Augun Jodis Blindheim

Bladder cancer from bench to bedside

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Doctoral thesis

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I denne avhandlingen presenteres resultater både fra basalforskning på blærekreft og blærekreft epidemiologi. I den epidemiologiske studien har jeg sett på hvordan en spesiell blærekreftgruppe behandles i Norge. Dette er pasienter med svulster som befinner seg mellom de helt overfladiske og de som vokser dypere inn i blæreveggen, og som utgjør tumorstadium T1. Dette er første gang behandlingsresultater for denne gruppen norske pasienter presenteres. Dette skyldes nok mye at registreringen ved Kreftregisteret har blitt utført på en måte som har gjort identifisering av gruppen vanskelig. Jeg har derfor i samarbeide med forsker og statistiker Bettina Andreassen ved Kreftregisteret laget et eget register over blærekreftpasienter i Norge diagnostisert i perioden 2008-2012 med videre oppfølging til 2017. Her har vi kunnet identifisere T1 gruppen og vi har også sammenholdt informasjon gitt Kreftregisteret med opplysninger fra Norsk pasientregister (NPR) som registrerer all behandling som gis ved sykehusene i Norge. Resultater sammenlignes så med anbefalinger i guidelines og med resultater fra andre land for å se om behandlingen vi gir i Norge holder mål.

I basalforskningen har jeg fått jobbe i en forskningsgruppe, APIM-gruppen, med leder professor Marit Otterlei, som har utviklet en mulig ny kreftbehandling. Medikamentet er utviklet på bakgrunn av gruppens tidligere oppdagelse av en ny bindingssekvens på proteiner, APIM. Denne sekvensen kan binde proteinene til et svært viktig molekyl, PCNA, som er helt vesentlig for celledeling, cellevekst og flere andre viktige cellulære prosesser.

En bærebjelke i all kreftbehandling er bruken av cytostatika; som skader kreftcellenes DNA og dermed deres evne til overlevelse. Blærekreft som har metastasert behandles hovedsakelig med cisplatin-basert kjemoterapi; imidlertid med kortvarig eller liten effekt på overlevelse. Tilbakefall skyldes blant annet cellenes evne til å reparere DNA skadene og angrep på cellenes reparasjonssystemer anses derfor å være et aktuelt mål i behandlingen av kreft. APIM ble først oppdaget på et reparasjonsprotein og effekten av medikamentet ble derfor først tenkt nettopp å hindre cellenes evne til reparasjon. Imidlertid viser nyere studier at APIM-peptidet også kan ha innvirkning på immunsystemet og på cellenes signalveier.

Vi har testet om cisplatin har økt behandlingseffekt når det kombineres med det nye medikamentet APIM-peptidet. Resultater fra studier på blærekreftcellelinjer og en dyremodell med muskelinvasiv blærekreft presenteres i avhandlingens første artikkel.

I innledningens Del I gir jeg en kort innføring i dagens behandlingsstrategier for blærekreft med hovedvekt på T1 og muskelinvasiv blærecancer (MIBC). I del II presenteres noen av de viktige funksjonene til PCNA og hvordan APIM-peptidet kan ha innvirkning på kreft- relaterte prosesser som reguleres av PCNA.

Table of contents

Acknowledgements

List of papers

Paper 1:

"Two hits – one stone"; increased efficacy of cisplatin-based therapies by targeting PCNA's role in both DNA repair and cellular signaling

Caroline K. Søgaard*, Augun Blindheim*, Lisa M. Røst, Voin Petrović, Anala Nepal, Siri Bachke, Nina-Beate Liabakk, Odrun A. Gederaas, Trond Viset, Carl-Jørgen Arum, Per Bruheim, Marit Otterlei
Oncotarget 9 (66), 32448-32465 (2018)
*Equal contribution

Paper 2:

T1 bladder cancer in Norway: treatment and survival

A Blindheim, S. Fosså, R Babigumira, T Å Myklebust, E Haug, C J Arum, B K Andreassen

Paper 3:

The use of reTURB in T1 bladder cancer, a Norwegian population-based study

A Blindheim*, S Fosså*, R Babigumira, B K Andreassen

*Equal contribution

Abbreviations

APIM	AlkB homologue 2 PCNA-interacting motif
APIM-peptide	APIM-containing peptide
ATM	Ataxia-telangiectasia-mutated
ATR	Ataxia-telangiectasia Rad3-related
BC	Bladder cancer
BCG	bacillus Calmette-Guerin
CRN	Cancer registry of Norway
CSS	Cancer specific survival
CPI	Checkpoint inhibitor
DM	Detrusor muscle
BER	Base excision repair
Bim	Bcl-2 homology 3-only protein
BRCA1/2	Breast cancer type 1/2 susceptibility protein
DDR	DNA damage response
DDT	DNA damage tolerance
DE	Differentially expressed
DSB	Double strand break
EGFR	Epidermal growth factor receptor
ERCC1	Excision repair cross-complementing 1
ERK	Extracellular signal regulated kinase
FA	Fanconi anaemia
FANC	FA core complex unit
FDA	U.S. Food and Drug Administration
GC	Gemcitabine/cisplatin
H	Histone
hABH2	Human AlkB homologue 2
HG	high grade
HR	Homologous recombination
ICLs	Interstrand crosslinks
IDCL	Interdomain connecting loop
MAPK	Mitogen activated protein kinase

MDT	Multi-disciplinary team
MIB	Multiplexed inhibitor bead
MIBC	Muscle-invasive bladder cancer
MMC	Mitomycin C
MMR	Mismatch repair
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
MVAC	Methotrexate/vinblastine/adriamycin/cisplatin
NAC	Neo-adjuvant chemotherapy
NER	Nucleotide excision repair
NK	Natural killer cell
NMIBC	Non-muscle-invasive bladder cancer
NPR	Norwegian patient registry
PARP1	Poly (ADP-ribose) polymerase 1
PCD	Programmed cell death
PCNA	Proliferating cell nuclear antigen
PCNA-I	PCNA-inhibitor 1
PD-1	Programmed death 1
PD-L1	Programmed death ligand 1
PI3K	Phosphoinositide 3-kinase
PIP-box	PCNA-interacting peptide box
POL	Polymerase
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
reTURB	repeated transurethral resection of the bladder
Rb	retinoblastoma
TLS	Translesion synthesis
TURB	Transurethral resection of the bladder
TS	Template switching
UV	Ultraviolet
VGFR	Vascular growth factor receptor
XP	Xeroderma pigmentosum

1. Introduction

Part one: Bladder cancer, challenges and treatment strategies

1.1 Bladder cancer, epidemiology and risk factors

Bladder cancer is a rather common disease with several unsolved problems. Approximately 500,000 new bladder cancer (BC) cases were registered worldwide in 2018, making the disease the seventh most common cause of cancer in men [1]. In Europe over 120,000, and in Norway about 1500 are diagnosed each year [2]. The incidence rates are highest in USA, Europe and Egypt. Three out of four patients are men, but research has not fully explained this gender difference. Molecular mechanism such as differences in sex hormones and gender differences in detoxification of carcinogens could be part of the explanation. In addition, differences in exposure to carcinogens and differences in smoking patterns between men and women could contribute as cigarette smoke and some industries harbor/produce carcinogens associated with bladder cancer [3, 4]. Arsenic and chloride in the drinking water is also an associated risk factor [5, 6]; however, tobacco smoking is the most significant risk factor and is associated in 50-60 % of BC cases [7] [8]. Bladder cancer costs are substantial for the health care system, mainly because patients often require multiple treatments and long follow-ups because of the high recurrence rates of up to 70 % [9, 10].

