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NOVEL PROGNOSTIC FACTORS FOR ADVANCED MELANOMA AND LOCALIZED RENAL CELL CARCINOMA

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To patients involved in clinical trials and academic studies

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ABSTRACT

This study aimed to evaluate prognostic and predictive factors in melanoma and renal cell carcinoma to tailor optimal treatment and follow-up for cancer patients.

Chemotherapy was the standard treatment for advanced melanoma before immune checkpoint inhibitors and targeted therapies. The median overall survival was 8.9 months (95% CI 7.5–10.4) and the five-year survival rate 13% in 146 patients who had received BOLD-IFN chemoimmunotherapy at Turku University Hospital in 1991─2010. Long-term survivors were found especially in patients without visceral metastases (five-year survival rate 28%).

The Finnish Melanoma Group conducted a prospective, multicenter trial enrolling 38 patients who received TOL-IFN (temozolomide, lomustine, vincristine, and interferone-alpha) ± vemurafenib for the first-line treatment of advanced cutaneous melanoma. Elevated LDH was associated with shorter overall survival unlike asymptomatic brain metastases. Undetectable circulating tumor DNA in baseline plasma samples correlated with longer progression-free survival and baseline ctDNA levels were inversely associated with overall survival. Patients with persistent detectable ctDNA during treatment had the shortest overall survival.

One-third of patients will develop disease recurrence after surgery for localized renal cell carcinoma. Tumor size, tumor grade (Fuhrman), and microvascular invasion were sufficient for the accurate prediction of metastasis-free survival in 196 patients operated for localized clear cell RCC. The three-feature prediction model was validated in an external cohort of 714 patients. It retained similar prediction accuracy as the Leibovich model (C-index 0.836 vs. 0.848, p=0.106) and had better prognostic value for long-term prediction in both cohorts

In conclusion, undetectable ctDNA is a novel biomarker indicating favourable prognosis in advanced melanoma. This study suggests that patients with persistent detectable ctDNA may require more frequent monitoring of treatment response and perhaps more intensive therapy. We also introduced a three-feature prediction model for metastasis-free survival as a tool for optimizing postoperative follow-up of localized RCC patients.

KEYWORDS: Biomarkers, ctDNA, Melanoma, Prognostic factors, Renal Cell Carcinoma

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Kliininen syöpätautioppi
KALLE E MATTILA: Edenneen melanooman ja paikallisen munuaissyövän uudet ennustetekijät
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TIIVISTELMÄ

Tämän väitöskirjatutkimuksen tavoitteena oli löytää uusia ennustetekijöitä, jotka voivat auttaa suunnittelemaan melanooma- ja munuaissyöpäpotilaiden yksilöllistä hoitoa ja seurantaa.

Ensimmäisessä osatyössä tutkittiin solunsalpaajahoidon ja alfainterferonin (DOBC-IFN) hyötyä ennustavia tekijöitä. 146 potilasta oli saanut DOBC-IFN-hoitoa TYKS:ssä edenneen ihomelanooman vuoksi vuosina 1991–2010. Potilaiden eliniän mediaani oli 8,9 kuukautta (95 prosentin luottamusväli 7,5–10,4 kk) ja viiden vuoden kohdalla elossa olevien potilaiden osuus oli 13 prosenttia. Jopa 28 prosenttia potilaista, joilla ei ollut todettu sisäelinetäpesäkkeitä, pysyi elossa viisi vuotta.

Toisessa ja kolmannessa osatyössä raportoitiin tulokset Suomen Melanooma-ryhmän toteuttamasta prospektiivisesta kansallisesta monikeskustutkimuksesta, jossa annettiin 38:lle edennyttä ihomelanoomaa sairastavalle potilaalle solunsalpaajien, alfainterferonin (TOL-IFN) ja vemurafenibin yhdistelmähoitoa. Korkea plasman laktaattidehydrogenaasipitoisuus ennusti lyhyempää elinaikaa, kun taas oireettomat aivometastaasit eivät olleet yhteydessä lyhyempään elinaikaan. Veressä kiertävä kasvain-DNA ennusti nopeampaa taudin etenemistä ja kasvain-DNA:n määrä oli kääntäen verrannollinen elinajan pituuteen. Lyhyin elinaika todettiin potilailla, joilla kasvain-DNA ei hävinnyt hoidon aikana toistetusti otetuista plasmanäytteistä.

Neljännessä osatyössä osoitettiin, että syöpäkasvaimen koko, syöpäsolujen erilaistumisaste ja leviäminen hiusverisuoniin ennustavat luotettavasti etäpesäkkeiden ilmaantumista paikallisen kirkassoluisen munuaissyövän leikkauksen jälkeen.

Johtopäätöksenä voidaan todeta, että veressä kiertävä kasvain-DNA ennustaa melanoomapotilaiden elinaikaa. Mikäli kiertävä kasvain-DNA ei häviä hoidon aikana, voidaan harkita hoidon tehostamista. Neljännessä osatyössä esitellyn uuden nomogrammin avulla voidaan arvioida potilaan riskiä sairastua levinneeseen munuaissyöpään ja tätä luokittelua voidaan käyttää, kun suunnitellaan potilaan seurantaa paikallisen kirkassoluisen munuaissyövän leikkauksen jälkeen.

AVAINSANAT: Ennuste, Kiertävä kasvain-DNA, Melanooma, Munuaissyöpä

Table of Contents

Abbreviations

ALK	Anaplastic Lymphoma Kinase
BAP1	BRCA1 Associated Protein 1
BEAM	Bead Emulsification Amplification and Magnetics
BOLD-IFN	Bleomycin, Vincristine, Lomustine, Dacarbazine, Interferon-Alpha
BRAF	v-Raf Murine Sarcoma Viral Oncogene Homolog B
CD8	Cluster of Differentiation 8
CDK	Cyclin Dependent Kinase Inhibitor
CEA	Carcinoembryonic Antigen
CNS	Central Nervous System
CR	Complete Response
CRP	C-Reactive Protein
CSS	Cancer Specific Survival
ctDNA	Circulating Tumor DNA
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
ddPCR	Droplet Digital PCR
DNA	Deoxyribonucleic Acid
DTIC	Dacarbazine
ECOG	Eastern Cooperative Oncology Group
EGFR	Epidermal Growth Factor Receptor
FDA	Food and Drug Administration
FDG	Fluorodeoxyglucose
HER2	Human Epidermal Growth Factor Receptor 2
HIF	Hypoxia Inducible Factor
ICI	Immune Checkpoint Inhibitors
IFN	Interferon
IMDC	International Metastatic RCC Database Consortium
ISUP	International Society of Urologic Pathology
LDH	Lactate Dehydrogenase
MAPK	Mitogen Activated Protein Kinase
MEK	Mitogen Activated Protein Kinase Kinase
MFS	Metastasis-Free Survival

MGMT	O^6-methylguanine DNA Methyltransferase
MIU	Million International Units
mTOR	Mammalian Target of Rapamycin
NF1	Neurofibromin 1
NGS	Next Generation DNA Sequencing
NRAS	Neuroblastoma RAS Viral Oncogene Homolog
NSCLC	Non-Small Cell Lung Cancer
ORR	Objective Response Rate
OS	Overall Survival
PBMR1	Polybromo 1
PCR	Polymerase Chain Reaction
PD	Progressive Disease
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death-Ligand 1
PET-CT	Positron Emission Tomography-Computed Tomography
PFS	Progression-Free Survival
PN	Partial Nephrectomy
PR	Partial Response
PSA	Prostate Specific Antigen
PTEN	Phosphatase and Tensin Homolog
RCC	Renal Cell Carcinoma
RCT	Randomised Clinical Trial
RECIST	Response Evaluation Criteria in Solid Tumours
RFS	Recurrence-Free Survival
RN	Radical Nephrectomy
RNA	Ribonucleic Acid
RWE	Real World Evidence
SD	Stable Disease
SETD2	SET Domain containing 2
TKI	Tyrosine Kinase Inhibitor
TMZ	Temozolomide
TNM	Primary **T**umor, Regional Lymph **N**odes and Distant **M**etastasis (Prognostic Classification of Malignant Tumors)
TOL-IFN	Temozolomide, Vincristine, Lomustine, Interferon-Alpha
TP52	Tumor Protein p52
UISS	UCLA Integrated Staging System
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
VHL	Von Hippel-Lindau
WHO	World Health Organization

List of Original Publications

I Mattila K, Raanta P, Lahtela V, Pyrhönen S, Koskivuo I, Vihinen P. Long-term Survival of Stage IV Melanoma Patients Treated with BOLD Combination Chemotherapy and Intermediate-dose Subcutanoeus Interferon-alpha. *Anticancer Research,* 2018; 38(11): 6393–6397.

II Mattila KE, Vihinen P, Ramadan S, Skyttä T, Tiainen L, Vuoristo MS, Tyynelä-Korhonen K, Koivunen J, Kohtamäki L, Mäkelä S, Hernberg M. Combination chemotherapy with temozolomide, lomustine, vincristine and interferon-alpha (TOL-IFN) plus vemurafenib or TOL-IFN as first-line treatment for patients with advanced melanoma. *Acta Oncologica,* 2020; 59(3): 310–314.

III Mattila KE, Mäkelä S, Kytölä S, Andersson E, Vihinen P, Ramadan S, Skyttä T, Tiainen L, Vuoristo MS, Tyynelä-Korhonen K, Koivunen J, Kohtamäki L, Aittomäki K, Hernberg M. Association of circulating tumor DNA with treatment outcomes in advanced cutaneous melanoma patients who received chemoimmunotherapy and vemurafenib. Manuscript.

IV Mattila KE, Laajala TD, Tornberg SV, Kilpeläinen TP, Vainio P, Ettala O, Boström PJ, Nisen H, Elo LL, Jaakkola PM. A three-feature prediction model for metastasis-free survival after surgery of localized clear cell renal cell carcinoma. *Scientific Reports,* 2021; 11(1): 8650.

1 Introduction

Treatment options for cancer patients have expanded in the early 21st century. In addition, there are currently more ways to characterize tumor- and patient-derived factors that affect treatment outcomes.

Traditional chemotherapy blocks cell cycle leading to apoptosis of rapidly proliferating cells. Increased knowledge on cancer biology (Hanahan et al, 2011) has led to pivotal novel therapies that selectively target pathways enhancing proliferation, invasion, and survival of cancer cells. Typical examples include therapies that block overactive growth factor signaling routes (anti-HER-2 antibodies in the treatment of cancers with HER-2 overexpression or BRAF and MEK inhibitors in cutaneous melanomas with $BRAF^{v600}$ mutation), inhibit tumor neovascularization (antiangiogenic receptor tyrosine kinase inhibitors ((TKI)) in solid tumors such as renal cell carcinoma), and prevent immune evasion (immune checkpoint inhibitors ((ICI)) in multiple solid tumors and lymphomas).

Novel therapies have succeeded in prolonging the survival of cancer patients. The median overall survival (OS) of advanced melanoma patients, which used to be less than one year with chemotherapy (Yang et al, 2009), has exceeded three or even five-years in clinical trials with BRAF and MEK inhibitors and ICI (Ascierto et al, 2019; Hamid et al, 2019; Larkin et al, 2019; Long et al, 2019; Ascierto et al 2020). Although novel therapies target cancer cells more selectively than chemotherapy, they still cause a variety of adverse events leading to diminished quality of life. Moreover, cancer therapies are often used in combinations to increase their efficacy, and this usually leads to increased toxicity and costs.

Hence, there is a continuing need for prognostic factors, predictive factors, and biomarkers that can be utilized to tailor optimal therapy and follow-up for each individual cancer patient. The aim is to further improve treatment outcomes and reduce harms. From patient`s perspective, the information on prognosis is also important to cope with their life-threatening disease and make plans for future.

2 Review of the Literature

This review will provide an overview on the current use of prognostic and predictive factors and the applications of biomarkers in the treatment of cancer patients with the focus on cutaneous melanoma and clear cell renal cell carcinoma (ccRCC).

2.1 Definition of key terms

Prognostic factors are tumor- or patient-derived features that can be used to predict patient´s chance to recover from cancer or the chance of cancer to recur. Hence, prognostic factors are related to outcomes such as the overall/cancer-specific survival (OS/CSS) or the recurrence/metastasis-free survival (RFS/MFS). The TNM classification of malignant tumors (Bierley et al, 2017) is a typical prognostic staging system. It is used to assess the risk for disease progression in patients with localized primary tumor to select patients for neoadjuvant and adjuvant therapies and to tailor postoperative follow-up. Prognostic algorithms are also commonly used to identify features indicating more aggressive course of the disease and shorter survival. The IMDC risk score is a typical example. It is used to classify metastatic RCC patients into favorable- (score 0), intermediate- (score 1–2), and poor-risk groups (score 3–6) and predicts overall survival (median OS 43.2 months vs 22.5 months vs 7.8 months, respectively) (Heng et al, 2009, Heng et al, 2013). In clinical practice, the IMDC risk score is used to guide the choice of the first line therapy for advanced ccRCC.

Predictive factors are used to predict treatment outcomes such as the objective response rate (ORR) or the progression-free survival (PFS). Positive predictive factors indicating good reponse could help to choose the optimal therapy, for example ICI for non-small cell lung cancer patients with high tumor PD-L1-expression (Planchard et al 2018) or temozolomide for patients with MGMT promoter methylation in their glioblastomas (Weller et al, 2021). Negative predictive factors indicating poor treatment outcomes are evenly important to avoid unnecessary interventions, e.g. cytoreductive nephrectomy for metastatic RCC patients with $\geq$2 IMDC risk factors (Méjean et al, 2018; Méjean et al, 2021).

Biomarker is defined as characteristics that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention (Califf, 2018). Tumor DNA containing tumor-spesific mutations is a

typical biomarker that can be analyzed from tumor biopsy or body fluids (liquid biopsy) and utilized in cancer diagnostics, as a prognostic or predictive factor, and for monitoring treatment response (circulating tumor DNA). Other examples inlcude numerous cancer-derived proteins ("tumor markers"), such as alpha-fetoprotein (AFP) and human chorionic gonadotropin (hCG) in testicular cancer, carcinoembryonic antigen (CEA) in colorectal cancer, and prostate-specific antigen (PSA) in prostate cancer. These biomarkers can be measured from blood samples and are used in cancer diagnostics, choosing optimal therapy, and monitoring treatment response.

2.2 Introduction to treatment of cutaneous melanoma

Cutaneous melanoma arises from melanocytes and causes the majority of skin cancer mortality. The incidence of cutaneous melanoma is rising worldwide. In 2020, the annual number of new cases was over 320000, and it caused more than 57000 deaths (GLOBOCAN, 2020). In Finland, 892 men and 766 women were diagnosed with cutaneous melanoma in 2018 (Pitkäniemi et al, 2020). Fortunately, newly diagnosed melanomas are often localized skin tumors that can be curatively treated with a surgical excision involving sentinel lymph node biopsy in melanomas thicker than 0.8 mm. The thicker melanoma (especially ulcerated tumors with high mitotic rate) the more frequently it sends metastases via lymph and blood vessels to local lymph nodes and distant sites. In Finland, 146 men and 88 women died of melanoma in 2018 and the five-year survival rates were 90% and 93%, respectively (Pitkäniemi et al, 2020).

2.2.1 Era of chemotherapy regimens

Until the 2010s, dacarbazine-based chemotherapy regimens were the standard treatment for patients with advanced cutaneous melanoma. Dacarbazine (DTIC) is an intravenously administered alkylating chemotherapy agent that has been used to treat advanced melanoma since 1970s. Single DTIC was associated with the modest ORR of around 13% (8–32%) in advanced melanoma patients. Durable responses were rare and the median OS ranged from 5.6 to 11 months (Yang et al, 2009; Cocconi et al, 1992; Thomson et al, 1993; Avril et al, 2004; Chapman et al, 1999; Bajetta et al, 1994; Falkson et al, 1998; Middleton et al, 2000). Temozolomide (TMZ) is an orally administered alkylating chemotherapy agent, which penetrates blood-brain-barrier, and is used to treat malignant central nervous system tumors (Brada et al, 2010). TMZ was studied in patients with melanoma brain metastases, but the results were poor (mOS only 3.5 months) (Agarwala et al 2004).

Chemotherapy was combined with interferon-alpha and antiangiogenic therapies to improve efficacy in advanced melanoma. Interferon-alpha is a cytokine that enhances immune response against cancer cells. A meta-analysis showed that the combination of DTIC and subcutaneous interferon-alpha (IFN) led to the increased ORR of 21.5% but significant survival benefit was not observed compared to single DTIC (Huncharek et al, 2001). The combination chemotherapy with BOLD (bleomycin, vincristine, lomustine, and dacarbazine) and IFN increased the ORR even up to 62% with 13% of complete responses (CR) and 49% partial responses (PR), as reported in a phase 2 trial, but the median survival with BOLD-IFN did not exceed one year (Pyrhönen et al, 1992; Vuoristo et al, 2005). Cisplatin and carboplatin-based combination chemotherapy regimens have also been used to treat advanced cutaneous melanoma. Carboplatin-paclitaxel resulted in the ORR of 18% and the mOS of 11 months in a phase III trial and the combination of antiangiogenic TKI sorafenib to carboplatin-paclitaxel did not improve efficacy (Flaherty et al, 2013). The combination of anti-VEGF antibody bevacizumab with DTIC and IFN was also studied with similar outcomes (ORR 23%, mOS 11.5 months) (Vihinen et al, 2010).

