600E/K mutated melanoma. The results showed a response rate of 22% and a prolonged PFS of 3 months when compared to chemotherapy (128).

Somewhat disappointingly, a phase I trial with trametinib including 16 patients with uveal melanoma metastases, showed no objective responses (8 patients with SD) and a median PFS of only 2 months (129). In contrast, at the 2013 ASCO meeting (American Society of Clinical Oncology) a randomized trial between the MEK inhibitor selumetinib and temozolomide was reported. The trial included 80 patients and the results showed a response rate of 15% for selumetinib, and a significant increase in PFS with 9 weeks. The overall survival was reported to be 10.8 months for selumetinib and 9.4 months for temozolomide (130).
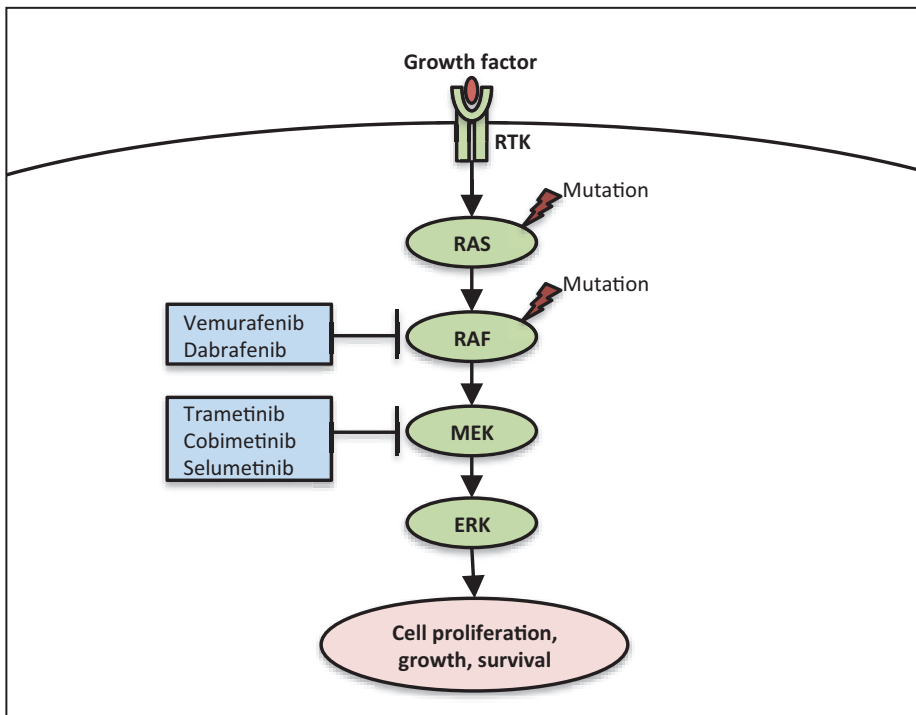


**Figure 7.** The mitogen-activated protein kinase (MAPK) pathway and BRAF and MEK inhibitors currently approved or undergoing trials. Growth factors bind to cell surface receptor tyrosine kinases (RTK), signalling down the MAPK pathway resulting in cell proliferation, growth and survival. Specific activating mutations in the MAPK pathway include BRAF (40–50%) and NRAS (20%).

## 1.3.2 Immunotherapy

William Coley (1862-1936) pioneered the use of immunotherapy as cancer treatment. Coley was convinced that a severe infection could cause the

regression of cancer, and reported in 1891 on the outcome of three patients with soft tissue sarcomas. The patients were treated by serial inoculations of streptococcal bacteria and he observed a clinical regression in all three patients (131).

In the 1950s, studies using immune cells to inhibit cancer growth showed that solid transplant rejection was mediated by cellular immunity (132). Later it was found that lymphocytes from immunized donors could mediate tumour regression in a syngeneic recipient (133).

The activation of antigen-specific T-cells requires two signals. The first signal occurs when an antigen-presenting cell (APC) presents an antigen in association with the major histocompatibility complex (MHC) to the T-cell receptor (TCR). The second signal is a necessary co-stimulatory signal which is provided by the binding of CD28 on T cells to the CD80/86 receptor on APCs. Both these signals are required for the activation of the T-cell, which then leads to a production of cytokines that enhance T-cell differentiation and proliferation. Many stimulatory and inhibitory molecules tightly regulate this process of T-cell activation. Among the inhibitory regulators is the T-cell antigen named cytotoxic T-lymphocyte antigen 4 (CTLA-4 or CD152). CTLA-4 inhibits T-cell activation via several mechanisms, including the competition with CD28 for CD80/86 ligation, thereby interrupting the necessary co-stimulatory signal (134) (Figure 8).

## Ipilimumab

Ipilimumab is a human monoclonal antibody against CTLA-4, leading to a continuous activation of the T-cells. Preliminary data from early phase I/II studies suggested that the combination of ipilimumab and a cancer vaccine could induce melanoma regression with acceptable toxicity (135). This constituted the basis for the landmark phase III trial by Hodi et al. (136) that assessed ipilimumab in combination with a peptide vaccine derived from the melanoma-related glycoprotein 100 (gp100). Patients with metastases from cutaneous melanoma (n=676) were randomized in a 3:1:1 ratio to the following three groups: ipilimumab with gp100, ipilimumab with placebo and gp100 with placebo. After a median follow-up of 20 months, the median survival time was 10 months for the combination group, 10 months for the ipilimumab group and 6 months for the vaccine group. The survival advantage with ipilimumab therapy marked the first time in the history of

metastatic melanoma research where a treatment had significantly improved survival in a randomized phase III clinical trial.
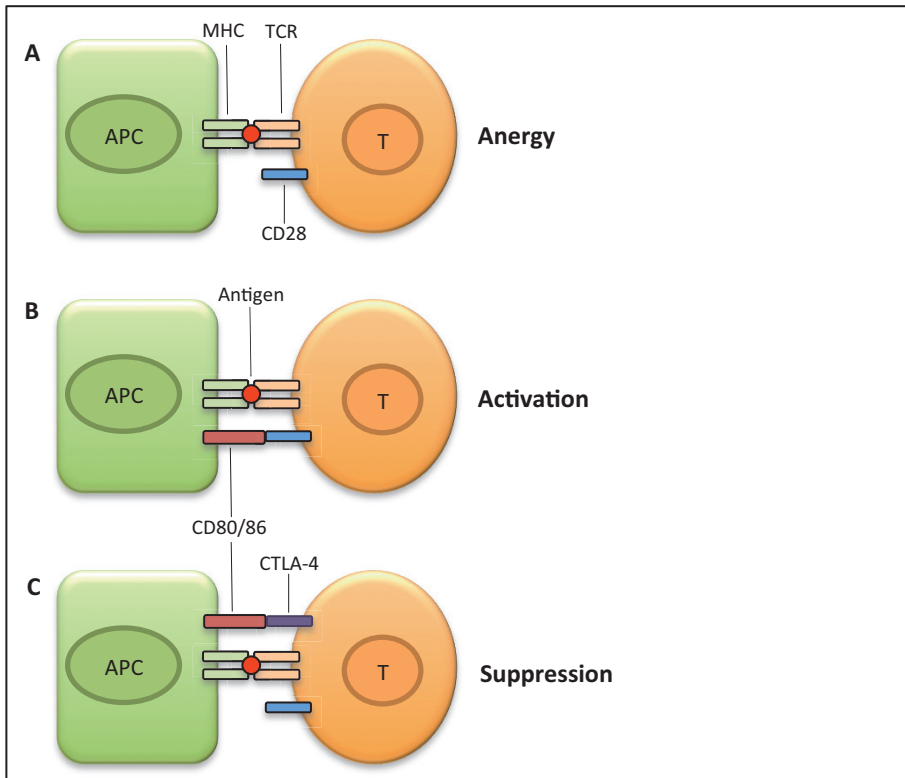


**Figure 8. (A)** Antigens are presented on the major histocompatibility (MHC) complex by antigen-presenting cell (APC) and binds to the T-cell receptor (TCR) on the T-cell. **(B)** In order to become activated, the T cell must not only bind to the MHC-antigen complex, but must also receive co-stimulation by the CD80/86 and CD28 interaction, otherwise the T-cell will go into anergy or apoptosis. The activation of the T-cell leads to cytokine production, proliferation and differentiation. **(C)** After T-cell activation, the T-cell up-regulates the cytotoxic T-lymphocyte antigen 4 (CTLA-4), resulting in cell-cycle arrest and suppression of the T-cell activation upon next encounter with the APC, preventing the T-cell activation to continue. Adapted from Alegre et al. (137)

Two small trials have been published evaluating efficacy and safety of ipilimumab in the treatment of uveal melanoma metastases. The response-rates have been low, with one PR and three SD in a total of 35 patients. The median survival was reported to be 9 months and 5 months respectively in the two trials (138, 139). On-going trials include the IPI-Trial, an open-label, multi-center, single-arm study aiming at further characterization of efficacy

and safety. The estimated study completion date is March 2014 (ClinicalTrials.gov Identifier: NCT01355120).

### 1.3.3   Immunogenic cell death

Cells can die through different pathways and with distinct morphological changes depending on the cell type and the cause of cell death. Apoptosis is primarily defined by its morphological hallmarks, including chromatin condensation, nuclear fragmentation, shrinkage of the cytoplasm and formation of apoptotic bodies (140). Apoptotic cell death is a widespread and constantly occurring mechanism, and is essential for normal development, tissue homeostasis and numerous other physiological processes. Phagocytic cells specifically recognize apoptosis, and apoptotic bodies are silently removed by phagocytosis during the release of potent anti-inflammatory mediators to avoid local inflammatory reactions. Therefore, apoptosis has been considered as an immunologically silent type of cell death (141).

However, recent evidence suggests that some chemotherapeutic agents, such as anthracyclines and the alkylating drug cyclophosphamide, induce an immunogenic type of apoptosis with some unique and distinctive properties. Systematic comparisons of cells undergoing immunogenic and non-immunogenic apoptosis have identified some of the major components involved in this process (142).

Calreticulin (CRT) is a $Ca^{2+}$-binding chaperone that is normally found in the lumen of the endoplasmatic reticulum, but early in the event of immunogenic cell death, CRT is translocated to the cell surface and serves as an "eat me" signal for phagocytes (143, 144).

Later in the process, large amounts of adenosine triphosphate (ATP) is released and serves as a "find me" signal, facilitating the recruitment of phagocytes into the tumour bed (142). Finally the nuclear protein High-Mobility Group Box 1 (HMGB1) is released. HMGB1 binds to macrophages and activates their release of pro-inflammatory cytokines, providing optimal antigen presentation to the T cells (145). All these processes result in a immune response involving CTLs, which eventually can lead to the eradication of chemotherapy-resistant tumour cells.
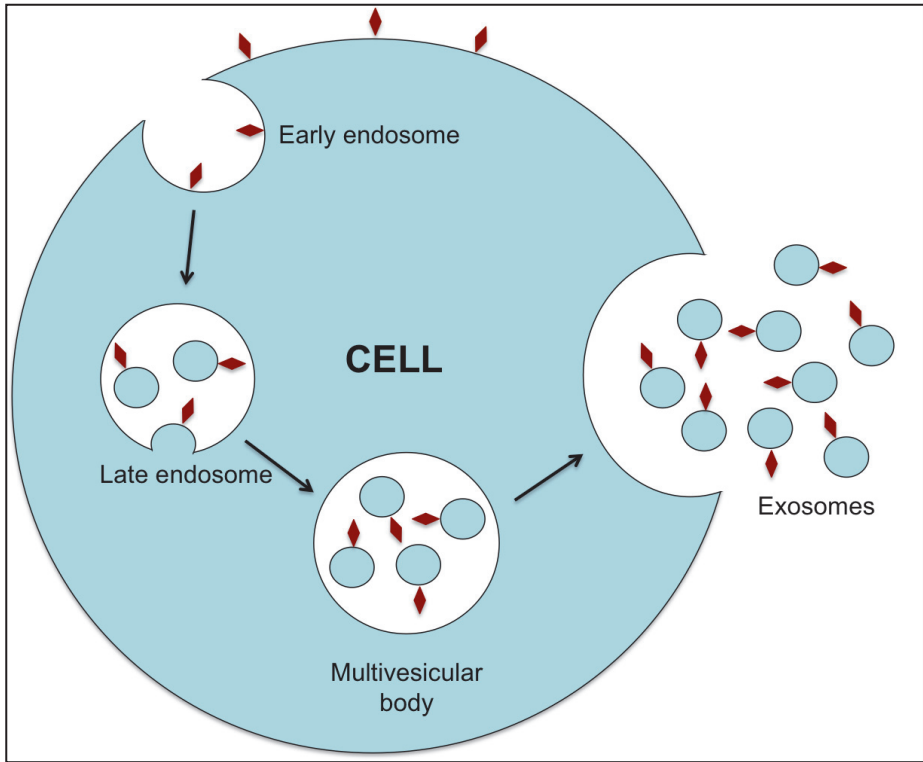
# 1.4 Exosomes

In the early 1980s, studies of reticulocytes identified the presence of a previously unknown system that discarded unwanted proteins from cells. It was found that the transferrin receptor was shed in small membrane vesicles via an unknown secretory process (146); these nano-sized membrane vesicles were later named exosomes (147). Exosomes are formed from multivesicular bodies containing intraluminal vesicles formed when the limiting membrane of an endosome buds inward and encapsulates cellular cytoplasmatic contents. Exosomes are therefore topologically equivalent to cells in that they have cellular cytoplasmic contents in the exosomal lumen and membrane protein domains on the exosomal surface (148). The size of an exosome has been calculated to be about 30-100 nm in diameter; indicating that the total cargo per exosome probably is less than 100 proteins and less than 10 000 nucleotides (149).

Virtually all cells release exosomes, and they are found in virtually all human body fluids. Exosomes contain proteins that are common for many types of exosomes (e.g. the tetraspanins CD9, CD63 and CD81) but also specific proteins based on cell origin, e.g. exosomes from melanocytes may contain Melan-A (150).

In the late 1990s it was shown that exosomes were important factors in cell-to-cell communication, with primarily immune regulatory functions (151, 152). But the versatility of this novel communication system was not fully appreciated until the pivotal study by Valadi et al. (153) was presented in 2007. It was then shown that cells incorporate RNA into exosomes and that this RNA was transferred to recipient cells where the RNA was translated into proteins. This RNA was named exosomal shuttle RNA (esRNA).

Exosomes contain both RNA and micro RNA (miRNA), the latter being a class of small (~22 nucleotides) non-coding RNA molecules acting as gene regulators. MiRNA regulate gene expression at the post-transcriptional level through RNA interference, where miRNA can bind to specific sequences of target mRNAs, resulting in either translational inhibition or mRNA degradation (154). In humans, approximately 2000 different miRNA have been described, and a single miRNA can regulate multiple mRNAs and a single mRNA can be targeted by several miRNAs. Through these

mechanisms, miRNAs are an essential component in the regulation of most cellular processes (155).



**Figure 9.** Schematic representation of the biogenesis of exosomes and the topological orientation of their content. Adapted with permission from Maria Eldh (156).

The packaging of RNA into exosomes seems not just to be the result of an engulfment of random RNA from the cytoplasm, but an active process. The mechanism behind this process is not fully known (157).

In 2010, Eldh and colleagues (158), showed that the RNA content of exosomes was dependent on the condition that they were released under. Exosomes released from cells under oxidative stress provided the recipient cells with a tolerance to further oxidative stress, showing that exosomes could transfer protective effects between cells.

# 1.4.1   Development of metastases

In the middle of the 19th century, Virchow introduced the idea that metastatic pattern is governed by mechanical/anatomical factors. The location of metastases simply was the result of a tumour-cell emboli stopping in the vasculature. In 1889 Steven Paget introduced the 'seed and soil' hypothesis for metastasis, a pivotal milestone in the study of malignant disease (159). It stated that certain organs, such as the liver, appeared to be particularly receptive to metastases due to a favourable microenvironment, and that this microenvironment was essential for malignant cells to form metastases. These results were based on the studies of autopsy records from 735 women with fatal breast cancer, with a discrepancy between the blood supply and frequency of metastasis to specific organs. Paget concluded that "When a plant goes to seed, its seeds are carried in all directions, but they can only live and grow if they fall on congenial soil" (159).

More recently it has been proposed that these soils might not be present from the beginning, but merely are created by specific factors produced by the primary tumour, prior to the seeding of metastatic cells (160). Kaplan et al. published one of the pivotal studies in this field where it was shown that bone marrow-derived cells (BMDCs) contribute to the metastatic spread by creating soils, currently known as metastatic niches. Mice were inoculated with a lung carcinoma and thereafter injected with cell culture media from a melanoma cell line. Following this, the lung cancer metastases were redirected to organs characteristic of melanoma metastasis (such as the spleen, intestine and kidney). It was shown that factors provided by the primary tumour induced BMDCs to enter the bloodstream and migrate to organ-specific sites. Perhaps the most striking finding was that this process actually preceded the arrival of the tumour cells, thereby creating the concept of pre-metastatic niche formation (161).

Growing evidence shows that one important factor coming from the primary tumour, during the formation of pre-metastatic niches, are tumour-derived exosomes. Peinado et al. showed in vivo that exosomes from highly metastatic melanomas increased the metastatic behaviour of primary tumours by both 'educating' BMDCs through the receptor tyrosine kinase MET and by inducing vascular leakiness at pre-metastatic sites. Exosomes, derived from a highly metastatic cancer cell line, could induce a larger metastatic tumour burden, compared to exosomes derived from a weakly metastatic cell line.

Reducing Met expression in exosomes also diminished the pro-metastatic behaviour of the bone marrow cells by limiting both tumour growth and metastasis (162).

Malignant melanomas primarily metastasize to regional lymph nodes. One hypothesis is that melanoma cells undergo simultaneous haematogenous and lymphatic spread, and that the presence of tumour cells in sentinel or regional lymph nodes is merely an indication of metastasis. Alternatively, there is an orderly progression of tumour cells with sentinel or regional nodes playing an active role in the development of metastasis (163). It has been shown that tumour spread to the lymph nodes is facilitated by preparation of a pre-metastatic niche within the lymph node by induction of lymphatic vessel growth (164). Hood et al demonstrated this in vivo, by using fluorescent exosomes to track melanoma-derived exosomes back to the sentinel lymph nodes. In the next step, tumour derived exosomes, or control liposomes, were injected into the footpads of mice. This was followed by an injection of melanoma cells three days later. The experiment showed an increase in the number of melanoma cells infiltrating the sentinel node in mice receiving the tumour-derived exosomes as compared to mice receiving liposome controls. In the sentinel nodes, which received tumour-derived exosomes, there was also an induction of genes associated with cell recruitment, extracellular matrix and vascular growth factors (165).

