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CHALLENGES IN THE MANAGEMENT OF UROTHELIAL CANCER: NOVEL TREATMENT, EVALUATION OF BIOMARKERS, AND IMAGING TECHNIQUES

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Front page: Ensemble de l'appareil urinaire (photocopy from *Traité Complet de L'Anatomie de L'Homme* by Bourgery et Claude Bernard, Paris 1867-1871). Original atlas stored at The Hagströmer Medico-Historical Library, Solna, Sweden.

To my parents and grandparents

Challenges in the Management of Urothelial Cancer: Novel Treatment, Evaluation of Biomarkers, and Imaging Techniques

Thesis for doctoral degree (Ph.D.)

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ABSTRACT

Urothelial cancer (UC) is the most common malignancy found in the urinary tract. The global annual incidence is approximately 430 000 new cases (Sweden: 3 200 new cases). Approximately one in four new UC patients being diagnosed has muscle-invasive disease. For curative intent, treatment involving surgical removal of the primary tumour remains the gold standard for locally advanced UC. Still, one in two patients relapses despite undergoing curative intended surgery or bladder-preserving radiotherapy. Platinum-containing regimens have been the standard treatment since the 1980s, despite only reaching an overall survival of about 1 year. During the last decade, merely one new chemotherapy has been approved for metastatic UC: vinflunine. The primary aim of this thesis was to improve the management of advanced and metastatic UC by evaluating experimental treatments and exploring predictive and prognostic biomarkers.

Paper I describes a patient with metastatic UC and with no available standard treatment options after failing platinum treatment. The patient received the tyrosine kinas inhibitor sorafenib in second-line for almost one year. Immunohistochemistry (IHC) analysis of this patient's tumour revealed intermediate expression of vascular endothelial growth factor receptor 2 (VEGFR2) and high expression of platelet-derived growth factor receptor β, two key targets of sorafenib.

In **Paper II**, the prognostic value of S100A4, S100A6, and VEGFR2, markers of metastasis, proliferation and angiogenesis, were analysed by IHC in tumour specimen from 83 UC patients following cystectomy of the urinary bladder. Expressions of these proteins were compared with overall and disease-free survival. High expression of VEGFR2 and low tumour stage were independently correlated with longer survival. No association was found for S100A4 or S100A6 in this cohort.

The Phase I trial Vinsor (**Paper III**) was the first clinical study to assess safety of vinflunine plus sorafenib in metastatic UC patients, refractory to platinum. Primary endpoint was to define the recommended Phase II dose (RPTD). In patients treated with a start dose of vinflunine 280 mg/m^2 the RPTD of sorafenib was 400 mg. In patients receiving vinflunine 320 mg/m^2, the RPTD was not determined because of toxicity. The median overall survival was 7.0 months and the overall response rate was 41%.

Predicting early response to treatment is of clinical importance in improving outcome. In **Paper IV** the predictive value of response evaluation with early ^{18}F-FDG PET scans and plasma exosomes were analysed in a subset of Vinsor trial patients (**Paper III**). Results demonstrated that early changes on ^{18}F-FDG PET predicted survival and RECIST based on subsequent CT scans. Plasma exosomes could be isolated and quantified, but analysis revealed no association to treatment response.

In **Paper V**, the cytotoxic properties of the peptidase-enhanced alkylating agent melflufen was studied *in vitro*. In UC cell lines melflufen increased cell death compared to melphalan. Aminopeptidases were found to be of importance for melflufen efficacy *in vitro* and high expression of aminopeptidase N expression in UC tumour specimens was associated with longer overall survival.

In summary, the results of this thesis indicate that subsets of UC patients may have a clinical benefit of sorafenib and that combined treatment with vinflunine is safe and possibly increases treatment efficacy. VEGFR2 appears to have prognostic potential besides being a target for therapy. Early treatment assessment of metastatic UC patients with ^{18}F-FDG PET holds predictive potential. Melflufen shows antitumoral effects in UC cell lines and could be a future novel chemotherapy against this cancer.

List of Scientific Papers Included in this Thesis

I. **Carl-Henrik Shah**, Kristina Viktorsson, Amir Sherif, Lena Kanter, Per Grybäck, Rolf Lewensohn, Per Sandström, Sten Nilsson, and Anders Ullén.
Clinical activity of sorafenib in a previously treated advanced urothelial cancer patient.
Anti-Cancer Drugs 2013, 24:648-652

II. **Carl-Henrik Shah**, Kristina Viktorsson, Lena Kanter, Amir Sherif, Jurate Asmundsson, Robert Rosenblatt, Rolf Lewensohn, and Anders Ullén.
Vascular endothelial growth factor receptor 2, but not S100A4 or S100A6, correlates with prolonged survival in advanced urothelial carcinoma.
Urologic Oncology: Seminars and Original Investigations 2014, 32:1215-1224

III. **Carl-Henrik Shah**, Helle Pappot, Mads Agerbæk, Karin Holmsten, Fredrik Jäderling, Jeffrey Yachnin, Per Grybäck, Hans von der Maase, and Anders Ullén.
Safety and Activity of Sorafenib in Addition to Vinflunine in Post-Platinum Metastatic Urothelial Carcinoma (Vinsor): Phase I Trial.
Accepted for publication in The Oncologist on November 15, 2018.

IV. **Carl-Henrik Shah**, Jacob Farnebo[†], Petra Hååg[†], Fredrik Jäderling, Vasiliki Arapi, Adam Sierakowiak, Per Sandström, Per Grybäck, Rolf Lewensohn, Kristina Viktorsson, and Anders Ullén.
Early Evaluation of Vinflunine and Sorafenib Treatment Responses in Metastatic Urothelial Cancer Patients by Use of Explorative ^{18}F-FDG-PET CT and Plasma Exosome Analyses.
Manuscript

V. Kristina Viktorsson, **Carl-Henrik Shah**, Therese Juntti, Petra Hååg, Katarzyna Zielinska-Chomeij, Adam Sierakowiak, Karin Holmsten, Jessica Tu, Jack Spira, Lena Kanter, Rolf Lewensohn, and Anders Ullén.
Melphalan-flufenamide is cytotoxic and potentiates treatment with chemotherapy and the Src inhibitor dasatinib in urothelial carcinoma.
Molecular Oncology 2016, 10:719-734

[†]= equal contribution

CONTENTS

List of abbreviations

^{18}F-FDG	^{18}F-fluorodeoxyglucose ($C_6H_{1118}FO_5$)
1L	first-line
2L	second-line
ALAT (=ALT)	alanine aminotransferase
ANPEP	aminopeptidase N
ASAT (=AST)	aspartate aminotransferase
b.i.d.	bis in die (twice daily)
bFGF	basic fibroblast growth factor
CD13	cluster of differentiation 13
CT	computed tomography
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CT scan	computed tomography scan
DCR	disease control rate
DFS	disease-free survival
ERK	extracellular signal-regulated kinases
FFPE	formalin-fixed paraffin-embedded
FGFR	fibroblast growth factor receptor
GC	gemcitabine plus cisplatin
GCP	gemcitabine, cisplatin, plus paclitaxel
IHC	immunohistochemistry
Hb	haemoglobine
HD-MVAC	high-dose intensity MVAC
melflufen	melphalan flufenamide
melphalan flufenamide	L-melphalanyl-L-p-fluorophenylalanyl ethyl ester hydrochloride
MAPK	mitogen-activated protein kinases
MVAC	methotrexate, vinblastine, doxorubicin, plus cisplatin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MIBC	muscle invasive bladder cancer
MTD	maximum tolerated dose
mUC	metastatic urothelial carcinoma
NACT	neoadjuvant chemotherapy
NMIBC	non-muscle invasive bladder cancer
NUCOG	Nordic Urothelial Cancer Oncology Group
OS	overall survival

PD	progressive disease
PDGFRβ	platelet-derived growth factor receptor β
PD1	programmed cell-death protein 1 receptor
PDL1	programmed cell-death ligand 1
PET	positron emission tomography
PERCIST 1.0	PET response criteria in solid tumours version 1.0
PFS	progression-free survival
PR	partial regression
Raf (c-Raf)	rapid accelerated fibrosarcoma, kinase (cellular Raf)
Ras	rat sarcoma; name of group of genes
RECIST 1.1	response evaluation criteria in solid tumours version 1.1
RIPA	radio immunoprecipitation assay
RPTD	recommended Phase II dose
S100A4	S100 calcium-binding protein A4
S100A6	S100 calcium-binding protein A6
SD	stable disease
SUV_{max}	maximal standardised uptake value in a voxel
SUV_{peak}	standardised uptake value in a sphere (VOI ⌀ 1 cm)
TBS	tris-buffer saline
TKI	tyrosine kinase inhibitor
TNM	tumour, (regional lymph) node(s), (distant) metastasis
TRIS	Tris(hydroxymethyl)aminomethane
TTP	time to progression
TURB	transurethral resection of the bladder
UC	urothelial carcinoma
VEGF (=VEGFA)	vascular endothelial growth factor A
VEGFR1, …2, …3	vascular endothelial growth factor receptor 1, …2, …3
VOI	volume of interest

Names of human genes are written in capital italics (*S100A4*), human proteins in capitals (S100A4), and mouse genes in italics, starting with an uppercase letter (*S100a4*).

1 Introduction urinary tract cancer

1.1 Definitions

In this thesis advanced urothelial cancer (UC) is defined as a primary cancer of the urinary tract of tumour stage 2 or above (T2+) as described in Classification by the Union Internationale Contre le Cancer, Editions VII and VIII [1,2]. In **Paper II**, a minority of cases with UC invading the subepithelial connective tissue (T1) were also included. Analysed in the papers are tumours with histology of pure or mixed UC, but predominantly of urothelial origin (90% or above).

1.2 Demography, epidemiology and risk factors

Urinary tract cancer is globally diagnosed in about half a million patients per annum [3]. This means that cancer derived from the urinary tract constitutes about 3% of all new cancer cases and is the sixth most common cancer for men, nineteenth for women, and eleventh for both genders combined worldwide in terms of incidence [3]. The incidence of urinary tract cancer varies around the world, mainly due to different environmental and lifestyle factors [4]. In Sweden, 3 156 new cases of urinary tract cancers including the renal pelvis were diagnosed in 2016 [5]. The risk of contracting urinary tract cancer varies widely among ethnicity, with Caucasians in South and West Europe, Middle East and North America at the top with a cumulative 0-74 year life risk of up to 2.5% for men and 0.6% for women. Lowest risks are found in and South-Central Asian and West African populations, with approximately a tenth of the formers' cumulative risks [3]. Globally, urinary tract cancers account for over 165 000 deaths per year [3]. The high mortality in urinary tract cancers commands a position as number thirteen of cancer related causes of death among all cancers worldwide [3]. The most common direct cause of UC is tobacco smoking [6]. Chemicals, mainly arylamines and polycyclic aromatic hydrocarbons, are known to cause cancer of the urinary tract [7]. Treatment with radiotherapy of cancers in the pelvic region and some chemotherapy may increase the risk of developing secondary UC [8-10]. Age and male gender are other well-known risk factors for UC with most patients diagnosed in their seventh or eighth decade [7]. Variation in the genes encoding N-acetyltransferase 2 and glutathione S-transferase-µ1 and single nucleotide polymorphisms have displayed a 10-50% increased risk of bladder cancer [4]. Urinary tract infection with schistosomiasis is a specifically causality for squamous cell carcinoma [11,12].

1.3 Classification according to TNM and diagnosis

UC is staged according to the worldwide accepted tumour, (lymph) node, and metastasis (TNM) nomenclature system by Union Internationale Contre le Cancer, with the latest edition (VIII) published last year [1]. The classification according to tumour infiltration depth was proposed in 1946 [13]. Basically, the TNM system separates non-muscle invasive bladder cancer (Ta, Tcis, T1) from muscle invasive bladder cancer (T2-T4). Staging is based on cystoscopy (via the urethra), the histopathology diagnosis and radiology (MRI and CT scans with functional imaging being optional but often performed) [14]. A transurethral resection of the bladder is performed to obtain a biopsy of the primary tumour. The proportion of incidence between NMIBC and MIBC is about 75-80% versus 20-25% at diagnosis [7]. Accurate staging is crucial as prognosis and treatment of NMIBC and MIBC differs significantly [14,15].

1.3.1 Histology and WHO grades

Cells of the urothelial (transitional) epithelium compose the outer cell layer in the urinary tract and bladder, and thus are exposed to the metabolites contained in the urine. Deriving from this cell layer, UC is the dominant type of cancer arising in the urinary tract, constituting about 90%

of all cancers. Much less frequently occurring are squamous cell carcinoma (4%), adenocarcinoma (2%), and sarcoma (~1%) [16]. The various histological subtypes are treated separately as their response to chemotherapy and radiation are different [14]. Along with the TNM system and histopathological classification, WHO grade adds information about the morphological differentiation of the constituting tumour cells [17,18]. Classification according to tumour grade was originally defined by the WHO in 1973 and updated in 1999, 2004, and 2016 [19]. Although the classification criteria varies between the versions, advantages with the updated versions have been the focus of debate [20]. However, more tumours are classified in the most aggressive group with the WHO 2004/2016 grading criteria and less differentiated (high grade or grade 3) UC tumours are more likely to have a higher T-stage [19].

