Isolated Regional Perfusion for Metastases of Malignant Melanoma

Clinical and Experimental studies

Roger Olofsson

Department of Surgery
Institute of Clinical Sciences at Sahlgrenska Academy
University of Gothenburg

Gothenburg, Sweden

2013
Correspondence:
Roger Olofsson, MD
Department of Surgery
Sahlgrenska University Hospital
41345 Gothenburg, Sweden
E-mail: roger.olofsson@surgery.gu.se

© 2013 Roger Olofsson. All rights reserved. No part of this doctoral thesis may be reproduced in any form without permission from the author.

ISBN 978-91-628-8747-6
http://hdl.handle.net/2077/33105
Printed by Ineko AB, Gothenburg, Sweden
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

Roger Olofsson
Department of Surgery, Institute of Clinical Sciences at Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

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

ISBN 978-91-628-8747-6 http://hdl.handle.net/2077/33105
SAMMANFATTNING PÅ SVENSKA


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 aktivering. 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

   *Isolated Hepatic Perfusion for Ocular Melanoma Metastasis - Registry Data Suggests a Survival Benefit.*

    *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

    *MicroRNA in exosomes isolated from the liver circulation in patients with uveal melanoma metastases.*
    Manuscript.
    *These authors contributed equally to this work.*
# CONTENT

1 INTRODUCTION ......................................................................................................................... 1

1.1 Malignant melanoma .............................................................................................................. 1

1.1.1 Cutaneous malignant melanoma ......................................................................................... 2

1.1.2 Uveal malignant melanoma .................................................................................................. 8

1.2 Isolated regional perfusion ..................................................................................................... 10

1.2.1 Melphalan ........................................................................................................................... 13

1.2.2 Hyperthermia ....................................................................................................................... 14

1.2.3 TNF-alpha .......................................................................................................................... 15

1.2.4 Technical development ....................................................................................................... 16

1.2.5 Toxicity ............................................................................................................................... 18

1.3 Systemic treatment for melanoma ......................................................................................... 20

1.3.1 Targeted therapies .............................................................................................................. 20

1.3.2 Immunotherapy .................................................................................................................. 21

1.3.3 Immunogenic cell death ....................................................................................................... 24

1.4 Exosomes .................................................................................................................................. 25

1.4.1 Development of metastases ............................................................................................... 27

1.4.2 Biomarkers ......................................................................................................................... 28

2 AIMS .................................. .................................................................................................. 30

3 PATIENTS AND METHODS ..................................................................................................... 31

3.1 Study populations .................................................................................................................. 31

3.2 Data retrieval ........................................................................................................................ 31

3.3 Isolated limb perfusion ......................................................................................................... 32

3.4 Isolated hepatic perfusion ..................................................................................................... 33

3.5 Response evaluation .............................................................................................................. 35

3.6 Statistical methods ................................................................................................................. 36

3.7 Laboratory analyses ............................................................................................................. 36
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>ALM</td>
<td>Acral lentiginous melanoma</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>BAC</td>
<td>Best alternative care</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived cells</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>CLND</td>
<td>Completion lymph node dissection</td>
</tr>
<tr>
<td>COMS</td>
<td>The Collaborative Ocular Melanoma Study</td>
</tr>
<tr>
<td>CR</td>
<td>Complete response</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
<td>DFS</td>
<td>Disease-free survival</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>esRNA</td>
<td>Exosomal shuttle RNA</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>ICD</td>
<td>International Classification of Diseases</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IHHP</td>
<td>Isolated hypoxic hepatic perfusion</td>
</tr>
<tr>
<td>IHP</td>
<td>Isolated hepatic perfusion</td>
</tr>
<tr>
<td>ILI</td>
<td>Isolated limb infusion</td>
</tr>
<tr>
<td>ILP</td>
<td>Isolated limb perfusion</td>
</tr>
<tr>
<td>IVC</td>
<td>Inferior vena cava</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopaedia of Genes and Genomes</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LMM</td>
<td>Lentigo maligna melanoma</td>
</tr>
<tr>
<td>M-ILP</td>
<td>Melphalan based isolated limb perfusion</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular bodies</td>
</tr>
<tr>
<td>NC</td>
<td>No change</td>
</tr>
</tbody>
</table>
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).