## 1.4.2   Biomarkers

Exosomes are released from tumour cells both in vitro and in vivo, and exosomes from cancer patients have been isolated from peripheral blood (162, 166), malignant effusion (167) and urine (168).

Patients with different malignant diseases have been shown to have circulating tumour-derived exosomes, making exosomes interesting as biomarkers. In a study by Skog et al, it was shown that exosomes from patients with glioblastoma contained a specific mutation of the epidermal growth factor receptor (EGFR), known to be present in about 50% of tumours. Interestingly, after tumour resection, the mutated EGFR could not be detected; indicating that the tumour was the true source of the exosomes and therefore the exosomal content might be utilized as a tumour marker (166).

Similar findings were reported by Silva et al, where exosomal miRNA expression profiles in patients with non-small cell lung cancer (NSCLC) were evaluated. It was shown that NSCLC patients and healthy controls had different exosomal miRNAs and that the presence of high levels of miRNA let-7f in NSCLC patients was associated with shorter overall survival (169).

Not only the content of exosomes might work as a tumour marker, Logozzi et al reported that patients with melanoma had a significantly higher concentration of exosomes in plasma when compared with healthy controls; and that higher protein concentration of exosomes was associated with worse outcome. They also showed that a there was a higher level of exosomes expressing the tumour marker Caveolin-1 when compared with healthy controls (170).

# 2 AIMS

The overall aim of this thesis is to evaluate the clinical outcome of isolated regional perfusion for extremity and liver metastases of malignant melanoma, investigate associated immunological mechanisms, and to explore the potential use of tumour-derived exosomes as future biomarkers.

The specific aims of the thesis are:

- To describe long-term clinical outcome and associated prognostic factors in patients with in-transit melanoma metastases treated with isolated limb perfusion.

- To analyse whether isolated hepatic perfusion increases overall survival when compared with a control group.

- To determine if isolated limb perfusion induces a tumour specific T-cell response.

- To isolate and characterise the miRNA content of tumour-derived exosomes from liver perfusate obtained during isolated hepatic perfusion.

# 3  PATIENTS AND METHODS

## 3.1  Study populations

### Paper I

During a 25-year period (January 1984 to December 2008) a total of 163 consecutive patients with in-transit metastases of malignant melanoma were treated with first-time ILP. Patients were referred from all over Sweden. There were 105 females and 58 males with a median age of 70 years.

### Paper II

Between April 2005 and March 2011, 34 patients with isolated liver metastasis from uveal melanoma were treated with IHP. There were 19 women and 15 men with a median age of 61 years. Inclusion criteria included less than 50% of the liver replaced by tumour and no evidence of extra-hepatic tumour manifestations.

### Paper III

Twelve patients with in-transit metastasis of malignant melanoma were recruited for the study between March 2008 and November 2009. There were six women and six men with a median age of 70 years. All patients were HLA-A2 positive and all tumours were stained positive for Melan-A.

### Paper IV

During the period from November 2010 to October 2012, twelve patients with isolated liver metastasis from uveal melanoma underwent treatment with IHP. Samples from peripheral blood and liver perfusate were collected.

## 3.2  Data retrieval

Data concerning response and progress was retrieved retrospectively from patient medical records obtained from each referral center. Data concerning survival and cause of death was retrieved from the Swedish National Cause of Death Register (Swedish National Board of Health and Welfare).

In Paper II, an overall survival comparison was made using data retrieved from the National Patient Register (Swedish National Board of Health and

Welfare). This register contains information on date and diagnoses for all inpatient and non-primary care outpatient visits in Sweden from 2002 and onwards. All patients with a primary ICD-10 diagnosis of C69.3 and C69.4 (malignant tumour in the choroidea and the ciliary body respectively) between September 2002 and September 2011 were retrieved from the register (n=1502). From this data, all patients that developed liver metastases (ICD-10 C78.7) were identified (n=132). Overall survival was calculated from the date of the first appearance of liver metastases to the date of death shown in the Swedish Cause of Death Register.

## 3.3   Isolated limb perfusion
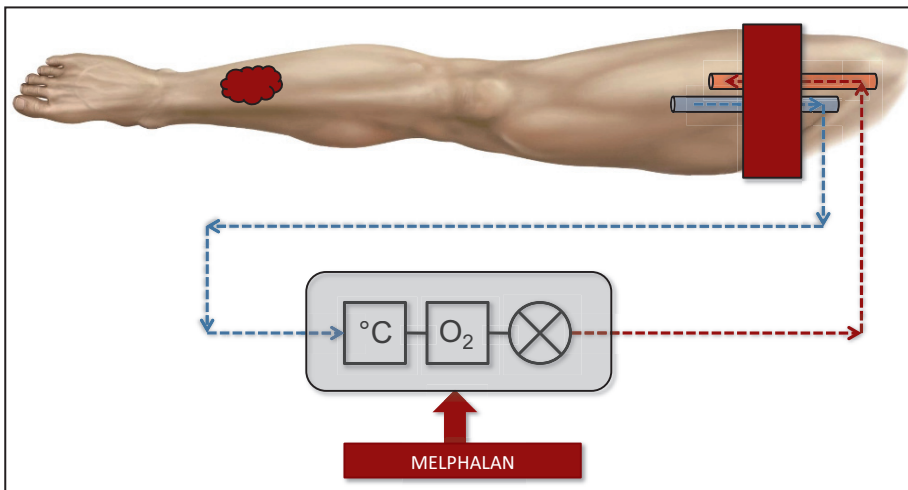


**Figure 10.** Schematic of the setup used in femoral isolated limb perfusion. Catheters are placed in the femoral artery and vein, and then connected to the perfusion circuit consisting of a heater, an oxygenator and a roller pump. An inflatable tourniquet around the thigh further completes the isolation of the limb.

ILP was performed via the axillary, brachial, subclavian, iliac, or femoral approach, with dissection and cannulation of the corresponding artery and vein. To achieve limb isolation in femoral perfusions, the remaining collateral vessels were compressed using an inflatable tourniquet (Zimmer disposable tourniquet). In iliacal and upper limb perfusions an Esmarch bandage secured around a Steinman pin, placed into either the anterior superior iliac spine or the humeral head, was used.

The cannulas were then connected to an oxygenated extracorporeal circuit. From October 2000, continuous leakage monitoring was performed using a precordial scintillation probe (MedicView, Sweden) to detect and measure leakage of technetium-99m labelled human serum albumin (Vasculosis, Cis Bio, France) injected into the perfusion circuit.

For M-ILP the dose of melphalan was 13 mg/L for upper limb and 10 mg/L for lower limb perfusions. Fifty per cent of the total dose was initially administered; thereafter the remaining 50% was administered as two equivalent doses with 30 minutes intervals. Between 1984 and 2005 the perfusion time was 120 minutes, which then was changed to 90 minutes. Between 1984 and 2003, the perfused tissue temperature was kept between 41-41.5°C, in 2003 this was changed to 39-40°C. At the end of the perfusion, the limb was irrigated with 1000 mL of low molecular weight dextran (Rheomacrodex, Meda, Sweden). Finally, one unit of erythrocytes was transfused into the treated limb.

With patients receiving TM-ILP, a bolus dose of TNF-alpha (Beromun, Boehringer Ingelheim, Germany) was injected into the arterial line (3 mg upper limb, 4 mg lower limb), when limb tissue temperatures had reached 38°C. After 30 minutes, the temperature was increased to 39°C-40°C and melphalan (13 mg/L for upper limbs, 10 mg/L for lower limbs) was administered during a 20 minute infusion. The total perfusion time was 90 minutes; the limb was then irrigated with 1000-2000 mL (upper limb) or 3000-4000 mL (lower limb) of Ringer's solution (Ringer Acetat, Baxter). Finally, one unit of erythrocytes was transfused into the treated extremity.

## 3.4   Isolated hepatic perfusion

The IHP procedure starts with ultra-sound guided cannulation of the femoral and the external jugular veins, which are then connected to an external veno-venous bypass pump. This allows for active shunting of blood around the clamped inferior vena cava (IVC) when the liver is later isolated. Thereafter, an exploratory laparotomy is performed and signs of extra-hepatic involvement are excluded. The liver is completely mobilized from the diaphragmatic and retrohepatic attachments. The IVC is extensively dissected from the level below the renal veins up to the diaphragm, with the ligation of all retroperitoneal venous tributaries including the right adrenal vein. A

perfusion outflow wire-reinforced catheter (18 Ch) is inserted through the right gonadal vein and placed in the retro-hepatic portion of the IVC. The hepatoduodenal ligament is dissected and the hepatic artery is cannulated using a wire-reinforced catheter (8-12 Ch) placed via the gastroduodenal artery. The liver is then isolated by applying tourniquets around the portal vein, the bile duct and around the IVC at a level above the renal veins. The IVC is clamped below the diaphragm using a vascular occlusion clamp and a small vascular clamp is placed on the hepatic artery. The cannulas are connected to an oxygenated extracorporeal circuit.
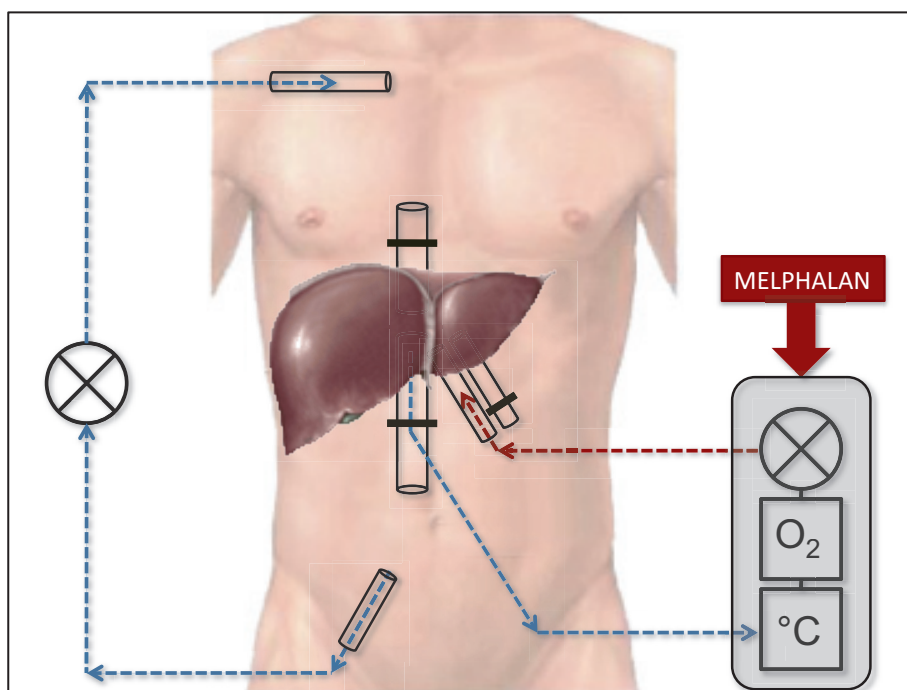


**Figure 11.** Schematic representation of the setup during isolated hepatic perfusion.

The perfusion is started and when stable conditions are reached, the leakage monitoring system is calibrated by injection of 10 MBq and 100 MBq technetium-99m labeled human serum albumin (Vasculosis, Cis Bio, France) into the systemic and the perfusion circuits, respectively. Melphalan (1 mg/kg body weight) is then administered into the perfusion circuit and the temperature is held at 40°C with a perfusion time of 60 minutes (Figure 1). During the procedure, continuous leakage monitoring is performed using a

scintillation probe (MedicView, Göteborg, Sweden), placed over the veno-venous bypass pump, to detect and measure leakage. After perfusion, the liver is washed with 1000-2000 mL of Ringer's solution (Ringer Acetat, Baxter) and one unit of erythrocytes is transfused into the liver. The catheters, tourniquets and vascular clamps are removed and the gastroduodenal artery and the gonadal vein are ligated.

# 3.5 Response evaluation

## Clinical response

Clinical responses (Papers I and III) are reported as the best response according to the WHO criteria (171). CR includes the disappearance of all lesions; partial response (PR) is defined as a decrease of more than 50% of the total tumour burden. Progressive disease (PD) is defined as an increase of more than 25% in existing lesions or the appearance of new lesions. No change (NC) is defined as a result where neither the criteria for CR, PR nor PD are met. Local progression is defined as the appearance of new lesions or progression of existing lesions within the treated limb, not including lymph-node metastases. The clinical evaluation was done by the treating physician, and the information was extracted from the patients medical record.

## Radiologic response

Radiologic response (Paper II and IV) was reported according to RECIST 1.1 criteria in which CR was the disappearance of all target lesions (172). PR was defined as at least a 30% decrease in the sum of diameters of target lesions taking as reference the baseline sum diameters. PD was defined as at least a 20% increase in the sum of diameters of target lesions. In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm. The appearance of one or more new lesions is also considered progression. Stable Disease (SD) was defined as neither a sufficient shrinkage to qualify for PR nor a sufficient increase to qualify for PD. In paper II, the response evaluation was done 8-12 weeks after IHP treatment with CT or MRI. Thereafter, the patients were evaluated every three to six months at the discretion of the treating physician. The response evaluation was done by one radiologist reviewing all patients.

# 3.6 Statistical methods

Survival estimates were made using the Kaplan-Meier method and compared using the log-rank test (Papers I, II). Univariate and multivariate analyses of prognostic factors for different survival analyses were made using Cox-regression and predictive factors for response were analysed using logistic regression. After univariate analysis, all variables with a significance level of less than 0.10 were included in a multivariate analysis with a stepwise backward algorithm to exclude factors without prognostic value. A p-value less than 0.05 were considered statistically significant. The data was analysed using SPSS version 19 (SPSS, Chicago, USA).

# 3.7 Laboratory analyses

## Paper III

### Major lymphocyte subset in peripheral blood
Freshly drawn EDTA blood was stained with the following fluorchrome-conjugated monoclonal antibodies: CD3-PerCP, CD4-PE, CD5-FITC, CD8-PE, CD14-PE, CD45-FITC, CD45RA-FITC, CD45RO-FITC, CD56-PE and HLA-DR-PerCP. After incubation, the erythrocytes were lysed and cells were washed. For absolute quantification BD TruCount beads were used according to the manufacturer´s protocol. The cells were analysed on a BD FACSCanto and CellQuest according to the manufacturer´s protocol.

### Pentamer staining
For detection of Melan-A specific CD8+ cytotoxic T cells, freshly drawn EDTA blood was incubated with a phycoerythrin conjugated ELAGIGTV-pentamer. Following incubation the blood was washed once and incubated with CD3-FITC, CD8-PerCP and CD19-APC for 20 minutes on ice. The blood was lysed, washed twice and resuspended in BD FACSFlow before flow cytometry analysis. The cells were analysed on a BD FACSCanto and CellQuest according to the manufacturer´s protocol.

## Paper IV

### Exosome isolation
Plasma was obtained from both liver perfusate and peripheral heparinized blood by centrifuging at 400 × g for 10 minutes followed by a second

centrifugation at 1 880 × g for 10 minutes. The purified blood plasma was then centrifuged at 29 500 × g for 20 minutes to remove remaining cells and cell debris. The supernatant was then filtered through 0.2 μm filters to remove particles larger than 200 nm. Exosomes were pelleted from the purified blood plasma by ultracentrifugation at 120 000 × g for 90 minutes (Ti70 rotor, Beckman Coulter, Brea, CA, USA).

### Flow cytometry analysis

Isolated plasma exosomes were resuspended in PBS and were incubated with anti-CD63 beads. The exosome-bead complexes were washed twice in FACS buffer and then incubated with PE-conjugated anti-CD9, anti-CD63, anti-CD81 antibody or isotype control and analysed by flow cytometry.

### Electron microscopy

Exosomes were loaded onto carbon-coated nickel grids and immunostained with anti-CD63 antibody followed by a 10 nm gold-labelled secondary antibody. The preparations were examined using a LEO 912AB Omega electron microscope (Carl Zeiss NTS, Jena, Germany).

### Western blot

Purified exosomes were treated with lysis buffer and subjected to sonication and vortex. Protein concentration was determined using a BCA protein assay kit. The exosomal proteins were then loaded on pre-casted gels and blotted to PVDF membranes. Melan-A was detected using anti-Melan-A antibody with a donkey-anti-rabbit horseradish peroxidase linked secondary antibody and then visualised by chemoluminescence .

### RNA analysis

RNA was extracted from exosomes and analysed for yield and size distribution using capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Foster City, CA, USA) with the total RNA 6000 Nano and 6000 Pico Kit according to the manufacturer's protocol.

Exosomes were also analysed for the presence of a panel of 88 cancer-related miRNA using RT2 miRNA PCR arrays (MAH-108A, QIAGEN, Germantown, MD, USA). As controls, exosomes purified from A375 cells, MML-1 cells, HTB-133 cells, HTB-177 cells and HMC-1 cells were analysed. Total RNA was DNase treated, reverse transcribed and subjected to

real-time PCR. The results were analysed using the CFX Manager™ software (Bio-Rad).

MiRNA expression levels were measured by the threshold cycle (Ct) and ranked accordingly. Cluster analyses were then performed with a hierarchical method using an average linkage and Euclidean distance metric.

Enriched KEGG pathway analyses for miRNAs was conducted via DIANA-miRPath v.2.0 software based on predicted targets by DIANA-microT-CDS (173, 174). Targets of miRNAs with a score of more than 0.8 were selected. Only KEGG pathways including at least 5 genes and displaying an enrichment p-value of less than 0.05 were considered significant.

# 4 RESULTS

## 4.1 Paper I

The aim of this paper was to describe long-term clinical outcome and associated prognostic factors in patients with in-transit melanoma metastases treated with first-time ILP. The study included 163 consecutive patients treated during a 25-year period with an almost complete follow-up.