2.2.2 Modern treatment options for melanoma

Fortunately, treatment options for advanced cutaneous melanoma have expanded since the approval of CTLA-4 inhibitor ipilimumab in Europe in 2011 (Figure 1).

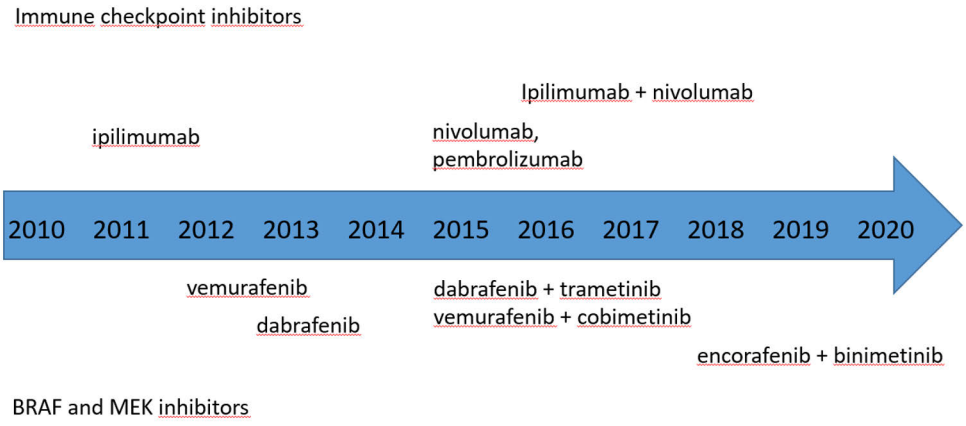


Figure 1. Timeline of approvals for ICI and BRAF and MEK inhibitors in Europe.

Currently, BRAF and MEK inhibitors and ICI have replaced chemotherapy from the first- and the second-line treatment but chemotherapy is still occasionally used as a palliative treatment after disease progression on other therapies or if patient is not

eligible for targeted therapies or ICI (BRAF wild type melanoma, patient requires active immunosuppressive treatment, poor ECOG performance status). Because of the rapid development of novel therapies, eligible melanoma patients are eagerly enrolled into available clinical trials.

2.2.2.1 Immune checkpoint inhibitors (ICI)

Immune checkpoints are mechanisms that restrict immune response against pathogens and protect against autoimmunity. Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) controls T-lymphocyte activation in lymph nodes and programmed death receptor-1−ligand-1 (PD-1–PD-L1) interaction maintains peripheral tolerance to self-reactive T-lymphocytes. Cancer cells have numerous ways to evade immune system for example by downregulating neoantigens recognized by immune cells or creating immunosuppressive microenvironment. Upregulated PD-L1 expression in the surface of cancer cells is an important mechanism to hamper immune response against cancer cells. Immune checkpoint inhibitors enhance antitumor immune response primarily by increasing the activity of cytotoxic T-lymphocytes. Anti-CTLA-4 antibody ipilimumab binds to CTLA-4 receptor in the surface of activated T-cells increasing their proliferation. Anti-PD-1 antibodies (nivolumab, pembrolizumab, and semiplimab) bind to PD-1 receptor in the surface of activated T-cells inhibiting T-cell anergy in the tumor microenvironment (Chen et al, 2013, Blank et al, 2005). Similarly, Anti-PD-L1 antibodies (atezolizumab, avelumab, and durvalumab) bind to PD-L1 and block the PD-1−PD-1-ligand interaction.

Durable responses and improved overall survival were first observed in advanced melanoma patients treated with anti-CTLA-4 antibody ipilimumab. The observed overall response rate (ORR) was 11–19% and 22% (20–26%) of the patients involved in phase II and III clinical trials were alive at three years (Hodi et al, 2010; Larkin et al, 2019, Schadendorf et al, 2015). Anti-PD-1 antibodies, nivolumab and pembrolizumab, further improved treatment outcomes and outperformed chemotherapy (Robert et al, 2015; Hamid et al, 2017) and ipilimumab (Robert et al, 2015) in the treatment of advanced melanoma. The ORR was 23–45% with nivolumab and pembrolizumab depending on prior therapies (Ascierto et al, 2019; Larkin et al, 2019; Robert et al, 2019) and 58% with the first-line combination of ipilimumab and nivolumab (Larkin et al, 2019). A significant group of patients achieved long-lasting responses. The three-year PFS rate exceeded 30% in clinical trials (32−33% with nivolumab/pembrolizumab monotherapy and 39% with ipilimumab and nivolumab combination therapy), and survival curves began to flatten thereafter (the five-year PFS rate 29−36%) (Carlino et al, 2021). The median OS was 33–37 months with nivolumab and pembrolizumab (Ascierto et al, 2019;

Larkin et al, 2019; Robert et al, 2019), and the mOS exceeded as much as 60 months in treatment-naïve patients receiving the combination of ipilimumab and nivolumab (Larkin et al, 2019).

2.2.2.2 BRAF and MEK inhibitors

$BRAF^{v600}$ mutation is found in approximately 50% of cutanoeus melanomas leading to constitutionally active mitogen activated protein kinase (MAPK) signaling pathway and excessive proliferation of melanoma cells (Flaherty et al, 2010; Schadendorf et al, 2018). Serine-threonine protein kinase inhibitors vemurafenib, dabrafenib, and encorafenib (Chapman et al, 2011; Hauschild et al, 2012; Dummer et al, 2018) bind to BRAF protein and block overactive MAPK pathway. Rapid responses were observed even in patients with high tumor burden (Figure 2) and single BRAF inhibitors yielded the unprecedented ORR of 50% in $BRAF^{v600}$ mutated melanoma patients (Chapman et al, 2011; Long et al, 2014). However, acquired resistance to single BRAF inhibitors developed usually within one year (mPFS 7−9 months) (Schadendorf et al, 2018). By combining MEK inhibitor (cobimetinib or trametinib) to BRAF inhibitor the ORR increased to 67–68% (Long et al, 2014; Larkin et al 2014) and the dual inhibition of MAPK pathway managed to delay acquired resistance to therapy (mPFS 11−15 months) (Schadendorf et al, 2018). BRAF and MEK inhibitors vemurafenib and cobimetinib, dabrafenib and trametinib, and encorafenib and binimetinib yielded the median OS of 26–34 months (Robert et al, 2019; Ribas et al, 2020; Ascierto et al, 2020) and the survival rate at 5 years was 34% (Robert et al, 2019) which is comparable to results achieved with ICI.

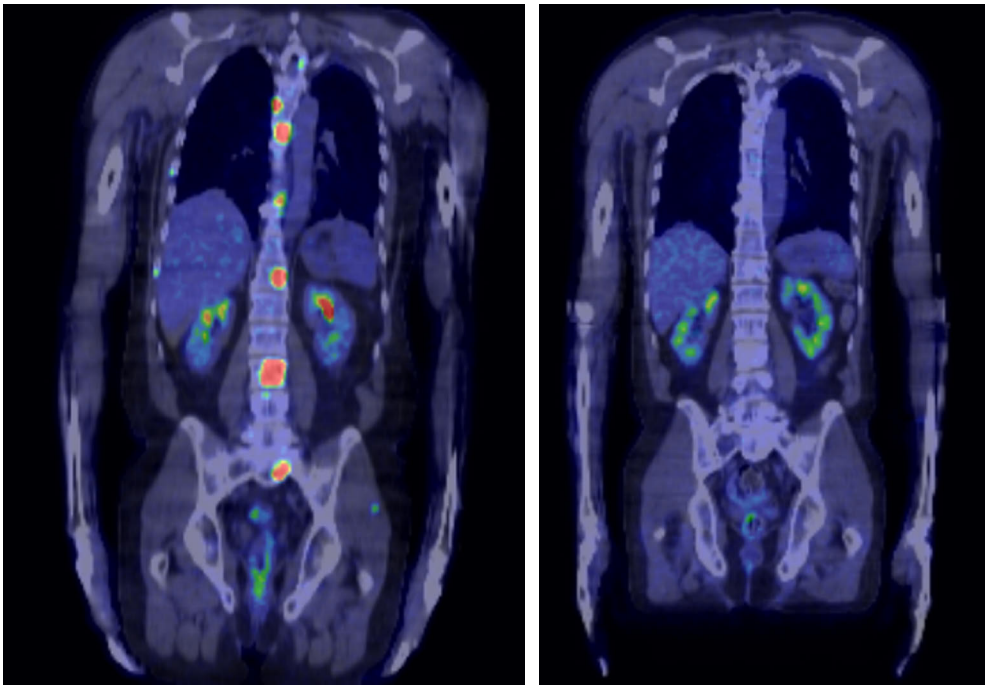


Figure 2. A patient with BRAF^{v600E} mutated metastatic melanoma was treated with vemurafenib. A complete metabolic response was achieved after three months on therapy (baseline FDG-PET-CT image on the left and the first response evaluation FDG-PET-CT on the right). Symptoms (fever and pain) were rapidly alleviated after the initiation of vemurafenib.

2.2.2.3 Medical treatment of brain metastases

ICI and BRAF and MEK inhibitors are used to treat melanoma brain metastases. The intracranial clinical benefit (CR, PR, and SD) was achieved in 57% of the patients treated with ipilimumab and nivolumab (Tawbi et al, 2018), and the combination therapy was superior to single nivolumab in patients with asymptomatic brain metastases (Long et al, 2018). In addition to ICI, BRAF and MEK inhibitors have been effective in the treatment of BRAF mutated melanoma brain metastases. The intracranial response rate of 44−59% has been observed with dabrafenib and trametinib in patients with melanoma brain metastases (Davies et al, 2017) and it is recommended for the treatment of BRAF^{v600} mutated symptomatic brain metastases (Keilholz et al 2020). Intracranial responses were also reported with encorafenib and binimetinib even in patients who had progressive disease on prior BRAF and MEK inhibitor therapy in a small retrospective case-series (Holbrook et al, 2020).

2.2.3 Current role of prognostic and predictive factors and biomarkers in the treatment of advanced melanoma

2.2.3.1 Traditional prognostic and predictive factors

Several prognostic and predictive features have been identified in clinical studies. Features indicating favorable survival outcomes include normal lactate dehydrogenase (LDH) level, less than three metastatic sites, good performance status (ECOG 0), higher relative eosinophil to lymphocyte count, and stage M1a or M1b (no visceral metastases outside lungs) in advanced cutaneous melanoma patients treated with BRAF and MEK inhibitors and ICI (Long et al, 2016; Weide et al, 2016). In addition to elevated LDH, elevated C-reactive protein (CRP) reflecting systemic inflammation reaction was also associated with worse survival outcomes in melanoma patients treated with ICI (Iivanainen et al, 2019).

Disease progression in the central nervous system has indicated shorter survival time (Long et al, 2016) and the patients with symptomatic brain metastases, especially leptomeningeal metastases, have had the median survival time of only 5 months (Raizer et al, 2008; Long et al, 2018). However, some patients with asymptomatic brain metastasis can achieve long survival with combination therapies (median OS 10−24 months with dabrafenib and trametinib and 18.5 months with ipilimumab plus nivolumab (Davies et al, 2017; Long et al, 2018)).

Generally, patients with good performance status, low tumor burden, normal LDH level, and without CNS metastases have favorable prognosis. Currently, there is no hard evidence, what is the best way to sequence ICI and targeted therapies for advanced melanoma patients (Keilholz et al, 2020). Patient`s clinical characteristics, age, medical history (autoimmune diseases etc.), and preferences have to be taken into account in treatment decisions. Considering possible long-lasting treatment responses, ICI are often the preferred first-line option for fit patients without immediately life-threatening metastases. However, targeted therapies can induce very rapid tumor responses and are used in the first-line e.g. for patients with symptomatic BRAF mutated brain metastases. The presence of poor prognostic features supports the use of combination therapies (ipilimumab + nivolumab or BRAF + MEK inhibitors) for fit patients without severe comorbidities. Different combinations of ICI and BRAF and MEK inhibitors or antiangiogenic TKIs are still experimental in advanced melanoma (Gutzmer et al, 2020; Ferrucci et al 2020; Nathan et al 2020; Fernandes et al, 2020)

2.2.3.2 BRAF and NRAS mutations

Currently, BRAF mutation is the only validated biomarker for BRAF and MEK inhibitor therapy, and there are no other established biomarkers adopeted into routine clinical practice (Keilholz et al, 2020).

$BRAF^{v600}$ is mutated in 40–60% of cutaneous melanomas (Flaherty et al, 2010; Lee et al, 2011; Heppt et al, 2017; Carlino et al, 2014; Jakob et al, 2012; Colombino et al, 2012). $BRAF^{V600E}$ is the most common mutation acounting for 70–80% of the cases and $BRAF^{V600K}$ for 10–20% of the cases. Non-E/K mutations and non-v600 mutations are rare and the benefit of MAPK inhibitors in these patients is inferior compared to $BRAF^{v600E}$ mutated melanomas (Menzer et al, 2019). ICI are also used to treat BRAF mutated melanoma patients eligible for immunotherapy. BRAF mutated melanoma patients have a trend towards better survival with ipilimumab and nivolumab combination therapy compared to BRAF wild type melanoma patients (Larkin et al, 2019; Atkins et al, 2019). Cutaneous melanomas of the head and neck region with the highest exposure to ultraviolet radiation tend to have different driver mutations, typically NF1, NRAS, TP53, and $BRAF^{non\text{-}v600E}$, than melanomas in the areas with less sun exposure, commonly driven by $BRAF^{v600E}$ mutation (Curtin et al, 2005; Craig et al, 2018).

Mutations in the NRAS gene (typically Q61K, Q61R, Q61L) are present in 15−24% of cutaneous melanomas and are associated with worse prognosis (Lee et al, 2011; Heppt et al, 2017; Carlino et al, 2014; Jakob et al, 2012; Colombino et al, 2012). NRAS mutations lead to downstream activation of the MAPK pathway irrespective of BRAF and therefore BRAF inhibitors do not have clinical activity in NRAS mutated melanomas. A minor increase in the progression-free survival (PFS) was achieved with the MEK inhibitors binimetinib and pimasertib compared to dacarbazine (2.8 vs 1.5 months and 3.3 vs 1.8 months, respectively), but no survival benefit was observed (Dummer et al, 2017; Lebbe et al, 2020). NRAS mutated melanoma patients are usually treated with ICI. The ORR of NRAS mutated patients treated with anti-PD-1 antibodies or ipilimumab and nivolumab combination therapy was comparable to the ORR of NRAS wild type patients but the mOS remained shorter (21 vs 33 months) (Kirchberger et al, 2018). There is still an unmet need for novel targeted therapies for NRAS mutated patients.

2.2.3.3 Pitfalls of precision medicine

Different methods to determine oncogenic driver mutations from tumor tissue samples or body fluids are more and more used to reveal tumor mutations that can be targeted by novel therapies and this information is also adopted into clinical decision-making. Tumors with similar histology can be categorized into subgroups

based on distinct oncogenic driver mutations. On the other hand, tumors with different histology can harbor similar mutations.

$BRAF^{v600}$ mutations are also found in 10% of metastatic colorectal carcinomas and have been associated with poor prognosis (Kopetz et al, 2019; Tran et al, 2011). However, vemurafenib did not yield clinically meaningful responses in colorectal carcinoma patients (Kopetz et al, 2015). The intrinsic resistance of $BRAF^{v600E}$ mutated colorectal carcinoma to vemurafenib was discovered to be mediated by the feedback activation of EGFR in colon carcinoma cells. Unlike melanoma cells, colorectal carcinoma cells usually have a high expression of EGFR which supports their proliferation even in the presence of vemurafenib (Prahallad et al, 2012). This could be overcome by combining anti-EGFR antibody (cetuximab or panitumumab) to BRAF and MEK inhibitor (encorafenib and binimetinib) in metastatic colorectal carcinoma patients (Kopetz et al, 2019). This underscores the need of prospective clinical trials to verify the efficacy of mutation-targeted therapy in tumors with different histology.

2.2.3.4 Tumor microenvironment and mutational load

Tumor microenvironment has complex interactions with immune cells affecting the response to ICI (Marzagalli et al 2019). Unlike in NSCLC, tumor cell PD-L1 expression level is not utilized to guide the choice of ICI therapy in advanced melanoma because PD-L1 expression level alone did not predict treatment outcomes and PD-L1 negative patients had also benefitted from ICI (Larkin et al, 2019). Tumor mutational load, tumor infiltrating lymphocytes, macrophages, and the expression of immune-related genes (e.g. IFN-gamma expression profile) have been observed to affect the outcome of patients treated with ICI (Riaz et al, 2017; Topalian et al, 2016), but the clinical relevance of these finding require further validation in prospective clinical trials. Tumors with mismatch-repair deficiency have a high rate of somatic mutations and are susceptible to ICI (Le et al, 2015). FDA has approved PD-1 inhibitor, pembrolizumab, for treatment-refractory cancers with a high tumor mutational burden (>10 mutations per megabase), but there is conflicting evidence to support the use of PD-1 inhibitors in patients without mismatch-repair deficiency (Rousseau et al, 2021). Cutaneous melanoma cells are associated with a high rate of somatic tumor mutations induced by chronic exposure to UV-radiation (Craig et al, 2018) and therefore tend to have high expression of neoantigens which makes them good targets for the immune system (Schumacher et al, 2015).