1.4   Molecular taxonomy and driving pathways in UC

Through the characterisation of mutations, gene expression, and epigenetic changes of UC, an integrated classification of UC is arising [21-23]. Thus, protein expression analyses of UC tumours from a group of 237 patients with muscle-invasive UC identified 20 proteins, collectively associated with significantly different molecular pathology and survival [23]. The proteins identified included cyclin B1, D1, E1, cyclin dependent kinase inhibitor 2A, desmocollin 2/3, E2F transcription factor, EGFR, ERBB2, FGFR3, E-, N- and P-cadherin, MKI67, TP63, RB1, uroplakin 3, cytokeratin 5, 6, 14, and 20 [23]. By combining these factors, three molecular subtypes of UC have emerged: urobasal, genomically unstable, and squamous cell carcinoma-like [23]. In a similar approach, 131 UC tumours had their mRNA, miRNA, and protein expressions analysed [24]. Mutations, copy number alterations, and RNA expression changes associated with UC included the PI3K-AKT and RTK-MAPK signalling pathways, amounting to 42-45% of the analysed cases. The most common alteration identified in each pathway was activating point mutations in PI3KCA (17%) and FGFR3 (17%). The authors identified four distinct subtypes of UC [24]. Two additional groups have performed discovery analyses on the molecular level for pathophysiological alterations in UC [21,22]. Pooled data from the published data sets have been compiled and analysed [25]. From this extensive analysis it was concluded that UC tumours can be classified into two major types: urothelial and squamous-like differentiation with extracellular matrix and immune related gene alterations. The smaller cohort ("basal" or squamous cell carcinoma-like) showed up-regulation of most of the previously identified 20 genes, whereas the larger cohort (luminal or urobasal A and genomically unstable) displayed an inverse pattern [25]. It is still unclear how the molecular alterations correspond to therapy outcome. The collective data has identified the most active pathways in UC development: the MAPK/ERK (RAS-RAF-MEK-ERK) and PI3K-AKT-mTOR signalling transduction routes [26].

1.5   Treatments

Initially cancer of the urothelial tract was solely treated by surgeons. With the advances in clinical oncology, megavoltage radiotherapy emerged as a treatment option in the 1950s [27]. Among the first systemic chemotherapeutic agents to be evaluated in UC were cisplatin and the anthracycline doxorubicin, both of which were tested in the late 1960s and early 1970s [28,29]. Chemotherapy combinations which included cisplatin were gradually introduced from the late 1970s [30,31]. Despite the increased incidence and latest pharmacologic developments including biologics, treatment options in locally advanced and metastatic UC remain limited [32,33].

1.5.1 Chemotherapies

Neoadjuvant Setting

In 1985 the first patient in the Nordic Cystectomy Trial I was randomised between two cycles of cisplatin and doxorubicin followed by low dose radiotherapy and cystectomy or radiotherapy and cystectomy alone [34]. This trial and the successor reported improved survival with NACT [34-36]. Subsequent trials have confirmed and established NACT as the standard protocol in advanced UC with curative intention [37]. This regimen typically consists of three to four courses of gemcitabine and cisplatin (GC) or methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) followed by cystectomy. The purposes of NACT are dual: to shrink the primary tumour and to treat micro-metastases. One meta-analysis has confirmed a 5 percentage-point gain in OS [37]. The PD1 inhibitor pembrolizumab and the PDL1 inhibitor atezolizumab were evaluated before radical cystectomy in separate single armed Phase II studies [38,39]. In the future, this could hopefully be improved with implementing a more precise strategy for UC patients based on biomarkers.

Adjuvant Setting

Several clinical trials have evaluated the effect of adjuvant chemotherapy in UC [40]. The results of these trials have not produced solid evidence for adjuvant chemotherapy in UC [41]. Currently, this treatment strategy is not recommended in clinical routine but only to selected patients who have not for any reason received neoadjuvant therapy and present with a histopathology of pT3/pT4 and/or pN+ [14].

Palliative setting – first-line

In the 1970s, single treatment with cisplatin and doxorubicin produced response rates of 20% to 35% in patients with advanced UC [31,42]. Further development of chemotherapy combinations eventually determined MVAC as the regime with the highest response rate at 55% [30]. This regimen has been improved and renamed HD-MVAC by optimising the doses and schedule besides minimising neutropenia by adding GCSF, consequently lifting the response rate to 62% [43]. However, significantly lower frequency of toxicity favours GC as first-line treatment in advanced UC [44]. The triplet GCP was found to improve the response rate to 56%, but without significant improvement in OS in the intention-to-treat population [45]. For cisplatin fit patients, the median OS is limited to 14-15 months [44]. However, 30-50% of UC patients are unfit to receive cisplatin due to renal dysfunction [46]. Patients with renal impairment, poor performance status or co-morbidity to whom cisplatin cannot be given, carboplatin-based combination regimens or gemcitabine monotherapy offers an alternative treatment option in addition to immunotherapies (see section 1.5.3) [47]. For cisplatin unfit patients, the median OS is 8-9 months with conventional chemotherapy [47].

Palliative setting – second-line

The short survival on first-line chemotherapy in UC clearly illustrates the need for further treatment options. Numerous drugs have been tested in second-line as monotherapy or as doublets [33]. Two of the most promising drugs tested were nab-paclitaxel and pemetrexed. Two small Phase II trials of these drugs claimed median OS of 9.6 and 10.8 months, respectively [48,49]. Later, larger trials in UC failed to confirm the effect of pemetrexed, resulting in an objective response rate of only 5% and median PFS of 2.4 months [50]. The vinca alkaloid vinflunine was initially tested in two Phase II trials, describing median OS of 6.6-8.2 months,

respectively [51,52]. In the Phase III Registration trial, vinflunine was concluded to add a median OS of 2.3 months in second-line UC treatment following platinum failure, compared with BSC only [53]. Presently, vinflunine is the only approved chemotherapy after cisplatin failure in UC within the European Union [14]. Nevertheless, multiple trials are investigating other drugs and also vinflunine treatment combinations [54]. Studies in mUC of vinflunine doublet combinations have resulted in unacceptable toxicity in second-line (pemetrexed, pazopanib) but promising ORR and OS in first-line (gemcitabine, carboplatin) [55-57].

1.5.2 The peptidase-enhanced alkylating agent melflufen

In the current thesis the novel peptidase enhanced alkylating agent melflufen (L-melphalanyl-L-p-fluorophenylalanine ethyl ester hydrochloride) has been explored (**Paper V**). Melflufen is a Swedish invention developed at Uppsala University and Karolinska Institutet [58-61]. Melflufen is a dipeptide of *para*-fluoro-L-phenylalanine and the active moiety melphalan and allows for rapid loading of the drug inside cells (**Figure 8**). Subsequently aminopeptidases cleave the peptide bond, releasing high amount of melphalan which alkylates DNA [62]. This increased amount of DNA damage causes increased cell death in tumour cells [30,58-69]. A role for aminopeptidases in the conversion of melflufen to free melphalan has been demonstrated using bestatin and siRNA against aminopeptidase N (ANPEP) [70, 64,69]. Melflufen (Ygalo®) is currently in clinical evaluation by Oncopeptides AB for treatment of multiple myeloma (MM) in several Phase II/III clinical trials (www.oncopeptides.se, [71]). A phase I/II trial of melflufen in MM has been completed and demonstrated that melflufen was well tolerated and had clinical efficacy in late stage MM patients [62]. A "first-in-man" Phase I/II clinical trial of melflufen in patients with solid tumours has also been carried out at Karolinska Institutet and Uppsala University. Results have demonstrated tolerability, yet with (expected) toxicity related to bone marrow cells [72].

Melflufen is converted to free melphalan by aminopeptidases. Aminopeptidases in general and ANPEP in particular, have been demonstrated to have increased expression in tumours of different origin, e.g. breast, lung, colorectal, and pancreatic cancer, and have in some tumour types been linked to poor prognosis or a metastatic phenotype [70,73]. In UC specimens, ANPEP has been described as localised to stromal cells [74]. Similarly to other malignancies, tumour stroma expression of ANPEP has also been described [70,75]. ANPEP has been explored for tumour therapy purposes [73]. The role of ANPEP in PET imaging using either labelled peptides, small molecules or affinity probes has been applied in a mouse model [70]. Thus, ANPEP is an interesting biomarker to further explore in UC specimens which was carried out in the current study.

1.5.3 Immunotherapies

During the past two years, immuno-oncology-based therapies including pembrolizumab, nivolumab, atezolizumab, durvalumab, and avelumab represent a major breakthrough for the treatment of metastatic UC patients within the post-platinum setting [76-80]. These therapies have reported an OS of up to 10.3 months and an ORR of up to 26% in second-line or beyond with durable responses [76,81]. For patients who are unfit to cisplatin-based combination chemotherapy, pembrolizumab and atezolizumab have been approved as treatment options, alongside with carboplatin-based regimens, for patients with high PDL1 expression [82-85]. Nonetheless, most metastatic UC patients do not respond to immunotherapy, and these patients need potent subsequent treatment, supporting further development of combination regimens of chemotherapy and other anticancer treatments.

1.5.4   Therapeutic antibodies

Several therapeutic antibodies have been examined in UC with different success. The monoclonal antibody bevacizumab, which blocks VEGFA, has been tested in addition to first-line palliative chemotherapy [86,87]. Addition of bevacizumab increased ORR but was also associated with increased toxicity. Last year, the RANGE study in second-line post-platinum in metastatic UC reported ORR of 24.5% with docetaxel and the monoclonal antibody ramucirumab targeting VEGFR2 [88]. Patients receiving ramucirumab and docetaxel had about 1½ months longer PFS than in patients receiving docetaxel plus placebo. HER2 is a confirmed negative prognostic factor for survival in breast, ovarian and gastric cancers [89-92]. In a Phase II trial on trastuzumab it was found that only 13.3% of UC patients overexpressed HER2 and no significant improvement in OS was confirmed when trastuzumab was added to GC in the HER2 group as first-line palliative treatment [93]. An earlier Phase II trial with trastuzumab in addition to paclitaxel, carboplatin, and gemcitabine found 49% of UC patients to express HER2 and 57% of the HER2 positive patients responded to the treatment with a median OS of 14.1 months [94].

1.5.5   The tyrosine kinase inhibitor sorafenib

Sorafenib, a TKI, initially approved for renal cell carcinoma, inhibits phosphorylation of key target proteins of the RAF-MEK-ERK signalling pathway including Raf-1, B-Raf, ERK1/2, PDGFRβ, VEGFR2/3, Flt3, and c-KIT.[95,96] The indication was subsequently extended to include hepatocellular carcinoma and thyroid cancer supported by phase 3 trials demonstrating improvement in survival.[97,98] Altered signalling in angiogenic pathways of UC tumours supported the hypothesis of sorafenib activity in UC, as well as individual cases with favourable outcome [99,100]. A Phase II first-line study in metastatic UC analysed the addition of sorafenib to GC [101]. The study reported an ORR of 52.5% versus 47% with standard treatment, and an OS of 11.3 months compared with 10.6 months. A single-armed neoadjuvant study combining GC with sorafenib in muscle invasive UC reached a pT0 rate of 43.5% with 26.1% grade 4 toxicity [102]. A phase 2 trial evaluated first-line sorafenib monotherapy in 17 patients with mUC [103]. The study reported a median OS of 5.9 months, but no responses were reported.
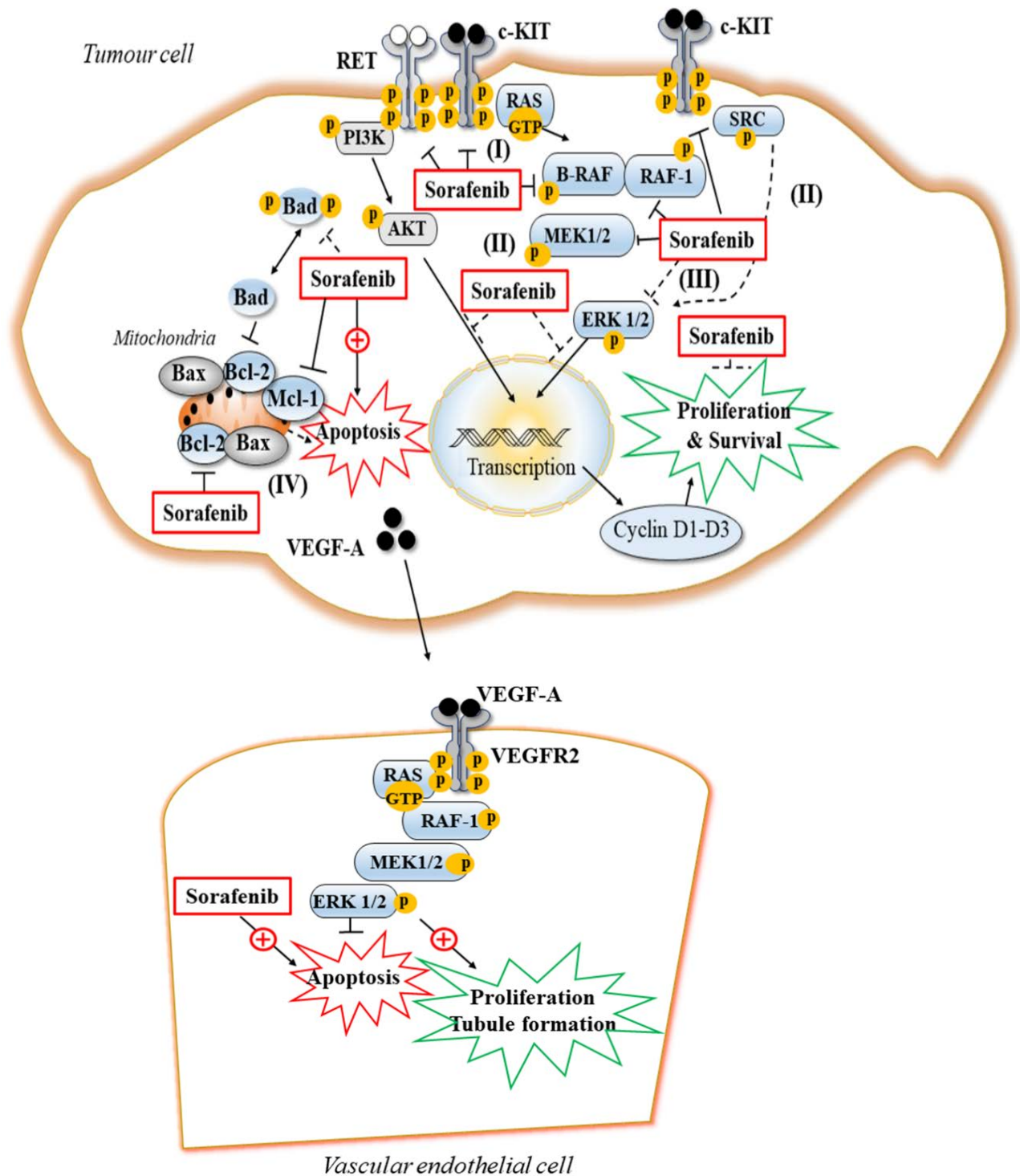
Figure 1. Anti-tumour effects of sorafenib. The multikinase inhibitor sorafenib directly targets the tumour expressed growth factor receptors c-KIT and RET, by inhibiting their autophoshorylation status (I). This influences their down stream targets, e.g. PI3K/AKT and SRC, resulting in partially inhibition of proliferation through transcriptional regulation (II). Sorafenib blocks RAF-1, B-RAF, MEK1/2 phosphorylation in cells and indirectly causes inhibition of ERK-mediated proliferative networks (III). Sorafenib alleviates blockers of mitochondria-mediated apoptotic signalling, e.g. Mcl-1 and Bcl-2, and enables pro-apoptotic signalling proteins, e.g. Bad, to work, resulting in increased cytochrome c release and promotion of apoptosis (IV). Sorafenib may cause a direct blockade of VEGFR2 autophosphorylation in vascular or lymphatic endothelial cells (V) and impair downstream Raf-1/MEK/ERK signaling cascade (VI). This reults in impairment of vascular endothelial cell proliferation, migration, and tubule formation thereby inhibiting angiogensis within the tumour (VII).