**Table 6.** Characteristics of the 163 consecutively included patients.

| | | |
|---|---|---|
| *Sex* | *Female* | *105 (64%)* |
| | *Male* | *58 (36%)* |
| *Age* | | *70 years (23-94)* |
| *Primary tumour location* | *Upper arm* | *6 (4%)* |
| | *Lower arm* | *6 (4%)* |
| | *Hand* | *2 (1%)* |
| | *Upper leg* | *11 (7%)* |
| | *Knee* | *5 (3%)* |
| | *Lower leg* | *95 (58%)* |
| | *Foot* | *33 (20%)* |
| | *Unknown* | *2 (1%)* |
| | *Missing* | *3 (2%)* |
| *Breslow thickness* | | *6.0 mm (0.8-137)* |
| *Bulky tumour (>4 cm)* | | *29 (18%)* |
| *N-stage* | *N2c* | *95 (58%)* |
| | *N3* | *68 (42%)* |
| *M-stage* | *M0* | *152 (95%)* |
| | *M1* | *8 (5%)* |
| *Chemo* | *Melphalan* | *148 (91%)* |
| | *Melphalan + TNF-alpha* | *15 (9%)* |
| *Vessel* | *Axillary* | *9 (6%)* |
| | *Brachial* | *3 (2%)* |
| | *Femoral* | *57 (35%)* |
| | *External iliac* | *92 (56%)* |
| | *Subclavian* | *2 (1%)* |

The sample can be regarded as population-based, as it is made up of nearly all patients in Sweden treated with ILP during these years (1984-2008), except for 5-10 patients treated at Norrland´s University Hospital (personal communication Mikael Öman).

The results showed CR in 101 patients (65%) and PR in an additional 31 patients (20%), giving an overall response rate of 85%. The only significant independent predictive factor for CR was the presence of less than ten in-transit metastases. When comparing response rates using different temperatures (41-41.5°C vs. 39-40°C) or perfusion lengths (120 min vs. 90 min), no significant differences were found; neither in univariate or multivariate analysis.
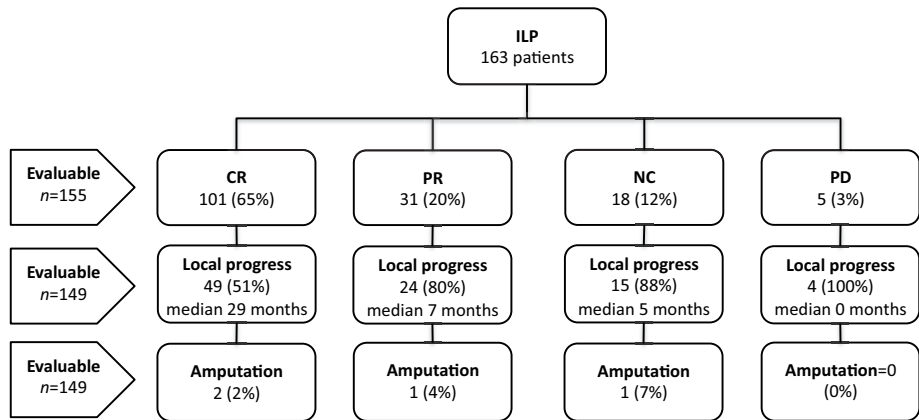


**Figure 12.** Flow chart of outcome for 163 consecutive patients. Reprinted with permission from Informa Healthcare.

Local progressive disease developed in 94 patients (63%) after a median time of 16 months (0-74 months). Independent risk factors for local progression were proximal location of the primary tumour and the absence of CR after ILP. Four patients (2%) were amputated due to progressive disease after a median of 19 months (10-36 months).

Eight patients (5%) had distant metastases at the time of ILP and another 108 patients (66%) developed distant metastases after a median period of 12 months (1-119 months). Median overall survival was 27 months with a 2-year, 5-year and 10-year survival of 53%, 26% and 8% respectively. Median cancer-specific survival was 30 months with significant independent negative

risk factors being positive lymph-node status, bulky tumour and the absence of CR after ILP. The cause of death was attributed to malignant melanoma for 105 of the 129 deceased patients (81%).

Using the Wieberdink classification (115), toxicity was classified as grade II in 103 (63%) patients and grade III in 53 patients (33%). Three per cent of the patients developed a grade IV toxicity with a long-lasting functional disturbance. Increasing age, longer perfusion time (120 vs. 90 minutes) and higher perfusion temperature (41°C vs. 40°C) were identified as predictive factors for increased toxicity.

# 4.2   Paper II

The aim of this paper was to describe clinical outcome and to analyse overall survival compared with a control group, in patients with isolated uveal melanoma liver metastases treated with IHP. The study included 34 patients treated between 2005 and 2011.

**Table 7.** Patient characteristics

| | | |
|---|---|---|
| Sex | Female | 15 (44%) |
| | Male | 19 (56%) |
| Age | | 61 (17-77 years) |
| Primary tumour localization | Choroidea | 31 (91%) |
| | Ciliary Body | 3 (9%) |
| Time from primary tumour to metastases | | 35 (19-395 months) |
| Previous treatment of liver metastases | Liver resection | 3 (9%) |
| | Chemotherapy | 6 (18%) |
| | No treatment | 26 (76%) |
| Largest liver metastases (diameter) | | 2.35 (1.0-6.4 cm) |
| Volume of liver occupied with metastases | <10% | 22 (65%) |
| | 10-25% | 9 (27%) |
| | 26-50% | 3 (9%) |
| Number of liver metastases | 1-4 | 7 (21%) |
| | 5-10 | 15 (44%) |
| | 11-100 | 9 (27%) |
| | >100 | 3 (9%) |

The overall response after IHP was 68% with four patients having a complete response (12%), and 19 patients having a partial response (PR 56%). Six patients had a stable disease (SD 18%) and 5 patients developed a progressive disease (PD 15%). The median time to local progression (TTLP) was 7 months (range 0-31 months), with two patients still being alive with CR after 23 and 69 months respectively. Independent significant prognostic factors for local progression was the radiological assessment of both the percentage of liver with metastases and the size of the largest metastasis, as well as the finding of more than 100 metastases during surgery.

Twenty-three patients (68%) developed extra-hepatic metastases after a median of 13 months (range 2-34 months). Twenty-five patients have died with a median survival of 24 months after IHP; with all deaths being attributed to uveal melanoma. Significant prognostic factors for survival in univariate analysis were related to tumour burden, measured as the radiological assessed volume of liver occupied with metastases and the diameter of the largest metastasis.

No post-operative mortality was observed in the study. There were three major complications; two patients with respiratory insufficiency requiring a prolonged stay at the intensive care unit (ICU) (38 and 25 days respectively) and one patient with a perforated duodenal ulcer. One other patient developed

**Table 8.** Post-operative complications within 30 days according to the classification of Clavien and Dindo.

| Grade | No of patients | Type |
|-------|----------------|------|
| 0 | 20 | - |
| I | 1 | Nausea |
| | 1 | Pain |
| | 1 | Hepatic artery thrombosis |
| | 2 | Pleural effusion |
| II | 1 | Pneumonia |
| | 1 | Pulmonary emboli |
| | 4 | Septicemia |
| IIIB | 1 | Perforated duodenal ulcer |
| IVA | 1 | Pulmonary effusion, thoracic drainage, pneumonia, reintubated. |
| IVB | 1 | Respiratory, cardiovascular and renal failure. |

a hepatic artery thrombosis leading to a subsequent cholestasis with a persistent bilirubin elevation (Table 8). In all other patients the liver parameters were normalized; most often within 7-10 days after IHP.

In an attempt to answer the question as to whether IHP prolongs survival, a register study using the Swedish National Patient Register was undertaken. From the register, 1502 patients with uveal melanoma were identified, and 161 of these patients had liver metastases. From this cohort, 30 patients that had undergone IHP were identified (*IHP group*; n=30) and these were compared to both all the remaining patients in the cohort (*Control group*; n=131) as well as the 30 longest survivors in the cohort (*Longest survivors group*; n=30). The median OS was 3.3 months, 12.3 and 26.0 months for the *Control group*, *Longest survivors group* and the *IHP group* respectively. There was a significant difference in survival between the *IHP group* and both the *Control group* ($p<0.0001$) and the *Longest survivors group* ($p=0.029$).
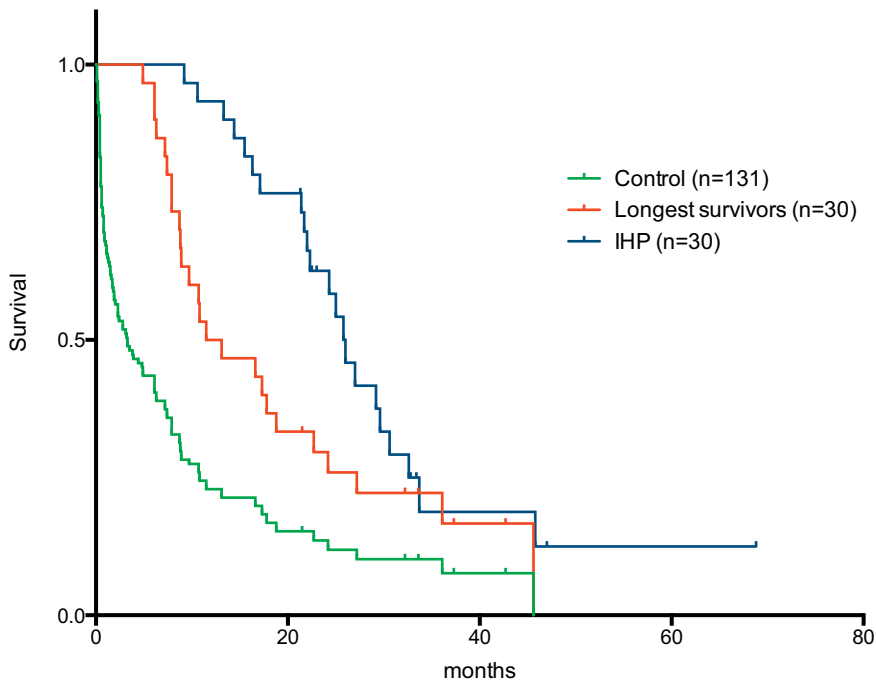
**Figure 13.** Overall survival comparison between patients treated with IHP (IHP; n=30), all the remaining patients in the cohort (Control; n=131) and the 30 longest survivors in the cohort (Longest survivors; n=30).

# 4.3 Paper III

The rationale for this study was preclinical data indicating that alkylating chemotherapeutic agents may give rise to an immunogenic cell death. In line with the hypothesis that part of the anti-tumour effect of conventional chemotherapy is caused by direct killing of tumour cells and part by indirect killing via stimulation of anti-tumour immunity (175), we tried to investigate the effect of hyperthermic ILP on the tumour specific immune response as measured by the number of Melan-A specific CD8+ T cells.

Twelve HLA-A2 positive patients with Melan-A positive melanoma in-transit metastases were included.

**Table 9.** Patient characteristics.

| Patient | Age | Gender | Local | Vessel | N-stage |
|---------|-----|--------|-------|--------|---------|
| 1 | 63 | Female | Foot | Femoral | N3 |
| 2 | 59 | Male | Lower arm | Axillary | N2c |
| 3 | 92 | Female | Lower leg | Femoral | N2c |
| 4 | 68 | Female | Foot | Femoral | N3 |
| 5 | 87 | Male | Lower leg | Femoral | N2c |
| 6 | 73 | Male | Foot | Femoral | N3 |
| 7 | 76 | Female | Lower leg | Iliacal | N2c |
| 8 | 63 | Male | Lower leg | Iliacal | N3 |
| 9 | 53 | Female | Lower arm | Axillary | N3 |
| 10 | 81 | Male | Whole leg | Femoral | N3 |
| 11 | 72 | Male | Whole leg | Femoral | N3 |
| 12 | 78 | Female | Whole leg | Femoral | N3 |

Four weeks following ILP there was a statistically significant (p=0.024) increase in Melan-A specific CD8+ CTLs (Figure 15A). However, the increase in absolute numbers (with a mean of 0.86% out of all CD8+ cells) was small. This is partly explained by the fact that out of the twelve included patients there were only four patients who could be regarded as immunological responders. These four patients showed an absolute increase between 0.84%-4.99% in Melan-A CD8+ specific CTLs 4 weeks after ILP, whereas the remaining patients showed no real difference (See Figure 15A).

At 12 weeks after ILP, the amount of Melan-A specific CTLs seems to return to baseline. However, samples were only available for five patients and only for two of the four patients considered to be immunological responders (See Figure 15A).

No statistical significant changes in major lymphocyte subpopulations were observed during the follow-up of the individual patients (See Figure 14).
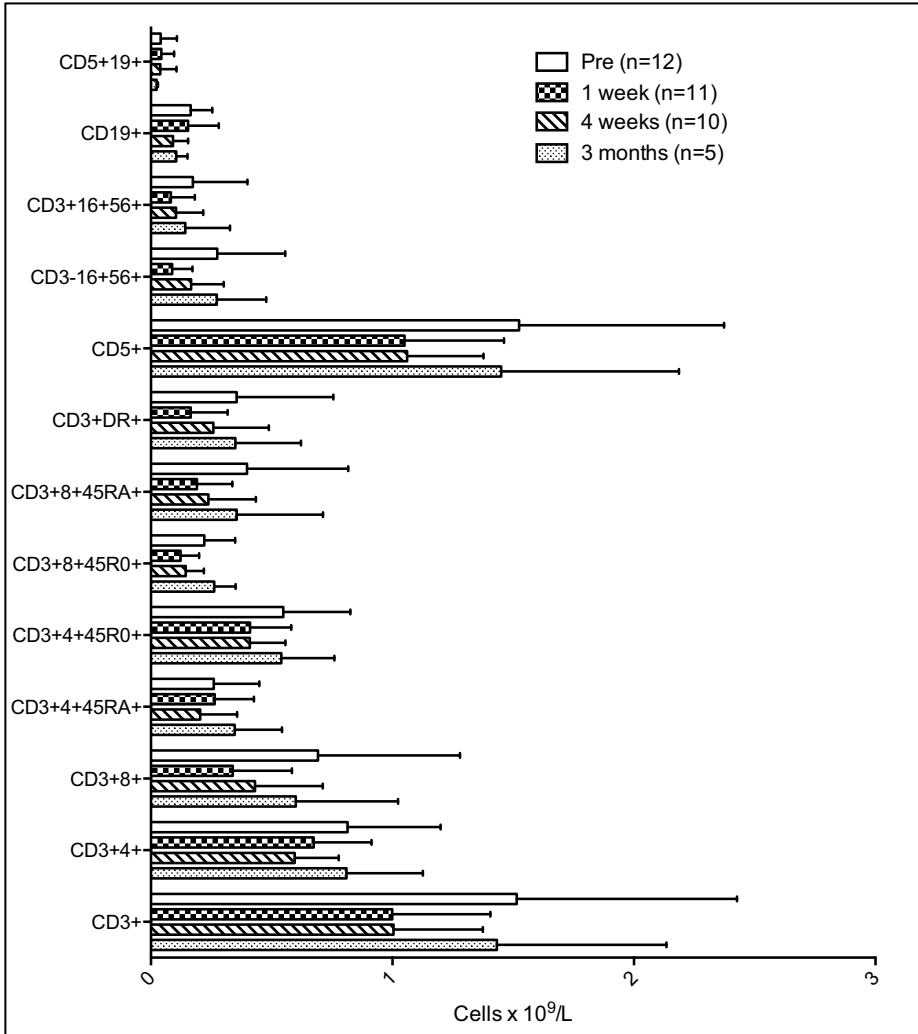


**Figure 14.** Levels (mean±SD) of different lymphocyte subpopulations before and after (1 week, 4 weeks and 3 months) isolated limb perfusion. No significant changes were observed. Reprinted with permission from Informa Healthcare.

When looking at predictive factors for clinical response, CD3+8+ cells, CD3+8+45RA+ cells and CD3+DR+ cells were significantly higher preoperatively in the group of patients with a CR after ILP (n=6) compared to patients without CR (NC+PR, n=6) (Figure 15B).
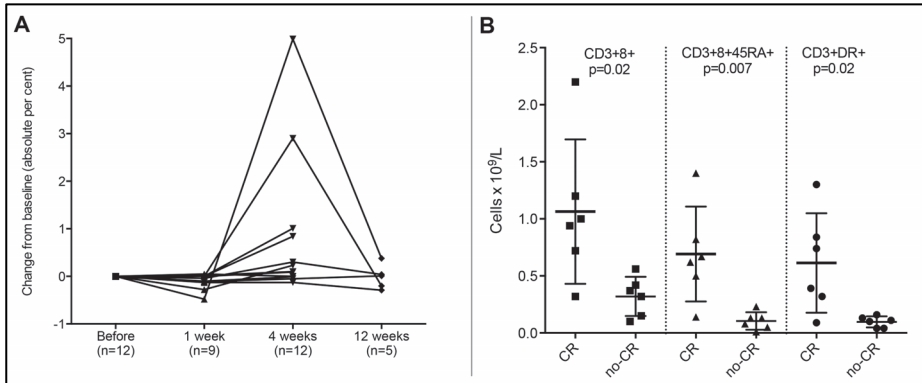


**Figure 15. (A)** Levels of Melan-A specific CD8+ T-cells during 12 weeks follow-up after isolated limb perfusion. **(B)** The levels of CD3+8+, CD3+8+45RA+ and CD3+DR+ cells were significant for clinical response. Reprinted with permission from Informa Healthcare.

## 4.4  Paper IV

The analyses of exosomal content as a tumour marker is in part diminished since only a smaller fraction of exosomes derived from peripheral blood of patients are from the tumour itself. The hypothesis was therefore that if exosomes could be derived directly from the tumour circulation in vivo, the amount of tumour-specific exosomes would increase. In the setting of IHP, this would then be possible by extracting exosomes derived directly from the perfusate of an isolated liver full of metastases.

By characterising these exosomes, insights could be gained into possible tumour specific markers. This hypothesis was explored in Paper IV where the main aim was to isolate exosomes and characterise the miRNA content from twelve patients with uveal melanoma undergoing IHP (Table 10).