2.2.3.5 Clinical applications of circulating tumor DNA

Circulating tumor DNA (ctDNA) is a potential biomarker for cancer patients. Fragments of tumor DNA are released into circulation after apoptosis and necrosis of tumor cells and by active secretion. Plasma sample containing these cell-free circulating tumor DNA fragments is easy to draw and "liquid biopsy" is particularly valuable when invasive tumor biopsy is not feasible or there is only limited amount of tumor tissue available. ctDNA analyses are currently adopted into clinical practice in advanced, treatment-naïve non-small cell lung cancer patients to reveal therapeutically actionable mutations and rearrangements in EGFR, ALK, ROS1, RET, KRAS, and BRAF genes especially in younger, non-smokers, and non-squamous NSCLC patients (Rolfo et al. 2018). Repeated tumor biopsies may be avoided using liquid biopsy to reveal mutations mediating resistance to therapy, e.g. EGFR T790M, at the time of disease progression on EGFR TKIs in NSCLC. ctDNA analyses are already integrated into biomarker analyses of current clinical trials in multiple different tumor types and may be used as a stratification factor in biomarker driven clinical trials in the future (Cescon et al, 2020).

ctDNA can be detected in body fluids (plasma, pleural effusion, ascites, cerebrospinal fluid, and urine) with multiple methods including polymerase chain reaction -based assays, such as droplet digital PCR (ddPCR) and bead emulsification amplification and magnetics (BEAMing) technology, or next generation DNA sequencing (NGS) -based multigene panels (Busser et al, 2017). BRAF or NRAS mutations are found in approximately two-thirds of the advanced melanomas (Cancer Genome Atlas, 2015). The sensitivity of ctDNA assays range from 37.5% to 86.6% and the specificity has been nearly 100% in advanced melanoma patients with a known tumor mutation (Ascierto et al, 2013; Santiago-Walker et al, 2016; Seremet et al, 2019; Rowe et al, 2018). A positive correlation with detectable plasma ctDNA and more advanced stage, higher tumor burden, higher number of metastatic sites, the presence of visceral metastases, and higher serum LDH levels have been observed in advanced melanoma patients (Ascierto et al, 2013; Santiago-Walker et al, 2016; Seremet et al, 2019; Rowe et al, 2018; Gonzales-Cao et al, 2015; Varaljai et al, 2019; Gray et al, 2015). Thus, ctDNA reflects tumor volume.

ctDNA levels and kinetics have reflected treatment outcomes in advanced melanoma patients. Undetectable baseline ctDNA was associated with longer PFS and OS in metastatic melanoma patients treated with PD-1 inhibitors (Seremet et al, 2019; Lee et al, 2017) and BRAF and MEK inhibitors (Santiago-Walker et al, 2016). The hazard ratio of death decreased after ctDNA became undetectable during sequential sampling (Seremet et al, 2019) and ctDNA levels paralleled radiological tumor responses in a case series of advanced melanoma patients treated with ICI (Seremet et al, 2018). Elevated baseline ctDNA levels predicted disease progression (PD) and rising ctDNA levels preceded radiological PD in advanced melanoma

patients receiving BRAF and MEK inhibitors and ICI (Varaljai et al, 2019). Ideally, rising ctDNA levels could reveal treatment failure before the radiological confirmation of disease progression. However, plasma ctDNA levels infrequently reflect intracranial disease progression and the early detection of brain metastases remains difficult (Bettegowda et al, 2014; Gray et al, 2015). Moreover, ddPCR may not be a sufficient method to monitor disease progression after initial response as resistant tumor cell clones might have different mutations and therefore remain undetectable with ddPCR. NGS analysis of plasma samples at the time of radiologically confirmed disease progression may reveal mutations that mediate resistance to therapy and are targetable by other drugs.

In addition to stage IV melanoma, detectable ctDNA before or after radical surgery of localized (stage II and III) melanomas has also predicted higher risk of disease recurrence and shorter survival (Tan et al, 2019; Lee et al, 2018) probably reflecting molecular residual disease not visible with current imaging methods. Thus, elevated plasma ctDNA levels may be a useful tool to define patients in need of adjuvant therapy and more frequent postoperative imaging during their follow-up after radical surgery for primary melanoma and regional lymph node metastases. Careful selection of patients at high-risk for disease recurrence is important as some patient will get potentially fatal, high grade adverse events (cardiac toxicity, neurological toxicity etc.) and long-lasting adverse events which require permanent hormone replacement therapies (insulin, levothyroxine etc.) after ICI adjuvant therapy.

2.3 Prognostic factors of renal cell carcinoma (RCC)

Renal cell carcinoma (RCC) is the third most common urogenital cancer after prostate and bladder cancers. Worldwide, the number of new kidney cancer diagnoses was over 400000 and it caused nearly 180000 deaths in 2020 (GLOBOCAN2020). In Finland, 633 men and 381 women were diagnosed with renal cell carcinoma in 2018 (Pitkäniemi et al, 2020). Despite advances in the medical treatment of advanced RCC, the survival rate of RCC is inferior to melanoma. In Finland, 213 men and 142 women died of RCC in 2018 and the five-year survival rates were 66% and 72%, respectively (Pitkäniemi et al, 2020).

Clear cell RCC (ccRCC) is the most common histological subtype accounting for 75−80% of RCCs. It originates from proximal renal tubular epithelial cells. Other histological RCC subtypes include papillary and chromophobe carcinomas (10−15% and 5%, respectively) (Moch et al, 2000; Leibovih et al, 2010; Delahunt B et al, 2013). Clear cell histology was observed to be independenlty associated with inferior

prognosis (the risk of metastases and death) compared to papillary and chromophobe RCC (Leibovich et al, 2010).

2.3.1 Treatment of localized and advanced RCC

Localized RCC can be treated with curative intent by radical (RN) or partial nephrectomy (PN). PN is preferred for smaller tumors (T1−2N0M0) if technically feasible without compromising the oncological outcome of surgery (negative surgical margins). There is only limited data comparing oncological outcomes of RN and PN. PN preserves kidney function which may lower the risk of postoperative cardiovascular disorders compared to RN. Small renal tumors might be eligible for radiofrequency ablation. Lymph node dissection (LND) is not routinely performed unless there is a suspicion of metastatic lymph nodes preoperatively or during surgery. If macrovascular invasion is present, tumor thrombus is removed from renal vein or inferior vena cava during surgery (Ljungberg et al, 2015; Motzer et al, 2020).

Unfortunately, approximately 20% of the RCC patients present with primarily metastatic disease and over one third of the patients will eventually develop distant metastases (Capitanio et al, 2016). Cytoreductive nephrectomy is considered for fit patients with primarily metastatic RCC if there are only few metastases and the disease does not present high risk group features (IMCD risk score 0–1, only one metastatic site) (Méjean et al, 2018; Méjean et al, 2021). Despite novel therapies for advanced RCC (antiangiogenic receptor tyrosine kinase inhibitors (TKI), immune checkpoint inhibitors (ICI), and TKI−ICI-combinations), metastatic disease will usually lead to death. Individualized, risk-based follow-up after surgery for localized RCC is recommended to detect disease recurrence early while the patient may still be surgically curable or eligible for oncologic therapies (Ljungberg et al, 2015; Motzer et al, 2020).

Systemic treatment options for metastatic ccRCC have increased in recent years. Antiangiogenic receptor tyrosine kinase inhibitors (sunitinib, sorafenib, pazopanib, axitinib, tivozanib, and cabozantinib) inhibit tumor neovascularization and have been the standard therapy for advanced RCC patients (Motzer et al, 2007; Strenberg et al, 2010; Motzer et al, 2013; Motzer et al, 2013; Choueiri et al, 2016; Choueiri et al, 2018) since the approval of sunitinib and sorafenib in 2006. Everolimus (MTOR inhibitor) and nivolumab (ICI) have showed efficacy after disease progression on VEGF targeted therapy (Motzer et al, 2008, Motzer et al, 2015). Recently, combination therapies (ipilimumab−nivolumab, pembrolizumab−axitinib, nivolumab−cabozantinib, and pembrolizumab−lenvatinib) have improved treatment outcomes in the first-line treatment of metastatic ccRCC (including sarcomatoid RCCs) compared to sunitinib (Motzer et al, 2019; Powles et al, 2020; Choueiri et al, 2021; Motzer et al, 2021). As much as one third of the patients seem to achive long-

term PFS (post 24 months) with these combinations. In addition to medical therapy, solitary metastases can be treated with surgical resection, stereotactic radiotherapy or other ablative techniques (e.g. radiofrequency ablation). Conventionally fractionated palliative radiotherapy is also used especially to alleviate pain from bone metastases.

The individual course of advanced RCC is highly variable. A subset of patients have very indolent growth of metastases while others have rapidly progressive, fatal disease. In spite of extensive biomarker research, the IMDC risk score (including Karnofsky performance status (KPS) <80%, time from diagnosis to treatment <1 year, hemoglobin less than the lower limit of normal (LLN), corrected calcium greater than the upper limit of normal (ULN), neutrophils >ULN, and platelets >ULN (Heng et al, 2009)) still remains the only clically adopted prognostic tool to guide the choice of the first line treatment in metastatic ccRCC. VEGF targeted TKIs or even initial surveillance without medical therapy may be sufficient for patients with 0−1 IMDC risk factors and low tumor volume (e.g. small lung metastases) (Rini et al, 2016). Combination therapies (ipilimumab−nivolumab or ICI−TKI) are especially beneficial for patients with IMDC intermediate (1−2) and poor risk score (≥3), or sarcomatoid features in their RCC indicating poorer outcomes of VEGF targeted TKI monotherapy. Hopefully, future biomarker research will provide more tools to choose between different treatment options. Furthermore, novel targeted therapies (e.g. HIF-2a inhibitors) and combinations are needed for patients who develop intolerable toxicity or disease progression on current therapies.

2.3.2 Histopathological prognostic factors of localized ccRCC

TNM classification of malignant tumors by the American Joint Committee on Cancer (AJCC) has been used since 1977 as prognostic staging system for multiple solid tumors (Swami et al, 2019). The staging of renal cell carcinoma based on pathological examination and radiological imaging provides crucial prognostic information. Stage I (T1N0M0, tumor ≤7 cm) and stage II (T2N0M0, tumor >7 cm) tumors are limited to kidney, whereas stage III (T3N0M0 or T1−3N1M0, tumor invades renal vein, perinephric tissues, or presents with regional lymph node metastases) and stage IV ($T4N_{any}M0$ or $T_{any}N_{any}M1$, tumor extends beyond Gerota fascia or presents with distant metastases) tumors extend beyond kidney (Bierley et al, 2017). TNM stage distribution and 5-year survival rates in large North American databases, National Cancer Database (1993−2004) and Surveillance, Epidemiology, and End Results (SEER) database (2004−2015), are presented in Table I (Kane et al, 2008, Cheaib et al 2020).

Table I TNM stage distribution and 5-year survival rates of RCC patients.

	stage I	stage II	stage III	stage IV
Stage distribution 1993–2004	54.7%	10.6%	16.1%	18.6%
Stage distribution 2004–2015	64.3%	10.9%	16.8%	8%
5-year survival 1993–2004	90.4%	83.4%	66.0%	9.1%
5-year survival 2004–2015	97.4%	89.9%	77.9%	26.7%

Stage I and II RCCs had significantly better 5-year survival rates compared to stage III and stage IV RCCs (Kane et al, 2008, Cheaib et al 2020). The proportion of stage I tumors has increased probably due to incidental detection of small renal tumors in abdominal imaging studies (Kane et al, 2008). The increase in the survival rate of stage III and IV tumors is probably driven by VEGF-targeted TKI-therapies introduced in the treatment of advanced RCC in the 21st century.

In addition to TNM stage, several histopathological factors affect the prognosis of localized ccRCC patients. Several tumor grading systems have been introduced to classify the histological differentiation of RCC cells. The Fuhrman and the WHO/ISUP grading systems are the most widely used. In 1982, Fuhrman developed a four-tiered grading system that is based on the assessment of nuclear size, nuclear shape, and nucleolar prominence. The estimated 5-year survival rate of RCC patients was 64% (grade I), 34% (grade II), 31% (grade III), and 10% (grade IV) (Furhman et al, 1982). In 2012, the International Society of Urological Pathology reformed the four-tiered grading system based on the prominence of nucleoli (grades 1–3) and grade 4 tumors showing extreme tumor nuclear pleomorphism, giant cells, or sarcomatoid/rhabdoid dedifferentiation (Delahunt et al, 2013).

Approximately 5% of RCCs undergo epithelial to mesenchymal transition and present with sarcomatoid differentiation. Sarcomatoid features have been observed in all major histological RCC subtypes including clear cell, papillary, and chromophobe RCCs (Blum et al, 2020). Sarcomatoid morphology is associated with more aggressive cancer behavior. Sarcomatoid RCCs (sRCC) often present with bulky primary tumor (higher size and stage) and higher tumor grade (Delahunt et al, 2013; Blum et al, 2020; Trudeau et al, 2016). Metastases are seen in as much as 60–80% of the newly diagnosed cases (Blum et al, 2020) and close to 80% of patients with localized sRCC developed disease recurrence within two years after nephrecotomy (Mian et al, 2002; Merrill et al, 2015). Sarcomatoid RCC has unfavorable prognosis compared to ccRCC regardless of TNM stage: 5-year cancer-specific mortality estimates were 32%, 63%, and 82% for stage I–II, III, and IV

sRCC patients compared to 6%, 20%, and 64% for stage I−II, III and IV ccRCC patients (Trudeau et al, 2016).

Tumor necrosis is another established adverse histological feature in RCC. It is associated with larger tumor size, higher grade, and higher proliferative activity and therefore considered to indicate biologically aggressive tumor behavior (Pichler et al. 2012; Lam et al. 2005). The presence of tumor necrosis has been reported in 21−32% of ccRCCs (Delahunt et al, 2013; Khor et al, 2016), and it has also been associated with inferior survival outcomes in multiple studies (Khor et al, 2016; Sengupta et al 2005; Katz et al, 2010; Foria et al, 2005; Lee et al, 2006; Renshaw et al, 2015). The combination of WHO/ISUP grading and tumor necrosis outperformed WHO/ISUP grading after adjusting for TNM stage. Researchers observed that the presence of tumor necrosis affected the prognosis especially in WHO/ISUP grade 3 tumors. The 10-year cancer-specific survival was 62% in grade 3 tumors without necrosis but only 30% in grade 3 tumors with necrosis (Delahunt et al, 2013).

RCCs are highly vascularized and microscopic vascular invasion is observed in 5.6−45% of tumors (Delahunt et al, 2013). Tumor cells can spread via blood and lymph vessels to distant sites (lungs, bones, liver etc.) and lymph nodes. Microvascular invasion is defined as tumor cells within small vessels in the tumor pseudocapsule, tumor, or renal parenchyma adjacent to the tumor (Delahunt et al, 2013). Microvascular invasion (MVI) was more commonly present in ccRCCs (29%) than in non-ccRCCs (12%) and it was associated with metastatic spread and inferior survival in ccRCC patients (Bedke et al, 2018). MVI was found to be associated with larger tumor size, higher Fuhrman grade, more advanced T stage, the presence of lymph node and distant metastases, along with shorter survival time in univariate but not in multivariate analysis (Lang et al, 2004). In another cohort of RCC patients (93% had ccRCC), MVI was observed to be associated with metastases and shorter disease-free survival as well as shorter cancer-specific survival (Kroeger et al, 2012).

Upregulated programmed death ligand-1 (PD-L1 or B7-H1) expression in the surface of tumor cells is an important mechanism of tumor immune evasion. The interaction of PD-L1 and PD-1 receptor in tumor-infiltrating lymphocytes (especially cytotoxic T-cells) hampers immune response against cancer cells (Blank et al, 2005). Although different studies had used variable methods to define PD-L1 positivity in RCC (different antibodies in immunohistochemisty, tumor cell or immune cell positivity, positivity cut-off % (usually ≥1% of cells positive)), PD-L1 expression has unequivocally been adverse prognostic feature in RCC. 20−24% of ccRCCs had demonstrated PD-L1 positive tumor cells and the 5-year cancer-specific survival rate of these patients was 42−47% compared to 66−83% in PD-L1 negative patients (Thompson et al, 2006; Abbas et al, 2016). PD-L1 expression can be found

in tumor cells and in tumor infiltrating lymphocytes (TILs) and both features were associated with inferior survival in RCC (Carlsson et al, 2020)

In addition to higher stage and higher tumor grade, sRCCs are found to have increased PD-L1 expression compared to ccRCCs. In the IMmotion151 trial evaluating bevacizumab and atezolizumab vs sunitinb in the first line metastatic RCC patients, sarcomatoid features were found in 16% (142/915) of the trial patients. 61% of sarcomatoid RCCs (86/142) were PD-L1 positive (≥1% of TILs positive) compared to 40% of PD-L1 positive disease among all study patient (362/915) (Rini et al, 2021; Rini et al, 2019). In the CheckMate 214 trial evaluating ipilimumab and nivolumab vs sunitinib in treatment naive metastatic ccRCC patients, 13% of all patients (145/1096) had sarcomatoid features and only 4% (6/145) of sRCCs had favorable IMDC risk score. Of 139 sRCC patients with intermediate or poor IMDC risk score, 50% were PD-L1 positive (≥1% of tumor cells positive) compared to 26% of all IMDC intermediate and poor risk patients (Motzer et al, 2018). Higher prevalence of PD-L1 positive tumors renders sRCCs more susceptible to ICI than to antiangiogenic TKI therapies (Rini et al, 2019; Motzer et al, 2018).