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1.5.6 Radiotherapy

Radiotherapy in UC has been a long-standing modality for selected indications, mainly for curative treatment of fragile and older patients with muscle-invasive disease and for palliation. For a suitable subset of UC patients with organ-confined disease (T2-3N0M0) combining radiotherapy with NACT or concomitant chemotherapy upfront produces response rates of 88% and a three-year disease-specific survival rate of 82% [104,105]. This approach offers the advantage of bladder preservation. Attempts to modify the dose regime in radiotherapy treatment of organ-confined MIBC (T2-4N0M0) have been undertaken [106]. The trend shows non-inferiority results with hypofractionated courses from conventional courses of 64 Gy/fx 2 Gy [107]. Adding chemotherapy to radiotherapy has been shown to improve efficacy with local disease-free rates of 67% without significant increase in side-effects [108].

1.5.7 Surgery

As early as in the 17th century, a very perceptive and enquiring surgeon named Fabrice de Hilden described a protruding mass in the bladder in one of his patients [109]. Modern surgical interventions are considered to have been developed in the late 19th century [110]. In the last century surgical techniques made substantial progress [111,112]. Localised non-muscle invasive UC is since long time ago treated with transurethral resection, alone or in combination with intravesical instillation of either bacillus Calmette–Guérin vaccine, epirubicin, or mitomycin, depending on risk of progression [113-115]. For low and intermediate grade tumours with medium and high risk of relapse or progression a single dose of mitomycin is instilled into the urinary bladder after resection [116]. For high grade tumours, 6 weekly induction therapies with bacillus Calmette–Guérin promoting an immune response and delaying relapse is recommended based on two meta-analyses [117,118]. For organ-confined muscle invasive UC, radical or in selective cases partial cystectomy including pelvic lymph node dissection is recommended as first-line treatment [14]. Following cystectomy approximately 50% of UC patients will relapse [119,120]. All relapsing patients should be considered for systemic treatment with the aim of prolonging overall survival and improving quality of life.

1.6 Evaluation of systemic treatment in advanced and metastatic UC

1.6.1 CT

The most utilised imaging system in UC is CT scans [121]. The CT technology provides relatively accurate and fast diagnostic radiology assessment of a patient's local or global tumour status [121]. Evaluation of systemic treatment is therefore routinely done with CT [14]. The anatomic assessment is based on the RECIST version 1.1 definitions [122-124]. However, CT has technical shortcomings, where other diagnostic techniques have evolved [125].

1.6.2 PET

A meta-analysis including six studies of PET in urinary bladder cancer concluded that ^{18}F-FDG PET improves diagnostic imaging accuracy of metastasis over CT and MRI [126]. Hence, imaging by PET has become more frequently used in bladder cancer and reports of ^{18}F-FDG PET CT restaging in up to 22% of MIBC patients leading to a change of therapy in 14% [127]. As most ^{18}F-FDG PET CT scanned UC tumours were upstaged, the two most common changes in therapy were adding NACT or converting the treatment indication from curative to palliative [127,128]. Apart from ^{18}F-FDG, other radiolabelled tracers evaluated in bladder cancer include ^{11}C-

acetate and ^{11}C-choline [129]. However, a meta-analysis found these tracers to have low detection rate of lymph node metastases [129].

1.7 Potential clinical and molecular biomarkers

Non-invasive ways to monitor treatment responses are needed, to optimise and individualise treatment.

1.7.1 Prognostic clinical parameters in metastatic disease

A few prognostic clinical parameters in UC have been identified [130,131]. Karnofsky performance status scale of less than 80% and lung, liver, or bone metastasis were found to be independent prognostic factors affecting OS in UC patients receiving MVAC [130]. Presence of 0, 1, or 2 factors resulted in median OS of 33, 13.4, and 9.3 months, respectively [130]. Performance status of 1 or higher, anaemia (Hb <10 g/dL), and presence of liver metastasis have been identified as negative prognostic factors in metastatic UC upon failure of platinum containing chemotherapy [131]. Another study also identified the presence of visceral metastases and low albumin levels as additional negative prognostic factors [132]. In second-line an additional negative prognostic factor has been identified in time from prior chemotherapy [133].

1.7.2 Development of molecular biomarkers

Yet, molecular biomarkers in UC are not utilised in clinical routine. Pioneering research in this field has lately been undertaken by several laboratories [21-24]. An interesting finding was a p53-like UC subtype, in which the p53 expression could predict between chemoresponsive and chemoresistant MIBC tumours [21] (see **section 1.4**). In the neoadjuvant setting, a report on molecular biomarkers in NACT treated MIBC patients (n=37) found that Glycerophosphodiester Phosphodiesterase Domain Containing 3 (*GDPD3*) and Sprouty-Related, EVH1 Domain-containing Protein 1 (*SPRED1*) gene expressions were predictive markers for GC treatment [134]. GDPD3 is involved in the metabolism of glycerol and SPRED1 down regulates the formation of lymphatic vessels via VEGFC and VEGFR3 by blocking ERK activation [135]. In the neoadjuvant setting, gene expression profiles of UC tumours have provided predictive data on response to MVAC treatment [136].

1.7.3 Liquid biopsy derived exosomes

The excretion of membrane-surrounded extracellular vesicles (EVs), e.g. microvesicles and exosomes, from different cells in the body into plasma has for cancer research generated a source of biomarkers [137-140]. Of particular interest in tumour biomarker perspectives are exosomes, vesicles <150 nm, which are generated from the cellular endosome system and in contrast to other EVs released by living tumour cells[139]. Another feature that makes exosomes attractive as a source of biomarkers is their content of protein, RNA, and to some extent DNA, which is thought to reflect the cell of origin [141,138,142]. Tumour derived exosomes (TDEs) are reported to act as communicators between the tumour and its surrounding tumour microenvironment, e.g. tumour stroma and infiltrating immune cells thereby regulating several hallmarks of tumours, including immune response and metastatic potential.

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2 Aims of the thesis

The overall aim of this thesis was to improve treatment and treatment evaluation methods in advanced and metastatic UC. Thus, focus was on evaluating novel treatments and treatment combinations with the long-term intention of decreasing disease burden and improving survival for these UC patients. Another focus of this thesis was to analyse potentially predictive and prognostic biomarkers to improve personalised treatment in advanced and metastatic UC. The specific aim for each paper was to:

- characterise a patient with metastatic UC with unexpected clinical benefit in response to treatment with sorafenib (**Paper I**).

- analyse the protein expression levels of VEGFR2, S100A4, and S100A6 in tumour specimen of cystectomy treated UC patients and correlate expression levels with survival outcome to identify possible prognostic biomarkers (**Paper II**).

- evaluate safety and to define the recommended Phase II dose of combined vinflunine and sorafenib second-line treatment in advanced and metastatic UC (**Paper III**).

- explore the potentially predictive value of early treatment response markers with 18F-FDG PET and plasma exosome analyses in advanced or metastatic UC patients treated with vinflunine plus sorafenib (**Paper IV**).

- evaluate the antitumoral effects, pharmacodynamic mechanism and potentiating ability of the novel peptidase activated alkylating agent melflufen in UC cell lines *in vitro* and analyse an enhancing peptidase, ANPEP, in UC tumour specimen and compare with survival outcome (**Paper V**).

3 Patients, material, and methods

3.1 Patient cohorts and analyses

The analysed UC patients were included at the Dept of Oncology, Karolinska University Hospital, Solna, Sweden (**Paper I-V**), Dept of Oncology, Rigshospitalet, University Hospitals of Copenhagen, Denmark (**Paper III**), and Dept of Oncology, Aarhus University Hospital, Aarhus, Denmark (**Paper III**). Two principal cohorts were used in the present thesis. In **Paper II** and **Paper V**, 83 patients with urothelial carcinoma or mixed histology with dominance of urothelial carcinoma, and who had undergone cystectomy were evaluated for putative prognostic immunohistochemical protein markers. Further, a second cohort of 22 patients with post-platinum metastatic disease with pure urothelial histology, was studied in **Paper III**, in the prospective dose-finding Phase I trial Vinsor, evaluating a novel treatment combination of vinflunine plus sorafenib. Thirteen of these patients also consented to participate in the explorative add-on study presented in **Paper IV**, which evaluated the putative predictive value of ^{18}F-FDG PET and exosomes as methods for early treatment evaluation. All preclinical and clinical studies were undertaken with approved ethical and regulatory permissions.

3.2 Immunohistochemistry

Immunohistochemistry (IHC) is an established method for examining protein expression *in situ* in tumour specimen using antibodies and was applied in **Paper I**, **II** and **V**. Prepared FFPE of UC tumour specimens were retrieved from the Biobank at Karolinska University Hospital in Solna. Primary antibodies used for the IHC were: anti-PDGFR-β (**Paper I**), anti-VEGFR2 (**Paper I**, **Paper II**), anti-S100A4 (**Paper II**), anti-S100 A6 (**Paper III**) and anti-ANPEP (**Paper V**). All antibodies apart from the anti-ANPEP were obtained from commercially sources and had previously been used for IHC on tumour specimens. The ANPEP antibody was generated in-house by immunising rabbits with a peptide towards ANPEP followed by isolation of monospecific IgG from rabbit serum (**Paper V**). All primary antibodies were tested in preparatory experiments to establish proper concentrations to be applied on the UC specimen. To visualise primary antibody binding anti-rabbit secondary antibody conjugated with biotin (Vector Labs) was applied followed by avidin-biotin peroxidase complex staining and 3,3-diaminobenzidine (DAB). DAB is in this reaction oxidized and a brown precipitate corresponding to primary antibody binding is generated in the specimen which can be visualised by light microscopy. The staining by the different primary antibodies were evaluated in a representative area of the tumour specimen as judged by the pathologists. On each slide, the number of stained cells (classified into score) and their staining intensity (negative (0), weak (1), moderate (2), and strong (3)) was evaluated by pathologists blinded to the patient characteristics. The product of score and intensity was used to dichotomise the patients into groups; low and high expression of the proteins studied (VEGFR2, S100A4, S100A6, and ANPEP) with subsequent analyses of relation to survival.

3.3 Phase I clinical trial Vinsor

In **Paper III**, a multicentre Phase I trial was set up to evaluate safety of sorafenib in addition to standard second-line therapy: vinflunine in advanced or metastatic UC. The trial was conducted at three sites within the framework of NUCOG and coordinated from the Dept of Oncology, Karolinska University Hospital, Solna.

3.3.1 Study design

The study implemented a classic stepwise dose-escalation design with 3+3 patients at each dose level with the primary endpoint to define the RPTD by evaluating safety. This practise is well described in early clinical trials and is a compromise to minimise the number of subjects exposed to unknown toxicity while keeping a steady flow of patients to find the maximum tolerated dose (MTD) of the experimental treatment combination. At each dose-step, a minimum of two and maximum of six patients will be treated. Special surveillance including overnight stay for the first patient treated within the Vinsor trial was carried out. Subsequently recruited patients were all closely monitored during the first two treatment cycles including weekly research nurse appointments. All patients were seen by a physician prior to starting every new treatment cycle. The MTD is used to define the RPTD which is one dose-step below the MTD. If at the highest planned dose level for sorafenib, no DLT is observed, then the RPTD will equal the MTD.

3.3.2 Dose-limiting toxicity

DLT was in **Paper III** defined as either a haematologic or non-haematologic toxicity.
Haematologic toxicity (according to CTCAE 4.0):
i/ grade ≥ 4 neutropenia (absolute neutrophil count <0.5 x 10^9 for ≥ 7 days or <0.1 x 10^9 for ≥ 3 days), or
ii/ febrile neutropenia of grade ≥ 3 (absolute neutrophil count <1.0 x 10^9 and temperature $\geq 38.5\degree$C), or
iii/ platelet count <25 x 10^9/L or thrombocytopenia with bleeding or requiring platelet transfusion.