The first step was to isolate exosomes from liver perfusate using ultracentrifugation. Electron microscopy showed a for exosomes typical appearance, with a small spherical shape with a diameter of about 50nm. Using immuno-gold staining it was also shown that the exosomes were

positive for CD63 (Figure 17A). Flow cytometry of the exosomes determined that they were also positive for the exosomal markers CD9, CD63 and CD81.

Additionally, Western blot analyses showed exosomes positive for the melanoma-specific marker Melan-A; indicating that, at least in part, the liver perfusate exosomes were of tumour origin (Figure 16B).

**Table 10.** Patient characteristics.

| Patient | Age | Sex | Tumour burden | Largest diameter | Response | Status | Laboratory characterisation of exosomes |
|---|---|---|---|---|---|---|---|
| 1 | 60 | F | 10-24% | 15 mm | PR | Alive 29 mo | RNA, FACS, PCR |
| 2 | 17 | F | 25-50% | 25 mm | PR | Dead 25 mo | RNA |
| 3 | 60 | F | 10-24% | 45 mm | PR | Dead 17 mo | RNA |
| 4 | 62 | M | <10% | 20 mm | PR | Alive 17 mo | EM |
| 5 | 76 | F | <10% | 55 mm | PR | Dead 14 mo | EC, RNA, PCR |
| 6 | 47 | F | 10-24% | 35 mm | PR | Alive 13 mo | EC, RNA, FACS |
| 7 | 64 | M | <10% | 23 mm | SD | Alive 13 mo | EC, RNA, PCR |
| 8 | 58 | F | 25-50% | 100 mm | PD | Alive 11 mo | EC, RNA, PCR, FACS |
| 9 | 67 | M | <10% | 40 mm | PR | Alive 10 mo | EC, RNA, PCR |
| 10 | 72 | F | <10% | 30 mm | SD | Alive 7 mo | EC |
| 11 | 69 | F | <10% | 15 mm | SD | Alive 6 mo | EC |
| 12 | 66 | M | 10-24% | 25 mm | PR | Alive 5 mo | EC |

*PR=Partial response; SD=Stable disease; PD=Progressive disease; EC=Exosome concentration (peripheral blood); RNA=RNA concentration (liver perfusate); FACS=Flow cytometry (liver perfusate); PCR=Exosomal miRNA PCR array (liver perfusate); EM=Electron microscopy (liver perfusate)*

To determine if the concentration of exosomes differed between patients and healthy controls, exosomes were isolated from peripheral blood before IHP. The exosome concentration, measured as total exosomal protein, differed significantly between the peripheral blood of healthy controls and patients (median 13.8 vs. 75.6 µg/ml plasma, p=0.003).

The RNA content was analysed using capillary electrophoresis and was shown to have the typical exosomal profile, lacking the 18S and 28S ribosomal subunits, and included RNA in the size of miRNA (Figure 17A).
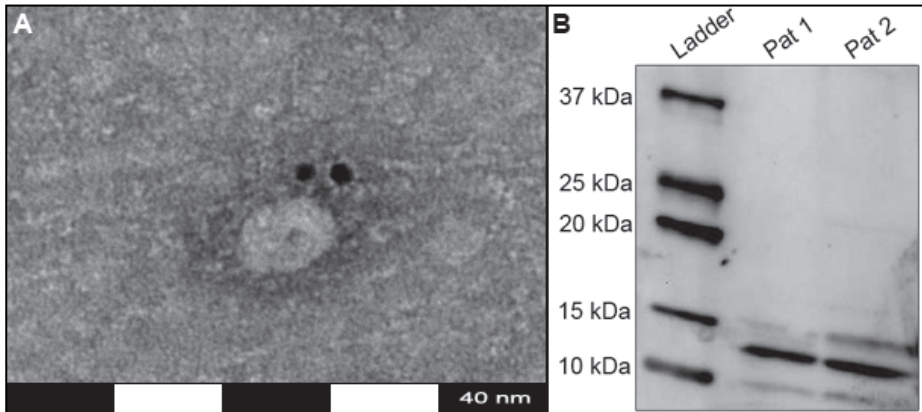


**Figure 16. (A)** A representative electron microscopy image of an exosome derived from the liver perfusate. The image shows a small vesicle, approximately 50 nm in diameter and immune-gold labelled with anti-CD63. **(B)** A representative Western blot analysis showing liver perfusate exosomes from two patients positive for the melanoma-specific marker Melan-A.

Exosomal miRNA profiles of patients were compared, using cluster analyses of exosomal miRNA, with two malignant melanoma cell lines (A375 and MML-1), one breast cancer cell line (HTB-133), one lung cancer cell line (HTB-177) and one human mast cell line (HMC-1). Comparison of the exosomal miRNA profiles revealed a clear relationship between the patient samples (Figure 17B).

The three top miRNA clusters were analysed in greater detail. *Cluster 1* consisted of four miRNAs (miR-216a, miR217, miR129-5p and miR-203) that were expressed almost equally in patients and the lung, breast and melanoma cell lines, but not in the human mast cell line HMC-1 (Figure 17C). KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis showed that the most significant predicted targets of these miRNA were metabolism and signalling pathways.

*Cluster 2* consisted of seven miRNAs (miR-9*, miR125a-5p, miR-25, miR-125b, miR-335, miR-19a and miR-9) that were also expressed almost equally in patients and the five tested cell lines (Figure 17C). KEGG pathway

analysis showed that biosynthesis and metabolism pathways were among the most common pathways.
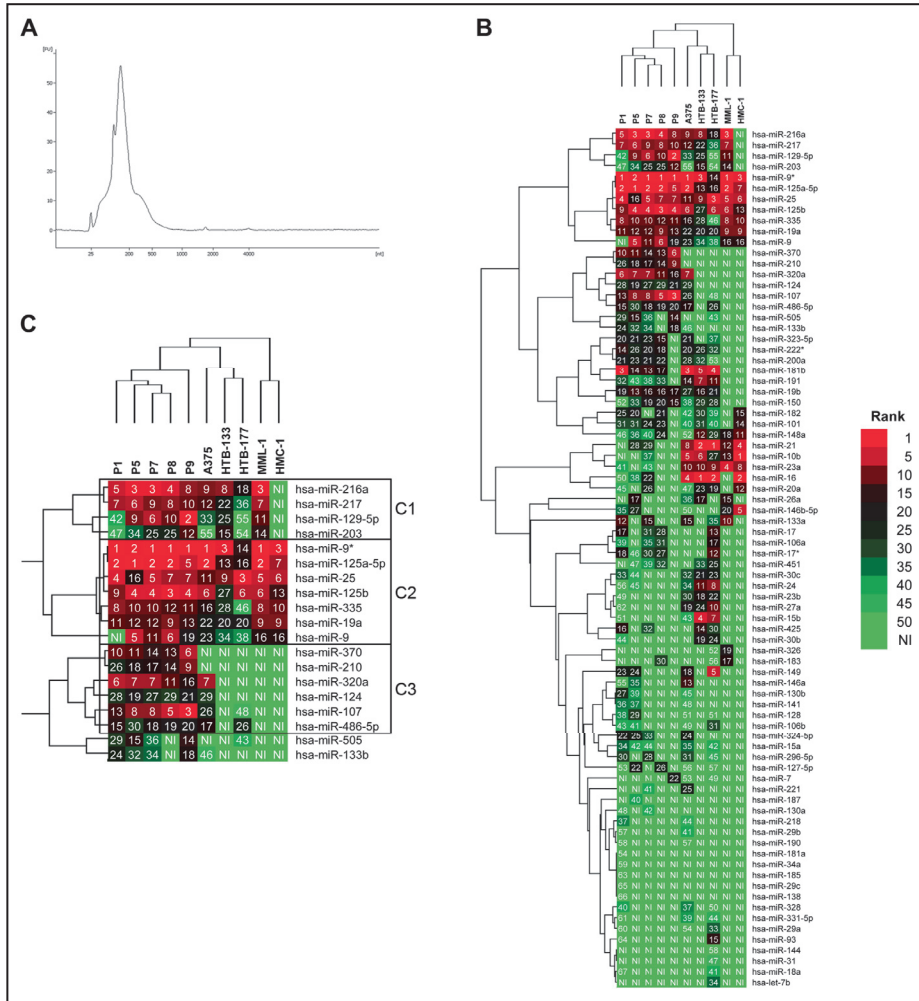


**Figure 17. (A)** A representative Bioanalyzer electropherogram of liver perfusate exosomes, showing a classical exosomal RNA profile, lacking the ribosomal 18S and 28S subunits, and enriched in lower molecular weight RNAs. **(B)** Cluster analysis of the miRNAs found in exosomes from patients and cell lines, showing a clear similarity between the patients. **(C)** A close-up of a specific portion of the cluster analysis, highlighting three miRNA clusters. Cluster 3 shows miRNAs that are more associated with patients than control cell lines. Abbreviations: P1-5 = Patient 1 to 5, C1-3 = Cluster 1 to 3, NI = Not included.

*Cluster 3* consisted of six miRNAs (miR-370, miR-210, miR-320a, miR-124, miR-107 and miR-486-5p) that were primarily present in patients, and

slightly in the A375 melanoma cell line, but not present in the other four cell lines (Figure 17C). KEGG pathway analysis showed that "melanoma", together with "glioma", "hedgehog signalling" and "prostate cancer" were the most significant pathways with p-values less than 0.01.

# 5   DISCUSSION

## 5.1   Paper I

### 5.1.1   Clinical response

The results in Paper I showed an overall response of 85% with previous studies reporting similar response rates (see Table 11). Taking into account the differences in both technique and patient populations among these studies, the variation is surprisingly small. Most studies show a CR rate between 60-70%; which is perfectly comparable to our result of 65%. Previously known independent prognostic factors were negative lymph node status, the absence of bulky tumour, low tumour burden and the addition of TNF-alpha to the perfusion (176-180). The only independent factor in our study was related to low tumour burden (less than 10 in-transit metastases).

Any conclusive comparisons concerning response rate in our study between M-ILP and TM-ILP is not possible; our only indication for TM-ILP was bulky tumour, a previously known negative prognostic factor for CR (181). In the subgroup analysis of bulky melanoma (29 patients) there was no improvement in response by the additional TNF-alpha. There are numerous retrospective analyses, summarized in a recent paper by Rossi et al. (179), which support the use of TM-ILP. However, the only randomized trial failed to demonstrate any improved short-term results (92), and the use of TNF-alpha is therefore debatable. The common critique about the randomized trial by Cornett and colleagues is that they did not analyse the impact of bulky melanoma in the trial (93). However, a new randomized trial addressing this specific issue in melanoma will most probably not be performed.

A randomized clinical trial using ILP in the treatment of soft tissue sarcoma has shown no difference in response rate between 0.5 mg and 4 mg TNF-alpha (182), an indication that if TNF-alpha has an effect, a lower dose is probably just as effective. A dose reduction of TNF-alpha would minimize the risk for severe systemic toxicity, but also make the procedure considerably cheaper (approximately 2500€ per mg).

**Table 11.** Clinical response after ILP.

| Study | Year | Chemo | Temp | n | OR | CR |
|---|---|---|---|---|---|---|
| Romics et al (176) | 2011 | M±T | H | 14 | 93% | 67% |
| Lasithiotakis et al (177) | 2010 | M+T | H | 14 | 100% | 62% |
| Alexander et al (178) | 2010 | M±T/IFN | H | 91 | 95% | 69% |
| Di Filippo et al (179) | 2009 | M±T | H | 113 | 88% | 63% |
| Rossi et al (180) | 2008 | M+T | H | 12 | 100% | 50% |
| | | T | H | 19 | 100% | 53% |
| Hayes et al (181) | 2007 | M+T | H | 27 | 77% | 41% |
| Cornett et al (91) | 2006 | M | H | 58 | 64% | 25% |
| | | M+T | H | 58 | 69% | 26% |
| Noorda et al (182) | 2006 | M±T | H/N | 17 | 77% | 65% |
| | | M±T RP | H/N | 21 | 72% | 62% |
| Knorr et al (188) | 2006 | M+Dac | H | 101 | 79% | 58% |
| Aloia et al (183) | 2005 | M | H | 58 | 88% | 57% |
| Grünhagen et al (189) | 2005 | M+T | H | 83 | 96% | 69% |
| Grünhagen et al (189) | 2005 | M+T low | H | 16 | 94% | 75% |
| Grünhagen et al (171) | 2004 | M+T | H | 100 | 95% | 69% |
| Noorda et al (184) | 2004 | M | N | 40 | | 45% |
| | | M+T | H | 90 | | 59% |
| Noorda et al (185) | 2004 | M±T | H/N | 43 | 84% | 64% |
| Rossi et al (186) | 2004 | M+T | H | 20 | 95% | 70% |
| Noorda et al (187) | 2002 | M±T | H/N | 57 | | 56% |
| | | M±T | H/N | 158 | | 58% |
| Liénard et al (193) | 1999 | M+T | H | 32 | 100% | 78% |
| | | M+T | H | 32 | 91% | 69% |
| | | M | H | 103 | 78% | 52% |
| Feldman et al (197) | 1999 | M+T | H | 6 | | 83% |
| Fraker et al (195) | 1996 | M+T 4mg | H | 26 | 92% | 76% |
| | | M+T 6mg | H | 12 | 100% | 36% |
| Klaase et al (190) | 1994 | M SP | N | 45 | 68% | 47% |
| | | M DP | N | 42 | 90% | 76% |
| Klaase et al (191) | 1994 | M | N | 216 | 67% | 42% |
| Vaglini et al (192) | 1994 | M+T | H | 22 | 77% | 64% |
| Liénard et al (194) | 1992 | M+T | H | 20 | 100% | 89% |
| Kettelhack et al (196) | 1990 | M±Cis | H | 54 | 94% | 60% |
| Weighted mean | | | | 1791 | 83 % | 57 % |
| Olofsson et al (Paper I) | 2013 | M+T | H | 163 | 85% | 65% |

*Abbreviations: Cis, cisplatin; CR, complete response; Dac, Dacarbazine; H, hyperthermia; N, normothermia; M, melphalan; IFN, interferon; SP, single perfusion; DP, double perfusion; RP, re-perfusion; T, TNF-alpha; T low, low dose TNF-alpha; ULAM, unresectable locally advanced melanoma; OR, overall response. Table adapted from Moreno-Ramirez (91)*

When comparing response rates using different temperature (41-41.5°C vs. 39-40°C) and perfusion length (120 min vs. 90 min), no significant response differences were found, neither in univariate or multivariate analysis. Cellular

uptake of melphalan is an energy-dependent process mediated by two distinct amino acid carrier systems. The process is temperature dependent and rapidly saturated. In cell culture studies the uptake reaches a plateau after about 10 min (183). This finding is supported in the clinical setting where studies have demonstrated that there is little, if any, tissue uptake of melphalan after about 20-30 minutes of perfusion (77). An in vitro model that examined the effect of a variety of factors on the sensitivity of melanoma cell lines showed that prolonging treatment beyond 60 minutes had little additional impact. Based on these findings, a possible future development is an even further reduction in perfusion times. Since 2008, when the series in Paper I was finished, we have decreased the perfusion time to 60 minutes, without evidence of decreasing response rates (unpublished data). It might be possible to decrease this further, to possibly 45 or even 30 minutes.

## 5.1.2　Survival

The median cancer-specific survival after ILP was 30 months, this is comparable to a systematic review that reported a median survival of 37 months (91). Based partly on data from the same review (91), overall survival was addressed in 9 studies with the results showing a 5-year survival of 38% and a median survival of 38 months (Table 12). The current study (Paper I) reports survival outcomes in the lower part of the previously reported studies. This could probably be explained by differences in patient populations between studies. Our study included stage IV patients (5% of the patients), and the median age was 70 years, compared with a median age between 57 and 65 years in the other studies (Table 12).

**Table 12.** Survival after ILP.

| Study | Year | n | 5-yr OS | Median OS |
|---|---|---|---|---|
| Noorda et al (184) | 2006 | 21 | 46% | 51 months |
| Knorr et al (185) | 2006 | 101 | 40% | 42 months |
| Grünhagen et al (186) | 2005 | 99 | 33% | |
| Grünhagen et al (178) | 2004 | 100 | 32% | 25 months |
| Noorda et al (187) | 2004 | 43 | 29% | |
| Noorda et al (188) | 2002 | 218 | 38% | |
| Zogakis et al (189) | 2001 | 50 | 50% | 70 months |
| Liénard et al (190) | 1999 | 167 | | 33 months |
| Klaase et al (191) | 1994 | 216 | 42% | |
| Weighted mean | | | 38 % (n=841) | 38 months (n=438) |
| Olofsson et al (Paper I) | 2013 | 163 | 26 % | 27 months |

Significant independent negative risk factors for survival were positive lymph-node status, bulky tumour and the absence of CR after ILP. These findings are similar to earlier reports and have mainly been attributed to underlying tumour biology (192). Studies reporting multivariate analyses of prognostic factors for survival after ILP have shown that the presence of lymph node metastases, CR after ILP, small tumour size, age and gender are independent prognostic factors for OS (178, 192, 193).

Whether ILP actually increases survival in patients with melanoma remains largely unanswered. There have been three randomized trials partly trying to address the issue.

The first study by Ghussen et al was reported in 1984 and included 107 patients with both high-risk primary tumours as well as local recurrences (194, 195). The patients were randomized to either wide excision or wide excision with adjuvant ILP. After almost a 6-year median observation time, there were 26 recurrences in the control group compared with 6 recurrences in the ILP group. They also reported an overall survival benefit with 11 patients dying from melanoma in the control group compared with 3 patients in the ILP group. This study has been criticized mainly for the unusually poor outcome in the control arm, patients with stage I disease randomized to the control group had a local recurrence rate of almost 50% (196).

The second study, by Hafström et al, was published in 1991 and included 69 patients with their first satellite/in-transit recurrence after wide resection of the primary melanoma. The patients were randomized to a wide re-resection with or without adjuvant ILP with melphalan. The results showed an increased DFS with 7 months (17 vs. 10 months), but no significant difference in overall survival (35 vs. 57 months) (197).