The current trend is a reduction of BC cases in the western world. This is probably a result of reduction in daily smoking and awareness of and protection against carcinogens. Unfortunately, in developing countries the incidence is increasing secondary to both increased smoking habits and industrialization. Increasing longevity in these countries will also contribute to increased incidence since age is a major risk factor for BC. The future will show how an aging population will contribute to incidence rates in the western countries [11, 12].

1.2 Bladder cancer diagnose

When suspecting a bladder tumor, the correct work-up is to recommend a computed tomography (CT) scan of the urinary tract and a cystoscopy of the bladder. When a tumor is detected the patients will have a transurethral resection of the bladder (TURB) which will give information about type of tumor, histological stage and grade. The aim of TURB is a complete removal of the tumor [13]; however, this

is not always possible because of the size of the tumor, the location and the risk of perforating the bladder wall. Despite macroscopic complete resection residual tumors are found at repeated resection of the bladder (reTURB) in more than 50 % of cases [14]. TURB is the initial step to obtain tissue for histological tumor (T) staging and World Health Organization (WHO) grading of the tumor, which forms the basis for further treatment recommendations and follow up. In the following chapters I will look at the challenges with obtaining the correct tumor stage and grade from TURB.

1.2.1 Histology

Histological investigations of bladder tumors show urothelial (transitional cell) carcinomas in 90-95 % of the cases. Other histological types are adenocarcinoma, squamous cell carcinoma, sarcomas, and rare histological variants which demands special treatment strategies and are not discussed in this thesis.

1.2.2 Tumor stage

About 60 % of BC patients have tumors confined to the epithelial layer of the bladder wall, stage Ta. These tumors will seldom progress or become life-threatening, but the rate of recurrences is up to 70 % [14]. Another malignancy, also confined to the epithelial layer, is carcinoma in situ (cis). This flat growing tumor entails a significant risk of progression, 50 % within 5 years if not treated. Cis as the only histological finding is rare, more commonly we find cis concomitant to other tumors in the bladder [8, 15, 16].

In this thesis, I will focus on the remaining 30-40 % of all BC patients which consists of two groups. The first group is muscle invasive bladder cancer (MIBC) where tumor infiltrates into the muscle layer of the bladder wall or beyond, defined as tumor stage T2 to T4. This group constitutes about 20 % of BC patients at initial diagnose. The second group has tumor infiltrating the subepithelial connective tissue, i.e. lamina propria, defined as tumor stage T1. This group also makes up about 20 % of BC patients at initial diagnosis [8]. There are several controversies and options in treatment of stage T1, likely because these tumors are highly heterogenic making prognostication as well as treatment recommendations difficult.

1.2.3 Tumor grade

Tumor grade together with tumor stage are the most clinically utilized prognostic factors in BC disease. The histological grading for bladder cancer consists of two classification systems, which are both in use. The 1973 WHO Grade 1-2-3 discriminates between well, medium and poorly differentiated tumors. The 2004/2016 WHO/International Society of Urological Pathology (ISUP), discriminates between papillary urothelial neoplasm of low malignancy potential (PUNLMP), high grade (HG) and low-grade (LG) malignancy. MIBC are always high-grade tumors whereas T1 cancers commonly are high grade, but in 5-10% of cases grade I, 2 or LG. The new grading from 2004 was introduced in order to have a more detailed and accurate description to reduce the variation between pathologic interpretation and diagnose. However, even the new system results in interobserver variation and needs further validation. According to guidelines both grading systems should be used until further clarification is available. Two grading systems in use at the same time is not optimal and might influence retrospective studies because some histological reports might use only one of the classifications, making comparison difficult [13, 17]. Dependable histological evaluation both in grading and staging have great implications for treatment decisions. Likewise, the surgeon's skills and experience at performing the TURB operation is essential to secure specimen of good quality for the pathologist. In the following I will look at the challenge's surgeons and pathologists face in staging and treatment of BC patients.

1.2.4 Challenges in surgery

The resection of a bladder tumor should include tissue from both the superficial epithelial layer and the deeper detrusor muscle (DM) of the bladder wall. At the same time the surgeon should carefully avoid perforating the bladder wall, which will increase the danger of spreading the tumor. DM in the specimen is recognized as a quality factor and important for correct BC staging [18]. TURB becomes challenging for large tumors, for extremely vascularized tumors and if the bladder wall is thin as might be the case in elderly women [19]. It is important that surgeons get supervision and training to perform this operation properly because the quality of the resection will impact staging and grading of the tumor. Studies show that experienced surgeons have more DM in the specimen, an accepted quality sign of the resection. Thus, TURB is a crucial factor in bladder cancer management [20-22].

When performing TURB the surgeon removes the tumor as small tissue chips using a diathermy loop. The current paradigm for complete removal of malignant tumors is free surgical margins and no tumor seeding. En-block resection of bladder tumors was introduced in 1997 by Kawada T [23] to increase

the quality of the tumor resection and to ease the pathologists' task of concluding the correct diagnosis. Currently, more studies are required to confirm the superiority of this method [24].

Photodynamic diagnostic (PDD) has been developed to increase the rate of complete tumor resection. PDD is based on the use of a photosensitizing agent, e.g. 5-aminolaevulinic acid (ALA) or hexaminolaevulinic acid (HAL), which is taken up by the tumor cells. When combined with a light source with appropriate wavelength the tumor cells are visualized. It is agreed that PDD can reveal tumors otherwise overlooked and thereby reduce the number of relapses when compared to standard white light TURB, but it is discussed whether PDD gives any survival benefits and whether it is cost-effective [25, 26]. Another tool to increase tumor visualization is narrow band imaging (NBI). NBI is an optical enhancement technology making microvascular structures in the bladder wall more visible. NBI has shown comparable results to PDD with much lower costs and it is in addition easier to use [27].

Although TURB is the most common urological cancer operation performed with huge implications for diagnose and treatment [18], there has so far not been any quality control of this procedure in Norway.

1.2.5 Challenges in pathology

The reliability of the pathologist's assessment is essential for correct treatment of bladder cancer. The TURB surgical technique of piecemeal resection using diathermy leads to difficulties for the pathologist both in orientating the tissue chips and in assessing tissue with thermal damage. Together with differences in the cellular assessments this might contribute to the substantial intra- and inter-observer variability among pathologists both in staging and grading of a tumor [13]. Several studies show both under- and over- staging of T1 tumors when a second assessment by uropathologist experts were performed. Studies report down-staging to Ta in 35-56 % and upstaging to T2 for 3-13 % of the T1 cases included [28] [29]. This variability may impact both treatment decisions and treatment results. It may also influence study results especially in retrospective studies where results rely upon assessment from several pathologists without any central evaluation. A close collaboration between surgeon and pathologist is needed to optimize correct tumor diagnosis [30].

1.2.6 Challenges in imaging

The routinely used imaging modalities for the staging of BC patients, which means search for nodal and organ metastasis, and to some extent assessment of the tumor, is CT and to some degree magnetic

resonance imaging (MRI). CT is the cheapest and fastest modality to perform, but CT cannot distinguish between different layers in the bladder wall. MRI seems to be better in differentiating layers in the bladder wall and to some extent distinguish muscle invasive from non-muscle invasive tumors [31]. The sensitivity for nodal metastasis is low for both CT and MRI, according to Crozier 40 and 60 % respectively, and neither can rule out pelvic metastasis [32, 33]. In addition, CT exposes patients to high doses of ionizing radiation with risk of DNA-damage. Both modalities are dependent on intravenous contrast and therefore have restrictions for patients with kidney failure [13]. Another imaging modality for staging is fluorodeoxyglucose (FDG) positron emission tomography (FDG-PET). However, FDG is not the best for BC since it is excreted through the kidneys. Studies on FDG-PET used for metastatic disease suggest that it could potentially reveal metastasis, but guidelines so far recommend CT or MRI as standard staging procedures for BC [34, 35]. Carbon-choline-PET/CT shows a higher sensitivity than FDG-PET for nodal staging, but the need for an in-house cyclotron due to short half-life of C-choline limits its use [36].