Non-haematologic toxicity was defined as a DLT (based on CTCAE 4.0) if:
liver toxicity (ALAT or ASAT) of grade ≥ 3 for >7 days, or
any other grade ≥ 3 major organ toxicity.

3.3.3 Primary and secondary endpoints

The primary endpoint of the Phase I study Vinsor presented in **Paper III**, was to define the RPTD by analysing safety parameters from treatment cycles 1 and 2. The secondary endpoints included data on safety parameters from all treatment cycles, duration of overall and progression free survival, overall response rate (including disease control rate and tumour response) measured according to RECIST 1.1 at every second treatment cycle. Additionally, readouts of early ^{18}F-FDG-PET CT in relation to conventional RECIST 1.1 assessments were collected for explorative analysis presented in **Paper IV**.

3.4 CT and PET Imaging

In **Paper I**, **III**, and **IV**, CT scans at pre-scheduled time points were conducted to assess treatment response. The scans followed clinical routine protocols and included intravenous contrast for optimal visualisation of tumours. Radiologic assessment on anatomical tumour response was undertaken according to RECIST 1.1 [122-124]. In **Paper IV**, patients underwent PET CT scans after receiving glucose labelled with the radioisotope ^{18}F. This procedure was repeated after 3 weeks treatment. Examination of metabolic ^{18}F-FDG response was done according to adapted PERCIST 1.0 [143]. Raw data was extracted with the Volume Viewer for Advantage Workstation (GE Medical Systems, Milwaukee, WI, USA) and separately analysed to examine different threshold SUV$_{max}$ and SUV$_{peak}$ levels for explorative early response evaluation.
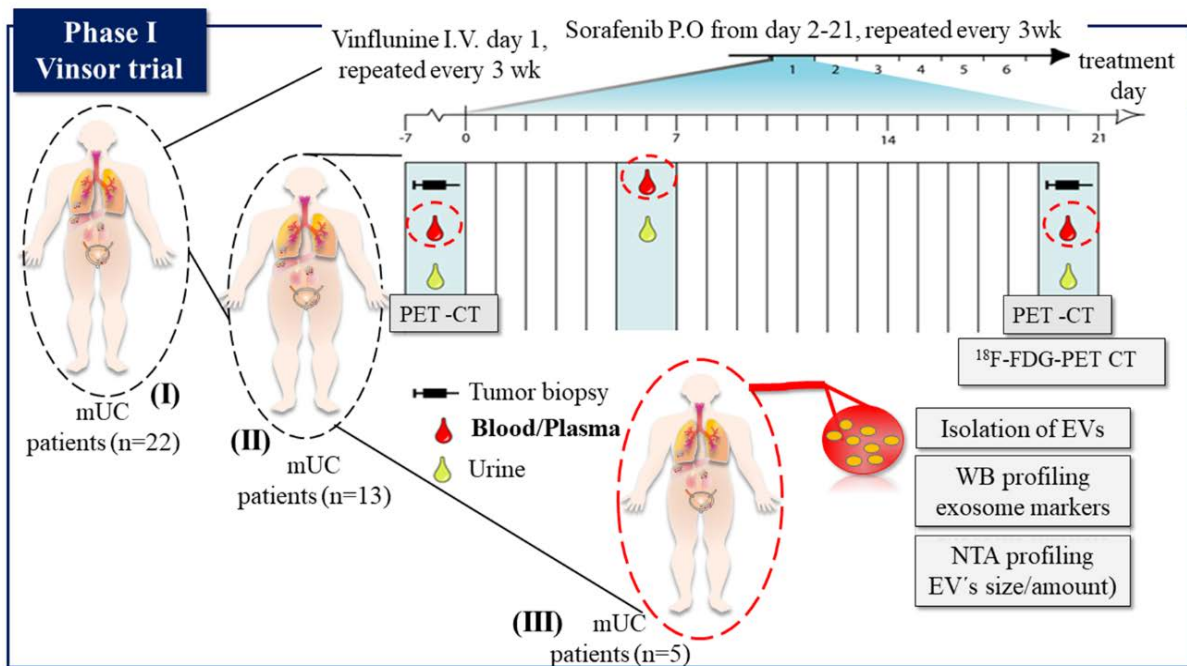
Figure 2. Experimental set up analyses of the Vinsor trial. (I). In the Phase I Vinsor trial (EudraCT Number: 2011-004289-14, NCT01844947) 22 patients with mUC were included, evaluated for safety and for establishing recommended Phase II dose of combined vinflunine and sorafenib second-line treatment (**Paper III**). (II). A subset of the Vinsor mUC patients (n=13) were analysed to explore the potentially predictive value of early treatment response markers with ^{18}F-FDG PET (**Paper IV**). Tumour and liquid biopsies (blood, plasma and urine) were taken at baseline, at day 8 and day 21 after treatment (**Paper IV**). (III). From plasma samples of some of the mUC patients (n=5) EVs were isolated at baseline, day 8 and day 21 using qEVoriginal size exclusion columns. EVs from fraction 8 were used to reveal amount EVs/ml and their using Nanoparticle Tracking Analysis. Data was analysed in relation to OS of the patients. Fraction 6-10 from the qEVoriginal size exclusion columns were applied in western blotting (WB) to verify expression of exosome markers (CD9 and CD63).

3.5   Exosome isolation and analyses

In **Paper IV** EVs were isolated from plasma from a subset of patients enrolled in the Phase I study Vinsor (**Figure 2**). For isolation of EVs from plasma either of two principal methods are used; ultracentrifugation and size exclusion chromatography both which separate out EVs of different sizes [140]. In **Paper IV** qEVoriginal size exclusion chromatography columns (Izon, Oxford, UK) was applied for isolation of EVs using 600 µL of filtered plasma. For the results presented in **Paper IV,** a fraction of 500 µL of eluting PBS was manually collected and analysed for EVs size and amount using Nanoparticle Tracking analyses (NTA) with a NS300 instrument (NanoSight, Malvern Panalytical, Malvern, UK). In NTA the sample to be analysed is put in a chamber through which a laser beam is passed. The particles in the sample will scatter light and this captured live on a 20x magnification microscope using a video camera. The particle size of the sample is presented in nm with a histogram showing the distribution into different sizes including exosomes (≤150 nm) and larger EVs [139,140]. To verify the presence of exosomes in the EV samples western blotting was carried in which two EV markers, CD9 and CD63, were probed for. The software of NTA can be used to calculate the number of particles in a fixed sample volume based on the particle movement and in **Paper IV** this generated EVs/ml which was corrected for extracted plasma volume to give EVs/ml plasma. The difference in EVs/ml of plasma was in **Paper IV** subsequently studied between the patients and during the treatment course.

3.6 Preclinical characterisation of melflufen response in UC cell lines *in vitro*

In **Paper V** melflufen and melphalan was evaluated in UC cell lines *in vitro* with respect to cell cytotoxicity, cellular accumulation/metabolism, induction of apoptosis, and capacity to alter the phosphorylation status of tumour growth regulating kinases (**Figure 8**). A brief summary of the methods is described below.

3.6.1 Cell lines and cell culture

In **Paper V** established human UC cell lines were used to study melflufen action mechanisms. The cell lines studied, which all were from obtained from American Type Culture Collection® (Manassas, VA, USA) were J82 (HTB-1™), TCC-SUP (HTB-5™), 5637 (HTB-9™), and RT4 (HTB-2™). J82 and TCC-SUP are both transitional cell carcinomas [144,145]. 5637 is a grade II carcinoma while RT4 is a transitional cell papilloma [145,146]. These cell lines have for long been used in experimental oncology research and have recently been profiled for mutations on a global scale with results presented in the Cell Strainer database: http://depmap.org/portal/cell_line. Prior to publication all cell lines were authenticated using Short-tandem repeats (STR) analyses by polymerase chain reaction (PCR). Cells were maintained in RPMI-1640 cell medium (Invitrogen, Carlsbad, CA, USA) and cultured as monolayer for the experiments.

3.6.2 Analysis of cell cytotoxicity

To assess the capacity of melflufen to induce cell death in UC cells, two different assays 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium salt bromide (MTT) assay and fluorometric micro culture cytotoxicity assay (FMCA) were used. For both assays UC cells were plated in 96-well plates at a density allowing proliferation during treatment with melflufen or melphalan (1h pulse or 72h of continuous treatment). MTT or FMCA assay was carried out at 72h after adding drug. In the MTT assay, cell viability is assessed by adding MTT solution which if cells are alive and have functional mitochondria, will be metabolised to formazan crystals that are solubilised and resulting absorbance measured in a microplate reader [147]. In the FMCA assay cell viability is monitored by addition of a fluorescein diacetate (FDA) probe which in viable cells with intact plasma membrane will be hydrolysed to fluorescein that is quantified by a microplate reader [148]. In both assays the measured absorbance is proportional to the number of viable cells and was used in **Paper V**.

3.6.3 Intracellular accumulation and metabolism of melflufen

Melflufen treatment has in other tumour cell types been demonstrated to result in rapid intracellular loading of melphalan which subsequently causes DNA damages (see section 1.5.2). Bestatin-inhibited aminopeptidases, including ANPEP, are involved in this biotransformation of melflufen to melphalan and esterases may also act on melflufen in tumour cells generating a deesterified form of melflufen [64,69]. In Paper V the biotransformation of melflufen to melphalan or deesterified form in UC cells were analysed. UC cells in suspension were exposed to melflufen or melphalan either alone or in presence of bestatin for different times (0-60 minutes). A sample was taken out and the biotransformation stopped by adding cold PBS. Proteins in the samples were precipitated by acetonitrile:ethanol and resulting supernatants taken for analyses of melflufen, melphalan or deesterified melflufen by HPLC-MS/MS. The MS spectra of the compounds was analysed, and drug concentration estimated using standards. To visualise the difference in melphalan amount after melflufen or melphalan treatment of the UC cells, the area under the curve over the entire treatment period was calculated from different biological replicates.

3.6.4 Profiling of apoptotic signalling

Induction of apoptosis is one way by which tumour cells may respond to chemotherapy. In **Paper V** apoptotic signalling after melflufen, melphalan and in some cases cisplatin treatment were analysed in UC cells by assessing apoptotic morphology of cell nuclei, cleavage of procaspase-9 into active caspase-9, cleavage of PARP1 as a measure for general caspase activity and conformational change/activation of the Bcl-2 proapoptotic proteins Bak or Bax (**Figure 8**).

Assessment of apoptotic morphology of cell nuclei

Induction of apoptotic morphology of cell nuclei in response to melflufen or melphalan in UC cells were studied by staining fixed cells with 4',6-Diamidine-2'-phenylindole dihydrochloride solution (DAPI). DAPI is a dye that binds certain regions in DNA and which can be monitored by fluorescence microscopy. In healthy cells the DNA in cell nuclei is homogenously stained by DAPI but as apoptosis starts the chromatin structure is altered and therefore DAPI will give another staining pattern of DNA with regions of bright staining (seen as dots).

Cleavage of procaspase-9 and PARP1 by western blotting

The apoptotic cascade involves activation of proteases so-called caspases which cleaves both signalling-and structural proteins which give rise to an apoptotic phenotype [149-152]. In **Paper V**, cleavage of procaspase-9 as well as PARP-1 was examined by western blotting in UC cells after melflufen- or melphalan treatment. An apoptotic response was mounted as disappearance of full-length procaspase-9 or PARP1 and appearance of shorter cleaved forms of either protein.

Analyses of proapoptotic conformation of Bax and Bax

Treatment of tumour cells with DNA damaging agents triggers apoptotic signalling via the mitochondria resulting in cytochrome release and activation of caspases [152-154]. It has been shown that DNA damages may trigger Bak and Bax to adopt a proapoptotic conformation allowing them to promote cytochrome c release from mitochondria [153,154]. In **Paper V** the pro-apoptotic conformational change of Bak and Bax after melflufen-, melphalan- or cisplatin treatment was monitored in UC cells using antibodies directed towards an N-terminal epitope in either protein only exposed in the conformational changed Bak or Bax. Primary antibody binding was visualized by a secondary FITC-conjugated antibody and the associated fluorescence signal monitored by flow cytometry.

Characterisation of alterations in kinome signalling

In **Paper V** melflufen-, melphalan-, or cisplatin induced effects on the kinome of UC cells was explored to reveal possible treatment combinations. The alterations in phosphorylation of about 40 different growth factor receptor tyrosine kinases and their downstream kinases was studied. Profiling was commenced utilising PathScan® receptor tyrosine kinase signalling antibody array (Cell Signaling Technology, Danvers, MA, USA). In this assay biotinylated antibodies recognising the phosphorylated form of the individual proteins are applied followed by a streptavidin conjugate labelled with linked fluorescence. The resulting signal was recorded on the Odyssey Sa Infrared Imaging System (LI-COR). A positive and negative control included in the assay served to normalise signal intensity and to subtract background from each sample and each protein spot on the array. To verify phosphorylation of Src, an antibody targeting the phosphorylated form p-Src Y416 was used in western blotting of UC cells treated with

melflufen, dasatinib, and a combination thereof. The generated signal from the western blot was quantified on the Odyssey Sa Infrared Imaging System after correcting for loading differences using GAPDH.

3.7   Statistical analyses

The statistical calculations included parametric tests. In **Paper II**, **III**, **IV**, and **V** the survival models were compiled with the Kaplan-Meier method and the log-rank test (Breslow) was applied to compare two or more groups [155]. The Cox proportional hazard model and the chi-squared tests were implemented to compare parameters for prediction accuracy and categorical variables, respectively, in **Paper II**. Statistical significance was defined as $p < 0.05$. All statistical analyses were performed in SPSS$^{©}$ version 25 (IBM Corp, Armonk, NY, USA) and Excel 365$^{©}$ (Microsoft Corp, Redmond, WA, USA).