The third study, by Koops et al, was a large randomized multi-centre trial including 832 patients with primary cutaneous melanoma >1.5 mm in thickness, that were randomized to either wide excision or wide excision with ILP. The result showed a decrease in the occurrence of in-transit metastases, which were reduced from 6.6% to 3.3%. However, there was no benefit from ILP in terms of time to distant metastasis or survival. The conclusion was that adjuvant ILP cannot be recommended in high-risk primary melanomas.

Notably, the one common problem among all these three studies, is the lack of power to detect a survival difference in the population that potentially has the most to gain - patients with in-transit metastases without evidence of distant metastases. In the Ghussen trial there were only 42 patients with recurrent melanoma, in the WHO study there were theoretically 55 patients (6.6% out of 832 patients) and in the Hafström trial 69 patients.

A 30-year follow-up of the Hafström trial (unpublished data) shows a 57 months median cancer specific survival difference between the control and ILP groups (Figure 18), however the difference was without statistical significance (p=0.24 using the Mantel-Cox log-rank test, and p=0.14 using the Gehan-Breslow-Wilcoxon test emphasizing earlier events).
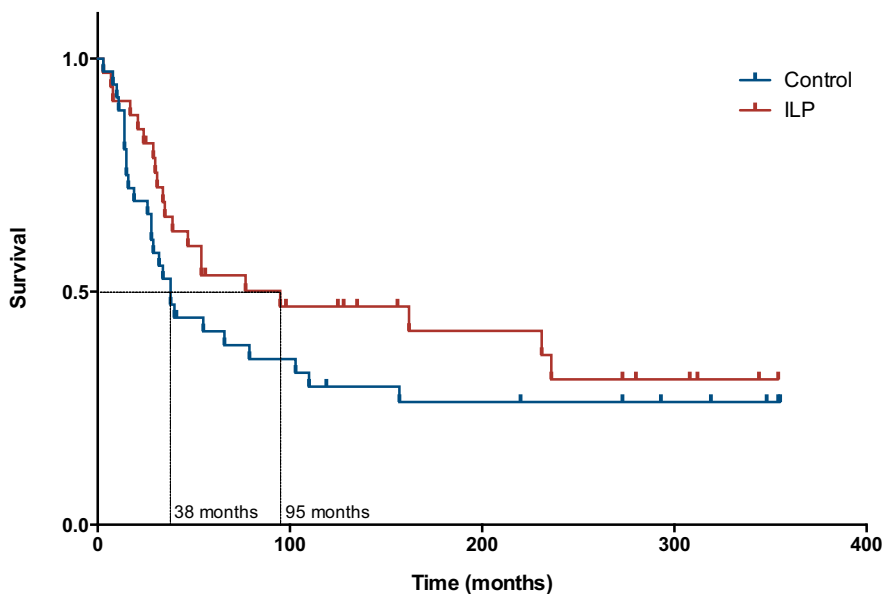


**Figure 18.** Long term follow-up of 69 patients randomized to wide excision with or without adjuvant ILP. The median overall survival difference is 63 months (38 vs. 95 months), however the difference was not statistically significant (log rank test p=0.24).

Taken together, there is no conclusive evidence that ILP prolong survival. However, the question might still be considered open for further debate. Studies have demonstrated a survival benefit in patients with CR after ILP, and several explanations may account for these findings (198). Firstly, and most likely, tumours responding well to ILP have a more beneficial tumour

biology with reduced metastatic potential correlating with prolonged survival. Secondly, elimination of in-transit disease may prevent metastatic spread. Thirdly, ILP may generate a beneficial systemic immune response affecting the development of distant metastases (discussed further in Paper III).

### 5.1.3   Future directions

Taken together, the response rate for ILP is quite uniform despite technical differences and different patient populations. A CR rate of 60-70% with an additional 20-30% achieving a PR is a very good result for any type of cancer treatment. The efforts to improve upon these results have historically focused on TNF-alpha, however, the difference in outcome has not been dramatic and strong data supporting the use of TNF-alpha is still missing. Technical improvements, with a main focus on making the procedure simpler, are also warranted. This could include dose-reduction of TNF-alpha and shorter perfusion times.

Another development of interest is the use of novel targeted therapies in the setting of isolated limb perfusion/infusion. An example is a trial that used ADH-1, a cyclic pentapeptide that disrupts N-cadherin adhesion complexes. It promisingly had a synergistic antitumor activity in a preclinical animal model; unfortunately, no improved outcome was reported in a clinical trial (199). The development of targeted therapies is increasing rapidly, but some fail in the clinical setting due to an unacceptable systemic toxicity. By using ILP, these effects could be minimized. The controlled setting of ILP might also very well prove to be a good model for the investigation of these new drugs.

## 5.2   Paper II

Patients with metastatic uveal melanoma have few therapeutic options. As noted earlier, many patients have isolated liver metastases and many loco-regional liver treatment strategies have been explored. For patients with few liver metastases, liver resection is an option. In the highly selected group of patients where an R0-resection can be achieved, a median overall survival of 27 months has been reported (200). However, surgical resection can only be offered to a minority of patients, and no patient in our material was eligible for resection due to extensive tumour burden. In the study by Mariani, less than 10% of the patients underwent radical resection. For patients with a

more extensive tumour burden, other loco-regional treatment strategies or systemic therapies have to be explored.

The liver is a unique organ with dual blood supply, deriving 70-80% of its supply from the portal vein and the remaining 20-30% from the hepatic artery. It is well established that both primary liver tumours and liver metastases predominantly have their blood supply derived from the hepatic artery (201), whereas the normal hepatocytes are predominantly nourished by the portal venous supply (202).

At this time, there are no evidence of improved survival for any type of treatment for uveal melanoma metastases. Current knowledge about IHP is primarily based on smaller, retrospective single institution series. Alexander and colleagues at the National Cancer Institute in the US have reported pioneering studies on IHP for uveal melanoma metastases. The initial report included 22 patients treated with melphalan (1.5-2.5 mg/kg) with or without TNF-alpha (1 mg). The results showed an overall response rate of 62%, including two patients with CR (10%), and a median overall survival of 11 months (95).

A subsequent study reported on the outcome of 29 consecutive patients treated using melphalan alone (1.5 mg/kg) (203). The outcome was very similar with a 62% overall response including 10% complete response and a median survival of 12 months. Prognostic factors for survival were the number of metastases, the size of the largest metastasis, the percentage of hepatic replacement and baseline level of lactate dehydrogenase (LDH).

In the study by Rizell et al, including 27 patients (20 patients with ocular melanoma), the patients were divided into three different time eras - depending on technical development. The first era (IHP I) used an internal caval shunt; both the hepatic artery and the portal vein were perfused. Also different combinations of melphalan, TNF-alpha and cisplatin, together with a high temperature of 41 degrees Celsius, were used. There was a 27% postoperative mortality, which led to changes in the surgical technique. The second era (IHP II) omitted the portal vein perfusion, used an external caval bypass and decreased the temperature to 40 degrees Celsius. There was still a high postoperative mortality and three patients (27%) died of multi-organ failure due to intractable liver insufficiency. This led to the exclusion of

patients with more than 50% of the liver parenchyma replaced by tumour, and there was also a dose reduction of melphalan to 1 mg/kg body weight (IHP III). Using this regimen, there has been no post-operative mortality in more than 50 patients treated (unpublished data).

**Table 13.** Response rate, survival and mortality after isolated hepatic perfusion

| Author | n | Type | ORR | CR | Median survival | Mortality |
|--------|---|------|-----|----|-----------------|-----------|
| Alexander et al 2000 (95) | 22 | IHP | 62 % | 10 % | 11 months | 5 % |
| Alexander et al 2003 (203) | 29 | IHP | 62 % | 10 % | 12 months | 0 % |
| Noter et al 2003 (204) | 8 | IHP | 50 % | 0 % | 10 months | 0 % |
| Pingpank et al 2005 (106) | 10 | PHP | 50% | 20 % | N/A | 0 % |
| Rizell et al 2008 (205) | 27 | IHP | 70 % | 7 % | 13 months | 22 % |
| Verhoef et al 2008 (206) | 4 | IHHP | 100% | 0% | 9 months | 0 % |
| van Iersel et al 2008 (207) | 12 | IHP | 33 % | 0 % | 10 months | 0 % |
| van Etten et al 2009 (100) | 8 | IHHP | 37 % | 0 % | 11 months | 0 % |
| Weighted mean | 120 | | 59% | 7% | 12 months | 6% |
| Olofsson et al (Paper II) | 34 | IHP | 68 % | 12 % | 24 months | 0 % |

*ORR=overall response rate; CR=complete response; IHP=isolated hepatic perfusion; IHHP=isolated hypoxic hepatic perfusion; PHP=percutaneous hepatic perfusion; N/A=Not available.*

## 5.2.1   Survival

The current study reports on 34 patients treated between 2005 and 2011. The overall response after IHP was 68% with four patients having a complete response (12%). These response rates are quite similar to previously reported studies (Table 13). However, there was a substantial improvement in median overall survival when compared to previously reported series (24 months vs. 9-13 months).

In an attempt to answer the question as to whether IHP prolongs survival, a register study using the Swedish National Patient Register was carried out. All patients in Sweden with uveal melanoma liver metastases were identified and a comparison between patients treated with IHP and the longest surviving patients during the same time period was done. There was an improved survival of 12.3 months for patients that underwent IHP (n=30) compared to a control group consisting of the longest survivors in Sweden during the same time period (n=30).

The hypothesis behind this approach was based on the fact that there are few reports of long-term survivors. In the COMS trial, more than 90% of the patients were dead after 2 years, and only 8 out of 2320 included patients survived for 5 years. This study also concluded that treatment (chemo, radiation, immunotherapy and other) did not have any impact on survival (62).

Another factor that supports the use of this approach is that other treatment trials report median survivals of about 12 months - in contrast to the 24 months survival described in Paper II. The 12 month average includes immunotherapy (9.6 months for ipilimumab (208)), targeted therapies (10.8 months for selumetinib (129)), chemotherapeutic (6.7 months for temozolomide (117) and 10.6 months for BOLD (118)), chemoembolization (9.0 months (209)), intra-arterial fotemustine (13.5 months (210)) and liver resection (14 months (200)).

The use of register data always has major drawbacks, mainly the risk of selection bias. One argument could be that we have selected the most favourable patients in Sweden for IHP treatment. In actual practice, far from all patients in Sweden were considered for IHP - the main reason being an uneven referral pattern between different areas of Sweden.

Another potential bias are the different prognostic factors between the IHP group and the control group - and especially the inability to adjust for the extent of liver metastases. However, when comparing with the longest survivors, any type of matching between cases and controls will only lead to an even more pronounced difference in survival rates. The control group is simply the toughest control group we could construct in Sweden, irrespective of prognostic factors.

## 5.2.2   Future directions

To verify the finding of a potential survival benefit of 14 months using IHP, a multicentre randomized phase III trial between IHP and BAC has recently been started in Sweden. The study (the SCANDIUM trial, ClinicalTrials.gov identifier NCT01785316, www.scandiumtrial.se) plans on recruiting 78 patients during 5 years  - with overall survival as the main endpoint. The first patient has already been included.

# 5.3 Paper III

To find evidence for the hypothesis that ILP might improve survival, and that this could be mediated by an immunological activation, we aimed at determining if ILP could induce a systemic tumour specific T-cell response.

During ILP, the high local concentrations of melphalan will most certainly induce local cell death in tumour cells, but would probably also negatively affect resident immune cells, including DCs. After restoration of the normal circulation, massively released tumour-derived components, including tumour-specific antigens, may gain access to secondary lymphoid organs, where adaptive immune responses may take place. The perfused tumour-containing limb will further become recolonized by new immune cells, including DCs and DC-precursors from blood, which may induce a tumour-specific T cell response.

In 1953, a rare phenomenon where local tumour irradiation caused regression of distant metastases was named the "abscopal effect" (Latin *ab=away from* and *scopus=target*) (211). The abscopal effect has been shown in several types of malignant tumours, including melanoma, lymphoma and renal cell carcinoma (212).

Recently there was a case report of a patient with melanoma metastases treated with ipilimumab. The patient had a progressive disease and the patient received local radiation therapy against a metastasis close to the spinal cord. After radiation, there was a reduction in size of also other metastasis, and this abscopal effect could be correlated to changes in peripheral blood immune cells, and also an increase in tumour specific antibodies (213). Partly based on this finding, a pilot study of ipilimumab in patients receiving palliative radiation therapy aims at studying the induction of immune responses (ClinicalTrials identifier NCT014492790).

Some early clinical experiments showed an activation of the complement cascade during ILP, which stimulated leucocytes to release lysosomal enzymes, interleukins and oxygen-derived free radicals (214, 215). In another study, there were signs of NK-cell activation during ILP, however the NK-cell activation was then suppressed one week after ILP. Additionally, there was a delayed T-cell augmentation after 1 week, especially the CD54+ (ICAM-1, Intercellular Adhesion Molecule 1) subpopulation. They also noted

increased levels of soluble ICAM-1 in serum after ILP, and hypothesized that it was caused by the shedding of the molecule from the up-regulated CD54+ T-cell population (216).

The clinical response after ILP is usually quite slow, and it may take 3-6 months before a CR is reached (98). One can speculate that tumours disappearing late (e.g. 2-3 months after ILP) might actually not have been killed by the melphalan itself, but rather have been removed by activated immunological processes. In this study (Paper III) Melan-A specific T cells were recorded and the results showed a statistically significant increase in specific CD8+ CTLs after 4 weeks. However, only 4 of the 12 patients could be regarded as immunological responders.

The results have to be seen as preliminary observations that have to be interpreted with considerable caution. More work is needed and should preferably be performed on larger patient groups. It should also include markers that detect circulating CTLs against other tumour-associated epitopes than Melan-A and also include immunohistochemical analyses of both tumour and draining lymph node infiltrating CTLs.

Interestingly, there was also a correlation between a higher preoperative concentration of cytotoxic T-cells (CD3+8+), naïve cytotoxic T-cells (CD3+8+45RA+) and activated T-cells (CD3+DR+), and the complete response rate after ILP. A prospective follow-up study is currently going on to validate this finding in a larger series.

## 5.3.1  Future directions

With the introduction of ipilimumab and anti-PD1 as effective immunotherapies for melanoma, the studies of immunological activation become even more interesting. Even a small and transient increase in tumour specific T-cells after ILP could act synergistically; leading to an increased immunologic response.

A phase II study is currently recruiting patients at Memorial Sloan-Kettering Cancer Center, where patients with unresectable extremity melanomas will receive ipilimumab 1-3 weeks after ILI with melphalan and dactinomycin. The primary objective will be to study progression free survival at one year; but immunological studies will also be performed (ClinicalTrials.gov

identifier NCT01323517). If this, or other studies, can show an enhanced systemic immunological activation after ILP, combination treatments with immunotherapy may become an important future development in the use of regional therapies.

# 5.4 Paper IV

The aim of Paper IV was to develop a system to isolate and characterize exosomes from the liver perfusate of patients with uveal melanoma metastases during IHP.

A problem studying exosomes from patients with cancer is that it takes a large amount of serum to extract enough exosomes for subsequent analyses, but also that only a smaller fraction of the exosomes in a serum sample are of tumour origin. The hypothesis was that both these two problems could be somewhat addressed by using the clinical setting of IHP. By perfusing the liver during 10 minutes (approximately flushing the total volume of perfusate through the liver ten times), we aimed at increasing the amounts of exosomes from tumour cells, and at the same time having the opportunity to collect a larger amount (200 ml) of the perfusate for the subsequent analyses.

## 5.4.1 Exosomes as biomarkers

Previous studies have shown that patients with cancer have higher levels of circulating exosomes compared with healthy controls (162, 217). By measuring the total exosomal protein concentration per millilitre of plasma, as an indirect measure of the amount of exosomes, our results confirmed these previous reports. In a larger context, this finding is quite unspecific; it is also well known that cancer patients have, for example, an increased erythrocyte sedimentation rate (218).

But, the use of exosomes as biomarkers has a much higher potential when studying the actual content of the exosomes. All body fluids seem to contain exosomes and due to their specific protein, RNA, and lipid content, they may prove useful for both early diagnosis and as a source for prognostic information. One example is that the oncogenic form of the epidermal growth factor receptor (EGFRvIII) could be detected in serum exosomes from glioblastoma patients, but not detected two weeks after tumour resection (166).

Also specific miRNA in serum have been used as biomarkers. For example, the independent prognostic value of four serum miRNAs (miR-22, miR-572, miR-638 and miR-1234) was shown in 512 patients with nasopharyngeal carcinoma (219). MiRNAs have also been used for the detection of cancer. Two serum miRNAs (miR-25 and miR-223) have been validated as potential biomarkers for the detection of non-small cell lung cancer in a trial of 75 healthy subjects and 152 cancer patients (220).

Partly based on this knowledge, the next step was to use real-time PCR to compare exosomal miRNA profiles obtained from liver perfusate with serum profiles obtained from healthy controls. Unfortunately, it was not possible to extract enough exosomal miRNA from peripheral blood of the healthy controls, which forced us to use exosomes from cell lines as controls. These cell lines included mast cells, lung cancer, breast cancer and cutaneous melanoma.

By cluster analyses of the most common miRNA in the samples, three different clusters were identified and analyses in more detail. *Cluster 1* consisted of four miRNAs expressed almost equally in patients and cell lines, with predicted targets towards pathways involved in metabolism and signalling. *Cluster 2* consisted of seven miRNAs that were also expressed almost equally in patients and the five tested cell lines, targeting pathways involved in biosynthesis and metabolism.*Cluster 3* consisted of miRNAs with great similarity between the patients, but with miRNAs almost completely lacking in the control cell lines. An interesting aspect of *Cluster 3* was that the predicted targets of the six included miRNAs were strongly correlated with the KEGG pathway of melanoma. The miRNA targets were directed towards both the MAPK and PI3K/AKT pathway (see Figure 19). The exact biological role of this finding has to be validated. However, a speculation is that this could be an important mechanism for cancer cells to escape regulatory mechanisms, without the need of having specific gene mutations themselves.