In conclusion we have no optimal imaging method to reveal lymph node metastasis prior to surgery or to currently predict how deep the tumor is invading into the bladder wall.

1.3 Treatment strategies for localized muscle invasive bladder cancer (MIBC)

1.3.1 Radical cystectomy

The standard recommended treatment for patients with localized MIBC is radical cystectomy (RC), which in addition to the urinary bladder includes removal of the prostate and seminal vesicles in men and uterus, ovaries and adjacent vagina in women. RC also includes removal of lymph nodes up to the aortic bifurcation, named extended lymph node dissection, which might be associated with survival benefits compared to lymph node dissection confined to the lower pelvis, although controversial [37, 38]. Upfront to RC, neoadjuvant chemotherapy (NAC) is recommended. Standard treatment recommendations therefore require that the patients are fit for both major surgery and chemotherapy [15]. RC is associated with a complication rate within 3 months of about 50-55 % and 3 months mortality rates of 3-4 % [39, 40]. Although the intention of RC is to gain disease control, about 18-25 % already have metastasis at the time of cystectomy and 50 % will develop metastasis and ultimately die within 5 years [15].

1.3.2 Neoadjuvant chemotherapy (NAC)

The recommended NAC prior to RC increases 5-years survival rate with 5-8 %, suggested to be due to a reduction of micro-metastatic burden [15, 41-43]. The number needed to treat (NNT) is reported to be 12.5 in a meta-analysis [44]. Of all MIBC patients treated with NAC, 25 % to 50 % achieved treatment response. Patients with complete response at RC, i.e. stage T0, had better overall and relapse-free survival compared to patients with remaining tumor at RC [45, 46]. A recent meta-analysis showed that NAC increased the numbers of complete response at RC from 20 % to 34 % in T2 cases and from 4 % to 24 % in T3-T4 cases [47]. The two most used NAC-regiments are methotrexate/vinblastine/adriamycin and cisplatin (MVAC) and gemcitabine/cisplatin (GC). Both MVAC and GC are limited by their renal, neural and cardiac toxicities. In BC 40 % of the patients are older than 70 years, often with severe co-morbidities and many are therefore unfit for chemotherapy [48]. Another problem is the imposed delay in surgery for those not responding to chemotherapy. Prediction of non-responders is currently not possible, nor is identification of patients with micrometastasis. This leads to both delay and unnecessary risk for toxic adverse events for non-responding patients [49]. NAC is therefore controversial and although it is recommended by the EAU guidelines utilization rates rarely exceed 25 % [50]. Recent studies have questioned improved survival for patients treated with NAC, and one study showed even reduced survival after NAC in patients with residual tumors in the cystectomy specimen [51]. Another study suggests that increased survival of NAC can be due to differences in the quality of the TURB [52]. Several randomized studies support the use of NAC but obviously, there is a need for better stratification to find patients who will clearly benefit from the treatment [53, 54]. Cisplatin is the main chemotherapeutic in NAC and the evolving understanding of cisplatin resistance (discussed later) may also impact NAC- treatment.

Preliminary results from studies using checkpoint inhibitors in neoadjuvant settings show similar or even increased survival compared to chemotherapy, giving hope for less toxic treatment in the future [55].

1.3.3. Multimodal bladder preserving treatment/ trimodal treatment (TMT)

Studies have shown a 5-year cancer specific survival (CSS) of 71 % for selected BC patients treated with a combination of TURB, radiation and chemotherapy, named trimodal treatment (TMT). This survival rate is like the results of RC [56, 57]. In line with this, a meta-analysis showed 5-years CSS of 78 % for patients with complete response after treatment [47]. Guidelines reserve TMT for patients for whom cystectomy is not an option or for patients refusing surgery. With an ageing population, this less

comprehensive treatment option may become more frequently recommended for a subgroup of patients [58].

1.4 Treatment strategies for stage T1 bladder cancer

Tumors progressing into MIBC and the risk of understaging tumors at the initial TURB are the two major concerns in the management of T1 BC. According to EAU guidelines a repeated TURB (reTURB) should therefore be included in the management of T1 patients within 4-6 weeks after the primary TURB. Patients eligible for conservative treatment should further be followed by cystoscopy surveillance combined with Bacillus Calmette-Guerin (BCG) bladder instillation treatment. Patients with special high risk of progression, should be assessed for immediate RC. Risk factors to be taken into account for progression are given by the EAU guidelines and include HG tumor, tumor > 3 cm, multiple tumors, difficult location e.g. the front wall behind the bladder neck, depth of lamina propria infiltration, lympho-vascular invasion, concomitant cis and persistent T1 at reTURB [13]. Primary assessment should also include general health performance and risk factors associated with RC. Despite these recommendations it is often a clinical dilemma to decide which patients should be offered early cystectomy and which can safely be followed with surveillance with the risk of both over- and under-treatment. In the following, some of the diagnostic and treatment challenges will be presented.

1.4.1 Risk of progression and understaging

For best possible treatment recommendation, the risk assessment for progression is crucial, however, this is difficult. Progression-rates for stage T1 varies in studies between 20-40 %, with the more recent studies showing lowest rates. This may be a result of more patients receiving both BCG and reTURB in later years [59-61]. In spite of a macroscopic complete resection of tumor at the initial TURB, up to 20-70 % of patients will at a reTURB still have residual tumor tissue and upstaging to MIBC is reported in 8 % of the cases [14, 62-64]. Studies have shown that the histopathological result at reTURB are of prognostic value and patients with remaining T1 at reTURB show high risk of progression. However, progression rates reported varies from 25-83 %, probably because of differences in study design, for example inclusion criteria [65-68]. A study by Palou *et al.* from 2018 presented a progression rate of 25 % for T1 at reTURB, while those with tumor stage below T1 had a progression rate of 11-14 % [68]. Even though several studies are showing MIBC at reTURB there is an ongoing discussion whether a reTURB is necessary in cases where DM is present in the primary TURB specimen. Studies mainly report survival benefits from reTURB [69, 70], although, one study by Gontero *et al* report survival benefit

only when muscle was missing in the primary TURB [71]. However, because of the risk of under-staging the tumor and high rates of remaining tumor tissue reTURB is recommended by international guidelines [13] [72].

1.4.2 Early vs delayed cystectomy

During surveillance, 20-25 % of T1 tumors will progress into MIBC leading to delayed RC for some of these patients. Denzinger *et al.* showed in a study from 2008 that in 105 T1 patients who all were offered immediate RC, those choosing delayed RC at tumor relapse (49 %) had a lower 10-years CSS than those with early RC (51 % vs 78 %) [73]. Studies have also shown that T1 patients who progressed to MIBC and then were treated with RC had a significantly lower survival compared to patients treated with RC for primary MIBC [74, 75]. In addition, studies report upstaging into MIBC in 40-50 % of T1 patients having RC some even with lymph node metastasis [76-78]. All these findings show that there is a great need for thorough assessment in the management of T1 patients.