4 Results & Discussion

4.1 Clinical Activity of Sorafenib in a Previously Treated Advanced Urothelial Cancer Patient (Paper I)

In the mid noughties, the newly approved TKIs sorafenib and sunitinib, turned around the treatment strategy in renal cancer [95]. For patients with adjacent tumours of the urinary tract, and with no other standardised treatment options, TKIs were an appealing avenue, nevertheless without being explored in UC until the end of that decade [156-158].

In **Paper I**, tumour specimen was analysed from a UC patient with unexpected clinical benefit following treatment with sorafenib. This patient had a platinum-progressive, locally advanced UC with lung metastases and was without any remaining approved treatment options. Sorafenib was initiated off-label at a dose of 400 mg twice daily in line with approved dose for sorafenib in renal cell carcinoma. At the first treatment evaluation a decrease in the lung metastases were observed. The shrinkage of the pulmonary metastases continued until the evaluation at 8.7 months, but at the following examination, at 10.5 months, the metastases had progressed in size (**Figure 3**). Interestingly the lung metastases, not only initially, decreased in size in response to treatment, but also showed cavitation which may indicate a more necrotic than solid tumour state. The recorded PFS for this patient exceeded the longest and median PFS in the previously reported sorafenib Phase II trials, respectively [157,158]. No studies have reported on sorafenib responses in metastatic UC patients, yet other TKIs including sunitinib and pazopanib have been shown to generate treatment benefit in selected cases [156,159]. There is however no defined tumour genotype or phenotype that has been correlated to treatment benefit following these treatments. In the present study, IHC analyses of two suggested key targets for sorafenib, VEGFR2 and PDGFR-β were analysed in the patient's tumour specimen and showed intermediate and high expression, respectively. For other diagnoses, including hepatocellular and renal cell carcinoma, it has been suggested that the expression of the expected targets for sorafenib, PDGFR-β and VEGFR2 may be correlated to treatment outcome, but this has never been clearly demonstrated [160]. The patient described in **Paper I** tolerated sorafenib well but developed hypertension and skin reaction grade 2 (CTCAE 3.0) early on. The correlation of hypertension and rash during treatment with sorafenib and other TKIs, and favourable outcome, has been reported for other malignancies [161,162].
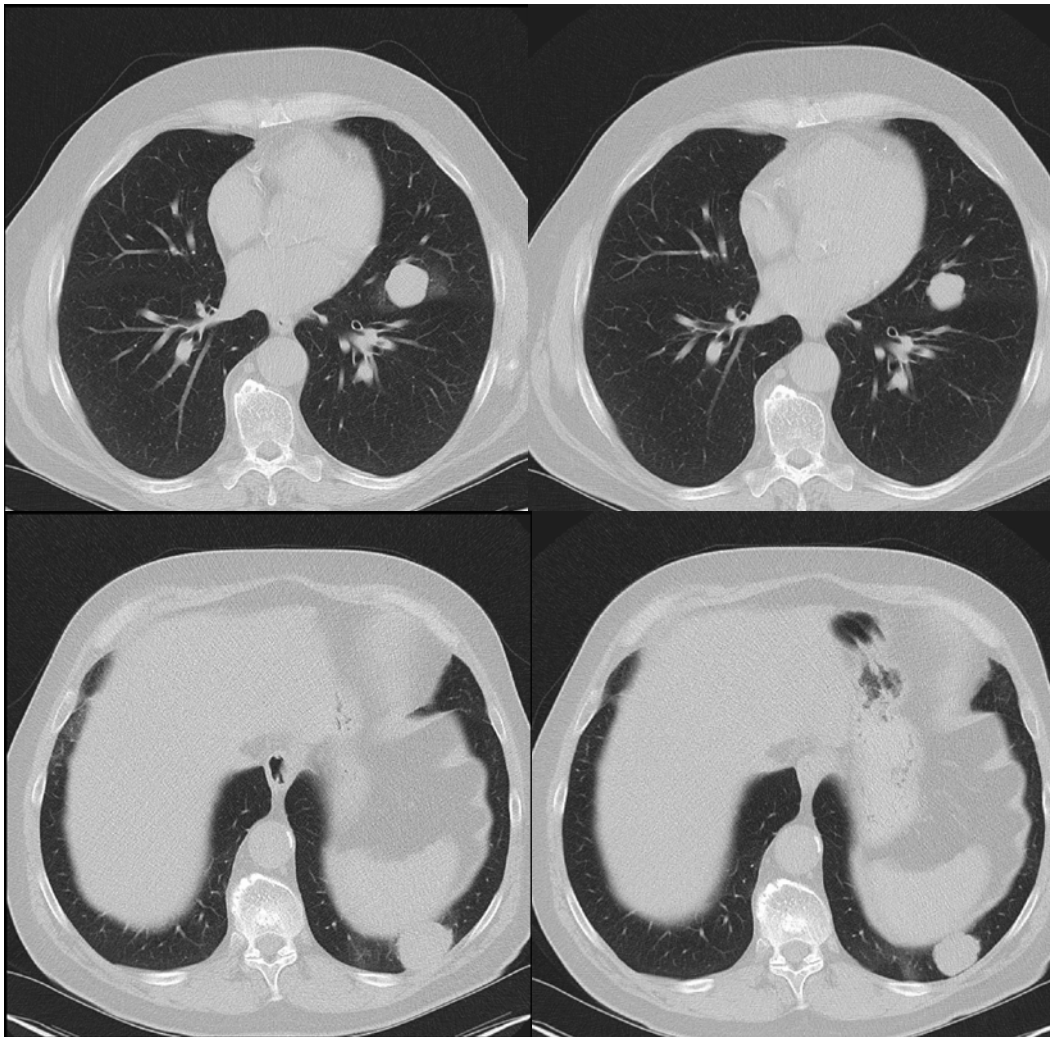
Figure 3. Computerised tomography scans demonstrating the radiological effects in response to treatment with sorafenib in a UC patient. CT images of the patient prior to treatment (left) and after 1.6 months (right) showing size reduction of lung metastases.

After 10.5 months the patient progressed on sorafenib treatment demonstrated by size increase in the lung metastases, which also again were more solid on the CT scans. Thereafter, the patient was offered further systemic treatment on-and-off with vinflunine for more than two years, in-between also receiving palliative radiotherapy.

In summary, the results in **Paper I** together with previous Phase II trials indicate that subset of patients may have clinical benefit following treatment with sorafenib [157,158]. Even though no radiological response was achieved in this patient, as defined by RECIST 1.1, it can be speculated if disease stabilisation demonstrated as size-reduction accompanied by changes in the composition of the metastases can translate into meaningful clinical benefit. Importantly, no firm conclusions can be drawn from case reports of individual patients, but the observations may still generate hypothesis which can be tested in subsequent prospective clinical trials. Following this pilot case and the subsequent approval of the vinca alkaloid vinflunine, attention was put on combining these two drugs as they have different mode of action and toxicity profiles [53,96,157].

4.2 Vascular endothelial growth factor receptor 2, but not S100A4 or S100A6, correlates with prolonged survival in advanced urothelial carcinoma (Paper II)

One in two patients with muscle-invasive UC undergoing cystectomy or local resection will inevitably relapse, pointing towards a micro-metastatic disease already at this stage [119]. In the clinical routine, the tools for predicting prognosis after the cystectomy is still, despite recent advances in tumour genome and proteome profiling, only based on anatomical and clincal features [14]. If at hand, such a set of biomarkers in tumour tissue and/or plasma could potentially be used to guide further clinical management of the patient. Adding treatments upfront in a personalised way could thereby, further improve outcome. Multiple studies have focused on prognostic biomarkers for UC. These includes markers of proliferation: Ki67, cell cycle regulators: p53, and cell death associated proteins: Bcl-2 and caspase-3 [163-166]. Focus has also been on angiogenesis, e.g. microvessel density, VEGFA, and its receptor VEGFR2 [167-170]. With respect to VEGFR2, prior results are contradictory in terms of association between VEGFR2 and clinical outcome [167,168,170]. In **Paper I**, the prognostic potential of VEGFR2 protein expression in UC tumour specimen from patients undergoing cystectomy for a muscle-invasive UC was studied with the aim to reveal if VEGFR2 could be used as a biomarker for prognosis. In tumour specimens from a cohort of 83 UC patients, VEGFR2 expression was analysed by IHC. VEGFR2 expression was mainly found in the cytoplasm of tumour and endothelial cells as illustrated in **Figure 4** (**upper panel**). The percentage of cells staining positive for VEGFR2 were generally high in all patients' specimen while the staining intensity varied from low to high.

Based on VEGFR2 expression, patients were divided into high or low groups with a cut-off at moderate intensity and high score ($\geq$75% of cells stained) (**Figure 4**, **lower panel**). Significantly longer OS (p = 0.014) was found in patients with high VEGFR2 tumour expression. An association between high VEGFR2 and outcome was also confirmed by applying Cox proportional hazards regression model, which further revealed that VEGFR2 expression within UC tumour specimen was an independent variable associated with OS (p = 0.046) and DFS (p = 0.04). These results are thus in line with findings of VEGFR2 expression in UC specimens retrieved by TURB from 114 patients and who observed increased OS in patients with >50% of UC cells expressing VEGFR2 [167].

In endothelial cells, VEGFR2 is known to be expressed on the cell surface and control endothelial proliferation, permeability, migration/invasion, and in the context of tumours enabling angiogenesis [171]. In the present study, VEGFR2 expression was in most of the cases only in the cytoplasm of UC tumour cells. This result is to some extent puzzling, yet it has been shown that VEGFR2 may, as consequence of alternative splicing, generate a soluble form of VEGFR2 that can, when secreted, act as a suppressor of lymphatic vessel generation [172]. One could speculate that the observed VEGFR2 expression in UC tumour specimen may in fact act as suppressor of lymphatic vessel formation and thereby influencing metastatic potential.
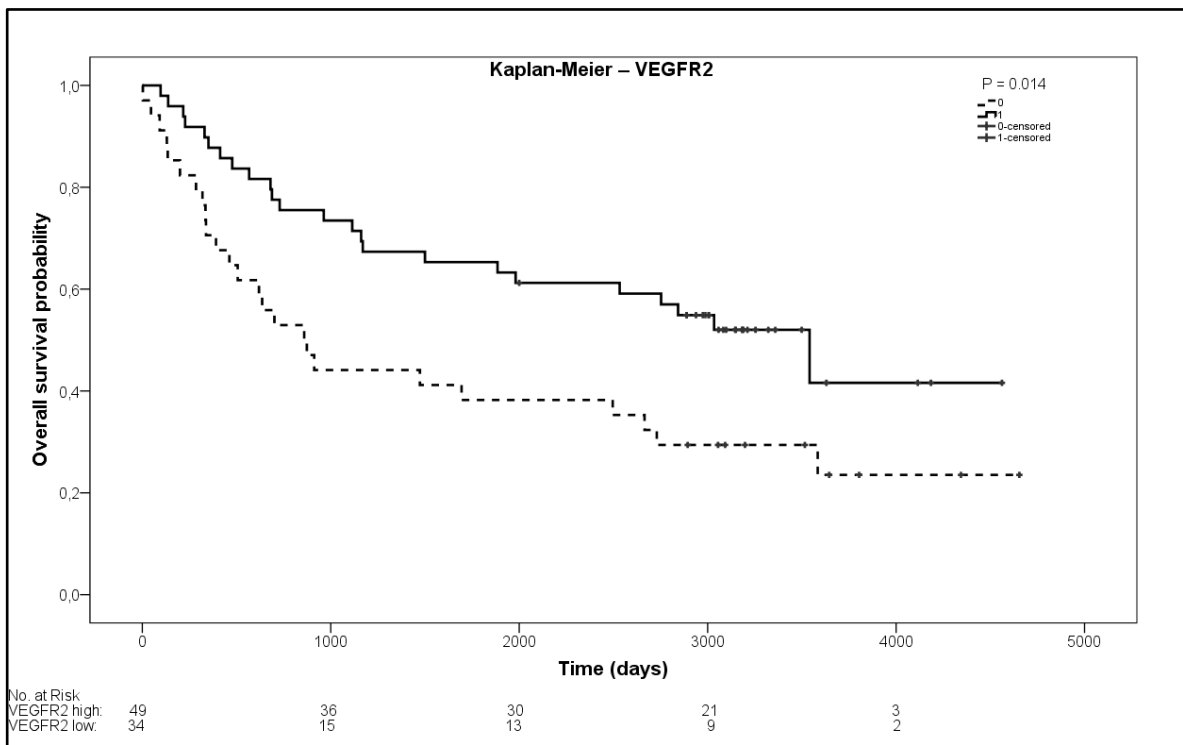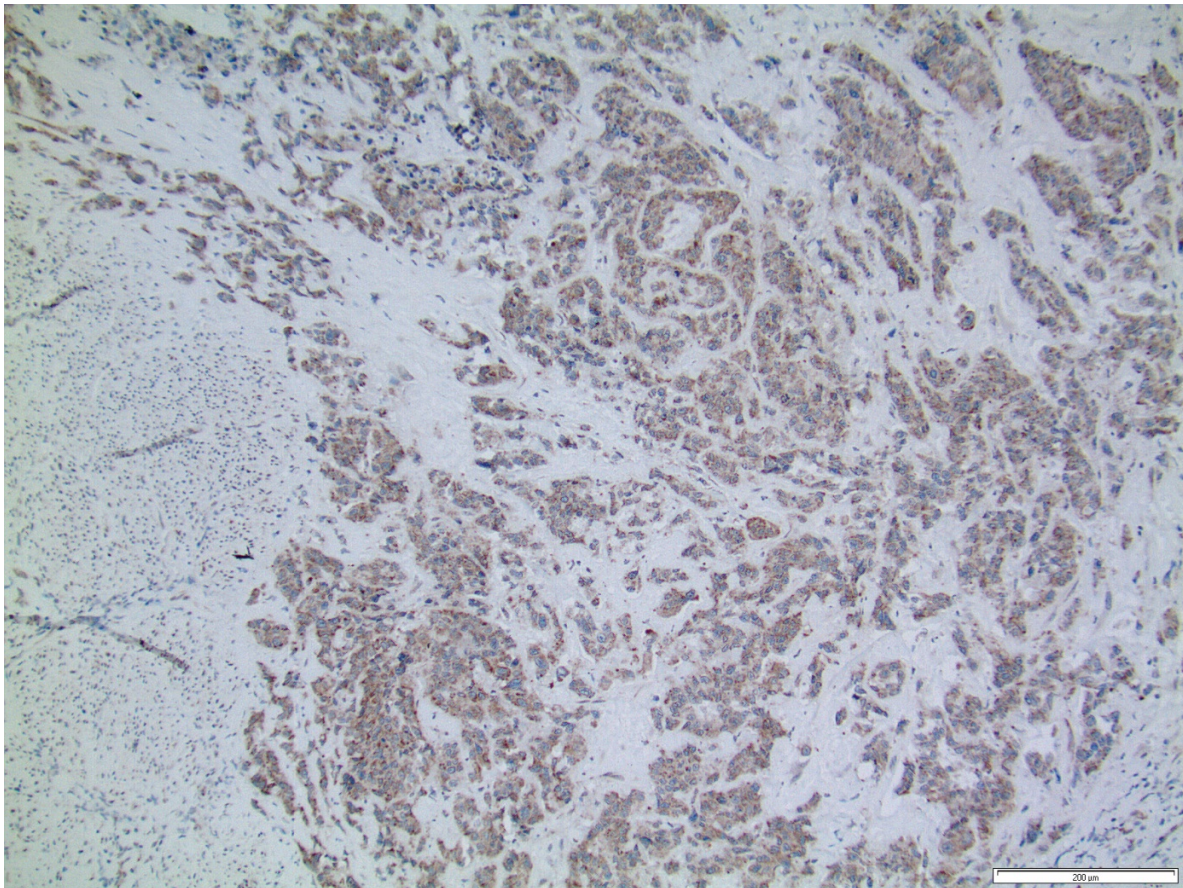
Figure 4. VEGFR2 expression in UC tumour specimen and association with overall survival. Immunohistochemistry (**upper panel**). Example of high VEGFR2 staining in UC specimen with high expression. Kaplan-Meier survival plot (**lower panel**). VEGFR2 expression was analysed in relation to OS probability dividing the cohort into low or high staining (by combined IHC score and intensity). Data presented from the publication with permission from the publisher.