For example, the PI3K/AKT pathway is constitutively activated in most uveal melanomas (221). In many cancers, including cutaneous melanoma, inactivating mutations and deletions of the PTEN gene is common, leading to an activation of the PI3K/AKT pathway (222, 223). But, in uveal melanoma actual mutations within the PTEN coding region seems to be uncommon

(224). In *Cluster 3* it is interesting to note that both miR-320a and miR-486-5p is predicted to target PTEN. These two miRNAs could then, hypothetically, be horizontally transferred via exosomes between tumour cells, leading to a suppression of PTEN. This would then increase the oncogenic potential in neighbouring cancer cells without the need of a deactivating PTEN mutation. Any functional analyses concerning these aspects has not yet been done in this study, but might be warranted for in future follow-up research.
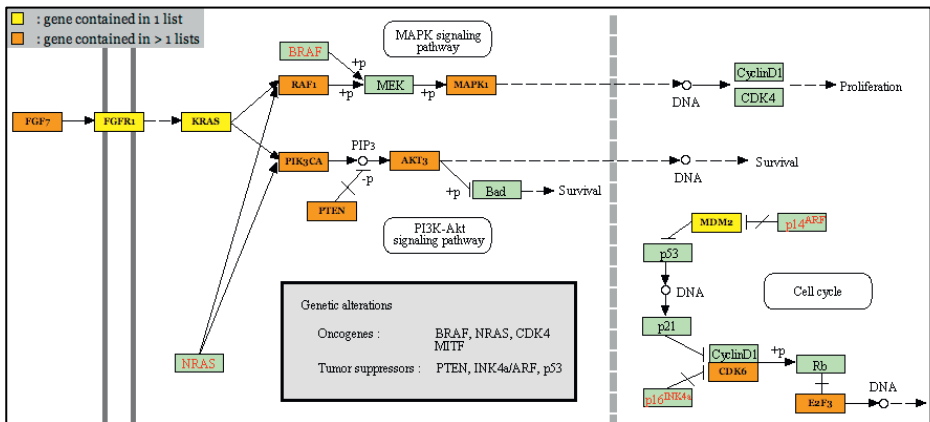


**Figure 19.** A section of the melanoma KEGG pathway (hsa05218) with the targets of the miRNAs in cluster 3 (miR-370, miR-210, miR-320a, miR-124, miR-107 and miR-486-5p) highlighted according to the DIANA-miRPath v2.0 tool (based on predicted targets by DIANA-microT-CDS) (174).

The current study has some major limitations, mainly attributed to the yield of exosomes in the control subjects. Any distinct conclusions about specific miRNA profiles and their potential role as future biomarkers cannot be drawn. However, it is the first study of exosomes isolated directly from a human organ in vivo, and as such it proves that isolated regional perfusion can be used in a translational research setting studying tumours in vivo.

## 5.4.2   Future directions

The next step in this project is to analyse the six different miRNA in *Cluster 3* in serum from patients with uveal melanoma metastases and compare these with both healthy controls and patients with other types of cancer. We will not be able to analyse exosomal miRNA exclusively, but will isolate miRNA

from serum to study if this subset of miRNAs may have a diagnostic potential.

# 6 CONCLUSIONS

Based on the studies presented in this thesis I conclude that:

- Isolated limb perfusion is a safe method with a high response rate for the palliative treatment of patients with in-transit metastases of malignant melanoma. Predictive factors for response are mainly attributed to tumour burden. Reductions in both perfusion time and temperature have decreased local toxicity without affecting the response rate.

- Isolated hepatic perfusion is a treatment option with a high response rate, acceptable surgical morbidity and with a potential survival benefit of more than one year.

- A small increase in Melan-A specific T-cells is induced after isolated limb perfusion, however the clinical significance needs to be more fully assessed.

- Tumour-derived exosomes can be isolated from liver perfusate during isolated liver perfusion. The miRNA characteristics of these exosomes could be a potential source for future biomarkers.

# 7  ACKNOWLEDGEMENT

Many people have contributed in numerous ways to this thesis. Colleagues, family and friends have shared their knowledge and inspiration throughout the work. To all of you, I would like to extend my warm and sincere gratitude. I would especially like to recognize:

My main supervisor, **Per Lindnér**, for your faith in my ability and for your support, for making things easy and for your friendship. For sharing your wisdom on how to combine work with what matters most.

My co-supervisor, **Jan Mattsson**, for inviting me to the field of regional perfusion and for teaching me every detail about it, in detail. I will always remember our discussions during surgery, always covering different aspects of life, always including at least one food recipe.

My co-supervisor, **Alex Karlsson-Parra**, whom I met during my final years as a medical student. You inspired me to become a researcher with your endless optimism and with your vast knowledge. Thank you for the kind guidance into the jungle of immunology.

The surgical equilibrists **Magnus Rizell** and **Christian Cahlin**, the radiant radiologists **Farida Hashimi** and **Mats Andersson,** and the ocular melanoma authority **Lotta All-Eriksson**. Thank you for a fruitful collaboration.

The masters of immunology, **Bengt Andersson** and **Erika Lindberg**, thank you for sharing your skills, there will be more flow cytometry in the future.

The all-knowing **Joar Svanvik** for introducing me to the fields of exosomes and quantum biology. The exosomal guru himself, **Jan Lötvall**, and all the kind colleagues at the Krefting Research Center. This collaboration did not just open up a completely new world in cell communication, it also resulted in a genuine friendship with co-author **Maria Eldh**.

The surgical nestor himself, **Lo Hafström**, for welcoming me into the fields of liver perfusion and steak tartare.

My sincere gratitude to **Peter Naredi**, you have given me the most valuable lessons for my academic future. I see you as my mentor.

The friendliest and most skilled orthopaedic surgeons **Björn Gunterberg**, **Örjan Berlin** and **Peter Bergh**. Thank you for your endless efforts in teaching me how to write a manuscript.

I have had the great pleasure in getting to know the medical oncologist, **Ulrika Stierner** and **Lars Ny**, amazing colleagues always being cheerful and enthusiastic. Together we have managed to team up with the brilliant couple of **Jonas** and **Lisa Nilsson**, with whom we have now started a journey with the greatest potential, the SATMEG (Sahlgrenska Translational Melanoma Group).

The writing of a thesis spans over a long time period, and the actual process started long before I knew I would end up writing one. I will always be grateful to **Staffan Edén** and **Birgitta Odén** at the Wallenberg laboratory for introducing me to research.

During the last years I have finished my surgical residency, and I wish to thank all the colleagues whom I've met. I have been welcomed with warmth at every single place. I have now kindly been invited to join the Surgical Oncology team, where **Rolf Sandström**, **Stig Holmberg** and colleagues have given me the opportunity to learn and develop as a surgical oncologist.

I had the luck of growing up with the best parents imaginable, **Karin** and **Johnny,** who always loved and cared for me. You have always been there for me with your warm support. We were once four siblings, but only three of us are left, **Magnus** will never be forgotten. I know that **Kenneth** and **Erika** will always be just a phone call away.

At my first year in medical school I met Ann-Sophie and suddenly my family became even bigger. I thank my parents in law, **Marianne** and **Tommy,** for generously inviting me to the family, my brother in law, **Kalle** for always being a jolly good chap, and my bonus father in law **Claes**, whom I remember with joy and miss with sadness.

**Ann-Sophie**, all of this work belongs to you. Since the first day we met, you have always cared for me, and we have evolved together through wonderful times and magnificent times. You are the love of my life and I look forward to the future with you.

**Charlie**, **Douglas** and **Bianca**, if you end up reading this book in the future, remember that family is what matters most. Love.

# 8 REFERENCES

1. Urteaga O, Pack GT. On the antiquity of melanoma. Cancer. 1966 May;19(5):607-10

2. Laennec R. Sur les melanoses. Bulletin de la Faculte de Medecine de Paris 1806;1:24-6

3. Norris W. Case of Fungoid Disease. Edinburgh Medical and Surgical Journal. 1820;16:562-5

4. Neuhaus SJ, Clark MA, Thomas JM. Dr. Herbert Lumley Snow, MD, MRCS (1847-1930): the original champion of elective lymph node dissection in melanoma. Ann Surg Oncol. 2004 Sep;11(9):875-8

5. Bodenham DC. Malignant melanoma. Br J Dermatol. 1968 Mar;80(3):190-2

6. Little EG, Eide MJ. Update on the current state of melanoma incidence. Dermatologic clinics. 2012 Jul;30(3):355-61

7. Claeson M, Andersson EM, Wallin M, Wastensson G, Wennberg AM, Paoli J, et al. Incidence of cutaneous melanoma in Western Sweden, 1970-2007. Melanoma Res. 2012 Oct;22(5):392-8

8. Registry TSC. Cancer Incidence in Sweden. 2011.

9. Mansson-Brahme E, Johansson H, Larsson O, Rutqvist LE, Ringborg U. Trends in incidence of cutaneous malignant melanoma in a Swedish population 1976-1994. Acta Oncol. 2002;41(2):138-46

10. Tsao H, Sober AJ. Ultraviolet radiation and malignant melanoma. Clinics in dermatology. 1998 Jan-Feb;16(1):67-73

11. Tucker MA, Goldstein AM. Melanoma etiology: where are we? Oncogene. 2003 May 19;22(20):3042-52

12. Pfeifer GP. Formation and processing of UV photoproducts: effects of DNA sequence and chromatin environment. Photochemistry and photobiology. 1997 Feb;65(2):270-83

13. Swerdlow AJ, English J, MacKie RM, O'Doherty CJ, Hunter JA, Clark J, et al. Benign melanocytic naevi as a risk factor for malignant melanoma. Br Med J (Clin Res Ed). 1986 Jun 14;292(6535):1555-9

14. Kanzler MH, Mraz-Gernhard S. Primary cutaneous malignant melanoma and its precursor lesions: diagnostic and therapeutic overview. J Am Acad Dermatol. 2001 Aug;45(2):260-76

15. Williams ML, Pennella R. Melanoma, melanocytic nevi, and other melanoma risk factors in children. J Pediatr. 1994 Jun;124(6):833-45

16. Goldstein AM, Tucker MA. Genetic epidemiology of cutaneous melanoma: a global perspective. Arch Dermatol. 2001 Nov;137(11):1493-6

17. Kopf AW, Hellman LJ, Rogers GS, Gross DF, Rigel DS, Friedman RJ, et al. Familial malignant melanoma. JAMA. 1986 Oct 10;256(14):1915-9

18. Bishop DT, Demenais F, Goldstein AM, Bergman W, Bishop JN, Bressac-de Paillerets B, et al. Geographical variation in the penetrance of CDKN2A mutations for melanoma. Journal of the National Cancer Institute. 2002 Jun 19;94(12):894-903

19. Singh AD, Bergman L, Seregard S. Uveal melanoma: epidemiologic aspects. Ophthalmol Clin North Am. 2005 Mar;18(1):75-84, viii

20. Mihajlovic M, Vlajkovic S, Jovanovic P, Stefanovic V. Primary mucosal melanomas: a comprehensive review. International journal of clinical and experimental pathology. 2012;5(8):739-53

21. Atmatzidis KS, Pavlidis TE, Papaziogas BT, Papaziogas TB. Primary malignant melanoma of the small intestine: report of a case. Surgery today. 2002;32(9):831-3

22. Tcheung WJ, Selim MA, Herndon JE, 2nd, Abernethy AP, Nelson KC. Clinicopathologic study of 85 cases of melanoma of the female genitalia. J Am Acad Dermatol. 2012 Oct;67(4):598-605

23. Liubinas SV, Maartens N, Drummond KJ. Primary melanocytic neoplasms of the central nervous system. Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia. 2010 Oct;17(10):1227-32

24. Lindholm C, Andersson R, Dufmats M, Hansson J, Ingvar C, Moller T, et al. Invasive cutaneous malignant melanoma in Sweden, 1990-1999. A prospective, population-based study of survival and prognostic factors. Cancer. 2004 Nov 1;101(9):2067-78

25. Newell GR, Sider JG, Bergfelt L, Kripke ML. Incidence of cutaneous melanoma in the United States by histology with special reference to the face. Cancer Res. 1988 Sep 1;48(17):5036-41

26. Markovic SN, Erickson LA, Rao RD, Weenig RH, Pockaj BA, Bardia A, et al. Malignant melanoma in the 21st century, part 1: epidemiology, risk factors, screening, prevention, and diagnosis. Mayo Clinic proceedings Mayo Clinic. 2007 Mar;82(3):364-80

27. Cox NH, Aitchison TC, Sirel JM, MacKie RM. Comparison between lentigo maligna melanoma and other histogenetic types of malignant

melanoma of the head and neck. Scottish Melanoma Group. Br J Cancer. 1996 Apr;73(7):940-4

28. Cascinelli N, Zurrida S, Galimberti V, Bartoli C, Bufalino R, Del Prato I, et al. Acral lentiginous melanoma. A histological type without prognostic significance. The Journal of dermatologic surgery and oncology. 1994 Dec;20(12):817-22

29. Barnhill RL, Gupta K. Unusual variants of malignant melanoma. Clinics in dermatology. 2009 Nov-Dec;27(6):564-87

30. Breslow A. Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. Ann Surg. 1970 Nov;172(5):902-8

31. Edge SB BD, Compton CC, Fritz AG, Greene FL, Trotti A. AJCC cancer staging manual (7th ed). New York, NY: Springer;; 2010.

32. Clark WH, Jr., From L, Bernardino EA, Mihm MC. The histogenesis and biologic behavior of primary human malignant melanomas of the skin. Cancer Res. 1969 Mar;29(3):705-27

33. Dickson PV, Gershenwald JE. Staging and prognosis of cutaneous melanoma. Surg Oncol Clin N Am. 2011 Jan;20(1):1-17

34. Allen AC, Spitz S. Malignant melanoma; a clinicopathological analysis of the criteria for diagnosis and prognosis. Cancer. 1953 Jan;6(1):1-45

35. Morton DL, Wen DR, Wong JH, Economou JS, Cagle LA, Storm FK, et al. Technical details of intraoperative lymphatic mapping for early stage melanoma. Arch Surg. 1992 Apr;127(4):392-9

36. Wong JH, Cagle LA, Morton DL. Lymphatic drainage of skin to a sentinel lymph node in a feline model. Ann Surg. 1991 Nov;214(5):637-41

37. Morton DL, Thompson JF, Cochran AJ, Mozzillo N, Elashoff R, Essner R, et al. Sentinel-node biopsy or nodal observation in melanoma. N Engl J Med. 2006 Sep 28;355(13):1307-17

38. Thomas JM. Concerns relating to the conduct and statistical analysis of the Multicenter Selective Lymphadenectomy Trial (MSLT-1) in patients with melanoma. Journal of plastic, reconstructive & aesthetic surgery : JPRAS. 2009 Apr;62(4):442-6

39. Pawlik TM, Ross MI, Johnson MM, Schacherer CW, McClain DM, Mansfield PF, et al. Predictors and natural history of in-transit melanoma after sentinel lymphadenectomy. Ann Surg Oncol. 2005 Aug;12(8):587-96

40. Karakousis CP, Choe KJ, Holyoke ED. Biologic behavior and treatment of intransit metastasis of melanoma. Surg Gynecol Obstet. 1980 Jan;150(1):29-32

41. Neuman HB, Patel A, Ishill N, Hanlon C, Brady MS, Halpern AC, et al. A single-institution validation of the AJCC staging system for stage IV melanoma. Ann Surg Oncol. 2008 Jul;15(7):2034-41

42. Damato B. Treatment of primary intraocular melanoma. Expert review of anticancer therapy. 2006 Apr;6(4):493-506

43. Shields CL, Kaliki S, Furuta M, Mashayekhi A, Shields JA. Clinical spectrum and prognosis of uveal melanoma based on age at presentation in 8,033 cases. Retina. 2012 Jul;32(7):1363-72

44. Bergman L, Seregard S, Nilsson B, Ringborg U, Lundell G, Ragnarsson-Olding B. Incidence of uveal melanoma in Sweden from 1960 to 1998. Invest Ophthalmol Vis Sci. 2002 Aug;43(8):2579-83

45. Virgili G, Gatta G, Ciccolallo L, Capocaccia R, Biggeri A, Crocetti E, et al. Incidence of uveal melanoma in Europe. Ophthalmology. 2007 Dec;114(12):2309-15

46. Eskelin S, Kivela T. Mode of presentation and time to treatment of uveal melanoma in Finland. Br J Ophthalmol. 2002 Mar;86(3):333-8

47. Singh AD, De Potter P, Fijal BA, Shields CL, Shields JA, Elston RC. Lifetime prevalence of uveal melanoma in white patients with oculo(dermal) melanocytosis. Ophthalmology. 1998 Jan;105(1):195-8

48. Singh AD, Kalyani P, Topham A. Estimating the risk of malignant transformation of a choroidal nevus. Ophthalmology. 2005 Oct;112(10):1784-9

49. Weis E, Shah CP, Lajous M, Shields JA, Shields CL. The association between host susceptibility factors and uveal melanoma: a meta-analysis. Arch Ophthalmol. 2006 Jan;124(1):54-60

50. Jovanovic P, Mihajlovic M, Djordjevic-Jocic J, Vlajkovic S, Cekic S, Stefanovic V. Ocular melanoma: an overview of the current status. International journal of clinical and experimental pathology. 2013;6(7):1230-44

51. Vajdic CM, Kricker A, Giblin M, McKenzie J, Aitken JF, Giles GG, et al. Artificial ultraviolet radiation and ocular melanoma in Australia. Int J Cancer. 2004 Dec 10;112(5):896-900

52. Lee DS, Anderson SF, Perez EM, Townsend JC. Amelanotic choroidal nevus and melanoma: cytology, tumor size, and pigmentation as

prognostic indicators. Optometry and vision science : official publication of the American Academy of Optometry. 2001 Jul;78(7):483-91

53. Conway RM, Chua WC, Qureshi C, Billson FA. Primary iris melanoma: diagnostic features and outcome of conservative surgical treatment. Br J Ophthalmol. 2001 Jul;85(7):848-54

54. Damato B, Duke C, Coupland SE, Hiscott P, Smith PA, Campbell I, et al. Cytogenetics of uveal melanoma: a 7-year clinical experience. Ophthalmology. 2007 Oct;114(10):1925-31

55. Onken MD, Worley LA, Tuscan MD, Harbour JW. An accurate, clinically feasible multi-gene expression assay for predicting metastasis in uveal melanoma. The Journal of molecular diagnostics : JMD. 2010 Jul;12(4):461-8