1.4.3 Bladder instillation treatments

The T1 patients at low risk of progression or at high-risk of progression but not wanting or unfit for cystectomy, are offered BCG treatment combined with regular cystoscopy surveillance. Various instillation agents such as mitomycin-C (MMC), epirubicin (EPI) and gemcitabine(G) are investigated but BCG has shown superiority both for preventing relapses and progression up to now [13, 79, 80]. BCG, an attenuated strain of the Mycobacterium bovis, was initially developed as a vaccine against tuberculosis by Albert Calmette and Camille Guerin in 1921 and is now also widely used as an instillation treatment in cis and T1 BC. It is generally accepted that BCG initiates a local inflammation by recruitment of immunocompetent cells. BCG starts a signaling cascade making urothelial cells and cancer cells secrete cytokines, which ultimately attract macrophages and activated lymphocytes from the immune system with the ability of destroying cancer cells [81, 82]. A paper published by Morales *et al*. in 1976 showed favorable effect on non-muscle invasive bladder cancer (NMIBC) which includes T1, Ta and cis tumors [83]. Lamm *et al.* reported the first randomized study showing clinical effect on NMIBC in 1980 [84]. In the first metaanalysis of BCG vs chemotherapy Sylvester *et al*. showed in 2002 that BCG reduced the progression rate of NMIBC from 14 % to 10 %, a risk reduction of 27 % [85]. BCG had than already been used for about 25 years and smaller studies showed diverging results regarding effect. Several metaanalysis have later confirmed the superiority of BCG regarding recurrence

compared to TURB alone [86, 87]; however, there is some debate as to whether BCG actually can prevent progression. Mitomycin C (MMC) and epirubicin (EPI) are the most used instillation treatments beside BCG. MMC is a natural product from species of the soil fungus Streptomyces. Activated MMC can react with DNA at the guanosine residue to form MMC-mono-guanosine adduct. MMC can further form both intra- and interstrand DNA crosslinks. If not repaired, these DNA adducts can block both DNA transcription and replication leading to apoptosis [88]. EPI acts by intercalating DNA strands i.e. binding of epirubicin in between planar pairs of DNA bases, and in this way, EPI disrupts the DNA double helix. Intercalation results in complex formation which inhibits DNA and RNA synthesis [89]. A recent Cochrane meta-analysis by Schmidt *et al.*, comparing BCG vs MMC including 12 randomized controlled trials (RCT) from 1995 to 2013 with 2932 T1 participants, concludes that BCG has no effect on risk of progression, shows more severe adverse events, and at best gives only a small decrease in risk of recurrence compared to MMC [90]. Although at best a modest effect, this is the only intravesical instillation treatment showing any impact on progression [85].

Another strategy to increase BCG efficacy is to combine BCG with other instillation drugs. A metaanalysis of randomized controlled studies from 2016 suggested that the combination of BCG and MMC or EPI may increase the efficacy of BCG [91]. Another study combining MMC and BCG in a sequential way concluded with higher efficacy of the combination than BCG as single agent but at the cost of higher toxicity. The combination was therefore only recommended for recurrent T1 patients [92]. Combination of for example MMC with microwave induced hyperthermia of the bladder is also reported to give promising effect on recurrence rates, but neither this technic or other combinations of drugs has been taken into clinical use on a regular basis [93].

It is a challenge that about 40 % of the patients are BCG non-responders and that we are lacking tools to decide who will benefit or not. Another major concern is the possibilities of adverse events due to systemic absorption of BCG with severe infection as a result. Although less than 5 % will experience severe adverse events, the use of BCG is not without problems and up to 40 % fail to complete the treatment because of cystitis and bladder pain [94].

Studies are ongoing to see whether immune checkpoint inhibitors (CPI), can become a future intravesical treatment option for T1 patients (discussed later) [95]. Anyhow, new strategies are needed in intravesical instillation treatment both to prevent progression and tumor relapses.

1.5 Treatment strategies for metastatic bladder cancer

Metastatic bladder cancer represents an incurable disease for the majority of patients and standard treatment with chemotherapy has only given months of increased survival [15]. For patients with lymph node metastasis 21 % are alive after 5 years, while only 7 % of patients with visceral metastasis are alive after 5 years [96]. Until 2018 cisplatin-based chemotherapy was the only first line medication for metastatic BC in Norway and vinflunine (targets tubulin) the second line drug when first line treatment fails. Today CPIs are available both as first line therapy for patients with metastatic disease unfit for chemotherapy and as second line treatment [55].

1.5.1 Metastatic bladder cancer and chemotherapy

The common drug in chemotherapeutic combination- treatment for metastatic BC is cisplatin. The therapeutic potential of cisplatin was first discovered by Dr. Barnett Rosenberg in 1965 who accidentally found that platinum products in electrolysis inhibited cell division in *Escherichia coli.* In 1969 he presented platinum- compounds as a potent antitumor agent for the first time [97-99]. Cisplatin (C) was approved for cancer treatment by the U.S. Food and Drug Administration (FDA) in 1978 and in several European countries the year after. Since then it has become one of the major drugs in cancer therapy. The standard care combinations used today is GC or MVAC. GC shows marginally lower median survival compared to MVAC (14 vs 15 months), but is still part of the standard treatment options because of the lower toxicity [96, 100, 101]. BC is a chemo sensitive tumor with overall response rates of 60-70 %, included the 20-30 % of patients with complete response on MVAC regimen for metastatic or locally advanced unresectable BC [102, 103]. The increase in survival rates for metastatic MIBC with chemotherapy are however only from 7-9 months without, to 12-14 months with chemotherapy [104]. The use is also restricted because of severe side effects and up to 50 % of BC patients are ineligible for cisplatin-based chemotherapy because of its toxicity [48]. Patients showing at least one of the following criteria are unfit: Eastern Cooperative Oncology group (ECOG) performance status 2, creatinine clearance less than 60 ml/min, severe hearing loss, severe neuropathy or severe heart failure. An alternative treatment combination is gemcitabine with carboplatin instead of cisplatin. This combination has inferior response rates because carboplatin exhibits a lower reactivity but is less toxic [105].

1.5.2 Cisplatin mode of action and resistance mechanisms

The cellular uptake of cisplatin is mediated by passive diffusion, cation transporter and/or together with copper transporter proteins (e.g. copper transporter CTR1) [106]. Cisplatin works as a cytotoxic drug both in cytosol and in the nucleus. In the cytosol cisplatin is activated by the replacement of the chloride atoms on cisplatin with water molecules which makes cisplatin a highly reactive molecule with the possibility of reacting with substrates such as proteins, lipids and RNA. For example, interruption of mitochondrial function via modification of the membrane may lead to apoptosis and oxidative stress. This is believed to be one of the most toxic effects of cisplatin as it leads to excessive reactive oxygen species (ROS) production. In normal cells there is a balance between production and elimination of ROS and high levels of ROS are toxic [107]. The ROS-balance is often disturbed in cancer cells and higher than in normal cells. This condition, part of what is named oxidative stress, can in cancer cells partly be explained by a higher metabolic rate, the influence of oncogenes and mitochondrial interruptions. Since cancer cells already often show elevated concentrations of ROS, and often have reduced abilities to cope with extra stress, cisplatin treatment leads cancer cells towards apoptosis [108]. In the cell nucleus, activated cisplatin can react with DNA inducing protein-DNA complexes and DNA inter- and intra- strand crosslinks. These DNA lesions inhibit DNA replication and transcription leading to apoptosis [109].