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In **Paper II** the prognostic value of the calcium-binding S100A proteins, S100A4 and S100A6, was also explored in the same UC patient cohort. The S100A proteins are known to control multiple hallmarks of tumours, including proliferation, differentiation, apoptosis and metastasis [173,174]. In UC, S100A4 has been linked to metastasis and poor outcome [175-178]. The analyses of S100A4 and S100A6 by IHC in **Paper II** of UC tumour specimen, revealed expression of both proteins in the cytosol of the specimen with some cases also showing nuclear localisation. Overall, the score (proportion of S100A4 and S100A6 positive cells) was high (median >75%), while signal intensity differed among the cases. Analysing a possible correlation between OS or DFS and S100A4 or S100A6 staining from the date of cystectomy did however not reveal any significant correlation ($p > 0.05$). Neither S100A4 nor S100A6 showed an association to clinical outcome (measured from the date of cystectomy). The results in **Paper II** are in disparity with a large retrospective analysis on a bladder localised UC (n=315 patients) which reported that S100A4 was significantly higher expressed in UC tumours of patients with lymph node or distant metastasis [179]. The same study also identified S100A4 to be a prognostic marker for survival, with significantly shorter OS among patients with high S100A4 expression in UC cells. No studies have so far analysed S100A6 in UC specimen. However, in lung squamous cell carcinoma (n=177) a high S100A6 expression was correlated with lower OS [180]. In sarcoma patients (n=50) a high expression of S100A6 was related with less disseminated disease [181]. In pancreatic tumours a high nuclear staining of S100A6 was associated with shorter OS [182]. S100A6 has also been evaluated as a serum biomarker for UC [183]. It was shown that higher serum levels of S100A6 in muscle invasive UC patients, relative to non-muscle invasive UC patients or healthy controls, indicating a putative role as a non-invasive biomarker.

In **Paper II** single biomarker expression by IHC has been explored, yet the path ahead is more likely global profiling of the genomic makeup of the individual tumour. The potential of such approaches is illustrated by both The Cancer Genome Atlas project (Cancer Genome Atlas research) and by studies of gene expression. Results from these studies have enabled sub group classification of UC thereby identifying signalling networks that could be targeted by small molecules with improved efficacy either alone or in combination with chemotherapy [184,185]. With respect to VEGFR2 expression, further analyses in context of the identified UC subgroups are warranted as it could identify patients that would benefit more on VEGFR2 targeting therapies, possibly including sorafenib.

4.3 Safety and Activity of Sorafenib in Addition to Vinflunine in Post-Platinum Metastatic Urothelial Carcinoma (Vinsor): Phase I Trial (Paper III)

Paper III describes the result of the academic prospective Phase I trial Vinsor trial (EudraCT Number: 2011-004289-14, NCT01844947) in which the safety of the combined therapy of vinflunine plus sorafenib in previously platinum-treated metastatic UC patients was evaluated. The trial included 22 subjects between April, 2012, and September, 2017. The primary endpoint was to define the RPTD of the treatment combination of vinflunine plus sorafenib by evaluating safety parameters during treatment cycles 1 and 2.

The MTD of vinflunine 280 mg/m^2 day 1 and sorafenib on days 2-21 Q3W, was sorafenib 600 mg (200 mg+ 400 mg). The DLTs presented in this cohort included febrile neutropenia (three patients, grade 3 (x2) and 4) and neutropenia (one patient, grade 4). All but one DLT in this cohort occurred at the highest dose level of sorafenib, 800 mg. Hence, for patients aged 75-80 years, or PS 1, or previous radiotherapy to the lower pelvic region, or with reduced renal function (creatinine clearance 40-60 ml/min) but adequate hepatic function, the RPTD was vinflunine 280 mg/m^2 day 1 and sorafenib 400 mg (200 + 200 mg) on days 2-21 Q3W.

The MTD of vinflunine 320 mg/m^2 day 1 and sorafenib on days 2-21 Q3W, could not be defined since three out of five patients had a DLT in the first dose cohort (sorafenib 400 mg). The observed DLTs were febrile neutropenia (two patients, grade 3 and 4) and hypertension (one patient, grade 3). Hence, for patients treated with vinflunine, 320 mg/m^2 adding sorafenib, even at a dose of 400 mg daily, resulted in intolerable toxicity.

Only two of the seven DLTs recorded resulted in permanent study treatment withdrawal: one patient had a DLT at cycle 1 day 3 (hypertension, grade 3) and one patient at cycle 1 day 7 (neutropenia, grade 4). In total, five patients discontinued treatment during cycle 1.

The most frequently reported side-effect was fatigue (16 patients/80%); including two grade 3. The second overall most frequent toxicity was constipation (13 patients/60%); however, none above grade 2. The most frequently observed grade $\geq$3 toxicities were neutropenia (six patients), febrile neutropenia (five patients), and hyponatremia (five patients). No grade 5 side-effects were recorded. Clinical side effects including hypertension and rash have been suggested to be correlated to treatment benefit in response to TKIs, by others as well as in **Paper I** but these adverse events were not more frequent in subjects responding to therapy in the present study [186,187].

The secondary endpoints were survival and response outcome. The ORR among the patients for whom efficacy could be evaluated was 41% (7 of 17 patients), all being partial responses by RECIST 1.1. Disease control rate (DCR) was 71% (12 of 17 patients). The measured changes in target tumour lesions over time are presented in **Figure 5**.

Median study treatment was 4.1 months (0.1-14.5) among the DLT evaluable patients. Including all patients, the median OS was 7.0 months (1.8-41.7) and PFS was 4.5 months (1.2-16.1). The validated baseline prognostic factors for second-line treatment: PS, liver metastasis, and anaemia, were analysed for impact on survival outcome [131]. No statistically significant divergence in OS or PFS based on number of prognostic factors at baseline was found among the patients in this trial.
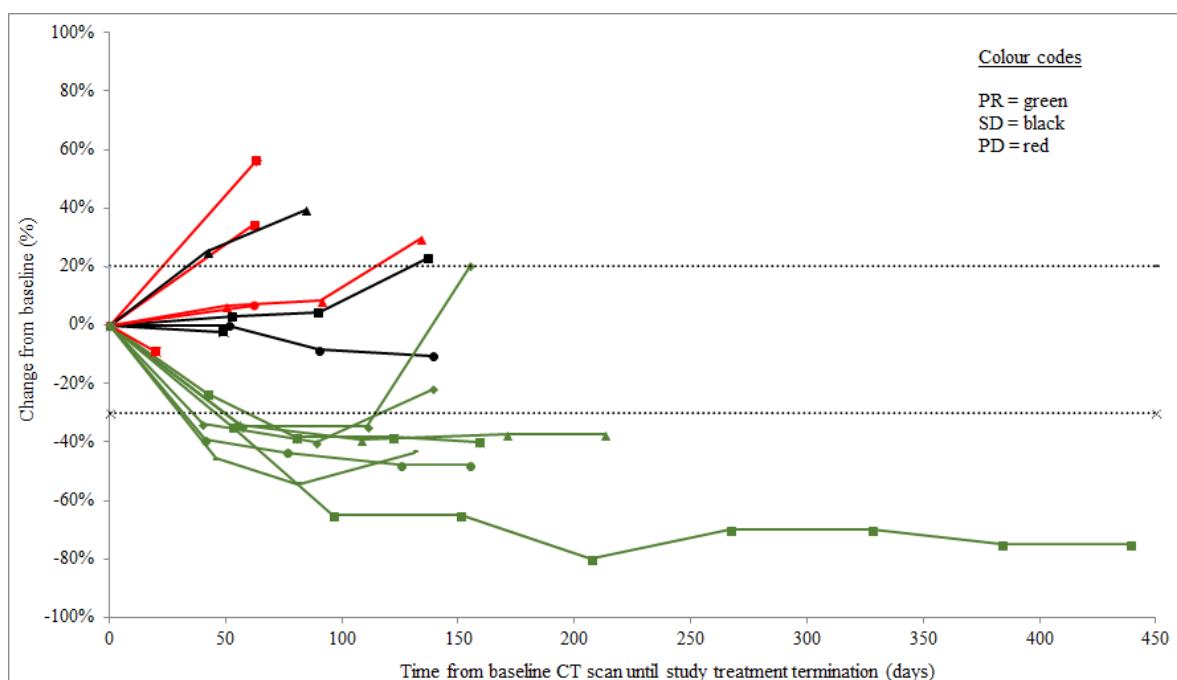
Figure 5. Visualisation of tumour response in the Vinsor study. The presented spider plot shows measured changes in target tumour lesions over time. Most of the treated patients within the Vinsor study had a size reduction of the target lesions in response to treatment; 41% of the patients fulfilled RECIST 1.1 for being partial responders. Colour codes: **PR** (partial regression), **SD** (stable disease), PD (progressive disease).

In the present study sorafenib was selected to be combined with vinflunine based on different action mechanisms, namely inhibition of angiogenesis and microtubule formation, respectively. The combination of sorafenib with vinflunine was also applied based on the potentially compatible toxicity profiles of the two drugs and the reports of individual patients responding to sorafenib ([100]; **Paper I**). The outcome of the Vinsor trial, in which a RPTD was defined with vinflunine 280 mg/m^2 and sorafenib 400 mg, provides a safe foundation for further clinical evaluation in metastatic UC patients.

Several previous trials of vinflunine doublets, including pazopanib, pemetrexed, and erlotinib have all terminated prematurely because of unacceptable toxicity (mainly neutropenia, thrombocytopenia, and fatigue) [55,57,188]. Only one study has reported acceptable toxicity with combined vinflunine *per os* and TKI; erlotinib with oral administration of vinflunine in second-line or beyond therapy of lung cancer [189]. Sorafenib has however been evaluated in combination with chemotherapies including gemcitabine and paclitaxel in platinum-resistant UC patients in which a single response (partial) in twenty treated patients was evident [190].

The ORR of 41% observed in the present Vinsor trial compares favourably with the ORR of 8.6%-18% in the efficacy evaluating Phase II and III studies of vinflunine in metastatic UC [51-53]. Neither of two reported Phase II trials on sorafenib monotherapy in metastatic UC (n=27 and n=20) demonstrated any treatment responses [157,158]. Though, the confirmed treatment responses in the present study did not translate into longer OS or PFS. This outcome is similar with previous vinflunine trials [53,157,158]. It can however be discussed whether a tumour response still may generate a clinically meaningful palliative benefit for the responding patients.

In summary, **Paper III** reports a RPTD for the treatment combination of sorafenib plus vinflunine in UC patients with early post-platinum progressive generalised disease. An additive anti-tumoral effect for this combination is plausible although the number of treated patients in the present study was too small to draw a definite conclusion on efficacy. Future randomised

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studies are needed to evaluate vinflunine and sorafenib in this setting alongside with approaches to identify biomarkers for treatment benefit.