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)**

<table>
<thead>
<tr>
<th>T Classification</th>
<th>Thickness (mm)</th>
<th>Ulceration/Mitoses</th>
</tr>
</thead>
</table>
| T1               | ≤1.0           | a: without ulceration and mitosis <1mm²  
|                  |                | b: with ulceration and mitosis ≥1mm²    |
| 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)**

<table>
<thead>
<tr>
<th>N Classification</th>
<th>No. of metastatic nodes</th>
<th>Micro/Macrometastases</th>
</tr>
</thead>
</table>
| 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 |
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)**

<table>
<thead>
<tr>
<th>M Classification</th>
<th>Site</th>
<th>Serum LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1a</td>
<td>Distant skin, subcutaneous or nodal metastasis</td>
<td>Normal</td>
</tr>
<tr>
<td>M1b</td>
<td>Lung metastasis</td>
<td>Normal</td>
</tr>
<tr>
<td>M1c</td>
<td>All other metastasis</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>Any distant metastasis</td>
<td>Elevated</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Pathologic staging</th>
<th>T</th>
<th>N</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IA</td>
<td>T1a</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IB</td>
<td>T1b</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2a</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIA</td>
<td>T2b</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3a</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIB</td>
<td>T3b</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T4a</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIIC</td>
<td>T4b</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIIA</td>
<td>T1-4a</td>
<td>N1a</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1-4a</td>
<td>N2a</td>
<td>M0</td>
</tr>
<tr>
<td>IIIB</td>
<td>T1-4b</td>
<td>N1a</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1-4b</td>
<td>N2a</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1-4a</td>
<td>N1b</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1-4a</td>
<td>N2b</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1-4a</td>
<td>N2c</td>
<td>M0</td>
</tr>
<tr>
<td>IIIIC</td>
<td>T1-4b</td>
<td>N1b</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1-4b</td>
<td>N2b</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1-4b</td>
<td>N2c</td>
<td>M0</td>
</tr>
<tr>
<td>IV</td>
<td>Any T</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>Any T</td>
<td>Any N</td>
<td>M1</td>
</tr>
</tbody>
</table>
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).

![Large choroidal melanoma pushing the retina.](image)

**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).
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).
1.2.1 Melphalan

Melphalan (L-phenylalanine mustard) is a phenylalanine derivative 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)**

IHHP 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)**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>No reaction</td>
</tr>
<tr>
<td>II</td>
<td>Slight erythema and/or edema</td>
</tr>
<tr>
<td>III</td>
<td>Considerable erythema and/or edema with some blisters; slightly disturbed motility permissible</td>
</tr>
<tr>
<td>IV</td>
<td>Extensive epidermolysis and/or obvious damage to the deep tissues, causing definite functional disturbances; threatened or manifest compartmental syndrome</td>
</tr>
<tr>
<td>V</td>
<td>Reaction that may necessitate amputation</td>
</tr>
</tbody>
</table>

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).

![Diagram](image)

**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
Isolated Regional Perfusion for Metastases of Malignant Melanoma

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 cytoplasmatic 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).

![Diagram of exosome biogenesis](image)

**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 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

![Image of isolated limb perfusion](image)

**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 ultrasound guided cannulation of the femoral and the external jugular veins, which are then connected to an external venovenous 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.

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

Figure 11. Schematic representation of the setup during isolated hepatic perfusion.
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 1880 × g for 10 minutes. The purified blood plasma was then centrifuged at 29500 × 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 120000 × 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.

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
<th></th>
<th></th>
<th></th>
<th>105 (64%)</th>
<th>58 (36%)</th>
<th>70 years (23-94)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female</td>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary tumour location</td>
<td>Upper arm</td>
<td>Lower arm</td>
<td>Hand</td>
<td>Upper leg</td>
<td>Knee</td>
<td>Lower leg</td>
<td>Foot</td>
<td>Unknown</td>
</tr>
<tr>
<td>Breslow thickness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulky tumour (&gt;4 cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-stage</td>
<td>N2c</td>
<td>N3</td>
<td></td>
<td></td>
<td></td>
<td>95 (58%)</td>
<td>68 (42%)</td>
<td></td>
</tr>
<tr>
<td>M-stage</td>
<td>M0</td>
<td>M1</td>
<td></td>
<td></td>
<td></td>
<td>152 (95%)</td>
<td>8 (5%)</td>
<td></td>
</tr>
<tr>
<td>Chemo</td>
<td>Melphalan</td>
<td>Melphalan + TNF-alpha</td>
<td></td>
<td></td>
<td></td>
<td>148 (91%)</td>
<td>15 (9%)</td>
<td></td>
</tr>
<tr>
<td>Vessel</td>
<td>Axillary</td>
<td>Brachial</td>
<td>Femoral</td>
<td>External iliac</td>
<td>Subclavian</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

39
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.