Development of resistance limits the effect of cisplatin treatment. Mechanisms associated with resistance can be divided into intrinsic resistance, which means that the tumor has features making treatment ineffective from the beginning, or acquired resistance, where resistance develops during treatment. Some key mechanisms of resistance are changes in drug transport over the cell membrane giving reduced uptake or increased efflux, and it is shown that cisplatin can trigger the degradation of copper transporter. Cisplatin might become deactivated in the cytosol and cisplatin induced mutations in the tumor might reduce its effect. For example, tumors can increase survival signaling and decrease cell death signaling and thus circumvent or adapt to treatments. Epigenetic factors and the tumor microenvironment are also contributing to cisplatin resistance [108, 110, 111]. Protective mechanisms are activated both in the cytoplasm and in the nucleus to attenuate cisplatin induced damage. In the nucleus, DNA lesions interfering with replication will rapidly lead to activation of the DNA damage response (DDR), resulting in either repair of the DNA lesions by activating DNA repair pathways and/or cell cycle arrest. Cell cycle arrest gives the cells time for repair before continuing the cell cycle or if the lesion levels are too high, initiating apoptosis. The DNA tolerance pathways (DDT) are also initiated upon DNA damage. This allows bypass of DNA lesions leading to mutations. The DNA damage response

and tolerance capacity will therefore be important for cisplatin efficacy. Tumors frequently develop mutations or dysfunction in the repair pathways leading to alteration in repair capacity [112].

A study looking for predictive biomarkers for NAC response showed that mutations in DNA repair associated genes like Ataxia-telangiectasia-mutated (*ATM*), retinoblastoma 1 (*RB1*) and Fanconi anemia core complex (*FANCC*) increased tumor sensitivity to cisplatin. Another study showed that Excision repair cross-complementing 2 (*ERCC2*) mutations was associated to cisplatin response [113-116]. So far these biomarkers for predicting cisplatin response are not in standard clinical use.

1.5.3 The MVAC and GC regimens

Combination of different chemotherapeutics is given to increase efficacy by attacking different targets simultaneously and to reduce severe adverse events by decreased doses of the most toxic drugs. A brief summary of the drugs in addition to cisplatin in the MVAC and GC regimens, focusing on the different targets they are attacking, is given below.

Methotrexate (M) is an antifolate and inhibits the metabolism of folic acid by inhibiting the enzyme dihydrofolate reductase from converting dihydrofolate to the active form tetrahydrofolate. Tetrahydrofolate is important in the metabolism of both nucleic and amino acids and inhibition by methotrexate leads to decreased synthesis of DNA, RNA, thymidylates and proteins. Therefore, tetrahydrofolate is especially important for rapidly dividing cells e.g. cancer cells. Like most chemotherapeutics, methotrexate therefore works mainly in the synthesis (S) phase of the cell cycle. Side effects such as bone marrow depression, inflammation in the digestive tract and kidney and liver failure are reported for methotrexate [117].

Vinblastin (V) is a microtubule targeting drug. Vinblastin works mainly in the mitotic (M) phase of the cell cycle where it binds to the tubulin molecule and thereby prevents proper microtubule formation. Microtubules are the main components of the cell cytoskeleton and important in the mitotic spindle necessary for separation of the chromosomes and thereby mitosis. Side effects of vinblastin includes bone marrow suppression and gastrointestinal toxicity. Vinblastine is reported to be an effective component of certain chemotherapy regimens that by allowing lower doses of the other cytostatic, e.g. methotrexate and vinblastin, thereby can reduce the overall treatment toxicity [118].

Adriamycin (A) (Doxorubicin) is like EPI a DNA intercalating drug. Topoisomerase II, which relaxes supercoils in DNA replication, is inhibited by adriamycin after the DNA brake and the resealing activity is blocked. This blocks DNA replication and leads to apoptosis. Severe side effects of adriamycin includes dilated cardiomyopathy, liver and bowel complications [119].

Gemcitabine (G) is a nucleoside analog for cytidine which gets incorporated during DNA replication leading to a termination of the DNA synthesis. Gemcitabine is also targeting ribonucleotide reductase (RNR), an enzyme necessary to produce DNA nucleotides, leading to nucleotide unbalance and thus impaired DNA replication and repair. Common toxic effects of gemcitabine include bone marrow suppression, liver, kidney and bowel complications [120].

1.5.4 Immune checkpoint inhibitors (CPI)

Development and progression of cancer are highly influenced by our immune system. Lymphocytes like cytotoxic T- lymphocytes, Natural killer (NK) cells and T- helper cells from our innate immune system can recognize and kill abnormal and stressed cells. However, these defense mechanisms are often inactivated, and tumor cells can evolve escape mechanisms. This includes both the ability to avoid damage from immune cells and development of mechanisms that suppress the immune system [110, 121, 122].

In recent years immunotherapy has become an important strategy for cancer treatment. Immunotherapy targets modulation of the immune system and not directly the cancer cells. Immune checkpoints are important in the control of autoimmunity and for regulation of the immune response. Therefore, drugs, often antibodies, binding to immune checkpoints receptors which comprise pathways that can either stimulate or inhibit immune responses, have been developed. These are called immune checkpoint inhibitors (CPI). Pathways inhibiting the immune response are often activated by cancer cells to attenuate the immune response and allow tumor growth. Immune checkpoints consist of receptors and its ligands, currently about 15-20 pairs are known. For example, programmed death receptor 1 (PD-1) and its inhibitory ligand PD-L1 are expressed on immune cell surface and are normally modulating the immune response to avoid overstimulation. However, PD-L1 is also expressed on cancer cells and the binding of PD-L1 to PD-1 receptor inhibits/downregulates the cytotoxic activity of T-cells. This is considered a key mechanism of tumor immune escape. By blocking the PD-L1/PD1 signaling with CPI's, tumor initiated immune suppression is inhibited and activated T cells can exert their cytotoxic function on the tumor cells. PD-L1/PD1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and its ligand CD80/CD86 are the best-known examples of immune checkpoint pathways targeted in current cancer immuno-therapy. However, several new CPIs against other immune checkpoints are now in clinical trials, e.g. the checkpoint T-cell immunoglobulin 3 (TIM3)/OX40L and lymphocyte activating gene 3 (LAG3)/MHC1/II. Also, agonists of stimulatory immune checkpoints are under investigation [123]. The regulation of the immune response is complicated and involves both signaling pathways, epigenetic and transcriptional factors. For example,

upregulation of PD-L1 as a response to oncogenic signaling via for example endothelial growth factor receptor (EGFR), mitogen activating receptor kinase (MARK) and phosphoinositide 3-kinases (PIK3)/ Akt/ mammalian target of Rapamycin (mTOR) signaling pathways are shown. This opens for the possibility of modulating the immune response via modulation of signaling pathways [124]. Several CPI's have shown great clinical effect and durable response for a subset of patients; especially for those with malignant melanoma and some types of lung cancer. Also, bladder cancer patients are responding to CPI treatment. In Norway the CPIs approved for metastatic BC includes anti-PD1 and anti-PD-L1. Treatment response is moderate with about 15-29 % responding; however, the response is reported for some patients to be durable [55, 125, 126]. To increase the response rates, combination treatment with CPIs and kinase inhibitors such as receptor tyrosine kinase (RTK) inhibitors, radiation- and chemotherapies are ongoing, but so far only experimental for BC patients. Biomarkers for identification of patients who will benefit from CPI treatment is needed [127-130].

The use of CPIs and chemotherapy are both restricted due to harm to normal cells, but also the side effects of CPI's are more unpredictable than of chemotherapy as autoimmunity can arise. Furthermore, our immune system is part of the tumor microenvironment and thus a risk of stimulating the immune system is increased tumor growth [131] [132].

1.5.5 Kinase inhibitors

 A comprehensive insight to the genetic variation of bladder cancer was for the first time given by The Cancer Genome Atlas (TCGA) project in 2014. This represented a landmark paper profiling 131 patients with MIBC [133]. TCGA has since then added patients to the study and is now including 412 patients [134]. Their analysis identified potential therapeutic targets in 69 % of the tumors and of these several targets were identified in signaling pathways such as PIK3/Akt/mTOR and the MAPK pathways. Some of the most frequently mutated genes were TP53, PIK3, Fibroblast growth factor receptor (FGFR) erythroblastic oncogene B (ERBB2)/ human epidermal growth factor receptor 2 (HER2) and retinoblastoma (Rb1). Chromatin regulatory genes were also frequently mutated. The main focus in molecular research for BC has up to now been on MIBC and kinase inhibitors are primarily tested in patients with metastatic MIBC [135].