Tumor invasion into perirenal tissues (perirenal fat, renal sinus fat), macrovascular invasion into renal vein or inferior vena cava (IVC), or local lymph nodes (T3N0−1, stage III) led to inferior survival compared to stage I and II tumors (Kane et al, 2008, Cheaib et al 2020). Perinephric fat, renal sinus fat, or renal vein invasion was present in 26%, 9%, and 29% of T3a tumors, respectively, and patients with multiple extrarenal extensions had inferior PFS and OS (Shah et al, 2019). The association of concomitant fat invasion and renal vein invasion with poorer cancer-specific survival has also been observed in other studies (Stuhler et al, 2021; da Costa et al, 2012; Baccos et al, 2013). Upper pole RCCs may invade directly into adrenal gland. Tumors with adrenal invasion along with tumors extending beyond Gerota fascia (T4, stage IV) have worse oncologic outcomes compared to stage I-III tumors (Kane et al, 2008, Cheaib et al 2020).

Tumor extension into renal vein and IVC has been observed in 23% and 7−13% of patients, respectively (Campbell et al, 2007; Ljungberg et al, 1995). Patients with venous invasion had significantly shorter survival compared to tumors limited to kidney (Ljungberg et al, 1995). The Mayo Clinic thrombus classification is commonly used to classify the level of tumor extension into IVC (Neves et al, 1987). The prognostic significance of tumor thrombus level is controversial. In a retrospective multicenter evaluation of tumor thrombus level, 78%, 16%, and 5% of the patients had tumor extension into renal vein, IVC below diaphragm, and IVC above diaphragm, respectively (Wagner et al, 2009). The level of tumor thrombus in IVC (below or above diaphragm) was not statistically significantly associated with the survival time, but patients with tumor thrombus in IVC had shorter survival

(18−26 months) compared to patients with tumor thrombus in renal vein (52 months) (Wagner et al, 2009; Ljungberg et al, 1995). In another multicenter study (89.9% had ccRCC), higher tumor thrombus level was independently associated with shorter cancer-specific survival (Tilki et al, 2014). Noteworthy, the patients in these studies were treated before modern TKI and ICI therapies. In a contemporary analysis of 6340 patients who underwent surgery for localized RCC (93.4% had ccRCC), only 3.6% of the patients had venous tumor thrombus and the level of thrombus was not associated with the risk of disease recurrence or death (Shiff et al, 2021).

Lymph node dissection is not routinely performed during nephrectomy unless there is a suspicion of metastatic lymph nodes preoperatively or during surgery. Therefore, regional lymph node status is usually unknown (Nx). LND has not been proven therapeutic but prognostic procedure to assess metastatic spread to regional (hilar, abdominal, para-aortic, and paracaval) lymph nodes. In the SEER database analysis, 24.8% of RCC patients underwent LND (59.4% had ccRCC). Regional lymph node metastases were observed in 17.1% of the patients (9.3% of T2 and 21.6% of T3) who underwent LND (Marchioni et al, 2018). In another study, regional lymph node metastases were present in 11% of localized RCC patients who underwent nephrectomy (90.7% had ccRCC) (Sun et al, 2013). The stage III RCC patients with regional lymph node metastases (T1−3N1) were observed to have as poor survival as stage IV RCC patients (Sun et al, 2013; Srivastava et al, 2020). The 5-year survival rates were 61.9%, 22.7%, and 15.6% for stage III lymph node negative, stage III lymph node positive, and stage IV patients, respectively (78.1% had ccRCC) (Srivastava et al, 2020).

2.3.3 Prognostic models for localized ccRCC

The assessment of individual risk for local recurrence and distant metastases after RN or PN is recommended in the international guidelines to tailor postoperative follow-up and to stratify patients for adjuvant therapy trials. Regular postoperative imaging follow-up with thoracic and abdominal CT at least for five years is recommended to detect local recurrences and distant metastases early while the patient may still be surgically curable or eligible for oncologic therapies (Ljungberg et al, 2015, Motzer et al, 2020).

There are several postoperative prognostic models to predict the risk of disease recurrence or death after surgery of localized RCC. The risk assessment is based on histopathological features and clinical manifestations, such as symptoms of the disease. Kattan et al. introduced the first nomogram in 2001 to assess the risk of disease recurrence for localized RCC (Kattan et al, 2001) which was followed by the UISS, the SSIGN, the Cindolo, the Leibovich, the MSKCC, and the Karakiewicz algorithms (Zisman et al, 2001, Frank et al, 2002; Cindolo et al, 2003; Leibovich et

al, 2003; Sorbellini et al, 2005; Karakiewicz et al, 2009). Most of the patients (88−100%) included in these models have had clear cell RCC although the Kattan, the UISS, the Cindolo, and the Karakiewicz models also included patients with papillary and chromophobe RCCs. Because of marked differences in the histopathology and prognosis of clear cell, papillary, and chromophobe RCC subtypes, similar prediction models are not optimal for different subtypes. Recently, Leibovich introduced different algorithms for each histological subtype aiming to improve the prediction accuracy. 75% of all patients included into the study had clear cell, 17% papillary, and 6% chromophobe RCC (Leibovich et al, 2018). The differences in the required prediction features and the prediction outcomes of prognostic models are described in Table II.

Table II. Postoperative prognostic nomograms for localized renal cell carcinoma.

Model	Prediction Outcome	C-index	Prediction features
Kattan (2001)	RFS	0.74	Symptoms (incidental, local, systemic symptoms), Histology (chromophobe, papillary, clear cell), Tumor size, 1997 pT-stage
UISS (2001)	OS	not defined	1997 TNM Stage, Fuhrman grade, ECOG performance status
SSIGN (2002)	CSS	0.84	1997 T stage, N stage, M stage, Tumor size, Fuhrman grade, Necrosis
Cindolo (2003)	RFS	not defined	Symptoms (asymptomatic, symptomatic), Tumor size
Leibovich (2003)	MFS	0.819	Tumor Stage, Regional lymph node status, Tumor Size, Fuhrman grade, Necrosis
Sorbellini MSKCC (2005)	RFS	0.82	Size, 2002pT, Fuhrman grade, Necrosis, Vascular invasion, Presentation (incidental, local symptoms, systemic symptoms)
Karakiewicz (2009)	CSS	not defined	Age, Gender, Symptoms (no, local, systemic), Tumor Size, T-stage, Metastasis
Leibovich (2018)	RFS	0.83	Constitutional symptoms (yes, no), WHO/ISUP 2016 tumor grade, Necrosis, Sarcomatoid differentiation, Tumor size, Perinephric or renal sinus fat invasion, Tumor thrombus level, Extension beyond kidney, Nodal involvement

The prediction accuracy (concordance index, C-index) of prognostic models for ccRCC has exceeded 0.8: SSIGN 0.82−0.84, Leibovich 2003 0.82, MSKCC 0.82, and Leibovich 2018 0.83−0.86 (Frank et al, 2002; Parker et al, 2017; Leibovich et al, 2013; Sorbellini et al, 2005; Leibovich et al, 2018). However, these prediction

models are based on the analysis of retrospective patient cohorts. A prospective validation of current prediction models in a cohort of 1647 nonmetastatic (≥T1b grade 3−4 or TanyN1M0) ccRCC patients enrolled into sorafenib adjuvant therapy trial (ASSURE) resulted in considerably lower C-indices (0.57−0.69) for the UISS, the SSIGN, the Leibovich 2003, the Kattan, the MSKCC, the Yayciogly, the Karakiewicz, the Cindolo, and the 2002 TNM staging systems. All models demonstrated the best prediction accuracy during the first two years of follow-up after surgery (Correa et al, 2019). Late disease recurrence after 5-years of follow-up have been observed in 5−11% of patients with localized RCC (Park et al, 2012) and the prediction of these late events remains imprecise with current prognostic models (Correa et al, 2019).

2.3.4 Current applications of biomarkers in localized ccRCC

Although clinically adopted prognostic models do not use any genetic or other biomarkers, several genetic alterations have been described for RCC. In ccRCC, the inactivation of the von Hippel-Lindau (VHL) gene is the best described. The inactivation of the VHL tumor suppressor can occur by a number of point mutations or by methylation of the promoter areas. The inactivation of VHL function results in activation of hypoxia-inducible transcription factors (HIF-1a and -2a) of the cellular oxygen sensing pathway leading to upregulation of proangiogenic genes, e.g. vascular endothelial growth factor (VEGF). In particular, HIF-2a has been shown to drive more aggressive phenotype in ccRCC. Since VHL inactivation has been detected from 80% to nearly all ccRCCs and is the first and universal genetic alteration in ccRCC (Jaakkola et al, 2001; Kaelin et al, 2007; Capitanio et al 2016)), it does not function as a prognostic factor.

Further analyses of tumor mutations and gene expression profiles have revealed other genetic prognostic features for ccRCC. Mutations in tumor suppressor genes PBRM1, BAP1, and SETD2, which function as chromatin and histone modifiers, and PI3K/AKT pathway have been identified in nephrectomy specimens included in The Cancer Genome Atlas (Cancer Genome Atlas Research Network 2013; Hakimi et al, 2013). PBRM1 and BAP1 mutations have been associated with unfavorable prognosis in ccRCC (Joseph et al, 2016; Carril-Ajuria et al, 2019). Patient with PBMR1 or BAP1 loss had increased risk of death from RCC but it was not statistically significant after adjusting for SSIGN score (Josepht et al, 2016). The association of gene expression profiles and RCC survival has been studied widely. A scoring system based on 16 genes discovered in gene expression analysis was observed to predict disease recurrence in localized ccRCCs that were stratified by stage and adjusted for tumor size, tumor grade, and the Leibovich score (Rini et al, 2015). Another gene expression signature biomarker (ClearCode34) was developed

to classify good and poor risk ccRCCs and was significantly associated with RFS, OS, and CSS (Ghatalia et al, 2018). Cell cycle proliferation (CCP) score assay, that measures the activation of 31 genes involved in cellular proliferation, was observed to be an independent predictor of disease recurrence after nephrectomy in 565 localized RCC patients (81% ccRCC) and it outperformed the prediction accuracy of the Karakiewicz nomogram (C-index 0.87 vs 0.84) (Morgan et al, 2018).

CtDNA has also been analyzed from plasma and urine samples of RCC patients. Studies are still scarce compared to NSCLC, colorectal cancer, melanoma, and urothelial cancer, and mostly done in metastatic RCC patients. Patients with metastatic ccRCC have had higher plasma levels of cell free DNA compared to localized ccRCC and healthy control patients and higher plasma cell free DNA levels have predicted disease recurrence after nephrectomy (Wan et al, 2013). Untargeted sequencing methods have revealed detectable ctDNA in plasma or urine samples of 30−40% of RCC patients with localized and metastatic disease. Detectable ctDNA in plasma, but not in urine, was more common in patients with larger tumors and with venous tumor thrombus (Smith et al, 2020). The rate of detectable ctDNA in RCC patients has varied substantially because of different methods and patient cohorts (localized or metastatic). Targeted analysis of ctDNA using RCC targeted NGS panel (including BAP1, KDM5C, MET, MTOR, PBRM1, PIK3CA, PTEN, SETD2, TP53, and VHL genes) has revealed detectable plasma ctDNA in only 18.6% of the patients (mostly metastatic ccRCC) (Smith et al, 2020). CtDNA analysis of plasma samples from 220 patients with metastatic RCC with a 74-gene panel has revealed genomic alterations in 79% of the patients. Most frequently observed mutations included TP53 (35%), VHL (23%), EGFR (17%), NF1 (16%), and ARID1A (12%) (Pal et al, 2017). In smaller series of metastatic RCC patients (76% ccRCC), 53% (18/34) of the patients have had detectable plasma ctDNA. In this study, ctDNA was associated with tumor burden (sum of longest diameters of all measurable lesions) but not with IMDC risk group or tumor histology (Maia et al, 2017).

In addition to gene expression profiles and ctDNA, the prognostic ability of other biomarkers, such as DNA methylation, expression of microRNAs, and long noncoding RNA are being studied. However, none of these novel biomarkers are yet recommended in the international RCC guidelines (Motzer et al, 2020; Escudier et al, 2019) nor adopted into widespread clinical use. The aim of future studies is to supplement current prognostic algorithms with novel biomarkers to improve their prediction accuracy and validate these findings in independent patient cohorts

2.3.5 Is there a role for adjuvant therapy in ccRCC

The efficacy of antiangiogenic TKI therapies and immune checkpoint inhibitors in the treatment of advanced ccRCC has led to adjuvant therapy trials aiming to reduce the risk of disease recurrence and improve the overall survival of patients with localized RCC after radical or partial nephrectomy. Before TKI and ICI therapies, cytokines (interferon-alpha and high-dose interleukin-2) showed modest clinical activity (ORR of 15−31%) in stage IV RCC (Janowitz et al, 2013). Cytokines and tumor vaccines were also studied in the adjuvant setting but these trials failed to improve the recurrence-free and overall survival in RCC patients (Janowitz et al, 2013; Pizzocaro et al, 2001; Messing et al, 2003; Clark et al, 2003).

The next attempt to improve RFS and OS was made with VEGF-targeted TKI adjuvant therapies. Five large, placebo-controlled, prospective, multicenter trials with sunitinib (S-TRAC), sunitinib and sorafenib (ASSURE), pazopanib (PROTECT), axitinib (ATLAS), and sorafenib (SORCE) were conducted (Ravaud et al, 2016; Haas et al, 2016; Motzer et al, 2017; Gross-Goupil et al, 2018; Eisen et al, 2020). The design of adjuvant therapy trials and results are described in Table III. The inclusion criteria for intermediate- and high-risk patients and the proportion of high risk (≥T3 or N1) patients were different across these trials. All trials aimed to show the DFS benefit, but only the S-TRAC trial yielded a positive result with a little over one-year improvement in the DFS of sunitinib arm (Ravaud et al, 2016). The S-TRAC, the PROTECT, and the ATLAS trials included only ccRCC patients and the majority of patients enrolled into the ASSURE and the SORCE trials had ccRCC (79% and 84%). Usually, the protocol specified duration of adjuvant TKI therapy was 12 months. The ATLAS and the SORCE trials included patient cohorts with adjuvant TKI therapy up to 36 months but longer duration of TKI therapy did not lead to improved DFS. Adjuvant TKI therapy caused substantial toxicity (grade 3−4 adverse events 49−72%) and a significant proportion of the patients (23−49%) discontinued adjuvant TKI therapy because of intolerable toxicity or refused to continue study therapy (Ravaud et al, 2016; Haas et al, 2016; Motzer et al, 2017; Gross-Goupil et al, 2018; Eisen et al, 2020). Therefore, adjuvant TKI therapy is not recommended after complete resection of the primary tumor in the international RCC guidelines (Motzer et al, 2020; Escudier et al, 2019).

Table III. Design and results of adjuvant TKI and ICI trials in RCC.

Trial	Treatment	Inclusion criteria	Median DFS / HR of disease recurrence or death	Discontinuation Rate Due to AE / (AE + patient withdrawal)[#]
S-TRAC (2016)	sunitinib vs placebo 12 months	≥T3N0 or TanyN+	6.8 years vs 5.6 years HR 0.76 (0.59–0.98)	28% vs 6%
ASSURE (2016)	sunitinib vs sorafenib vs placebo 12 months	≥T1b(gr3–4)N0 or TanyN+	5.8 years vs 6.1 years vs 6.6 years HR 1.17 (0.90–1.52) sunitinib vs placebo HR 0.97 (0.75–1.28) sorafenib vs placebo	44%[#] vs 45%[#] vs 11%[#]
PROTECT (2017)	pazopanib vs placebo 12 months	T2(gr3–4)N0, T3–4N0 or TanyN+	HR 0.86 (0.70–1.06)	35% vs 5%
ATLAS (2018)	axitinib vs placebo 12–36 months	≥T2N0 or TanyN+	HR 0.87 (0.66–147)	23% vs 11%
SORCE (2020)	sorafenib 12 months vs sorafenib 36 months vs placebo	intermediate risk (score 3–5) or high risk (score ≥6) according to Leibovich 2003	HR 0.94 (0.77–1.14) sorafenib 12 months vs placebo HR 1.01 (0.82–1.23) sorafenib 36 months vs placebo	44%[#] vs 49%[#] vs 12%[#]
KEYNOTE-564 (2021)	pembrolizumab vs placebo 12 months	T2(gr3–4 or sarcomatoid)N0 or T3–4N0 or TanyN+, or resected M1	HR 0.68 (0.53–0.87)	21% vs 2%

The efficacy of different adjuvant therapies should not be directly compared to each other because of differences in patient populations and trials designs.