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

---

**Figure 12.** Flow chart of outcome for 163 consecutive patients. Reprinted with permission from Informa Healthcare.
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>
<thead>
<tr>
<th>Table 7. Patient characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
</tr>
<tr>
<td><strong>Primary tumour localization</strong></td>
</tr>
<tr>
<td>Choroidea</td>
</tr>
<tr>
<td>Ciliary Body</td>
</tr>
<tr>
<td><strong>Time from primary tumour to metastases</strong></td>
</tr>
<tr>
<td><strong>Previous treatment of liver metastases</strong></td>
</tr>
<tr>
<td>Liver resection</td>
</tr>
<tr>
<td>Chemotherapy</td>
</tr>
<tr>
<td>No treatment</td>
</tr>
<tr>
<td><strong>Largest liver metastases (diameter)</strong></td>
</tr>
<tr>
<td><strong>Volume of liver occupied with metastases</strong></td>
</tr>
<tr>
<td>&lt;10%</td>
</tr>
<tr>
<td>10-25%</td>
</tr>
<tr>
<td>26-50%</td>
</tr>
<tr>
<td><strong>Number of liver metastases</strong></td>
</tr>
<tr>
<td>1-4</td>
</tr>
<tr>
<td>5-10</td>
</tr>
<tr>
<td>11-100</td>
</tr>
<tr>
<td>&gt;100</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Grade</th>
<th>No of patients</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>I</td>
<td>1</td>
<td>Nausea</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Pain</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Hepatic artery thrombosis</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Pleural effusion</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>Pneumonia</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Pulmonary emboli</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Septicemia</td>
</tr>
<tr>
<td>IIIb</td>
<td>1</td>
<td>Perforated duodenal ulcer</td>
</tr>
<tr>
<td>IVA</td>
<td>1</td>
<td>Pulmonary effusion, thoracic drainage, pneumonia, reintubated.</td>
</tr>
<tr>
<td>IVB</td>
<td>1</td>
<td>Respiratory, cardiovascular and renal failure.</td>
</tr>
</tbody>
</table>
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).

![Survival curve](image)

**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>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>Local</th>
<th>Vessel</th>
<th>N-stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63</td>
<td>Female</td>
<td>Foot</td>
<td>Femoral</td>
<td>N3</td>
</tr>
<tr>
<td>2</td>
<td>59</td>
<td>Male</td>
<td>Lower arm</td>
<td>Axillary</td>
<td>N2c</td>
</tr>
<tr>
<td>3</td>
<td>92</td>
<td>Female</td>
<td>Lower leg</td>
<td>Femoral</td>
<td>N2c</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>Female</td>
<td>Foot</td>
<td>Femoral</td>
<td>N3</td>
</tr>
<tr>
<td>5</td>
<td>87</td>
<td>Male</td>
<td>Lower leg</td>
<td>Femoral</td>
<td>N2c</td>
</tr>
<tr>
<td>6</td>
<td>73</td>
<td>Male</td>
<td>Foot</td>
<td>Femoral</td>
<td>N3</td>
</tr>
<tr>
<td>7</td>
<td>76</td>
<td>Female</td>
<td>Lower leg</td>
<td>Iliacal</td>
<td>N2c</td>
</tr>
<tr>
<td>8</td>
<td>63</td>
<td>Male</td>
<td>Lower leg</td>
<td>Iliacal</td>
<td>N3</td>
</tr>
<tr>
<td>9</td>
<td>53</td>
<td>Female</td>
<td>Lower arm</td>
<td>Axillary</td>
<td>N3</td>
</tr>
<tr>
<td>10</td>
<td>81</td>
<td>Male</td>
<td>Whole leg</td>
<td>Femoral</td>
<td>N3</td>
</tr>
<tr>
<td>11</td>
<td>72</td>
<td>Male</td>
<td>Whole leg</td>
<td>Femoral</td>
<td>N3</td>
</tr>
<tr>
<td>12</td>
<td>78</td>
<td>Female</td>
<td>Whole leg</td>
<td>Femoral</td>
<td>N3</td>
</tr>
</tbody>
</table>

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).

![Graph A](image)

**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.

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

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.](image)

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.

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.

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

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

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

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.

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.
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.
REFERENCES

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


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


184. Noorda EM, Vrouenraets BC, Nieweg OE, van Geel AN, Eggermont AM, Kroon BB. Repeat isolated limb perfusion with TNFalpha and
of uveal melanoma metastases confined to the liver. Melanoma Res. 2004 Feb;14(1):67-72


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