Cellular signaling commonly starts with activation of receptors on the cell surface by ligands as e.g. growth factors. Of special interest in bladder cancer are two RTKs, EGFR and ERBB2, also named HER2. EGFR and ERBB2 are both often upregulated in bladder cancer [136, 137]. When stimulated, these RTKs activates signaling pathways downstream like the PIK3/Akt/mTOR and the MAPK pathway [138,

139]. These pathways contribute to cell cycle progression, proliferation, anti-apoptosis, autophagy and are found dysregulated in 40 % of bladder cancer tumors [140].

Although several kinase inhibitors have been investigated, only one kinase inhibitor, a pan-FGFR inhibitor, has been approved for metastatic BC as second line treatment by FDA [141]. Both EGFR, FGFR and vascular endothelial growth factor receptor (VGFR) are RTKs activated by growth factors, hormones and cytokines and are often involved in cancer progression. However, clinical trials with mTOR, ERBB2/3 and EGFR inhibitors have only showed minimal benefit for patients [142-144]. When inhibiting kinases, one mechanism of resistance is oncogenic bypass, which means that the cell can circumvent the inhibition of one pathway by using alternative pathways. Together with other tumor escape mechanisms, this crosstalk between signaling pathways makes kinases as single agents less effective in cancer treatment [145]. New treatment strategies with multiple inhibitors or inhibitors in combinations with chemotherapy, CPI's and radiation are now evolving [146, 147].

1.5.6 Molecular subclassification of bladder cancer

The purpose of making new BC subclassifications is to connect specific molecular signatures to important differences in tumor biology to improve treatment strategies as well as prognostication. Some early studies showed that papillary superficial tumors were highly enriched with activating mutations in FGFR3 and non-papillary tumors enriched with inactivating mutations of *TP53* and *RbI.* This resulted in the first subclassification of bladder cancer, named luminal vs basal since they showed similarity to this original breast cancer classification [148, 149]. Several research groups that had developed their own sub-classification, are now cooperating to find new overlapping subgroups for consensus [150]. The key mutations used in contemporary studies are *FGFR3*, cytokine dependent kinase inhibitor 2A (*CDKN2A*), peroxisome proliferator-activated receptor gamma (*PPARG*), *ERBB2*, the E2 transcription factor 3 gene (*E2F3*), *TP53* and *Rb1.* These represent genes encoding proteins important for controlling tumor growth [151]. Molecular characterization of non-muscle invasive bladder cancer is also in progress and interestingly, alterations in ERBB2/HER2 was found in 57 % including T1 HG tumors and DNA damage repair alterations such as mutations in *ERCC2* gene are present in 17 % of HG NMIBC tumors and in 20 % of MIBC [152].

Part two: Basic research in PCNA and the APIM-peptide

1.6. Roles of proliferating cell nuclear antigen
1.6.1 PCNA

PCNA was discovered in 1978 and first described as upregulated in patients with systemic lupus erythematosus. It was found highly expressed in the S-phase of the cell cycle, making PCNA a frequently used proliferation marker. Later PCNA is best known for its essential role in DNA replication, chromatin remodeling, epigenetics and DNA repair [153-155]. PCNA was initially thought to be exclusively located in the cell nucleus, but PCNA is also found in cytosol in multiple cells [156-159] [160]. In cytosol, PCNA is involved in regulation of apoptosis and in regulation of multiple cellular signaling pathways, e.g. the PI3K/Akt pathway, essential for cell proliferation. PCNA in cytosol has also been linked to glycolysis and immune evasion [157, 158, 161, 162].
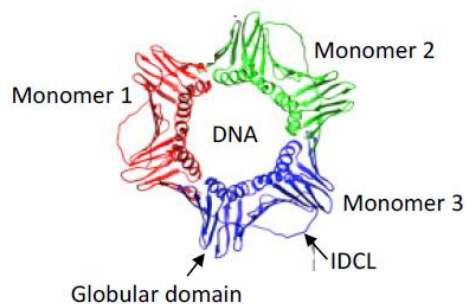
Figure 1: Structure of human PCNA. Three PCNA monomers, each consisting of two globular domains connected by the interdomain connecting loop (IDCL), link head-to-tail and assembles into a ring structure able to encircle DNA and slide along DNA strands. Figure adapted from[161].

PCNA is a homotrimer and has no enzymatic activity itself but works as a "scaffold-protein" which organizes processes by binding, and thereby activating and presenting proteins needed during multiple cellular processes. The PCNA-protein interactions take place via PCNA-interacting motifs located at the

protein surfaces which connects directly to the binding- pocket located below the inter domain connecting loop (IDCL) at the PCNA molecule. **Figure 1**. Two PCNA interacting motifs are known. The first motif discovered was named PIP-box (PCNA interacting peptide-box) [163]. The second motif, APIM (AlkB homolog 2 PCNA-interacting motif), was discovered by our research group on the DNA repair protein human AlkB homolog 2 (hABH2) [164] The five amino acids of APIM constitute together with a linker and a cell penetrating peptide a new drug candidate hereby called the APIM-peptide. The APIM- peptide binds to PCNA and inhibits binding of PCNA interacting proteins [165]. **Figure 2.** About 600 proteins contain either PIP-box or APIM and are thus possible interacting partners for PCNA [158, 164].
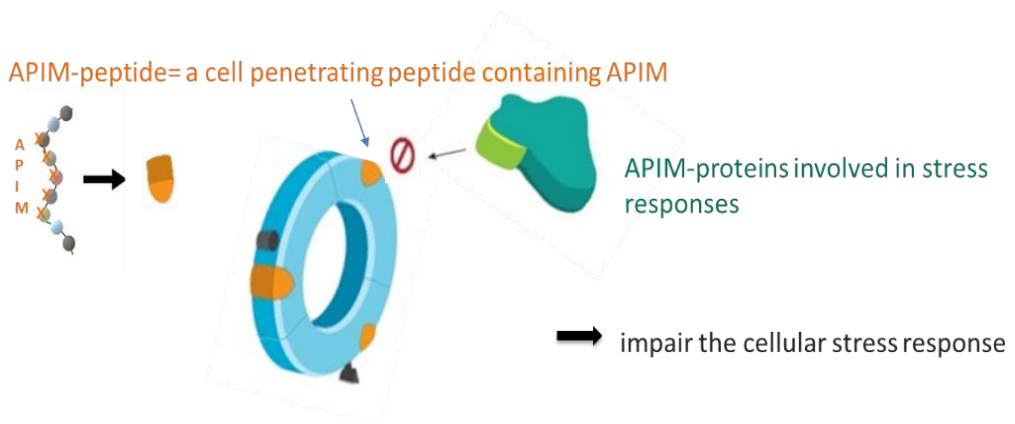
Figure 2. APIM was identified in a DNA repair protein that bound to PCNA

In the nucleus PCNA consists of 3 monomers coupled in a ring-shaped structure which is loaded on the DNA strand where it is working as a sliding clamp collecting the proteins needed for replication or repair processes. In the cytosol multiple proteins including kinases and regulators of apoptosis contain APIM or PIP-box motifs, suggesting that targeting PCNA may influence multiple cellular pathways simultaneously [160, 164]. Several research groups are trying to develop new anticancer drugs targeting PCNA because of its essential functions [166, 167].