Immune checkpoint inhibitors (ICI) have replaced cytokines in the immune therapy of advanced RCC and are also being studied in randomized, placebo-controlled, prospective clinical trials in the adjuvant and neoadjuvant setting. The IMmotion010 trial is evaluating 12-month adjuvant therapy with PD-L1 inhibitor atezolizumab, the PROSPER trial neoadjuvant therapy (nivolumab two doses) followed by 9-month adjuvant therapy with PD-1 inhibitor nivolumab, and the CheckMate 914 trial 6-month adjuvant therapy with the combination of CTLA-4 inhibitor ipilimumab and PD-1 inhibitor nivolumab in resected localized ccRCC patients. The first results from these trials are expected to be published in 2022−2024 (www.clinicaltrials.gov). The results from KEYNOTE-564 trial evaluating 12-month adjuvant therapy with pembrolizumab in resected intermediate- or high-risk ccRCC patients showed statistically significantly longer recurrence-free survival rate in the pembrolizumab arm compared to placebo at 24 months (77.3% vs 68.1%, HR

for recurrence or death 0.68 (0.53−0.87) (Table III) (Choueiri et al, 2021). As this was the first analysis, longer follow-up will be needed to confirm the survival outcomes of pembrolizumab adjuvant therapy. However, ICI may finally become practice-changing adjuvant treatment option for intermediate- or high-risk RCC patients after complete resection of the primary tumor and lymph node or distant metastases.

Although the proportion of patients who discontinued 12-month adjuvant pembrolizumab therapy due to adverse events was smaller compared to adjuvant TKI trials (Table III), ICI therapy may cause severe, long-lasting toxicities and significant economic burden. Prognostic models and predictive biomarkers are urgently needed to guide patient selection for adjuvant therapy in RCC as well as in cutaneous melanoma. Interestingly, detectable ctDNA was shown to predict the DFS-benefit from adjuvant atezolizumab therapy in the IMvigor010 trial (Powles et al, 2021). Atezolizumab did not improve the DFS in unselected patient population after surgical resection of localized urothelial carcinoma (HR 0.89 (0.74-1.08)). However, ctDNA positive patients had significantly better DFS in the atezolizumab adjuvant therapy arm compared to the observation arm (HR for DFS 0.58 (0.43–0.79) and HR for OS 0.59 (0.41–0.86)) (Powles et al, 2021). Hopefully, ongoing RCC adjuvant therapy trials will also provide novel biomarkers for clinicians to guide treatment decisions.

3 Aims

The aim of this study was to evaluate prognostic and predictive factors and biomarkers in advanced melanoma and localized clear cell renal cell carcinoma. Increased understanding on prognostic and predictive factors may help to choose the optimal therapy and follow-up strategy for each individual cancer patient. The specific aims of this study included:

I. To evaluate factors associated with the long-term survival of advanced cutaneous melanoma patients who had received chemoimmunotherapy (BOLD-IFN) before modern treatment options

II. To evaluate prognostic and predictive factors in patients with advanced cutaneous melanoma who received chemoimmunotherapy (TOL-IFN) and vemurafenib in a prospective clinical trial

III. To evaluate the association of circulating tumor DNA with treatment outcomes of chemoimmunotherapy and vemurafenib in advanced melanoma patients

IV. To develop an easy-to-use and accurate model to predict metastasis-free survival after radical or partial nephrectomy for localized clear cell renal cell carcinoma. Focus was on finding a minimal set of features that would still optimally predict the development of metastases

4 Materials and Methods

4.1 Study design

Studies I, II, and III focused on advanced cutaneous melanoma patients who were treated with chemoimmunotherapy and BRAF inhibitor. The study IV evaluated clear cell RCC patients after radical surgery for localized kidney tumor.

The Study I evaluated advanced cutaneous or unknown primary melanoma patients who had received BOLD (bleomycin, vincristine, lomustine, and dacarbazine) combination chemotherapy and subcutaneous interferon-alpha (IFN) at Turku University Hospital in 1991–2010. Patient characteristics, treatment for melanoma, and treatment outcomes were retrospectively collected from the medical records of Turku University Hospital. The primary objective was to describe treatment outcomes, especially long-term survival, before modern therapies (BRAF and MEK inhibitors and ICI) were available. The secondary objective was to identify clinical and histopathological factors that were associated with survival outcomes in a real-life patient cohort treated outside clinical trials.

The metastasis stage (M stage) was classified according to AJCC7th version in Studies I and II. In 2017, melanoma staging was updated. In the Study III, the M stage was classified according to AJCC8th version as described in Table IV (Keung et al, 2018).

Table IV. M stage classification according to AJCC7th and 8th edition.

M stage AJCC7th	**M stage AJCC8th**
M1a: Distant skin, subcutaneous, or nonregional nodal metastases	M1a: Distant skin, soft tissue, or nonregional lymph node metastases
M1b: Lung metastases	M1b: Lung metastases
M1c: All other visceral metastases.	M1c: All other non-CNS visceral metastases
	M1d: CNS metastases

Studies II and III displayed the results of a prospective clinical trial (COBRA) enrolling 38 treatment-naïve, advanced cutaneous melanoma patients for the first-line chemoimmunotherapy TOL-IFN (temozolomide, lomustine, vincristine, and

subcutaneous interferon-alpha) and BRAF inhibitor vemurafenib (VEM) between 2014–2016. The COBRA trial was initiated by the Finnish Melanoma Group and conducted at Helsinki, Turku, Tampere, Kuopio, and Oulu University Hospitals. The primary objective was to evaluate the efficacy and safety of TOL-IFN ± VEM. The secondary objective was to discover traditional clinicopathological features and biomarkers (tumor mutations, ctDNA) that could be used to predict treatment outcomes. The trial flow-chart and TOL-IFN regimen are described in Figure 3.

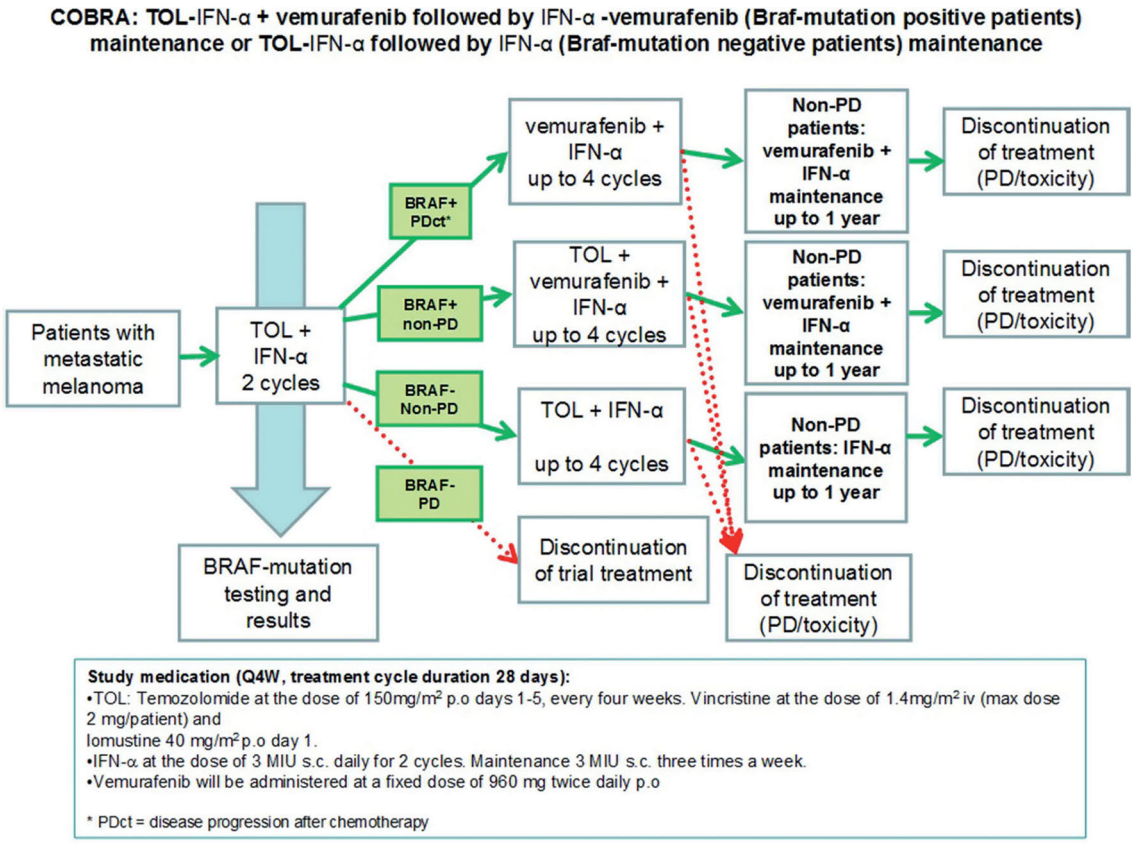


Figure 3. Flow-chart of the prospective COBRA-trial (Study II, III).

The Study IV evaluated clinicopathological features associated with the metastasis-free survival (MFS) of localized clear cell RCC. The primary objective was to build an easy-to-use and accurate prognostic model to guide follow-up after surgery of localized clear cell RCC. The focus was placed on selecting a minimal set of features that would still optimally predict MFS. The list of all features included in our analysis is described in the original article. A prognostic nomogram and a visual

prediction surface were constructed to stratify patients into clinically relevant risk groups.

The feature extraction for proposed model was performed in a cohort of localized clear cell RCC patients operated with RN or PN at Turku University Hospital between 2005–2014. To validate our proposed prognostic model, it´s prediction accuracy was tested in an independent cohort of localized clear cell RCC patients operated at Helsinki University Hospital between 2006–2015 and benchmarked against the Leibovich model (Leibovich et al, 2003). The Leibovich model was chosen as the reference model because it has been thoroughly validated by other researchers (Beisland et al, 2015; Pichler et al, 2011) and is commonly used in clinical practice for planning postoperative follow-up (e.g. at Turku and Helsinki University Hospitals). Patient selection for the study IV is described in Figure 4.

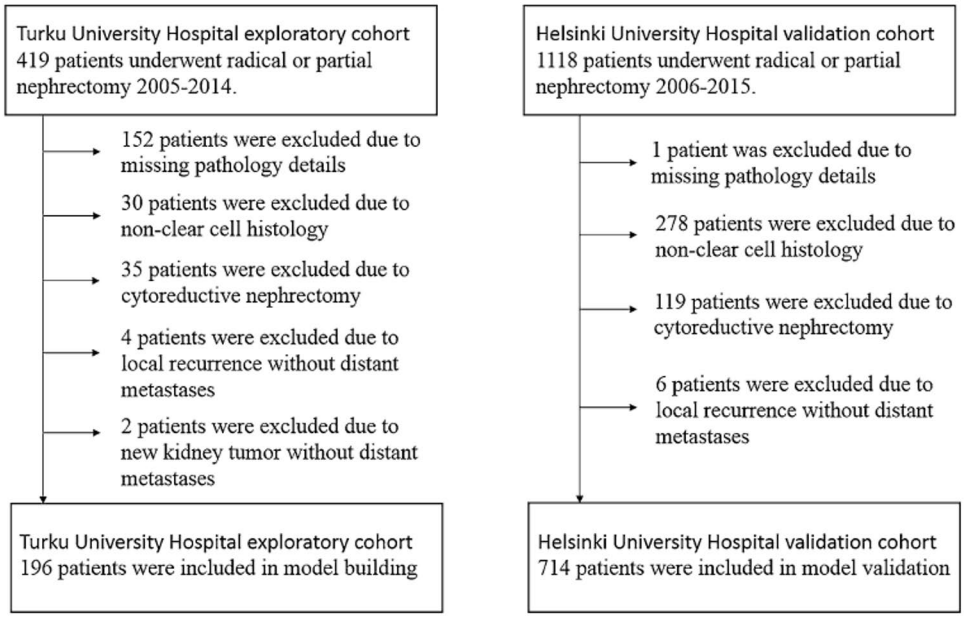


Figure 4. Patient selection for Turku University Hospital training cohort and Helsinki University Hospital validation cohort (Study IV).

4.2 Study patients

A cohort of 146 patients that had received at least one dose of BOLD were identified for Study I. 134 patients (92%) had received BOLD with subcutaneous IFN-alpha and 12 patients (8%) BOLD alone. The patient cohort represents a typical population of advanced melanoma patients treated outside clinical trials including patients with

ECOG performance status ≥2 (13%). Clinical characteristics and treatment for advanced melanoma are described in Table V.

Table V. Characteristics of 146 stage IV melanoma patients treated with BOLD-IFN (Study I)

Age	58 (21–79)
Charlson Comorbidity Index	6 (6–12)
Gender	
Male / Female	97 (66%) / 49 (34%)
ECOG Performance Status	
0 / 1 / 2-3 / unknown	22 (15%) / 82 (56%) / 19 (13%) / 23 (16%)
Primary melanoma	
Cutaneous / Unknown	128 (88%) / 18 (12%)
M stage*	
M1a / M1b / M1c	39 (27%) / 39 (27%) / 68 (46%)
Treatment	
BOLD-IFN / BOLD	134 (92%) / 12 (8%)
Number of BOLD ± IFN cycles	4 (1–13)
Oncologic therapy before BOLD ± IFN	
Yes / No	24 (16%) / 122 (84%)
Oncologic therapy after BOLD ± IFN	
Yes / No / Unknown	71 (49%) / 72 (49%) / 3 (2%)
Radiotherapy of metastases	
Yes / No / Unknown	83 (57%) / 62 (42%) / 1 (1%)
Operation of metastases	
Yes / No	82 (56%) / 64 (44%)

* Stage is determined according to AJCC version 7 (2009)
Continuous variables are presented with median (range) and others number (percentages)

Fourteen patients (37%) with BRAF mutations (cohort 1), eight patients (21%) with NRAS mutations, and sixteen (41%) $BRAF^{v600}$ and NRAS wild type (WT) patients (cohort 2) were enrolled into the COBRA-trial (Study II and III). Baseline clinical characteristics were balanced across BRAF, NRAS and WT patients (no statistically significant differences in age, gender, ECOG PS, M-stage (AJCC 8^{th}), or LDH level) Patient characteristics and treatment delivered in the trial are described in Table VI.

Table VI. Baseline clinical characteristics and treatment in the COBRA-trial (Study II, III)

	Cohort 1 (14 BRAF-positive patients)	**Cohort 2** (24 BRAF-negative patients)
Age*	59 (55–70)	62 (30–74)
Gender		
male	10 (71%)	12 (50%)
female	4 (29%)	12 (50%)
ECOG performance status		
0	5 (36%)	5 (21%)
1	8 (57%)	15 (62%)
2	1 (7%)	4 (17%)
M stage (AJCC 7th)		
M1a	0 (0%)	2 (8%)
M1b	1 (7%)	6 (25%)
M1c	13 (93%)	16 (67%)
LDH		
normal	7 (50%)	6 (25%)
elevated	7 (50%)	18 (75%)
Brain metastases		
present	2 (14%)	7 (29%)
absent	12 (86%)	17 (71%)
Number of TOL-IFN cycles*	2 (1–6)	2 (1–6)
Number of TOL-IFN cycles/patient		
1	2 (14%)	4 (17%)
2	8 (57%)	13 (54%)
3	-	-
4	-	3 (13%)
5	1 (7%)	-
6	3 (21%)	4 (17%)
Duration of IFN (months)*	2.3 (0.7–20.1)	1.1 (0.5–7.3)
Duration of vemurafenib (months)*	4.3 (0–25.5)	-

*median (range)

The clinical characteristics of localized clear cell RCC patients in the Study IV are described in Table VII.

Table VII. Patient characteristics (Study IV).

	Turku University Hospital training cohort (N=196)	*Helsinki University Hospital validation cohort (N=714)*
Age (years)[1]	67 (37–89)	66 (21–89)
Male Female[2]	118 (60%) 78 (40%)	393 (55%) 321 (45%)
T Stage[2]		
1	104 (53%)	424 (59%)
2	32 (16%)	50 (7%)
3–4	59 (30%)	240 (33%)
Unknown	1 (<1%)	-
Regional Nodal Status[2]		
Nx/N0	194 (99%)	703 (98%)
N1	2 (1%)	11 (2%)
Tumor Size (mm)[1]	57 (10–160)	48 (8–200)
Histologic Tumor Necrosis[2]		
Yes	71 (36%)	148 (21%)
No	7 (4%)	563 (79%)
Unknown	118 (60%)	3 (<1%)
Microvascular Invasion[2]		
Yes	33 (17%)	127 (18%)
No	152 (78%)	587 (82%)
Unknown	11 (6%)	-
Tumor Grade (Fuhrman)[2]		
1	32 (16%)	102 (14%)
2	87 (44%)	389 (55%)
3	60 (31%)	193 (27%)
4	17 (9%)	27 (4%)
Unknown	-	3 (<1%)

Values reported as: [1] Median (Range), [2] Absolute amount (Percentage

4.3 Biomarker analyses

In Studies II and III, BRAF mutations were analyzed from tumor tissue specimen of the COBRA-trial patients using the fully integrated, real-time PCR-based Idylla™ system (Biocartis, Belgium) or a cancer targeted NGS panel (Ion Ampliseq Cancer Hotspot Panel v2, ThermoFisher Scientific, USA). Non-BRAF mutations, including

NRAS mutations, were analyzed from tumor tissue specimen using the same NGS panel at Helsinki University Hospital. Plasma samples were drawn at screening (baseline sample) and before radiological response evaluations (sequential samples). The centralized analysis of plasma samples was retrospectively performed at Helsinki University Hospital. Droplet digital PCR (ddPCR) was used to quantify tumor specific mutation allele frequency (MAF%) from baseline and sequential plasma samples of the patients with a known tumor mutation. Baseline plasma samples of the patients without a known tumor mutation were analyzed with NGS (Ion AmpliSeq Cancer Hotspot Panel v2) to reveal potentially undetected tumor mutations. Blood sample preparation, NGS, and ddPCR analyses were performed as described previously (Holm et al, 2020). Targeted mutation probes for BRAF, NRAS, IDH-1 and KRAS mutations were designed and prevalidated by Bio-Rad (Bio-Rad Laboratories, USA).