4.4 Early Evaluation of Vinflunine and Sorafenib Treatment Responses in Metastatic Urothelial Cancer Patients by Use of Explorative ^{18}F-FDG PET CT and Plasma Exosome Analyses (Paper IV)

Early treatment evaluation approaches which can separate patients that benefit from the given treatment from non-responders, remains an unmet medical need for patients with late-stage, metastatic UC. In **Paper IV** the putative predictive value of early response assessment of combined vinflunine plus sorafenib treatment by use of early ^{18}F-FDG-PET CT scans and longitudinal plasma exosome analyses (**Figure 2**). From the Vinsor trial (**Paper III**) thirteen patients that had consented to participate were included. These patients were analysed with ^{18}F-FDG-PET CT scans at baseline and prior to initiate treatment cycle two (= three weeks later). The metastatic lesions observed in these patients reflected the typical pattern of metastasis seen in patients with advanced UC with distribution to lymph nodes, visceral organs including lungs, adrenal glands and bone. Patients were dichotomised into partial responders and non-responders based on changes in maximal ^{18}F-FDG PET calculated SUV_{max} and SUV_{peak} in tumour lesions in comparison to the baseline examination in which various thresholds were tested. Patients with a SUV_{max} and SUV_{peak} decline of ≥10% after one cycle of treatment had a significant longer OS than patients with <10% SUV_{max} or SUV_{peak} reduction at the same time point (p = 0.039). Metabolic response on ^{18}F-FDG PET at 3 weeks, by use of this adapted threshold, could predict best response by RECIST 1.1 in 11 out of 13 patients as seen in **Figure 6**.
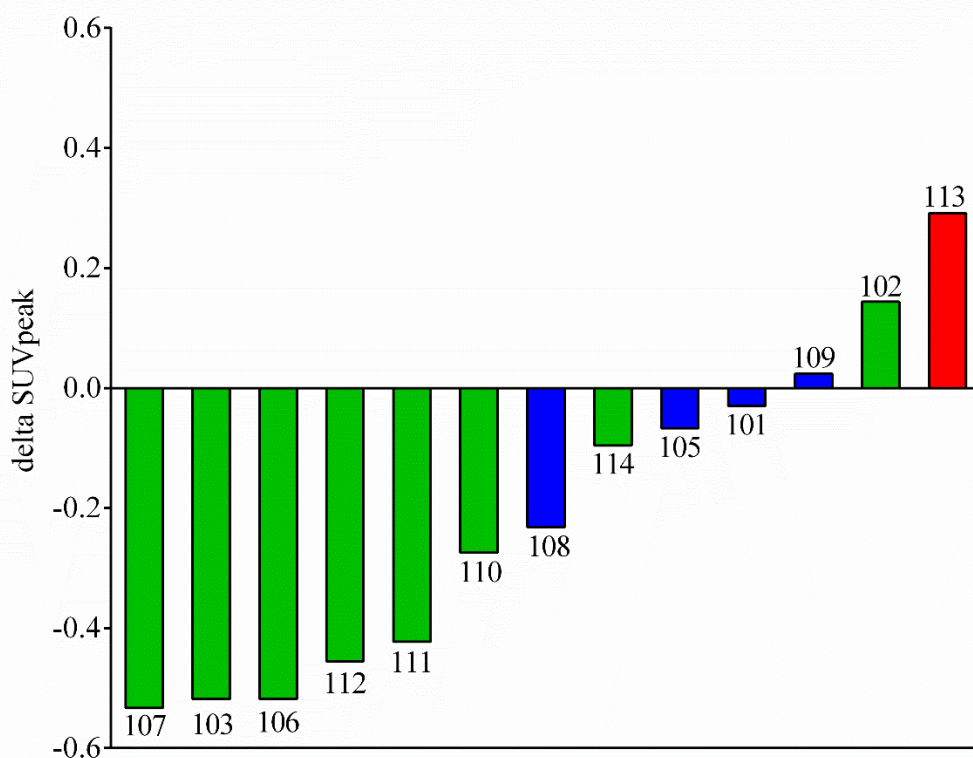

Figure 6. Explorative early treatment evaluation by use of ^{18}F-FDG-PET CT after one treatment cycle of combined sorafenib and vinflunine treatment in UC patients. Relative changes in delta SUV_{peak} in comparison to the baseline values are shown for each patient. The corresponding best response as assessed by conventional CT and RECIST 1.1 is shown with the following colour codes: partial regression (green), stable disease (blue), progressive disease (red).

On the contrary, and for unclear reasons, there was no significant correlation between metabolic response and PFS was observed. It is reasonable to anticipate a time-dependent dynamic process of tumour metabolic activity and response, why early evaluations may need modified thresholds for defining treatment response. By use of a similar approach, with the aim of optimising the threshold for defining response and OS, a 10 % reduction of the sum of long axis diameter evaluated by CT, was found to be superior to standard RECIST criteria in UC patients treated with vinflunine in monotherapy [191]. The potential value of early ^{18}F-FDG PET/CT assessment during treatment with TKIs and chemotherapy has been demonstrated in other cancer diagnoses including renal and colorectal [192,193]. If other PET tracers, such as ^{11}C-choline and ^{18}F-FLT can further improve this early treatment evaluation approach for patients with metastatic UC, remains unclear but should be explored. Similarly, the optimal timing for the early ^{18}F-FDG-PET CT evaluation during the treatment course needs to be addressed in future studies.

Further, in **Paper IV**, isolated EVs (at baseline, day 8 and day 21) from plasma samples of five UC patients in the Vinsor trial (**Paper III**) with short or long survival were analysed (**Figure 2**). NTA demonstrated that all plasma samples from the UC patients analysed contained EVs with sizes 100-120 nm, in line with earlier reported exosome sizes in other tumour types [138,194,195]. One analysed patient had EVs of a larger size (200-350 nm). Western blot analyses of these samples confirmed the expression of at least one of the exosome markers CD9 and CD63, yet with no consistent variation seen during the treatment course. To reveal if differences in concentration of exosome-sized EVs in plasma could be linked to treatment outcome, NTA was used to quantify the number of EVs in a defined volume of plasma. Results showed that the level of EVs at baseline differed between the analysed subjects, ranging from $\sim 2 \times 10^9$ to $\sim 2 \times 10^{10}$. There was also a clear alteration in amount of EVs during the treatment course, and at day 8 four out of five patients showed increased level of plasma EVs which was not linked to treatment outcome (**Figure 7**).

As the exosome isolation method did not separate tumour specific exosomes from exosomes generated from other cells it may well be so that exosomes from infiltrating immune cells and/or other normal cells also contributed to the observed alterations. Further analyses should use tumour and/or immune specific surface markers to sort out subsets of exosomes on which global miRNA or protein profiling could be applied to reveal individualised biomarkers for treatment monitoring. Global miRNA profiling of exosome from UC patients has already been demonstrated to be feasible and a biomarker subset linked to non-invasive UC and metastatic UC have been reported [137,196]. Thus, focusing on these miRNAs in the context of treatment with combined vinflunine and sorafenib could be one way forward. Interestingly, for one of the biomarkers analysed in **Paper II**, S100A4, it has been demonstrated that exosomes isolated from UC cells *in vitro* and urine from C patients express this protein. Besides, adding such exosomes to UC cells promoted their epithelial to mesenchymal transition process [197]. Another path forward could therefore be to profile for S100A4 and associated EMT signalling of the plasma derived exosomes from UC patient plasma in the Vinsor trial [198].
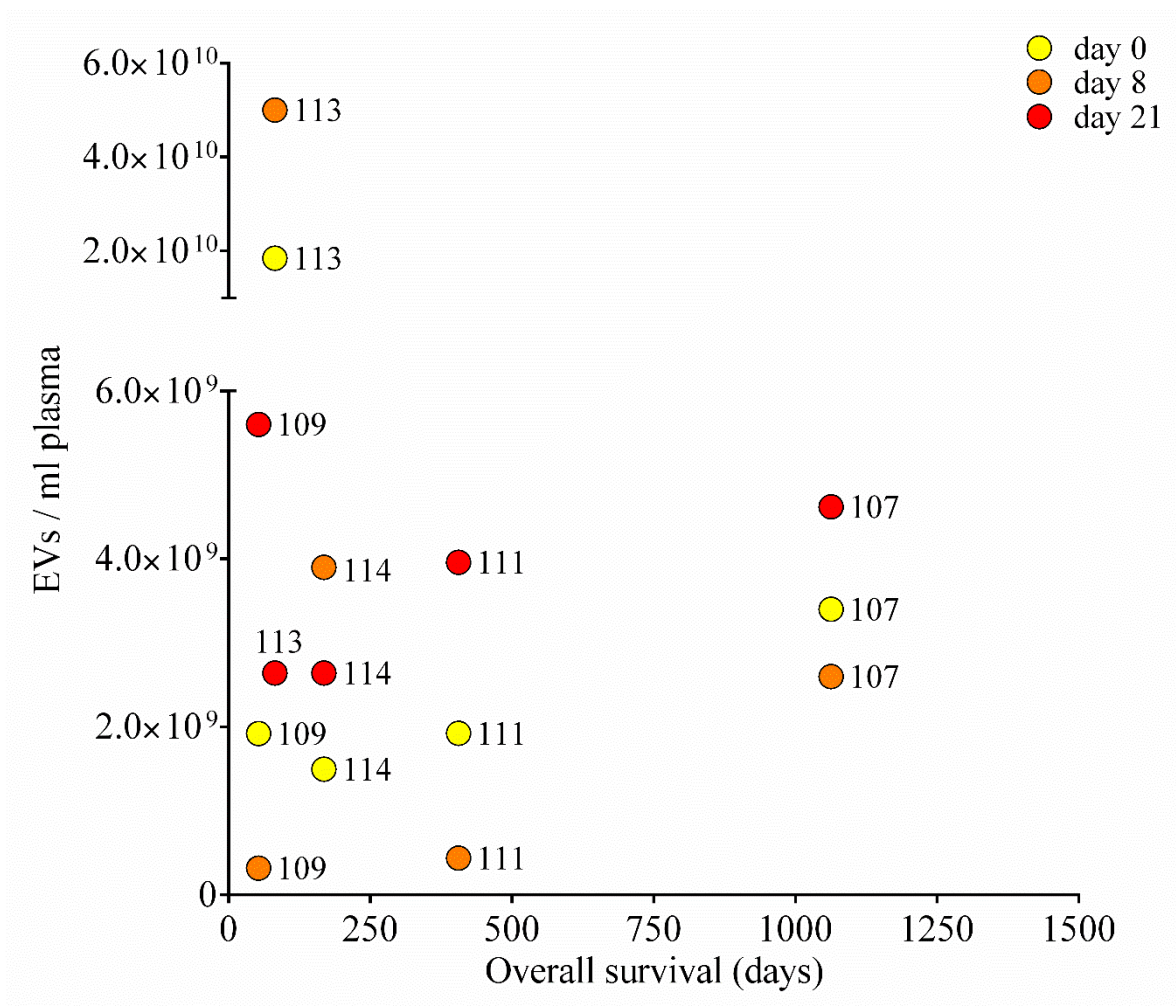
Figure 7. Analysis of EVs from patients with metastatic UC treated within the Vinsor trial. Plasma samples were taken from UC patients with short (109, 113) or long (107, 111, 114) treatment response and EVs were isolated by size exclusion chromatography. Isolated EVs were analysed with respect to counts per ml plasma at baseline (day 0), and during treatment (day 8, day 21). Results are shown in relation to OS of each patients.

4.5 Melphalan-flufenamide is cytotoxic and potentiates treatment with chemotherapy and the Src inhibitor dasatinib in urothelial carcinoma (Paper V)