1.6.2 Regulation of binding to PCNA

The interactions between proteins containing APIM or PIP-box and PCNA are likely regulated both by affinity-driven competition and post-translational modifications (PTMs) on both PCNA and the PCNA-binding proteins [164, 168]. Affinity is also influenced by the cellular context of PCNA, e.g. cytosol vs nuclear location, cellular replication vs cellular repair. The amino acid sequences outside the PIP-box or APIM can also contribute to variation in affinity [169]. Several proteins essential for replication contains the PIP-box, e.g. the replicative polymerases, while DNA repair and damage tolerance proteins often contain APIM. It is shown that APIM has increased affinity for PCNA during cellular stress and after poly-ubiquitination of PCNA [164, 165, 168, 170, 171]. This is supported by a study showing that immunoprecipitated PCNA pulled down by tagged APIM-peptides have a different isoelectric profile than total PCNA [164].

PIP-box and APIM have overlapping binding sites on PCNA [165, 172], but APIM has lower affinity towards PCNA than the PIP-box in the absent of stress, e.g. under normal replication, and the APIM-peptide is therefore not affecting normal replication [158, 165, 169, 173]. A model of this change in interacting protein partners is illustrated in **Figure 3.** This switch is crucial for the low toxicity of the APIM-peptide in cells in the absence of cellular stress.
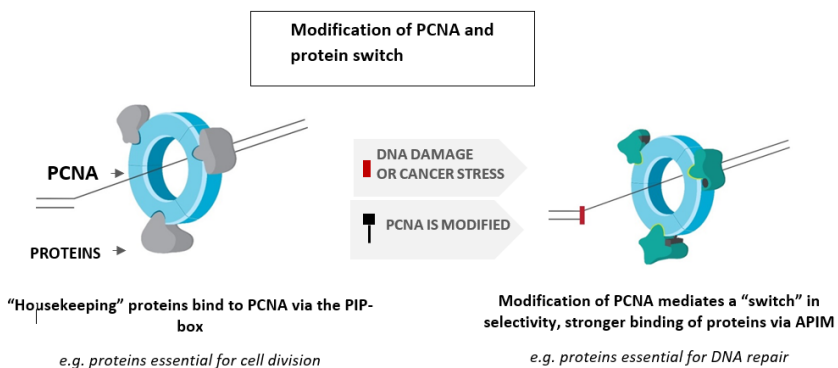
Figure 3. PCNA is essential for cellular proliferation and binds housekeeping proteins via PIP-box motif for replication. Cellular stress, induced by DNA damage or mutations, leads to an affinity switch with increased affinity for the APIM containing proteins.

1.6.3 The DNA damage response (DDR)

Our DNA continuously experiences both endogenous and exogenous DNA damage which can give rise to genome instability, mutations and cancer development. Spontaneously induced DNA lesions are estimated to happen in a number of 20-50 000 per cell per day, and in addition, external factors such as chemicals and radiation may induce DNA lesions [174]. DNA lesions may block replication and cause strand break, leading to chromosome rearrangements if not repaired [175, 176]. Multiple DNA repair and tolerance systems have evolved to take care of these DNA lesions to preserve the genome and avoid disease and cell death. These pathways are commonly termed the DNA damage response (DDR). Normally, repair and tolerance mechanisms are wanted, but after chemotherapy they may reduce the efficacy of treatments. Therefore, the DNA damage response has emerged as a potential anticancer target [175].

Jim Cleaver studied the rare syndrome xeroderma pigmentosum (XP) and was the first to identify that a defect in repair of DNA lesions caused by UV-light gave the patients an over 1000-fold increased risk of developing skin cancer. He found that XP was caused by mutations in genes encoding proteins in the nucleotide excision repair pathway (NER) [177]. Since then a complex picture of multiple DNA repair pathways have been identified and increased insight of the link between DNA repair activities and cancer has evolved [178].

Important DNA repair pathways are: i) mismatch repair (MMR) which corrects mis- incorporated bases during replication, ii) base excision repair (BER) which removes damaged single bases and iii) NER which is responsible for removing bulky DNA lesions and important for recognition and repair of both UV-lesions and cisplatin crosslinks [111, 175]. In addition, homologous recombination (HR) and non-homologous end joins (NHEJ) are repair pathways important for repair of DNA double strand break, for example induced by cisplatin. HR is based on the use of homologous sequences in the sister chromatid of the damaged chromatid as template for DNA synthesis while NHEJ just "glue" available ends together [179].

To secure replication in presence of unrepaired DNA lesions, cells also have strategies to avoid replication arrest and double strand breaks. This process is named DNA damage tolerance (DDT) and includes template switch (TS) and translesion synthesis (TLS). TLS allows replication bypass of DNA lesions via the use of so-called TLS polymerases. The TLS polymerases are less accurate i.e. the risk of inserting incorrect base is high, and this makes them intrinsically mutagenic [180, 181]. Ubiquitination of PCNA as a response to stalled replication forks controls to a large degree the initiation of the DDT pathway [182].

Central proteins in DDR in addition to DNA repair proteins include the checkpoint kinases Ataxia-telangiectasia mutated (ATM) kinase, ATM- and Rad3-Related (ATR) kinase, and the DNA-dependent protein kinase (DNA-PK) as well as poly ADP-ribose polymerases 1 (PARP1). These are all activated by single and double strand breaks or stalled replication forks [183]. The tumor suppressors TP53 and Rb1 are downstream effectors of ATM which are important for regulation of cell cycle arrest, repair, or apoptosis depending on the severity of the damage [184, 185]. PCNA is involved in all these stress induced cellular processes via its interaction with multiple proteins and thereby organizing the many different steps involved [161, 186].

Interestingly, multiple proteins with important roles in handling of replicative stress, DNA repair and DNA damage tolerance bind to PCNA via APIM: hABH2, involved in direct repair of base lesions; Topoisomerase II alfa, important to avoid replications collapse; XPA, essential for NER; RAD51B, important in HR [164, 170] and five proteins involved in DDT: ZRANB3, FBH1, the catalytic unit of the polymerase zeta (REV3L), HLTP and SHPRH [168, 187-189].

1.6.4 The immune system and PCNA

Targeting PCNA does not only affect DDR and DDT, but also the immune system because PCNA plays a role in cellular signaling. Monocytes are antigen-presenting cells as well as important responders to pathogen- associated molecular patterns (PAMPs), e.g. lipopolysaccharide (LPS) from bacteria, and damage-associated molecular patterns (DAMPs), e.g. molecules generated during tissue injury. As a response to stimulus, monocytes secrete cytokines which can influence tumor progression via inflammatory mediators [190]. Targeting PCNA with the APIM-peptide is shown to reduce Akt-phosphorylation and cytokine secretion in LPS activated monocytes, suggesting that PCNA may play a role in immune signaling [158].

Another mechanism where inhibiting PCNA could impact immune response is through modulation of natural killer (NK)-cell activities. NK- cells play an important role in protection against cancer via expression of natural cytotoxicity receptors (e. g NKp44) which when activated can bind to and lyse cancer cells. PCNA is recruited to cancer cell surfaces where it can bind to class 1 human leucocyte antigen (HLA-1). This inhibits the binding between HLA-1 and NKp44 and in this way PCNA inhibits lysis of cancer cells [157]. Impairing PCNA functions could therefore affect the immune system important for our defense mechanisms against cancer in several ways.