4.4 Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics version 21 and 27 in Studies I–III. The results of continuous variables are presented as median (range) and categorical variables as numbers and percentages. OS was measured from day 1 of chemoimmunotherapy to the date of death or the last follow-up visit. PFS was calculated similarly to the date of disease progression or the end of follow-up. Kaplan-Meier curves were used to illustrate univariate analyses of PFS and OS. The Kaplan-Meier estimates of OS and PFS are presented with 95% confidence intervals (95%CI) and the log-rank test was used to calculate statistical significance. The Pearson two-sided Chi-Square test was used to calculate statistical differences in categorical variables and the one-way analysis of variance (ANOVA) was used for continuous variables. The Cox regression analysis was performed to analyze the association of clinical characteristics with PFS and OS.

In the Study IV, R statistical software v3.5.2 was utilized for all statistical analyses. Turku University Hospital training cohort was subjected to regularized Cox regression using the LASSO L1-norm for identifying optimal features required to predict metastases after surgery. After testing for C-index in the validation cohort in comparison with the Leibovich score, the final proposed Cox model was fit using the predetermined features for observations from both cohorts.

4.5 Ethical considerations

Studies I and IV were retrospective register studies and approved by the Institutional Review Boards of Turku and Helsinki University Hospitals (Turku Clinical Research Centre Licence number T06/035/15, date 30.10.2015 and Turku Clinical Research

Centre Licence number T06/032/15, date 28.9.2015, respectively). All patients in the prospective COBRA trial (Studies II and III) provided written informed consent and the trial was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines. The trial was approved by the Institutional Review Board and Ethics Committee of Helsinki University Hospital and it was registered to the European Union Drug Regulating Authorities Clinical Trials Database (Eudra CT study number 2013-000280-84). All studies were performed in accordance with the institutional guidelines and regulations. Data was anonymized before statistical analyses and handled in a manner that meets the EU General Data Protection Regulation 2016/679 (GDPR) on data protection.

5 Results

5.1 Prognostic factors for long-term survival in advanced melanoma treated with BOLD-IFN (Study I)

The median number of BOLD ± IFN cycles were 4 (range 1–13). 91 patients (62%) received 1–4 cycles and 55 patients (38%) 5–13 cycles of BOLD ± IFN-alpha. The ORR reached 29% (PR 23% (33 patients) and CR 7% (10 patients)). Progressive disease (PD) was the best response in 46% of the patients (68 patients) and the median PFS was only 3.8 months (95% CI 3.0–4.6 months). The most common reasons for discontinuing the treatment were disease progression in 61% (89 patients), adverse events (AE) in 12% (18 patients), and preplanned discontinuation after 6 or 8 chemotherapy cycles in 12% of study patients (18 patients).

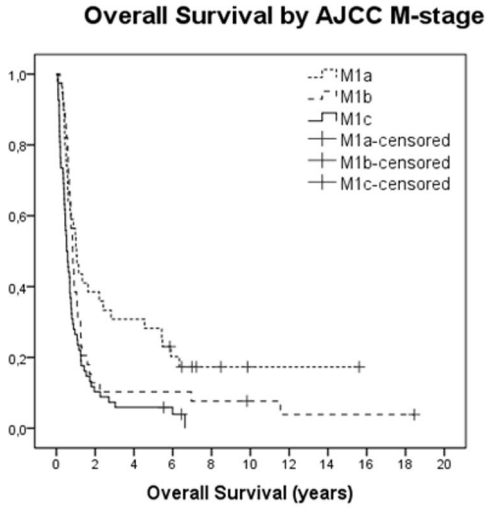
After the median follow-up of 8.9 months (range 0.8–224.5 months), 135 deaths had occurred by the end of March 2016. Only two causes of deaths were not melanoma related (heart failure and hemorrhagic gastritis). The median OS was 8.9 months (95% CI 7.5–10.4 months). The 1-year survival rate was 36% (53 patients), 2-year 18% (27) and 5-year 13% (19). 7% (11) of the patients were alive at the end of the follow-up. Clinical characteristics of five-year survivors are presented in Table VIII. The M-stage according to American Joint Committee on Cancer 7th version (2009) was significantly associated with survival (log-rank p=0.001) in the univariate Kaplan-Meier analysis. Survival curves along with one-, two-, and five-year survival rates and the median overall survival according to M-stage are described in Figure 5.

A multivariable Cox regression analysis was performed to evaluate independent prognostic factors for death. Smaller number of BOLD ± IFN cycles (1–4 vs 5–13, p>0.001), worse baseline ECOG performance status (0 vs 1 vs 2 vs 3, p=0.001), male gender (p=0.001), the absence of chemotherapy after BOLD ± IFN (p=0.006), and more advanced M-stage (M1a vs M1b vs M1c, p=0.012) were independently associated with shorter survival in the multivariable analysis.

Table VIII. Characteristics of 19 patients who survived 5 years after the initiation of BOLD-IFN.

Age	59 years (40–79)
Gender	
Male / Female	7 (37%) / 12 (63%)
ECOG performance status	
0 / 1 / unknown	7 (37%) / 10 (53%) / 2 (10%)
M-stage	
M1a / M1b / M1c	11 (58%) / 4 (21%) / 4 (21%)
Number of BOLD ± INF cycles	5 (1–10)
Oncologic therapy before BOLD ± INF	
Yes / No	5 (26%) / 14 (74%)
Oncologic therapy after BOLD ± INF	
Yes / No	11 (58%) / 8 (42%)
Surgical therapy of metastases:	
Yes / No	16 (84%) / 3 (16%)
Radiotherapy of metastases	
Yes / No	12 (63%) / 7 (37%)

Continuous variables are presented with Median (Range) and numbers n (%).



	1-year	2-year	5-year	Median OS (95% CI)
M1a (n=39)	51% (20)	38% (15)	28% (11)	12.7 months (8.7–16.6)
M1b (n=39)	38% (15)	13% (5)	10% (4)	10.3 months (7.9–12.6)
M1c (n=68)	26% (18)	10% (7)	6% (4)	6.4 months (4.7–8.2)
Total (n=146)	36% (53)	18% (27)	13% (19)	8.9 months (7.5–10. 4)

Figure 5. Overall survival and 1-, 2-, and 5-year survival rates by M-stage (AJCC 7th Edition).

5.2 Prognostic and predictive factors for treatment outcomes of TOL-IFN ± vemurafenib in advanced melanoma (Study II and III)

After two initial cycles of TOL-IFN, 71% of all study patients had discontinued TOL-IFN because PD. Only 21% (3/14) of BRAF mutated patients, 0% (0/8) of NRAS mutated, and 25% (4/16) of WT patients completed all six cycles of chemoimmunotherapy. The ORR was 57% in $BRAF^{v600}$ mutated patients treated with TOL-IFN and vemurafenib, whereas the ORR of TOL-IFN was 0% in NRAS mutated patients and 19% in WT patients (p=0.009). Responses to treatment are described in Table IX.

Table IX. Responses to TOL-IFN ± VEM by tumor mutations.

	All patients	**Cohort 1**	**Cohort 2**	
	(38)	$BRAF^{v600}$ mutated (14)	NRAS mutated (8)	WT (16)
ORR	11 (29%)	8 (57%)	0 (0%)	3 (19%)
Best Objective Response				
CR	4 (11%)	3 (21%)	0 (0%)	1 (6%)
PR	7 (18%)	5 (36%)	0 (0%)	2 (13%)
SD	5 (13%)	1 (7%)	1 (13%)	3 (19%)
PD	22 (58%)	5 (36%)	7 (87%)	10 (62%)

After the median follow-up of 10.3 months (range 0.7–67.8 months), the median progression-free survival (PFS) was 5.5 months (0.7–64.5) in cohort 1 and 1.7 months (1.2–65.5) in cohort 2 (p=0.019). Six patients (16% of all patients), four BRAF-positive (29% of cohort 1) and two wild type (8% of cohort 2), were alive in September 2019. The median OS was 15.1 months (0.7–67.8) in cohort 1 and 7.2 months (1.3–65.5) in cohort 2 (p=0.047). The Kaplan-Meier survival curves of OS and PFS are shown in Figure 6A and B.

Four patients (three $BRAF^{v600}$ mutated and one WT patient, 11% of all patients) had not experienced PD on study therapy at the end of the follow-up in September 2019. The estimated PFS was significantly better in $BRAF^{v600}$ mutated patients compared to NRAS mutated and WT patients: 5.4 months (1.6–9.1) vs. 1.6 months (1.4–1.9) vs. 1.8 months (1.3–2.2), p=0.011. Six patients (four $BRAF^{v600}$ mutated and two WT patient, 16% of all patients) were alive at the end of the follow-up. Despite the difference in PFS, there was not a statistically significant difference in the OS of $BRAF^{v600}$ mutated patients compared to NRAS mutated and WT patients: 13.1 months (5.5–20.6) vs. 6.9 months (0–15.0) vs. 6.1 months (2.9–9.2), p=0.105.

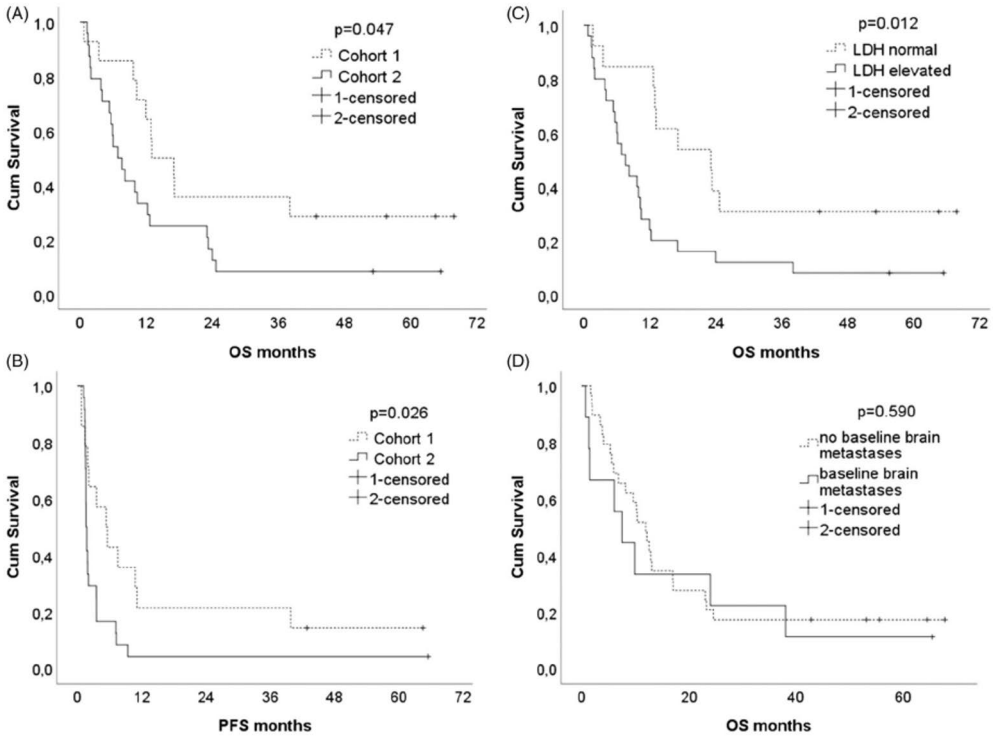


Figure 6. Kaplan–Meier survival curves: (A) OS in cohort 1 and 2; (B) PFS in cohort 1 and 2; (C) OS in patients with normal LDH and elevated LDH; (D) OS in patients with and without brain metastases.

Elevated baseline LDH was associated with shorter survival: the median OS of patients with elevated baseline LDH was 7.6 months (95% CI 4.1–11.0 months) compared to 23.1 months (95% CI 11.0–35.1 months) in patients with normal baseline LDH (log-rank p=0.012), Figure 6C.

Nine patients (24%) had asymptomatic baseline brain metastases: two (14%) in cohort 1 and seven (29%) in cohort 2. The survival of patients with asymptomatic baseline brain metastases wasn`t statistically significantly shorter compared to patients without baseline brain metastases (mOS 7.6 months (95% CI 3.2–11.9) versus 12.0 months (95% CI 8.5–15.4), log-rank p=0.590), Figure 6D. Two patients with baseline BRAF-negative brain metastases had unexpectedly long survival (24 months and +65.5 months).

5.3 Associations of ctDNA with treatment outcomes in advanced melanoma (Study III)

14 $BRAF^{v600}$ mutated (12 V600E and 2 V600K mutations), 8 NRAS mutated (4 Q61K, 3 Q61R, and 1 Q61L mutations), and 16 BRAF and NRAS wild type (WT) melanoma patients were treated in the COBRA trial. After analyzing baseline plasma samples retrospectively with NGS, one patient with a $BRAF^{V600E}$ mutated melanoma was discovered to harbor a concurrent IDH-1 mutation and one patient with a WT melanoma had additionally a KRAS (A146V) mutation. Four patients (1 $BRAF^{v600E}$, 2 NRAS Q61K, and 1 NRAS Q61L) were excluded from ctDNA analyses due to the lack of baseline and follow-up plasma samples. Altogether 74 plasma samples from 19 patients with a known tumor mutation (13 BRAF, 5 NRAS, and 1 KRAS mutation) were analyzed with ddPCR to quantify tumor specific MAF% during study therapy. The median number of samples per patient was 3 (range 1–10).

Baseline mutation allele frequency (MAF) values ranged from 0% to 62% (median MAF 3.1%). Detectable baseline ctDNA levels (MAF >0%) were associated with diminished ECOG performance status (0 vs 1 vs ≥2) (p=0.004) but not with other baseline demographic variables. Furthermore, a statistically significant correlation with subsequent cancer therapy (p=0.75) and subsequent ICI (p=0.23) was not observed in patients with undetectable and detectable baseline ctDNA levels. Although patients with diminished ECOG performance status, elevated LDH, and NRAS/KRAS mutated melanomas had higher mean MAF values, statistically significantly differences in mean MAF values were not observed.

The patients with a known tumor mutation and undetectable baseline ctDNA levels (MAF 0%) had longer PFS compared to patients with detectable levels of BRAF mutated ctDNA and detectable NRAS/KRAS mutated ctDNA: median PFS 3.5 months (not evaluable) vs 3.5 months (0–8.9) vs 1.7 months (1.2–2.2), p=0.016. However, in a multivariate analysis including age (<60, ≥60), gender (male, female), mutational status (BRAF mutated, NRAS/KRAS mutated), baseline ctDNA level (undetectable, detectable), LDH level (normal, elevated), ECOG performance status (0, 1, ≥2) and M-stage (M1a, M1b, M1c, M1d), none of these features were independently associated with PFS

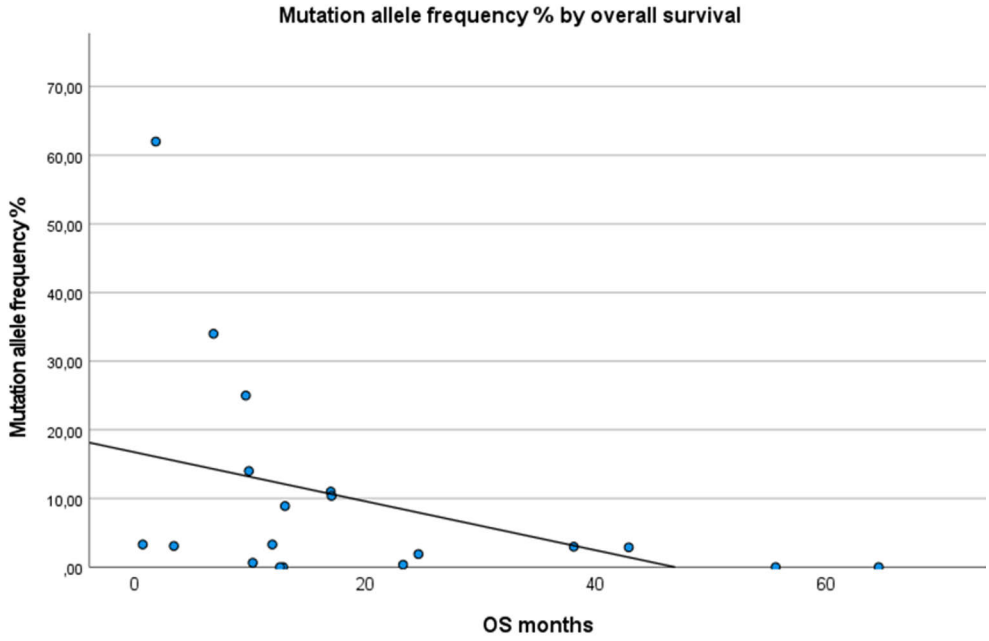


Figure 7. Association of ctDNA (MAF%) with OS.