56. Pereira PR, Odashiro AN, Lim LA, Miyamoto C, Blanco PL, Odashiro M, et al. Current and emerging treatment options for uveal melanoma. Clin Ophthalmol. 2013;7:1669-82

57. Diener-West M, Earle JD, Fine SL, Hawkins BS, Moy CS, Reynolds SM, et al. The COMS randomized trial of iodine 125 brachytherapy for choroidal melanoma, III: initial mortality findings. COMS Report No. 18. Arch Ophthalmol. 2001 Jul;119(7):969-82

58. Bergman L, Nilsson B, Lundell G, Lundell M, Seregard S. Ruthenium brachytherapy for uveal melanoma, 1979-2003: survival and functional outcomes in the Swedish population. Ophthalmology. 2005 May;112(5):834-40

59. Virgili G, Gatta G, Ciccolallo L, Capocaccia R, Biggeri A, Crocetti E, et al. Survival in patients with uveal melanoma in Europe. Arch Ophthalmol. 2008 Oct;126(10):1413-8

60. Kujala E, Makitie T, Kivela T. Very long-term prognosis of patients with malignant uveal melanoma. Invest Ophthalmol Vis Sci. 2003 Nov;44(11):4651-9

61. Finger PT, Kurli M, Reddy S, Tena LB, Pavlick AC. Whole body PET/CT for initial staging of choroidal melanoma. Br J Ophthalmol. 2005 Oct;89(10):1270-4

62. Diener-West M, Reynolds SM, Agugliaro DJ, Caldwell R, Cumming K, Earle JD, et al. Development of metastatic disease after enrollment in the COMS trials for treatment of choroidal melanoma: Collaborative Ocular Melanoma Study Group Report No. 26. Arch Ophthalmol. 2005 Dec;123(12):1639-43

63. Kath R, Hayungs J, Bornfeld N, Sauerwein W, Hoffken K, Seeber S. Prognosis and treatment of disseminated uveal melanoma. Cancer. 1993 Oct 1;72(7):2219-23

64. 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 Oct;132(4):811-32

65. Creech O, Jr., Krementz ET, Ryan RF, Winblad JN. Chemotherapy of cancer: regional perfusion utilizing an extracorporeal circuit. Ann Surg. 1958 Oct;148(4):616-32

66. Krementz ET, Carter RD, Sutherland CM, Muchmore JH, Ryan RF, Creech O, Jr. Regional chemotherapy for melanoma. A 35-year experience. Ann Surg. 1994 Oct;220(4):520-34; discussion 34-5

67. Ausman RK, Aust JB. Isolated perfusion of the liver with HN2. Surg Forum. 1960;10:77-9

68. Aust JB, Ausman RK. The technique of liver perfusion. Cancer Chemother Rep. 1960 Dec;10:23-33

69. Bartlett DL, Libutti SK, Figg WD, Fraker DL, Alexander HR. Isolated hepatic perfusion for unresectable hepatic metastases from colorectal cancer. Surgery. 2001 Feb;129(2):176-87

70. Grover A, Alexander HR, Jr. The past decade of experience with isolated hepatic perfusion. Oncologist. 2004;9(6):653-64

71. Feldman ED, Wu PC, Beresneva T, Helsabeck C, Rodriguez M, Bartlett DL, et al. Treatment of patients with unresectable primary hepatic malignancies using hyperthermic isolated hepatic perfusion. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2004 Feb;8(2):200-7

72. Luck JM. Action of p-[di(2-chloroethyl)]-amino-l-phenylalanine on Harding-Passey mouse melanoma. Science. 1956 Jun 1;123(3205):984-5

73. Coates AS. Systemic chemotherapy for malignant melanoma. World J Surg. 1992 Mar-Apr;16(2):277-81

74. Minor DR, Allen RE, Alberts D, Peng YM, Tardelli G, Hutchinson J. A clinical and pharmacokinetic study of isolated limb perfusion with heat and melphalan for melanoma. Cancer. 1985 Jun 1;55(11):2638-44

75. Roberts MS, Wu ZY, Siebert GA, Thompson JF, Smithers BM. Saturable dose-response relationships for melphalan in melanoma treatment by isolated limb infusion in the nude rat. Melanoma Res. 2001 Dec;11(6):611-8

76. Hafstrom L, Hugander A, Jonsson PE, Westling H, Ehrsson H. Blood leakage and melphalan leakage from the perfusion circuit during regional hyperthermic perfusion for malignant melanoma. Cancer Treat Rep. 1984 Jun;68(6):867-72

77. 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 Apr;45(4):1885-9

78. Moyer HR, Delman KA. The role of hyperthermia in optimizing tumor response to regional therapy. Int J Hyperthermia. 2008 May;24(3):251-61

79. Cavaliere R, Ciocatto EC, Giovanella BC, Heidelberger C, Johnson RO, Margottini M, et al. Selective heat sensitivity of cancer cells. Biochemical and clinical studies. Cancer. 1967 Sep;20(9):1351-81

80. Pelz J, Mollwitz M, Stremmel C, Goehl J, Dimmler A, Hohenberger W, et al. The impact of surgery and mild hyperthermia on tumor response and angioneogenesis of malignant melanoma in a rat perfusion model. BMC cancer. 2004 Aug 23;4:53

81. Stehlin JS, Jr. Hyperthermic perfusion with chemotherapy for cancers of the extremities. Surg Gynecol Obstet. 1969 Aug;129(2):305-8

82. Dahl O. Interaction of hyperthermia and chemotherapy. Recent Results Cancer Res. 1988;107:157-69

83. Omlor G, Gross G, Ecker KW, Burger I, Feifel G. Optimization of isolated hyperthermic limb perfusion. World J Surg. 1992 Nov-Dec;16(6):1117-9

84. Clark J, Grabs AJ, Parsons PG, Smithers BM, Addison RS, Roberts MS. Melphalan uptake, hyperthermic synergism and drug resistance in a human cell culture model for the isolated limb perfusion of melanoma. Melanoma Res. 1994 Dec;4(6):365-70

85. Eikesdal HP, Bjorkhaug ST, Dahl O. Hyperthermia exhibits anti-vascular activity in the s.c. BT4An rat glioma: lack of interaction with the angiogenesis inhibitor batimastat. Int J Hyperthermia. 2002 Mar-Apr;18(2):141-52

86. Fajardo LF, Prionas SD. Endothelial cells and hyperthermia. Int J Hyperthermia. 1994 May-Jun;10(3):347-53

87. Di Filippo F, Anza M, Rossi CR, Cavaliere F, Botti C, Lise M, et al. The application of hyperthermia in regional chemotherapy. Seminars in surgical oncology. 1998 Apr-May;14(3):215-23

88. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci U S A. 1975 Sep;72(9):3666-70

89. Sugarman BJ, Aggarwal BB, Hass PE, Figari IS, Palladino MA, Jr., Shepard HM. Recombinant human tumor necrosis factor-alpha: effects on proliferation of normal and transformed cells in vitro. Science. 1985 Nov 22;230(4728):943-5

90. Watanabe N, Niitsu Y, Umeno H, Kuriyama H, Neda H, Yamauchi N, et al. Toxic effect of tumor necrosis factor on tumor vasculature in mice. Cancer Res. 1988 Apr 15;48(8):2179-83

91. Moreno-Ramirez D, de la Cruz-Merino L, Ferrandiz L, Villegas-Portero R, Nieto-Garcia A. Isolated limb perfusion for malignant melanoma: systematic review on effectiveness and safety. Oncologist. 2010;15(4):416-27

92. Cornett WR, McCall LM, Petersen RP, Ross MI, Briele HA, Noyes RD, et al. Randomized multicenter trial of hyperthermic isolated limb perfusion with melphalan alone compared with melphalan plus tumor necrosis factor: American College of Surgeons Oncology Group Trial Z0020. J Clin Oncol. 2006 Sep 1;24(25):4196-201

93. Lejeune FJ, Eggermont AM. Hyperthermic isolated limb perfusion with tumor necrosis factor is a useful therapy for advanced melanoma of the limbs. J Clin Oncol. 2007 Apr 10;25(11):1449-50; author reply 50-1

94. Grunhagen DJ, de Wilt JH, ten Hagen TL, Eggermont AM. Technology insight: Utility of TNF-alpha-based isolated limb perfusion to avoid amputation of irresectable tumors of the extremities. Nat Clin Pract Oncol. 2006 Feb;3(2):94-103

95. Alexander HR, Libutti SK, Bartlett DL, Puhlmann M, Fraker DL, Bachenheimer LC. A phase I-II study of isolated hepatic perfusion using melphalan with or without tumor necrosis factor for patients with ocular melanoma metastatic to liver. Clin Cancer Res. 2000 Aug;6(8):3062-70

96. Thompson JF, Kam PC, Waugh RC, Harman CR. Isolated limb infusion with cytotoxic agents: a simple alternative to isolated limb perfusion. Seminars in surgical oncology. 1998 Apr-May;14(3):238-47

97. Beasley GM, Caudle A, Petersen RP, McMahon NS, Padussis J, Mosca PJ, et al. A multi-institutional experience of isolated limb infusion: defining response and toxicity in the US. J Am Coll Surg. 2009 May;208(5):706-15; discussion 15-7

98. Testori A, Verhoef C, Kroon HM, Pennacchioli E, Faries MB, Eggermont AM, et al. Treatment of melanoma metastases in a limb by isolated limb perfusion and isolated limb infusion. J Surg Oncol. 2011 Sep;104(4):397-404

99. van Etten B, Brunstein F, van IMG, Marinelli AW, Verhoef C, van der Sijp JR, et al. Isolated hypoxic hepatic perfusion with orthograde or retrograde flow in patients with irresectable liver metastases using percutaneous balloon catheter techniques: a phase I and II study. Ann Surg Oncol. 2004 Jun;11(6):598-605

100. van Etten B, de Wilt JH, Brunstein F, Eggermont AM, Verhoef C. Isolated hypoxic hepatic perfusion with melphalan in patients with irresectable ocular melanoma metastases. Eur J Surg Oncol. 2009 May;35(5):539-45

101. Ku Y, Saitoh M, Nishiyama H, Fujiwara S, Iwasaki T, Ohyanagi H, et al. [Extracorporeal adriamycin-removal following hepatic artery infusion: use of direct hemoperfusion combined with veno-venous bypass]. Nihon Geka Gakkai zasshi. 1989 Oct;90(10):1758-64

102. Curley SA, Byrd DR, Newman RA, Carrasco CH, Cromeens D, Ellis HJ, et al. Hepatic arterial infusion chemotherapy with complete hepatic venous isolation and extracorporeal chemofiltration: a feasibility study of a novel system. Anti-cancer drugs. 1991 Apr;2(2):175-83

103. Beheshti MV, Denny DF, Jr., Glickman MG, Bodden W, Marsh JC, Strair R, et al. Percutaneous isolated liver perfusion for treatment of hepatic malignancy: preliminary report. Journal of vascular and interventional radiology : JVIR. 1992 Aug;3(3):453-8

104. Ravikumar TS, Pizzorno G, Bodden W, Marsh J, Strair R, Pollack J, et al. Percutaneous hepatic vein isolation and high-dose hepatic arterial infusion chemotherapy for unresectable liver tumors. J Clin Oncol. 1994 Dec;12(12):2723-36

105. Curley SA, Newman RA, Dougherty TB, Fuhrman GM, Stone DL, Mikolajek JA, et al. Complete hepatic venous isolation and extracorporeal chemofiltration as treatment for human hepatocellular carcinoma: a phase I study. Ann Surg Oncol. 1994 Sep;1(5):389-99

106. Pingpank JF, Libutti SK, Chang R, Wood BJ, Neeman Z, Kam AW, et al. Phase I study of hepatic arterial melphalan infusion and hepatic venous hemofiltration using percutaneously placed catheters in patients with unresectable hepatic malignancies. J Clin Oncol. 2005 May 20;23(15):3465-74

107. Pingpank JF, Hughes MS, Alexander HR, Faries MB, Zager JS, Royal R, et al. A phase III random assignment trial comparing percutaneous hepatic perfusion with melphalan (PHP-mel) to standard of care for patients with hepatic metastases from metastatic ocular or cutaneous melanoma. . J Clin Oncol. 2010;28 (18 suppl):LBA8512

108. Klaase JM, Kroon BB, van Geel AN, Eggermont AM, Franklin HR. Systemic leakage during isolated limb perfusion for melanoma. Br J Surg. 1993 Sep;80(9):1124-6

109. Thom AK, Alexander HR, Andrich MP, Barker WC, Rosenberg SA, Fraker DL. Cytokine levels and systemic toxicity in patients undergoing isolated limb perfusion with high-dose tumor necrosis factor, interferon gamma, and melphalan. J Clin Oncol. 1995 Jan;13(1):264-73

110. Stehlin JS, Jr., Clark RL, Jr., Dewey WC. Continuous monitoring of leakage during regional perfusion. Arch Surg. 1961 Dec;83:943-9

111. Lejeune FJ, Lienard D, Ewalenko P. Hyperthermic isolation perfusion of the limbs with cytostatics after surgical excision of sarcomas. World J Surg. 1988 Jun;12(3):345-8

112. Sandrock D, Horst F, Gatzemeier W, Ghorbani M, Rauschecker H, Munz DL, et al. Leakage measurement during selective limb perfusion using a gamma probe. European journal of nuclear medicine. 1996 May;23(5):534-8

113. van Ginkel RJ, Limburg PC, Piers DA, Koops HS, Hoekstra HJ. Value of continuous leakage monitoring with radioactive iodine-131-labeled human serum albumin during hyperthermic isolated limb perfusion with tumor necrosis factor-alpha and melphalan. Ann Surg Oncol. 2002 May;9(4):355-63

114. Perry MC. The chemotherapy source book. Philadelphia, Pa.; London: Lippincott Williams & Wilkins; 2007.

115. Wieberdink J, Benckhuysen C, Braat RP, van Slooten EA, Olthuis GA. Dosimetry in isolation perfusion of the limbs by assessment of perfused tissue volume and grading of toxic tissue reactions. Eur J Cancer Clin Oncol. 1982 Oct;18(10):905-10

116. Bedikian AY, Legha SS, Mavligit G, Carrasco CH, Khorana S, Plager C, et al. Treatment of uveal melanoma metastatic to the liver: a review of the M. D. Anderson Cancer Center experience and prognostic factors. Cancer. 1995 Nov 1;76(9):1665-70

117. Bedikian AY, Papadopoulos N, Plager C, Eton O, Ring S. Phase II evaluation of temozolomide in metastatic choroidal melanoma. Melanoma Res. 2003 Jun;13(3):303-6

118. Kivela T, Suciu S, Hansson J, Kruit WH, Vuoristo MS, Kloke O, et al. Bleomycin, vincristine, lomustine and dacarbazine (BOLD) in combination with recombinant interferon alpha-2b for metastatic uveal melanoma. Eur J Cancer. 2003 May;39(8):1115-20

119. Schmittel A, Scheulen ME, Bechrakis NE, Strumberg D, Baumgart J, Bornfeld N, et al. Phase II trial of cisplatin, gemcitabine and treosulfan in patients with metastatic uveal melanoma. Melanoma Res. 2005 Jun;15(3):205-7

120. Schmittel A, Schuster R, Bechrakis NE, Siehl JM, Foerster MH, Thiel E, et al. A two-cohort phase II clinical trial of gemcitabine plus treosulfan in patients with metastatic uveal melanoma. Melanoma Res. 2005 Oct;15(5):447-51

121. Pyrhonen S, Hahka-Kemppinen M, Muhonen T, Nikkanen V, Eskelin S, Summanen P, et al. Chemoimmunotherapy with bleomycin, vincristine, lomustine, dacarbazine (BOLD), and human leukocyte interferon for metastatic uveal melanoma. Cancer. 2002 Dec 1;95(11):2366-72

122. Goel VK, Lazar AJ, Warneke CL, Redston MS, Haluska FG. Examination of mutations in BRAF, NRAS, and PTEN in primary cutaneous melanoma. The Journal of investigative dermatology. 2006 Jan;126(1):154-60

123. Jakob JA, Bassett RL, Jr., Ng CS, Curry JL, Joseph RW, Alvarado GC, et al. NRAS mutation status is an independent prognostic factor in metastatic melanoma. Cancer. 2012 Aug 15;118(16):4014-23

124. Van Raamsdonk CD, Griewank KG, Crosby MB, Garrido MC, Vemula S, Wiesner T, et al. Mutations in GNA11 in uveal melanoma. N Engl J Med. 2010 Dec 2;363(23):2191-9

125. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med. 2011 Jun 30;364(26):2507-16

126. Hauschild A, Grob JJ, Demidov LV, Jouary T, Gutzmer R, Millward M, et al. Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. Lancet. 2012 Jul 28;380(9839):358-65

127. Chapman PB, Hauschild A, Robert C. Updated overall survival (OS) results for BRIM-3, a phase III randomized, open-label, multicenter trial

comparing BRAF inhibitor vemurafenib (vem) with dacarbazine (DTIC) in previously untreated patients with BRAFV600E-mutated melanoma. J Clin Oncol. 2012;30 No (18 suppl):8502

128. Flaherty KT, Robert C, Hersey P, Nathan P, Garbe C, Milhem M, et al. Improved survival with MEK inhibition in BRAF-mutated melanoma. N Engl J Med. 2012 Jul 12;367(2):107-14

129. Falchook GS, Lewis KD, Infante JR, Gordon MS, Vogelzang NJ, DeMarini DJ, et al. Activity of the oral MEK inhibitor trametinib in patients with advanced melanoma: a phase 1 dose-escalation trial. Lancet Oncol. 2012 Aug;13(8):782-9

130. Carvajal RD, Sosman JA, Quevedo F. Phase II study of selumetinib (sel) versus temozolomide (TMZ) in gnaq/Gna11 (Gq/11) mutant (mut) uveal melanoma (UM). J Clin Oncol. 2013;31 (suppl):CRA9003

131. Coley WB. Contribution to the Knowledge of Sarcoma. Ann Surg. 1891 Sep;14(3):199-220

132. Mitchison NA. Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. J Exp Med. 1955 Aug 1;102(2):157-77

133. Eberlein TJ, Rosenstein M, Rosenberg SA. Regression of a disseminated syngeneic solid tumor by systemic transfer of lymphoid cells expanded in interleukin 2. J Exp Med. 1982 Aug 1;156(2):385-97