In **Paper V** the aim was to analyse if the peptidase enhanced alkylating agent melflufen which has demonstrated efficacy in solid tumours and haematological malignant cells (**see section 1.5.2**) could be a putative chemotherapy for UC. For that purpose, the effect of melflufen on UC cell lines *in vitro* was examined. The results of **Paper V** are summarised in **Figure 8**.
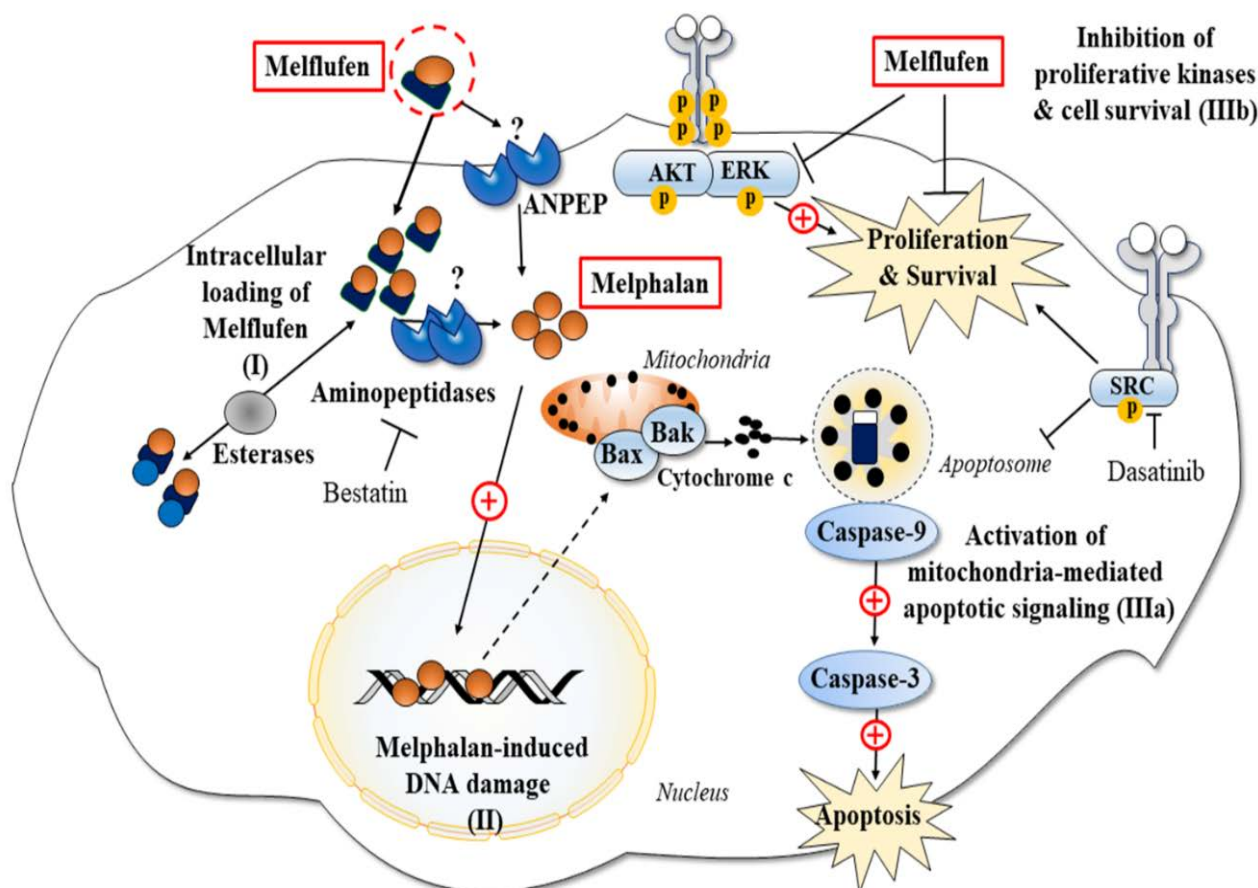
Figure 8. Melflufen-induced signalling in UC cells *in vitro*. Melflufen treatment of UC cells results in intracellular accumulation of intact melflufen which either may be deesterified by esterases cleaved by aminopeptidases in cytosol to generate melphalan (I). Inhibition of aminopeptidases by bestatin reduces melphalan accumulation and partially blocks melflufen-induced cell death. Melphalan may in theory encounter ANPEP expressed on the cell surface (I). Free melphalan alkylates the DNA resulting in DNA damage (II). The subsequent DNA damages trigger activation of Bak/Bax, release of cytochrome c and subsequent caspase-9/3 activation and apoptotic morphology (IIIa). Signals from the melphalan-induced DNA damage may block growth factor receptor-regulated kinases and proliferative signalling (IIIb). Phosphorylated Src still remains after melflufen-treatment of UC cells and promotes cell survival. Treatment with dasatinib combined with melflufen blocks Src phosphorylation and increases cell death signalling.

Profiling of melflufen cytotoxicity and induction of apoptosis in UC cells revealed higher degree of cell death compared to melphalan. Thus, melflufen activated pro-apoptotic Bcl-2 proteins Bak and Bax, followed by cleavage of pro-caspase-9/PARP-1 resulting in apoptotic nuclear morphology.

Combined treatment of melflufen and cisplatin or gemcitabine demonstrated additive cytotoxic effects with increased cell death in line with published data on other tumour types[64,67]. For gemcitabine and cisplatin, the additive effect when combined with melflufen may directly be attributed to inhibition of DNA repair processes required for repair of melphalan-inflicted DNA damages, e.g. inhibition of DNA polymerase by gemcitabine, or alteration of cell cycle distribution by cisplatin [199-201]. The additive effect could also be a result of blockade of growth factor survival kinases of either agent, tilting the UC cells towards apoptosis.

To further understand possible effects of melflufen on the UC cell signalome as well as to reveal novel treatment combinations PathScan RTK signalling antibody array was applied. Multiple growth factor receptors previously linked to UC and/or sorafenib response, e.g. PDGFR, FGFR3 and c-Kit, showed reduced phosphorylation after melflufen treatment [26,198]. In line with previous results from cisplatin-treated UC cells, phosphorylation of Src remained high after melflufen treatment [202]. By adding dasatinib, a Src-family kinase inhibitor, a reduction in Src phosphorylation was evident and when combined with melflufen a significant increased cell death seen. Interestingly, a Phase I clinical trial of dasatinib in a neoadjuvant setting of UC and prior to cystectomy, was successfully conducted and the primary endpoint (feasibility) reached [78]. Albeit the metastatic UC tumour specimen in that study showed reduced phosphorylation of Src after dasatinib exposure, this monotherapy did not reduce tumour cell proliferation or induced apoptosis. Nevertheless, it illustrates that Src may be a target that could be further explored in mUC for chemotherapy sensitisation.

Melflufen results in melphalan loading in tumour cells of different origin and that is partly controlled by bestatin-blocked aminopeptidases due to peptidase activating in the tumour [58,62,64,73]. In **Paper V** it was similarly demonstrated that exposure of UC cells to melflufen results in a time dependent increase of melphalan. Importantly, comparing melflufen with melphalan revealed a ~20-fold higher accumulation of melphalan after adding melflufen to UC cells, illustrating that the concept of peptidase enhancement also works in UC cells [58,64,73]. There was also some intact melflufen as well as a deesterified form observed in UC cells which decreased over time accompanied by increased level of melphalan, suggesting that there are maybe peptidases with different efficiency or kinetics which act on melflufen in UC cells. To verify that aminopeptidases were involved in the biotransformation of melflufen to melphalan in UC cells, bestatin, an inhibitor of multiple aminopeptidases was applied. A partial reduction (~40%) of melphalan accumulation after melflufen exposure was found and a 50% inhibition of melflufen-induced cytotoxicity was recorded. These results demonstrate that aminopeptidases are involved in the biotransformation of melphalan from melflufen in UC cells. In other cell systems, a critical role for ANPEP has been observed. [58,64,73]. It remains however to be elucidated, which aminopeptidases that are operative on melflufen in UC cells. Interestingly, a recent report showed that bestatin (Ubenimex), an inhibitor of ANPEP and likely of other aminopeptidases, may *per se* inhibit UC cell viability and migration/invasion capacity illustrating that targeting ANPEP may also be therapeutic way for UC [203].

ANPEP shows deregulation in tumours and may be used for imaging or therapy purposes (**see section 1.5.2**). ANPEP expression was therefore analysed in tumour specimen (n=83) from the same UC patient cohort used in **Paper II**. Results showed that all, but one case, were positive for ANPEP and had a higher expression in tumour relative to non-tumour tissue. ANPEP was mainly localised to the tumour cells *per se* and not to the stroma as previously been shown in other studies [74,75]. Both ANPEP staining intensity, as well as the %-positive cells varied between the UC specimen with most of the cases (n=77) having 76-100% positive cells. The staining was both cytosolic and membranous in 20 cases, only cytosolic in 60 cases while two cases showed only membranous staining. ANPEP expression was subsequently analysed in relation to survival of the patients by dividing the cohort into high ANPEP (histoscore >5) or low ANPEP (histoscore< 5) expression. It was demonstrated that patients with high ANPEP

expression had a longer OS (p = 0.02; median OS 8.1 years vs 3.2 years; mean OS 7.9 years vs 5.7 years) while no difference in DFS was found. ANPEP has in some tumour types been linked to poor prognosis but in others not been linked to outcome at all [70,73,75]. In **Paper V** it is demonstrated that high ANPEP expression may be correlated to favourable OS, which also has been shown in gastric carcinoma and prostate cancer [204] [205]. Given the recent subgroup classification of UC tumours by genomic methods it would be appealing to test if ANPEP expression is linked to a certain subclass of UC, as it may for the future guide therapies [184,185].

In summary, **Paper V** shows that melflufen may be a novel therapy for UC either alone but more likely combined with conventional chemotherapy or Src blockade. Guiding of ANPEP in UC specimen suggest ANPEP to be a putative biomarker for directing melflufen-based therapies in UC.

5 Conclusions

In **Paper I** the presented clinical data indicated that a benefit may be achieved with sorafenib in metastatic UC even though no definitive response as defined by RECIST was observed. IHC expression profiling of key targets for sorafenib, VEGFR2 and PDGFR-β, may be of relevance for treatment gain but prospective and properly sized biomarker-driven clinical trials are needed to verify this observation in the context of other possible biomarkers.

In **Paper II**, low VEGFR2 expression was associated with the risk of disease relapse and shorter survival in UC patients, treated by cystectomy. Hence, if the prognostic property of VEGFR2 can be verified, this protein may hold potential as a biomarker. Pre-cystectomy S100A6 expression differed among patients surviving beyond 18 months, suggesting that this protein may intervene in the development of micro-metastasis. Further studies are warranted of VEGFR2 including re-evaluation of S100A4 and S100A6 to confirm their prognostic value to UC patients.

In **Paper III** the Phase I trial Vinsor determined the RPTD for the treatment combination of vinflunine plus sorafenib in patients (n = 22) with post-platinum progressive disease; vinflunine 280 mg/m^2 day 1 and sorafenib 400 mg (200 + 200 mg) on days 2-21 Q3W. Combining vinflunine 320 mg/m^2 with sorafenib was too toxic. Side-effects were manageable and expected but with higher incidence of hyponatremia than previously reported. The ORR to this treatment combination was 41% and the median OS was 7.0 months.

In **Paper IV** it was demonstrated that patients on combined treatment with vinflunine and sorafenib, can be evaluated for early treatment response by use of ^{18}F-FDG-PET. Here a modified response-threshold of PERCIST ($\geq$10% SUV$_{max}$ or SUV$_{peak}$ reduction) could predict treatment response and was correlated to OS. Plasma derived exosomes with intrapatient differences in absolute numbers during treatment were found, but with no clear correlation to outcome in a subset of patients.

In **Paper V** the peptidase-activated alkylating agent melflufen was for the first time reported to induce cytotoxicity in UC cell lines. It was demonstrated that melflufen could be combined with standard chemotherapy but also that a blockade of Src offers an alternative combination regimen. The pharmacodynamic profiling of melflufen revealed a higher concentration of melphalan in UC cells, involving activity of aminopeptidases. One of these peptidases, ANPEP was characterised in UC clinical specimens and expression was associated with improved clinical outcome.

6 Future perspectives

Advanced and metastatic UC was the focus of this thesis. Management of this disease in the palliative setting requires careful consideration of the best treatment strategy for each patient [14]. Up till now, the optimal therapy decisions have been based on general evidence, with few clinical and no genetic or phenotype parameters in the decision models [206]. The purpose of this thesis was to address these shortcomings by exploring potential prognostic and predictive biomarkers along with novel treatments and imaging technology.

As more treatment options emerge in late stage UC, prognostic and predictive biomarkers for treatment response will, for certain, have an increased impact on the selection of therapy. Learning from the collected data in The Cancer Genome Atlas, attention is focusing on delivering precise and tailored drug candidates in UC [207]. Examples are emerging of trials in advanced UC prospectively analysing the predictive value of biomarkers, e.g. PDL1 and tumour mutation burden in the neoadjuvant setting with pembrolizumab [38]. Prospective trials in metastatic UC may include platform studies, as new drug classes with different pharmacodynamic principles are added to chemotherapy.

Following the results of the Phase I trial Vinsor (**Paper III**), the next reasonable goal is to set up a randomised study to evaluate the additive efficacy value of sorafenib to standard second-line chemotherapy. Clinical research on developing chemotherapy regimens with targeted therapy along with immunotherapies will remain a key area for identifying future regimens in second-line and beyond. Monotherapy with PD1/PDL1 inhibitors have reported response rates of up to 24% in second-line, which is impressive but leaves room for improvement in overall response outcome [208]. In a recently reported Phase I/II study in metastatic UC patients treated with combined PD1 and CTLA-4 inhibitors, subgroup stratification based on PDL1 expression found a response rate of 58% in patients with high PDL1 expressing UC tumours [209]. Worth noting are the recently enforced limitations to the treatment indications sanctioned by EMA for atezolizumab and pembrolizumab. These drugs are presently only approved in UC patients unfit for cisplatin-based therapy with PDL1 score ≥5% or PDL1 combined positive score ≥10, respectively [83,85]. This decision to curb the indications of immunotherapies reinstitutes chemotherapies, most notably carboplatin, as the drug of choice for treatment combinations. This will keep the field open to more interesting combinations with chemotherapy and targeted drugs for the foreseeable future.

The availability of precise and high-definition functional imaging is fundamental to understand, evaluate, and develop new treatments in cancer [210,211]. Reports of molecular imaging with reactive probes, including ANPEP, are generating promising results with detailed specificity in pre-clinical models [70]. This technology should be expanded into clinical research on UC patients with the aim to select optimal treatment on a tumour individual basis.

The results from **Paper V** showing efficacy of the aminopeptidase activated alkylating agent melflufen in UC cells *in vitro* highlights the priority to setup an early stage trial in metastatic UC. The recent achievements in late-stage myeloma of melflufen versus standard-of care pomalidomide, which significantly increased response and prolonged survival in a Phase II trial further supports pharmacodynamic properties of melflufen [62].

During the last decade advances in bioinformatics and nanotechnology have contributed to the knowledge and research possibilities in UC. As this momentum in medical research is increasing, new tools to diagnose, profile, and ultimately tailor treatment will influence future treatment decisions in complex diseases, such as cancer, and especially in cancers with high mutational burden [212]. Exosomes as biomarkers and diagnostic tools is one example of an emerging application under development to possibly dynamically monitor and describe treatment response in UC [213,214].

Finally, as an acknowledgement of the intense research focusing on UC, there are over one hundred registered clinical studies worldwide trying to identify new treatments, indications or combinations in metastatic UC [207]. A future prospective trial in metastatic UC patients may advantageously implement an adaptive randomisation in the study design. This trial strategy would more rapidly provide the newly obtained predictive information applicable to other study subjects. Developing new treatments and improved evaluation methods in metastatic UC are top priorities to increase survival outcome.

7 Acknowledgements

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