MAF values were inversely associated with OS: Spearman`s rho correlation coefficient -0.588, p=0.008 (Figure 7). However, none of the risk factors included in the multivariable analysis (age, gender, mutational status, baseline ctDNA level, LDH level, ECOG performance status, M-stage, subsequent cancer therapy, and subsequent ICI) were independently associated with OS.

ctDNA levels (MAF values) in sequential samples paralleled the best radiological tumor response in twelve out of fifteen patients (in nine out of ten BRAF-mutated melanomas and in three out of five NRAS/KRAS mutated melanomas). Interestingly, ctDNA kinetics was associated with OS (Figure 8). Patients with undetectable ctDNA levels throughout the study had the longest survival. The patients whose ctDNA disappeared in sequential plasma samples had longer survival (mOS 17.1 months (2.7–31.5)) compared to the patients whose ctDNA remained detectable during the study (mOS 9.7 months (3.7–15.7)), p=0.003.

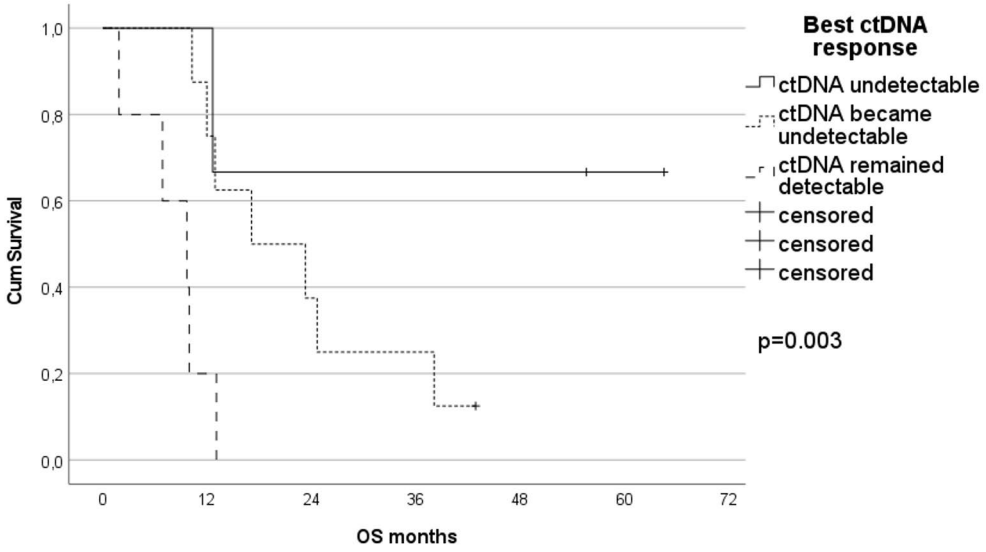


Figure 8. OS by ctDNA response.

5.4 Prognostic factors for metastasis-free survival after surgery of localized clear cell RCC (Study IV)

After RN or PN for localized clear cell RCC, the median follow-up was 76.1 (interquartile range 40.9–103.9) months in the training cohort and 65.4 (47.7–90.8) months in the validation cohort. 55 patients (28%) in the training cohort and 134 patients (19%) in the validation cohort developed distant metastases during postoperative follow-up. The median time to distant metastases was 25.5 (11.2–49.9) months in the training cohort and 21.9 (8.8–42.8) months in the validation cohort. The most common sites of metastases were lungs (47% of the patients with metastases), lymph nodes (36%), and brain (16%) in the training cohort and lungs (68%), bone (16%), and lymph nodes (12%) in the validation cohort.

Only three features were essential for the accurate prediction of MFS in the training cohort: tumor size, tumor grade (Fuhrman), and microvascular invasion. The final proposed Cox model is displayed in Table X.

Table X. The final Cox regression model obtained after feature extraction.

Feature	Levels	Parameter estimate	p-value	Hazard Ratio* [95% CI]
Tumor max diameter	Increment in millimetres	0.017326	< 0.0001	1.017 [1.014–1.021]
Tumor grade (Fuhrman)	Increment in levels from 1 to 4	0.685226	< 0.0001	1.984 [1.604–2.454]
Microvascular invasion status	Positive finding reported	0.236717	0.0034	1.267 [1.081–1.485]

CI = confidence interval; * ratio increment in hazard of event happening per level of feature when all other features are held constant

Based on these three features, a prognostic nomogram was introduced to stratify patients into low-, intermediate-, and high-risk group (Figure 9). In addition to the prognostic nomogram, a visual prediction surface (heat map) was constructed to determine risk group easily (Figure 10).

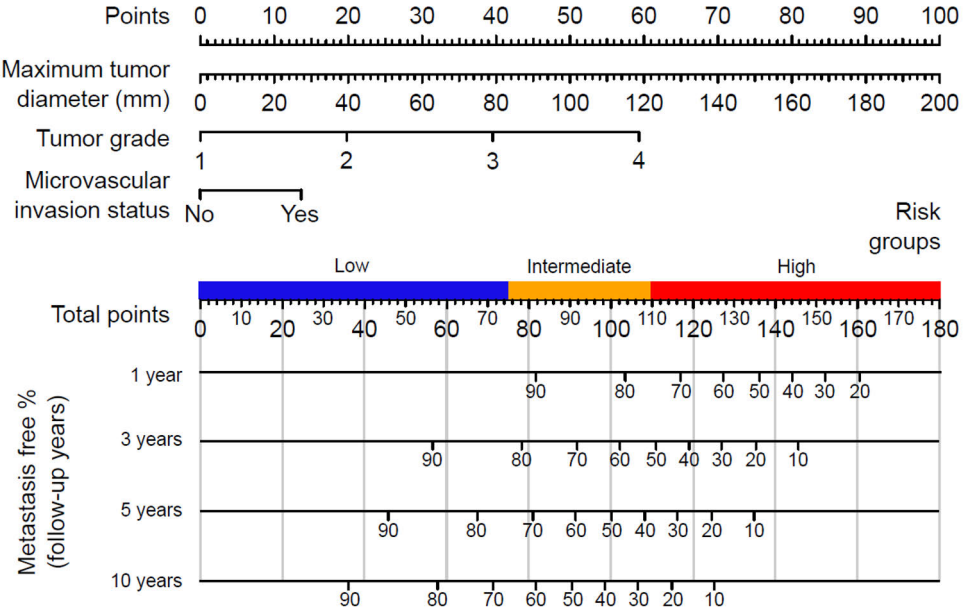


Figure 9. A prognostic nomogram to stratify patients to the low-, intermediate-, and high-risk of metastases after nephrectomy based on tumor size, tumor grade, and microvascular invasion.

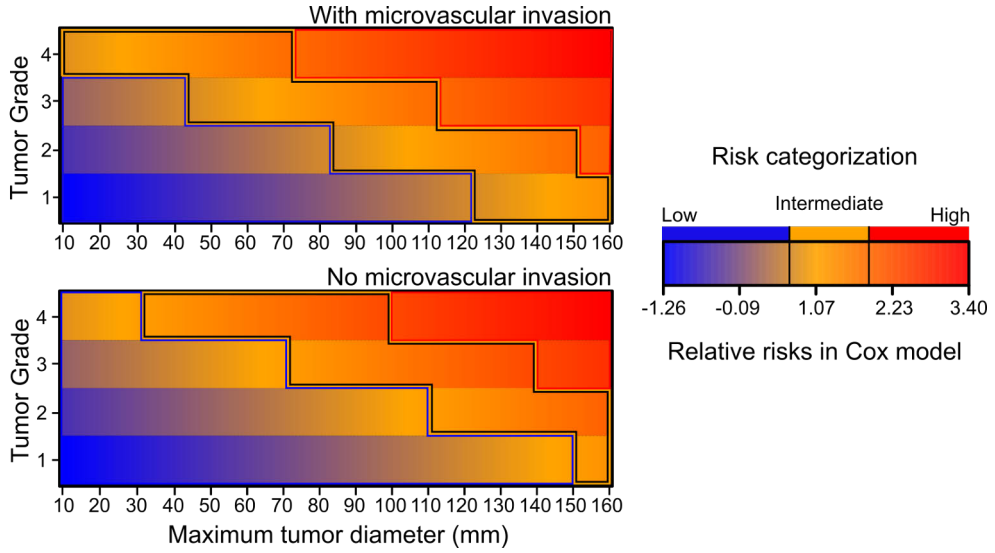


Figure 10. A visual prediction surface to determine risk group according to tumor size, tumor grade and microvascular invasion.

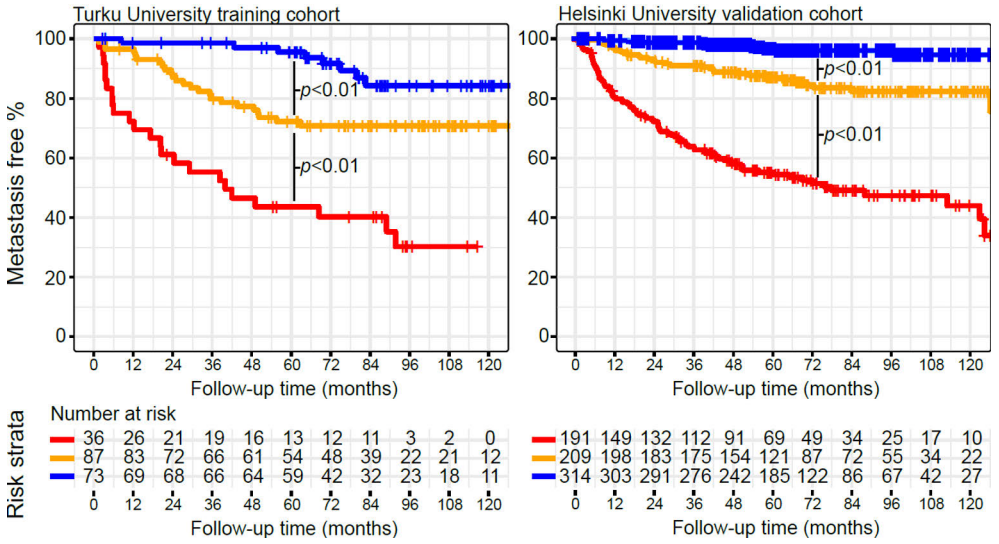


Figure 11. Kaplan-Meier curves for MFS and MFS rates in low-, intermediate-, and high-risk groups.

According to the proposed three-feature prediction model, 73 (37%), 87 (44%), and 36 (18%) patients in the training cohort and 314 (44%), 209 (29%), and 191 (27%) in the validation cohort were stratified into low-, intermediate-, and high-risk groups. During postoperative follow-up, distant metastases were discovered in 8 (11%) low-risk, 24 (28%) intermediate-risk, and 23 (64%) high-risk patients of the training

cohort and in 12 (4%) low-risk, 31 (15%) intermediate-risk, and 91 (48%) high-risk patients of the validation cohort. The median time to distant metastases was 66.6 (52.5–75.9) months (low-risk group), 26.0 (18.2–39.8) months (intermediate-risk group) and 17.5 (4.7–39.0) months (high-risk group) in the training cohort and 46.6 (19.7–57.9) months (low-risk group), 24.2 (13.0–50.9) months (intermediate-risk group) and 18.5 (7.2–36.0) months (high-risk group) in the validation cohort, respectively. MFS rates and survival curves according to the proposed risk group are described in Figure 9 and 11.

The proposed three-feature prognostic model yielded high prediction accuracy. The C-index and standard error was 0.755 ± 0.029 in the training cohort and 0.836 ± 0.015 in the validation cohort. The three-feature prediction model was compared to the original Leibovich model (Leibovich et al, 2003). While our model outperformed the Leibovich model in the training cohort (C-index 0.734 ± 0.035), there was no statistically significant difference between our proposed prediction model and the Leibovich model (C-index 0.848 ± 0.017) in the validation cohort (p=0.106). The sensitivity and specificity of our proposed model and the Leibovich model is illustrated by time-dependent ROC-AUC-curves in Figure 12. Noticeably, our novel model retained higher predictive accuracy in the later time points (24 to 90 months, Figure 12, Panels A and B), suggesting that the smaller set of prognostic features was more robust for long-term predictions.

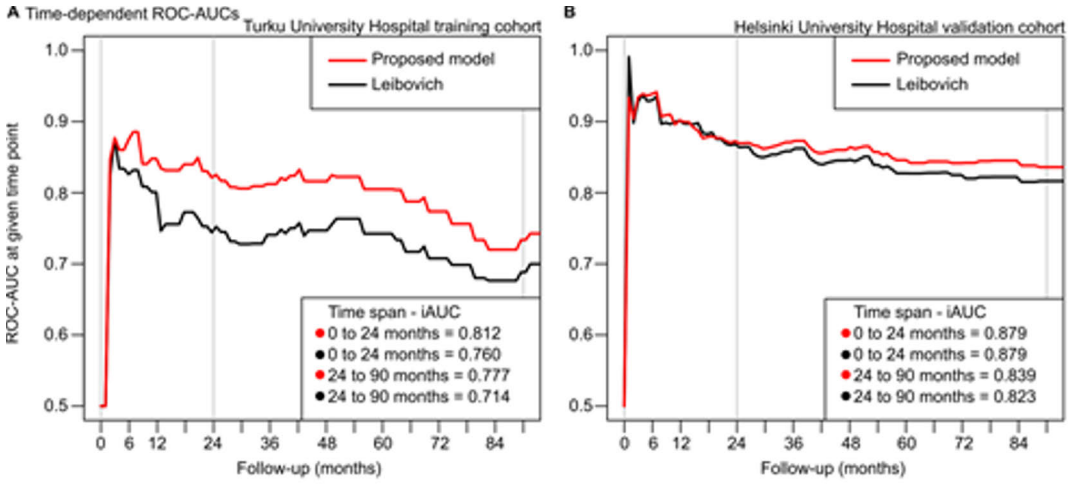


Figure 12. Time-dependent ROC-AUC performance for the proposed three-feature model and the Leibovich model in the training cohort (**panel A**) and the validation cohort (**panel B**).

6 Discussion

6.1 Traditional prognostic and predictive factors in advanced melanoma patients treated with chemoimmunotherapy and vemurafenib

Historically, long-term survival was uncommon in advanced melanoma patients treated with chemotherapy-based regimens and the five-year survival rate ranged from 6 to 9% (Vuoristo et al, 2005, Eigentler et al 2003, Maio et al, 2015). In study I, prognostic and predictive factors and treatment outcomes were evaluated in advanced melanoma patients who received chemoimmunotherapy (BOLD-IFN) outside clinical trials. Patients with poor performance status (ECOG ≥2), significant comorbidities, and symptomatic brain metastases are usually excluded from randomised clinical trials and treatment outcomes in everyday clinical practice tend to be inferior compared to results observed in RCTs. In a study of patients included in the Danish Metastatic Melanoma Database (DAMMED), only 39% of the patients were considered "trial-like" and 61% were considered "trial-excluded" (Donia M et al., 2019). Thus, results from retrospective real-world evidence studies may better reflect treatment outcomes achieved in a less selected patient population and offer another perspective for everyday clinical practice.

Even in a historical real-world patient cohort treated with chemoimmunotherapy (including 13% ECOG PS ≥2 patients) before ICI and BRAF and MEK inhibitors, there were some long-term survivors. In the Study I, the five-year survival rate was 13% and 7% of the patients were still alive at the end of the follow-up, which is comparable to survival outcomes achieved with chemotherapy in earlier clinical trials (Vuoristo et al, 2005, Eigentler et al 2003, Maio et al, 2015). Multimodal treatment was common in long-term survivors: 84% of five-year survivors had undergone surgery and 63% radiotherapy for the treatment of metastases in addition to chemoimmuntherapy. Male gender, poor ECOG PS, more advanced M-stage, lower number of chemotherapy cycles (1–4 compared to ≥5), and the absence of subsequent chemotherapy were independently associated with shorter survival in a multivariate analysis. Similarly, a pooled analysis of ECOG trials has also showed that female gender was associated with longer survival whereas higher number of metastatic sites and ECOG performance status ≥1 was associated with shorter survival

(Manola et al, 2000). In study I, the survival of patients with more advanced disease (M1b and M1c) was shorter than in patients M1a patients, as expected. Interestingly, the plateau in the survival curve of M1a patients was reached at the survival rate of 18% indicating that a small proportion of metastatic melanoma patients without visceral metastases achieved durable clinical benefit from chemoimmunotherapy before modern therapies (ICI and BRAF and MEK inhibitors).