134. Rudd CE, Taylor A, Schneider H. CD28 and CTLA-4 coreceptor expression and signal transduction. Immunological reviews. 2009 May;229(1):12-26

135. Phan GQ, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzentruber DJ, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci U S A. 2003 Jul 8;100(14):8372-7

136. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010 Aug 19;363(8):711-23

137. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. Nat Rev Immunol. 2001 Dec;1(3):220-8

138. Danielli R, Ridolfi R, Chiarion-Sileni V, Queirolo P, Testori A, Plummer R, et al. Ipilimumab in pretreated patients with metastatic uveal melanoma: safety and clinical efficacy. Cancer immunology, immunotherapy : CII. 2012 Jan;61(1):41-8

139. Kelderman S, van der Kooij MK, van den Eertwegh AJ, Soetekouw PM, Jansen RL, van den Brom RR, et al. Ipilimumab in pretreated metastastic uveal melanoma patients. Results of the Dutch Working group on Immunotherapy of Oncology (WIN-O). Acta Oncol. 2013 Apr 23

140. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972 Aug;26(4):239-57

141. Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: clearance of apoptotic cells regulates immune responses. Nat Rev Immunol. 2002 Dec;2(12):965-75

142. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. Annu Rev Immunol. 2013;31:51-72

143. Tesniere A, Panaretakis T, Kepp O, Apetoh L, Ghiringhelli F, Zitvogel L, et al. Molecular characteristics of immunogenic cancer cell death. Cell Death Differ. 2008 Jan;15(1):3-12

144. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med. 2007 Jan;13(1):54-61

145. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med. 2007 Sep;13(9):1050-9

146. Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. The Journal of cell biology. 1983 Aug;97(2):329-39

147. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J Biol Chem. 1987 Jul 5;262(19):9412-20

148. Huotari J, Helenius A. Endosome maturation. The EMBO journal. 2011 Aug 31;30(17):3481-500

149. Vlassov AV, Magdaleno S, Setterquist R, Conrad R. Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. Biochimica et biophysica acta. 2012 Jul;1820(7):940-8

150. van der Pol E, Boing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. Pharmacological reviews. 2012 Jul;64(3):676-705

151. Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nat Med. 1998 May;4(5):594-600

152. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, et al. B lymphocytes secrete antigen-presenting vesicles. J Exp Med. 1996 Mar 1;183(3):1161-72

153. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007 Jun;9(6):654-9

154. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nature reviews Genetics. 2004 Jul;5(7):522-31

155. Taft RJ, Pang KC, Mercer TR, Dinger M, Mattick JS. Non-coding RNAs: regulators of disease. J Pathol. 2010 Jan;220(2):126-39

156. Eldh M. Exosomes and Exosomal RNA – A Way of Cell-to-Cell Communication: University of Gothenburg; 2012.

157. Gibbings DJ, Ciaudo C, Erhardt M, Voinnet O. Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. Nat Cell Biol. 2009 Sep;11(9):1143-9

158. Eldh M, Ekstrom K, Valadi H, Sjostrand M, Olsson B, Jernas M, et al. Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA. PLoS One. 2010;5(12):e15353

159. Paget S. The distribution of secondary growths in cancer of the breast. 1889. Cancer Metastasis Rev. 1989 Aug;8(2):98-101

160. Psaila B, Lyden D. The metastatic niche: adapting the foreign soil. Nature reviews Cancer. 2009 Apr;9(4):285-93

161. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature. 2005 Dec 8;438(7069):820-7

162. Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med. 2012 Jun;18(6):883-91

163. van Akkooi AC, Verhoef C, Eggermont AM. Importance of tumor load in the sentinel node in melanoma: clinical dilemmas. Nature reviews Clinical oncology. 2010 Aug;7(8):446-54

164. Rinderknecht M, Detmar M. Tumor lymphangiogenesis and melanoma metastasis. Journal of cellular physiology. 2008 Aug;216(2):347-54

165. Hood JL, San RS, Wickline SA. Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis. Cancer Res. 2011 Jun 1;71(11):3792-801

166. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol. 2008 Dec;10(12):1470-6

167. Andre F, Schartz NE, Movassagh M, Flament C, Pautier P, Morice P, et al. Malignant effusions and immunogenic tumour-derived exosomes. Lancet. 2002 Jul 27;360(9329):295-305

168. Nilsson J, Skog J, Nordstrand A, Baranov V, Mincheva-Nilsson L, Breakefield XO, et al. Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. Br J Cancer. 2009 May 19;100(10):1603-7

169. Silva J, Garcia V, Zaballos A, Provencio M, Lombardia L, Almonacid L, et al. Vesicle-related microRNAs in plasma of nonsmall cell lung cancer patients and correlation with survival. The European respiratory journal. 2011 Mar;37(3):617-23

170. Logozzi M, De Milito A, Lugini L, Borghi M, Calabro L, Spada M, et al. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. PLoS One. 2009;4(4):e5219

171. World Health Organization. WHO handbook for reporting results of cancer treatment. Geneva: World Health Organization; 1979. 45 p. p.

172. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). European Journal of Cancer. 2009;45(2):228-47

173. Paraskevopoulou MD, Georgakilas G, Kostoulas N, Vlachos IS, Vergoulis T, Reczko M, et al. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. Nucleic acids research. 2013 Jul;41(Web Server issue):W169-73

174. Vlachos IS, Kostoulas N, Vergoulis T, Georgakilas G, Reczko M, Maragkakis M, et al. DIANA miRPath v.2.0: investigating the

combinatorial effect of microRNAs in pathways. Nucleic acids research. 2012 Jul;40(Web Server issue):W498-504

175. Galluzzi L, Senovilla L, Zitvogel L, Kroemer G. The secret ally: immunostimulation by anticancer drugs. Nat Rev Drug Discov. Mar;11(3):215-33

176. Deroose JP, Grunhagen DJ, van Geel AN, de Wilt JH, Eggermont AM, Verhoef C. Long-term outcome of isolated limb perfusion with tumour necrosis factor-alpha for patients with melanoma in-transit metastases. Br J Surg. 2011 Nov;98(11):1573-80

177. Di Filippo F, Calabro A, Giannarelli D, Carlini S, Cavaliere F, Moscarelli F, et al. Prognostic variables in recurrent limb melanoma treated with hyperthermic antiblastic perfusion. Cancer. 1989 Jun 15;63(12):2551-61

178. Grunhagen DJ, Brunstein F, Graveland WJ, van Geel AN, de Wilt JH, Eggermont AM. One hundred consecutive isolated limb perfusions with TNF-alpha and melphalan in melanoma patients with multiple in-transit metastases. Ann Surg. 2004 Dec;240(6):939-47; discussion 47-8

179. Rossi CR, Pasquali S, Mocellin S, Vecchiato A, Campana LG, Pilati P, et al. Long-term results of melphalan-based isolated limb perfusion with or without low-dose TNF for in-transit melanoma metastases. Ann Surg Oncol. 2010 Nov;17(11):3000-7

180. Vrouenraets BC, Hart GA, Eggermont AM, Klaase JM, van Geel BN, Nieweg OE, et al. Relation between limb toxicity and treatment outcomes after isolated limb perfusion for recurrent melanoma. J Am Coll Surg. 1999 May;188(5):522-30

181. Di Filippo F, Giacomini P, Rossi CR, Santinami M, Garinei R, Anza M, et al. Hyperthermic isolated perfusion with tumor necrosis factor-alpha and doxorubicin for the treatment of limb-threatening soft tissue sarcoma: the experience of the Italian Society of Integrated Locoregional Treatment in Oncology (SITILO). In Vivo. 2009 Mar-Apr;23(2):363-7

182. Bonvalot S, Laplanche A, Lejeune F, Stoeckle E, Le Pechoux C, Vanel D, et al. Limb salvage with isolated perfusion for soft tissue sarcoma: could less TNF-alpha be better? Ann Oncol. 2005 Jul;16(7):1061-8

183. Begleiter A, Lam HY, 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 Feb;39(2 Pt 1):353-9

184. Noorda EM, Vrouenraets BC, Nieweg OE, van Geel AN, Eggermont AM, Kroon BB. Repeat isolated limb perfusion with TNFalpha and

melphalan for recurrent limb melanoma after failure of previous perfusion. Eur J Surg Oncol. 2006 Apr;32(3):318-24

185. Knorr C, Meyer T, Janssen T, Goehl J, Hohenberger W. Hyperthermic isolated limb perfusion (HILP) in malignant melanoma. Experience with 101 patients. Eur J Surg Oncol. 2006 Mar;32(2):224-7

186. Grunhagen DJ, de Wilt JH, van Geel AN, Graveland WJ, Verhoef C, Eggermont AM. TNF dose reduction in isolated limb perfusion. Eur J Surg Oncol. 2005 Nov;31(9):1011-9

187. Noorda EM, Takkenberg B, Vrouenraets BC, Nieweg OE, van Geel BN, Eggermont AM, et al. Isolated limb perfusion prolongs the limb recurrence-free interval after several episodes of excisional surgery for locoregional recurrent melanoma. Ann Surg Oncol. 2004 May;11(5):491-9

188. Noorda EM, Vrouenraets BC, Nieweg OE, van Geel AN, Eggermont AM, Kroon BB. Safety and efficacy of isolated limb perfusion in elderly melanoma patients. Ann Surg Oncol. 2002 Dec;9(10):968-74

189. Zogakis TG, Bartlett DL, Libutti SK, Liewehr DJ, Steinberg SM, Fraker DL, et al. Factors affecting survival after complete response to isolated limb perfusion in patients with in-transit melanoma. Ann Surg Oncol. 2001 Dec;8(10):771-8

190. Lienard D, Eggermont AM, Koops HS, Kroon B, Towse G, Hiemstra S, et al. Isolated limb perfusion with tumour necrosis factor-alpha and melphalan with or without interferon-gamma for the treatment of in-transit melanoma metastases: a multicentre randomized phase II study. Melanoma Res. 1999 Oct;9(5):491-502

191. Klaase JM, Kroon BB, van Geel AN, van Wijk J, Franklin HR, Eggermont AM, et al. Limb recurrence-free interval and survival in patients with recurrent melanoma of the extremities treated with normothermic isolated perfusion. J Am Coll Surg. 1994 Jun;178(6):564-72

192. Noorda EM, Vrouenraets BC, Nieweg OE, van Geel BN, Eggermont AM, Kroon BB. Isolated limb perfusion for unresectable melanoma of the extremities. Arch Surg. 2004 Nov;139(11):1237-42

193. Alexander HR, Jr., Fraker DL, Bartlett DL, Libutti SK, Steinberg SM, Soriano P, et al. Analysis of factors influencing outcome in patients with in-transit malignant melanoma undergoing isolated limb perfusion using modern treatment parameters. J Clin Oncol. 2010 Jan 1;28(1):114-8

194. Ghussen F, Kruger I, Smalley RV, Groth W. Hyperthermic perfusion with chemotherapy for melanoma of the extremities. World J Surg. 1989 Sep-Oct;13(5):598-602

195. 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 Dec;200(6):764-8

196. Monson JR, Donohue JH. Long-term results of a randomized trial of hyperthermic limb perfusion (HLP) with chemotherapy for extremity melanoma. World J Surg. 1990 Nov-Dec;14(6):845-6

197. Hafstrom L, Rudenstam CM, Blomquist E, Ingvar C, Jonsson PE, Lagerlof B, et al. Regional hyperthermic perfusion with melphalan after surgery for recurrent malignant melanoma of the extremities. Swedish Melanoma Study Group. J Clin Oncol. 1991 Dec;9(12):2091-4

198. Sanki A, Kam PC, Thompson JF. Long-term results of hyperthermic, isolated limb perfusion for melanoma: a reflection of tumor biology. Ann Surg. 2007 Apr;245(4):591-6

199. Beasley GM, Riboh JC, Augustine CK, Zager JS, Hochwald SN, Grobmyer SR, et al. Prospective multicenter phase II trial of systemic ADH-1 in combination with melphalan via isolated limb infusion in patients with advanced extremity melanoma. J Clin Oncol. 2011 Mar 20;29(9):1210-5

200. Mariani P, Piperno-Neumann S, Servois V, Berry MG, Dorval T, Plancher C, et al. Surgical management of liver metastases from uveal melanoma: 16 years' experience at the Institut Curie. Eur J Surg Oncol. 2009 Nov;35(11):1192-7

201. Breedis C, Young G. The blood supply of neoplasms in the liver. Am J Pathol. 1954 Sep-Oct;30(5):969-77

202. Ackerman NB, Lien WM, Kondi ES, Silverman NA. The blood supply of experimental liver metastases. I. The distribution of hepatic artery and portal vein blood to "small" and "large" tumors. Surgery. 1969 Dec;66(6):1067-72

203. Alexander HR, Jr., Libutti SK, Pingpank JF, Steinberg SM, Bartlett DL, Helsabeck C, et al. Hyperthermic isolated hepatic perfusion using melphalan for patients with ocular melanoma metastatic to liver. Clin Cancer Res. 2003 Dec 15;9(17):6343-9

204. Noter SL, Rothbarth J, Pijl ME, Keunen JE, Hartgrink HH, Tijl FG, et al. Isolated hepatic perfusion with high-dose melphalan for the treatment

of uveal melanoma metastases confined to the liver. Melanoma Res. 2004 Feb;14(1):67-72

205. Rizell M, Mattson J, Cahlin C, Hafstrom L, Lindner P, Olausson M. Isolated hepatic perfusion for liver metastases of malignant melanoma. Melanoma Res. 2008 Apr;18(2):120-6

206. Verhoef C, de Wilt JH, Brunstein F, Marinelli AW, van Etten B, Vermaas M, et al. Isolated hypoxic hepatic perfusion with retrograde outflow in patients with irresectable liver metastases; a new simplified technique in isolated hepatic perfusion. Ann Surg Oncol. 2008 May;15(5):1367-74

207. van Iersel LB, Hoekman EJ, Gelderblom H, Vahrmeijer AL, van Persijn van Meerten EL, Tijl FG, et al. Isolated hepatic perfusion with 200 mg melphalan for advanced noncolorectal liver metastases. Ann Surg Oncol. 2008 Jul;15(7):1891-8

208. Luke JJ, Callahan MK, Postow MA, Romano E, Ramaiya N, Bluth M, et al. Clinical activity of ipilimumab for metastatic uveal melanoma: A retrospective review of the Dana-Farber Cancer Institute, Massachusetts General Hospital, Memorial Sloan-Kettering Cancer Center, and University Hospital of Lausanne experience. Cancer. 2013 Aug 2

209. Sharma KV, Gould JE, Harbour JW, Linette GP, Pilgram TK, Dayani PN, et al. Hepatic arterial chemoembolization for management of metastatic melanoma. AJR Am J Roentgenol. 2008 Jan;190(1):99-104

210. Leyvraz S, Suciu S, Piperno-Neumann S, Baurain J, Zdzienicki M, Testori A, et al. Randomized phase III trial of intravenous (IV) versus hepatic intra-arterial (HIA) fotemustine in patients with liver metastases from uveal melanoma: Final results of the EORTC 18021 study. J Clin Oncol. 2012;30 (15 suppl):Abstract 8532

211. Mole RH. Whole body irradiation; radiobiology or medicine? Br J Radiol. 1953 May;26(305):234-41

212. Kono K, Mimura K, Kiessling R. Immunogenic tumor cell death induced by chemoradiotherapy: molecular mechanisms and a clinical translation. Cell death & disease. 2013;4:e688

213. Postow MA, Callahan MK, Barker CA, Yamada Y, Yuan J, Kitano S, et al. Immunologic correlates of the abscopal effect in a patient with melanoma. N Engl J Med. 2012 Mar 8;366(10):925-31

214. Arnestad JP, Bengtsson A, Bengtson JP, Henriksson BA, Stenqvist O, Naredi P, et al. Isolated hyperthermic liver perfusion with cytostatic-

containing perfusate activates the complement cascade. Br J Surg. 1992 Sep;79(9):948-51

215. Arnestad JP, Bengtsson A, Bengtson JP, Hafstrom L, Redl H, Schlag G. Leukocyte activation by isolated hyperthermic liver and limb perfusion due to malignancy. World J Surg. 1995 Nov-Dec;19(6):861-6

216. Nakayama J, Guan XC, Tominaga R, Nagae S, Matsuda K, Nakao T, et al. Increase in the peripheral lymphocyte populations expressing CD54 (ICAM-1) after hyperthermic isolated limb perfusion in patients with malignant melanoma: an analysis of four cases. J Dermatol Sci. 1998 May;17(1):33-8

217. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecologic oncology. 2008 Jul;110(1):13-21

218. Terzic M, Dotlic J, Likic I, Ladjevic N, Brndusic N, Mihailovic T, et al. Predictive factors of malignancy in patients with adnexal masses. European journal of gynaecological oncology. 2013;34(1):65-9

219. Liu N, Cui RX, Sun Y, Guo R, Mao YP, Tang LL, et al. A four-miRNA signature identified from genome-wide serum miRNA profiling predicts survival in patients with nasopharyngeal carcinoma. Int J Cancer. 2013 Sep 2

220. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell research. 2008 Oct;18(10):997-1006

221. Babchia N, Calipel A, Mouriaux F, Faussat AM, Mascarelli F. The PI3K/Akt and mTOR/P70S6K signaling pathways in human uveal melanoma cells: interaction with B-Raf/ERK. Invest Ophthalmol Vis Sci. 2010 Jan;51(1):421-9

222. Zebary A, Omholt K, Vassilaki I, Hoiom V, Linden D, Viberg L, et al. KIT, NRAS, BRAF and PTEN mutations in a sample of Swedish patients with acral lentiginous melanoma. J Dermatol Sci. 2013 Aug 8

223. Aguissa-Toure AH, Li G. Genetic alterations of PTEN in human melanoma. Cellular and molecular life sciences : CMLS. 2012 May;69(9):1475-91

224. Naus NC, Zuidervaart W, Rayman N, Slater R, van Drunen E, Ksander B, et al. Mutation analysis of the PTEN gene in uveal melanoma cell lines. Int J Cancer. 2000 Jul 1;87(1):151-3