In Studies II and III, chemoimmunotherapy (TOL-IFN) reached similar efficacy in patients with BRAF-negative advanced melanomas (ORR 13%, median PFS 1.7 months (1.2–65.5), and median OS 7.2 months (1.3–65.6) in cohort 2) than dacarbazine-based chemotherapy in earlier studies (Yang et al, 2009). As expected, patients with BRAF-positive advanced melanomas who received TOL-IFN and vemurafenib had better treatment outcomes (ORR 57%, median PFS 5.5 months (0.7–64.5), and median OS 15.1 months (0.7–67.8) in cohort 1). The combination of TOL-IFN and vemurafenib was tolerable after dose reductions. However, this experimental combination did not exceed the efficacy of single BRAF inhibitors (Chapman et al, 2011; Long et al, 2014). Overall, there is very scarce data published reporting outcomes of chemotherapy combined with BRAF inhibitors (Flaherty et al, 2006). However, BRAF and MEK inhibitors have been more effective and well tolerated in large phase III RCTs yielding the median OS of 26-34 months (Robert et al, 2019; Ribas et al, 2020; Ascierto et al, 2020), and are currently the standard treatment for BRAF-positive advanced melanoma patients in addition to ICI. Triple combinations of ICI plus BRAF and MEK inhibitors (atezolizumab, vemurafenib, and cobimetinib; pembrolizumab, dabrafenib, and trametinib; spartalizumab, dabrafenib, and trametinib) have not unequivocally outperformed the efficacy of BRAF and MEK inhibitors (Gutzmer et al, 2020; Ferrucci et al 2020; Nathan et al 2020) and remain experimental.

In the Study II, elevated LDH was significantly associated with shorter survival in the univariate Kaplan-Meier analysis similarly as seen with BRAF and MEK inhibitors and ICI (Long et al, 2016, Weide et al, 2016). Melanoma brain metastases have been associated with short survival, especially in the era of chemotherapy: the median OS was only 3.5 months with temozolomide (Agarwala et al, 2004). Some patients with asymptomatic brain metastasis can achieve long survival with BRAF- and MEK-inhibitors or ipilimumab plus nivolumab (Davies et al, 2017; Tawbi et al, 2018). Interestingly in the Study II, asymptomatic baseline brain metastases were not associated with shorter survival in the univariate Kaplan-Meier analysis, and two BRAF-negative patients with baseline brain metastases had unexpectedly long survival (24 and +65.5 months) suggesting that TOL-IFN might have intracranial activity. However, larger studies are needed to confirm this finding.

6.2 ctDNA as a prognostic and predictive biomarker in advanced melanoma

In addition to traditional prognostic and predictive factors, novel biomarkers are required to identify patients with poor prognosis requiring combination therapies (e.g. combinations of ICI and targeted therapies). Evenly important is to avoid more toxic and costly combination therapies for patients with favorable prognosis.

Circulating tumor DNA has been studied in multiple tumor types and it has turned out to be a useful tool to support clinical decision-making. Liquid biopsies are already used in clinical practice to supplement or even replace tumor biopsy revealing potential target mutations in NSCLC. Blood sample is safe and easy to draw and ctDNA may reflect tumor heterogeneity better than single-site tissue biopsy. ctDNA is a promising prognostic and predictive biomarker for melanoma patients too. Undetectable ctDNA in plasma samples have predicted longer recurrence-free and overall survival in patients with completely resected primary melanoma with or without regional lymph node metastases (stage II/III) (Lee et al, 2018; Tan et al, 2019) and in advanced melanoma patients treated with ICI and BRAF and MEK inhibitors (Seremet et al 2019, Santiago-Walker et al, 2016; Syeda et al 2021).

Earlier studies have shown that higher plasma ctDNA levels were associated with higher proliferative activity of tumor cells and higher tumor burden (metabolic tumor volume assessed by ^{18}FDG-PET-CT, number of metastatic sites, sum of baseline lesion diameters, and LDH levels) (Seremet et al 2019; Syeda et al, 2021) and all of these features predict inferior oncologic outcomes (ORR, PFS, and OS). In Study III, detectable ctDNA levels in baseline plasma samples were associated with diminished ECOG performance status as observed earlier (Lee et al, 2017) but significant association of baseline plasma ctDNA with LDH or M-stage was not found in this study. Patients with undetectable baseline ctDNA levels (MAF 0%) had longer PFS on chemoimmunotherapy and vemurafenib compared to patients with detectable BRAF, NRAS, or KRAS mutated ctDNA. Moreover, higher baseline ctDNA levels (MAF%) were associated with shorter OS.

ctDNA kinetics in sequential plasma samples during treatment may predict radiological treatment response and reflect survival outcomes. In the Study III, ctDNA levels paralleled the best radiological tumor response to chemoimmunotherapy and vemurafenib in 80% of the patients as seen in studies with BRAF and MEK inhibitors and ICI (Seremet et al, 2018; Lee et al, 2020; Syeda et al 2021). In advanced BRAF^{v600} mutated melanoma patients, the conversion of detectable pretreatment plasma BRAF mutated ctDNA concentration to undetectable at week 4 has indicated significantly better radiological ORR of dabrafenib and trametinib therapy (ORR 81% in patients with “zero conversion” vs 53% in patients with detectable ctDNA at week 4) (Syeda et al 2021).

However, there are some caveats to bear in mind. Ideally, ctDNA could reveal treatment failure (persistent ctDNA level during therapy or rising ctDNA level after initial response to therapy) before radiological confirmation of disease progression. While ctDNA has been shown to reflect extracranial melanoma, it is not an accurate biomarker for intracranial disease (Seremet et al, 2019; Lee et al, 2020). In addition, ddPCR may not be sufficient method for monitoring disease progression after initial treatment response as resistant tumor cell clones might have different driver mutations and remain undetectable with ddPCR. NGS analysis of plasma samples at the time of radiologically confirmed disease progression may reveal mutations that mediate resistance to therapy and are targetable by other drugs. This will be the target of our future studies. After all, longitudinal ctDNA sampling during cancer treatment may be used along with radiological imaging to supplement response evaluation.

Interestingly, patients with persistent detectable plasma ctDNA levels during chemoimmunotherapy and vemurafenib seemed to have the shortest OS in the Study III. Similar findings were observed in melanoma patients treated with ICI and BRAF and MEK inhibitors (Seremet et al, 2019, Syeda et al 2021). This phenomenon has also been observed in other cancers, as early reduction of ctDNA levels in longitudinal plasma samples was associated with favorable OS in NSCL patients treated with ICI (Wang et al, 2021). Thus, detectable ctDNA in longitudinal samples during cancer treatment seem to indicate poorer treatment outcomes and underline a population of patients in need of more aggressive therapy.

6.3 Clinical applications for novel postoperative prognostic model in clear cell RCC

The prediction of metastasis-free survival after surgery of localized RCC is important to guide postoperative follow-up and to stratify high-risk patients for adjuvant therapy trials. So far, adjuvant therapy after surgery of localized RCC is recommended in clinical trials only although neoadjuvant or adjuvant ICI therapy may become a relevant treatment option as the results from ongoing RCTs mature.

The Leibovich model (Leibovich et al, 2003) is thorouhgly validated (Pichler et al 2011; Beisland et al 2015) and clinically relevant tool for urologists to determine the frequency of radiological surveillance with thoracic and abdominal CT based on the individual risk for distant metastases after radical or partial nephrectomy. It requires five features for the prediction of metastasis-free survival (T-stage, N-stage, tumor size, nuclear grade, and tumor necrosis). In the updated Leibovich model, the number of prediction features was increased to nine to improve the prediction accuracy even more (constitutional symptoms, tumor grade, coagulative necrosis, sarcomatoid differentiation, tumor size, perinephric or renal sinus fat invasion, tumor thrombus level, extension beyond kidney, and nodal involvement) (Leibovich et al,

2018). On the contrary, we introduced a prediction model requiring only tumor size, tumor grade (Fuhrman), and microvascular invasion for the accurate prediction of MFS in the Study IV.

Tumor size had the largest effect on MFS in our proposed model. In addition to tumor size and tumor grade, microvascular invasion increased the risk of disease recurrence as noted in earlier studies (Bedke et al, 2018; Klatte et al 2018). Despite smaller number of features required for the prediction task, the Concordance index (C-index) of the three-feature model reached 0.836 and was comparable to the C-index of 0.848 for the Leibovich model (Leibovich et al, 2003) in the validation cohort (p=0.106). The reported C-indices for other prediction models have not exceeded 0.83 (Klatte et al, 2018). The prediction accuracy of the three-feature model was also comparable to the reported C-index of 0.83 of the updated Leibovich model (Leibovich et al, 2018). Due to the lack of information on constitutional symptoms and tumor thrombus level in our dataset, we were not able to perform direct comparison of the three-feature model against the updated Leibovich model. Interestingly, our three-feature model had better prognostic value for long-term prediction than the Leibovich model (2003), as it retained a higher integrated AUC for the time-dependent ROC-AUC post 24 months in Turku and Helsinki University Hospital patient cohorts.

To facilitate the use of the proposed three-feature prediction model in clinical practice, a nomogram and a visual prediction surface were introduced to stratify patients into low-, intermediate- and high-risk groups for metastases after surgery. This classification may be utilized to target more frequent postoperative radiological surveillance for intermediate- and high-risk patients and even stratify patients for adjuvant therapy trials.

6.3 Limitations and future directions

There are some limitations in this work. The limitations of Study I include it`s retrospective single-centre design which makes it vulnerable to selection bias. An external patient cohort treated with chemoimmunotherapy in another hospital would have been beneficial to increase the number of patients and verify our findings. However, the results of Study I were consistent with earlier studies of chemotherapy and interferon-alpha in advanced melanoma patients (Vuoristo et al, 2005, Eigentler et al 2003, Maio et al, 2015). Chemotherapy was the standard first line treatment option for stage IV melanoma patients in 1991–2010 and ICI and BRAF and MEK inhibitors were not available outside clinical trials. Thus, the results of this study can`t be extrapolated to the present-day patients but rather represent a historical reference for novel therapies.

Based on real-life experiences and the results of clinical trials with cytokines and ICI, patient´s immune system plays a significant role in eradicating metastatic melanoma. Patients who have small tumor burden and no visceral metastases are still the ones most likely to achieve long-lasting responses with novel targeted therapies and ICI. Sometimes, these patients might be even eligible for surgical resection of metastases or other local treatment options, such as isolated limb perfusion, intralesional T-VEC therapy, or radiotherapy. Unfortunately, tumor biopsies or blood samples were not available to study histopathological features or biomarkers (gene expression profiles, immune cell profiles, ctDNA etc.) among long term-survivors in the Study I.

The limitations of the prospective COBRA trial (Study II and III) include small number of patients enrolled into the trial although it was a nationwide, multicenter study coordinated by the Finnish Melanoma Group. BRAF and MEK inhibitors and ICI became available in routine clinical practice after 2015 slowing especially the enrollment of BRAF-positive patients in cohort 1 and the trial was closed before the preplanned number of 48 patients was recruited. Novel therapies have replaced chemoimmunotherapy from the first-line therapy of advanced melanoma patients and a larger study required to confirm the efficacy of TOL-IFN plus vemurafenib is irrelevant in the first-line setting. Treatment options after disease progression on ICI and BRAF and MEK inhibitors are still occasionally needed, and chemoimmunotherapy regimens (e.g. TOL-IFN) might be considered as a salvage therapy if the patient is not eligible for ongoing clinical trials.

Small number of patients and missing baseline and longitudinal plasma samples in four patients reduced statistical power to compare ctDNA levels with treatment outcomes. There was also a lack of centralized radiological review of response evaluation CT images. Tumor volume (sum of longest diameters of measurable lesions) was not determined. Hence, we were not able to evaluate the association of ctDNA directly with tumor burden. The results of ctDNA analyses in Study III are in line with earlier studies establishing undetectable ctDNA (before and during treatment) as a biomarker indicating favorable treatment outcomes in advanced melanoma patients.

One may argue that ctDNA does not add any value to other well-established prognostic factors, such as ECOG performance status or LDH level, in advanced cutaneous melanoma patients. While higher baseline ctDNA levels (MAF%) seemed to be associated with shorter OS and detectable baseline ctDNA with shorter PFS in the univariate analysis in our small patient cohort, we could not confirm that baseline ctDNA level was an independent risk factor for shorter PFS and OS in the multivariate analysis including ECOG performance status and LDH. However, elevated pretreatment $BRAF^{v600}$ mutated ctDNA levels have been associated with worse overall survival independent of LDH level in 345 advanced cutaneous

melanoma patients who had received dabrafenib and trametinib in large, multicenter Combi-D and Combi-MB trials. Moreover, undetectable $BRAF^{v600}$ mutated ctDNA level at week 4 anticipated longer PFS and OS, particularly in patients with elevated LDH (Syeda et al, 2021). Thus, longitudinal ctDNA sampling during therapy could provide additional prognostic information for advanced cutaneous melanoma patients beyond traditional prognostic factors. Biomarker-driven, randomized clinical trials with ctDNA as a stratification factor would be needed to support the use of ctDNA analyses in everyday clinical practice.

Detectable ctDNA (molecular residual disease) may provide even more valuable prognostic information in earlier stage melanoma patients indicating higher risk for disease recurrence and distant metastases after radical surgical resection of the primary tumor and regional lymph node metastases. Elevated plasma ctDNA levels could be a useful tool in selecting stage II and III cutaneous melanoma patients for adjuvant or even neoadjuvant therapy. Longitudinal ctDNA sampling during adjuvant therapy may also help to evaluate the benefit from adjuvant therapy while there is no visible disease to assess with current imaging methods. Unfortunately, there is no ctDNA information available from the adjuvant therapy trials conducted in stage III cutaneous melanoma and this issue should be studied in prospective clinical trials.

The limitations of Study IV include its retrospective nature, the small number (196 patients) of patients in the training cohort and the lack of centralized re-review of the original pathology reports. However, the number of patients in the training cohort was compensated by the size of the validation cohort (714 patients) and by fitting the final Cox model utilizing patients from both cohorts to obtain more reliable estimates. Pathologic features were collected from the original pathology reports and therefore the traditional Fuhrman grading was used instead of the modern WHO/ISUP grading. The information on tumor necrosis was missing in 60% of the original pathology reports of the Turku University Hospital cohort but it was routinely reported in the Helsinki University Hospital cohort (<1% missing). Because of the retrospective nature of our study, there was no standardized follow-up protocol and postoperative imaging was performed according to local clinical practice at Turku and Helsinki University Hospitals. The proportion of patients that underwent PN increased from 8% in 2007 to 23% in 2014 in the Turku University Hospital cohort. Although surgical technique has not been observed to affect the risk of distant metastases (Van Poppel et al, 2011), we decided to exclude patients with local recurrence in the kidney after PN and in the retroperitoneal space after RN without distant metastases from our study to eliminate the effect of surgical technique on our analysis,

A systematic, prospective evaluation of the three-feature model with other prognostic models is required to support wider clinical use of our model as

retrospective prognostic models have been found to overestimate their accuracy in prospective settings (Correa et al, 2019). Furthermore, our current training data may have underrepresented otherwise informative clinical factors, such as necrosis, and may therefore present some bias towards features that were widely available in our training cohort. Our future research projects will evaluate the association of molecular biomarkers (such as HIFs) with traditional histopathologic prognostic factors and treatment outcomes in localized ccRCC patients.

7 Conclusions

1. Long-term survival was uncommon in advanced melanoma patients who had received BOLD-IFN chemoimmunotherapy before modern treatment options (the median OS was 8.9 months and the five-year survival rate 13%). Long-term survivors were found especially in the subgroup of patients without visceral metastases (the five-year survival rate 28% in the M1a subgroup). Other factors predicting longer survival included female gender, good performance status, higher number of BOLD-IFN cycles (≥5), and subsequent chemotherapy.

2. The combination of TOL-IFN and vemurafenib was feasible in $BRAF^{v600}$ mutated melanoma patients after dose reductions. The efficacy of TOL-IFN-VEM in BRAF mutated melanomas was superior compared to TOL-IFN alone in NRAS mutated and WT melanomas, as expected, but did not exceed the efficacy of single BRAF inhibitors observed in phase III RCTs. Elevated LDH was associated with shorter survival whereas the presence of asymptomatic brain metastases did not deteriorate survival. Undetectable ctDNA in baseline plasma sample indicated favorable PFS and higher baseline ctDNA levels were inversely associated with OS. Patients with persistent detectable ctDNA during study therapy had the shortest OS.

3. Tumor size, tumor grade (Fuhrman), and microvascular invasion were sufficient for the accurate prediction metastases after radical or partial nephrectomy for localized clear cell RCC. A nomogram and a prediction surface were constructed to stratify patients into clinically meaningful risk groups for metastasis-free survival. The accuracy of the proposed three-feature model was validated in an external patient cohort. The three-feature model retained similar accuracy as the Leibovich model and had even better prediction value after 24 months from surgery.

Acknowledgements

A lot of water has flowed under the Aura Bridge since I started this thesis project in 2016. Three years earlier, I had found my way into the Department of Oncology and Radiotherapy at Turku University Hospital and luckily started working with urogenital and skin cancer patients. Finally, I also started my academic research project to gain deeper insight into renal cell carcinoma and melanoma.

First of all, I´m extremely grateful for my supervisors professor Panu Jaakkola, docent Pia Vihinen, and professor Heikki Minn for their ideas, guidance, and support in intellectual and technical aspects of this project. Panu is a great role model in combining a career as a scientist unraveling pivotal mechanisms of cell oxygen sensing and clinical work. Pia has led me to melanoma and clinical trials and guided me through my early years at the Turku University Hospital chemotherapy unit. Heikki has given me invaluable advice for research work, life, and birdwatching.

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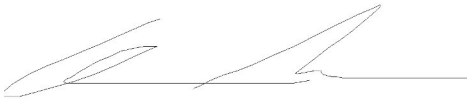

Kalle Mattila

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