1.6.5 Drugs targeting PCNA

PCNA has emerged as a potential drug target because of its prominent function in proliferation and cellular stress responses. The PCNA targeting approaches includes blocking of the protein binding site in the C-terminal hydrophobic pocket, interfering with the IDCL region, or targeting the PCNA trimer stability [166, 191]. Potential drugs include an inhibitor called PCNA-I which binds to PCNA and inhibits the opening of PCNA for loading onto DNA. Cell studies show reduced proliferation of different cancer cells (breast and prostate), but also reduced proliferation of normal cells [192, 193]. The Y211F-peptide targets the stability of PCNA on chromatin and by doing so reduces phosphorylation of PCNA which is important for binding to DNA during replication and repair. Phosphorylated PCNA is enhanced in cancer cells and the Y211F-peptide therefore has some selectivity towards cancer cells. The peptide is shown to reduced tumor growth of aggressive breast and prostate cancer in xenograft models [194-196]. Another approach is to reduce the PCNA-chromatin association, and thus cell replication, by DNA aptamers. Aptamers are short DNA sequences able to compete with DNA for PCNA and DNA polymerase binding [197]. A PIP-box containing peptide targeting the same C-terminal hydrophobic pocket as APIM is also reported [198], but the development of this peptide to a cancer drug was stopped due to toxicity. Further, a small molecule T2AA, an inhibitor of the PIP-box-PCNA interaction is reported to increase sensitivity against cisplatin by reducing TLS and the repair of ICLs [199]. However, the two latter mentioned drug candidates are binding to PCNA with high affinity also in absence of cellular stress and are therefore inhibiting replication. Finally, a recent reported small molecule AOH1160 targets the protein binding pocket of PCNA but without completely inhibiting PIP-box proteins and replication. AOH1160 shows induction of apoptosis as well as enhanced effect of cisplatin therapy. This study suggested that inhibiting PCNA interactions can be useful combined with cytostatic drugs [200].

The APIM-peptide represents another way of targeting PCNA. APIM is mainly the binding motif of proteins interacting with PCNA in stressed cells and thus does not interfere with replication of non-stressed cells [164, 165, 173]. The APIM-peptide has increased the efficacy of different chemotherapeutics in multiple cell lines as well as yeast cells [160, 164, 165, 170]. Increased effect is also reported in a study with prostate cancer in combination with docetaxel both in a mouse model of prostate cancer and in prostate cancer cell lines [173]. Tumor growth inhibition is also reported when adding the APIM-peptide to standard chemotherapy treatment both in two rat models of NMIBC [171], MIBC [201] and in a xenograft model with multiple myeloma cells [165]. Recently even the combination of the APIM-peptide with an EGFR inhibitor showed increased treatment effect *in vivo* in a breast

cancer mouse model [202]. These results suggest that the APIM-peptide could increase the efficacy of different treatments in multiple different tumors. The APIM-peptide is currently in a clinical Phase 1 dose escalating study in cancer patients (all-comers) (ANZCTR: 12618001070224). The drug shows good tolerability without any myeloproliferative impact, which supports the suggestion that inhibition of tumor growth is not primarily caused by inhibition of replication.

2. Aims of the study

Our main aims:

1. To explore the effect and the molecular mechanisms of the APIM-peptide- cisplatin- based chemotherapy.

2. To explore how treatment management affects disease outcome in T1 BC.

3. To explore the use of reTURB and the impact on treatment and survival of T1 BC.

3. Summary of papers

"Two hits-one stone"; increased efficacy of cisplatin-based therapies by targeting PCNA's role in both DNA repair and cellular signaling

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* shared first authorship

Bladder cancer is normally cisplatin sensitive, but cisplatin has often limited anticancer effect over time because of acquired resistance. In this study we tested if the APIM-peptide increased the anti-cancer effect of cisplatin. Eight different bladder cancer cell lines, seven human and one rat, were treated with the APIM-peptide and with the cisplatin-based combinations commonly used in human medicine, MVCA and GC, *in vitro* cell survival assay. The cell lines showed different sensitivity against the APIM-peptide as a single agent while their sensitivity against cisplatin were more similar. The APIM-peptide increased the efficacy of cisplatin as well as GC and MVAC in all cell lines.

Next, we tested the cisplatin-APIM-peptide combination *in vivo* in a bladder cancer rat model were medication was given intravenously. Treatment effect was defined as bladder weight lower than the average bladder weight of the vehicle group. The treatment effects were found to be 100 % for the cisplatin-APIM-peptide combination, compared to 81 % for cisplatin and 43 % for the APIM-peptide as single treatment. In addition to significantly lower bladder weight, histological evaluation showed less invasive disease in the combination group compared to the cisplatin only group.

To investigate the molecular mechanisms behind the increased efficacy of cisplatin when combined with the APIM-peptide we examined the responses at several omics-levels, i.e. transcriptome, proteome/kinome and metabolome in two different BC cell lines with high or low sensitivity to the APIM-peptide. Gene expression analysis revealed an increase of ~1200 differentially expressed (DE) genes in the two combination groups compared to cisplatin single agent treatment in both cell lines. 75 % of DE genes in the cisplatin only treated cells were also found in cisplatin-APIM-peptide treated cells. The majority (~1000) of the increased DE genes found in the combination group were downregulated and included several pathways regulating cell cycle, DNA damage response, EGFR/VEGFR signaling, transcription and apoptosis.

Genes often upregulated or mutated in bladder cancer such as *VEGFR, EFGR and ERBB2* important for PI3K/Akt/mTOR and MAPK signaling and thereby regulation of growth were downregulated. Furthermore, genes encoding proapoptotic factors such as Bim and Caspase3 were upregulated, while antiapoptotic factors often upregulated in cancer were downregulated.

Changes in 522 signaling proteins (the "kinome") were found in cells treated with the cisplatin-APIM-peptide combination compared to untreated cells and 148 of these not found in cisplatin or APIM-peptide only treated cells. The kinome analysis confirmed the results from the gene expression analysis, e.g. reduced pulldown of EFGR/ERBB2 and several proteins in the MAPK and PIK3/Akt pathway were detected in cisplatin-APIM-peptide treated cells.

Gene expression analysis indicated that the cisplatin-APIM-peptide treatment downregulated genes encoding enzymes important in glycolysis. To investigate whether this was reflected in the metabolome, we measured glucose and glutamine consumption, and lactate production and applied targeted metabolomic profiling on the central carbon metabolism. The combination treatment significantly increased glucose and glutamine consumption as well as lactate excretion. Thus, the lactate/glucose ratio was decreased, and this may indicate Warburg effect in these cells. Significant changes in metabolite pool sizes such as increased levels of amino acids and deoxynucleotides were detected, supporting systemic response to the treatment.

Cisplatin resistance is a major issue in bladder cancer treatment. Genes associated with multidrug resistance are often overexpressed in bladder cancer and our gene expression analysis indicated that many of these genes were downregulated in combination treated cells. We also showed that APIM-peptide treatment increased the sensitivity for cisplatin in a cisplatin resistant cell line. This sensitizing effect could be mediated via cellular signaling and altered gene expression or via direct reduction in the cells ability to repair DNA lesions, or both. To test the latter, we measured if APIM-peptide treatment increased the level of DNA damage by impairing DNA repair. The levels of DNA lesions after cisplatin treatment were lower in the cisplatin resistant cells compared to the original cell. After cisplatin-APIM-peptide treatment; however, the levels of DNA lesions were increased to the same level as in the original cisplatin sensitive cell line. This supports that at least part of the cisplatin sensitizing effect of the APIM-peptide is mediated via inhibition of DNA repair.

This paper showed that the PCNA- interacting APIM-peptide increased the efficacy of cisplatin treatment both *in vitro* and *in vivo*. Omics analysis suggest that several pathways often upregulated in bladder cancer are downregulated in cisplatin-APIM-peptide treated cells. Targeting PCNA with APIM-peptides block multiple protein-PCNA interactions, thereby impairing PCNA scaffold functions. Because the APIM-peptide has increased affinity towards PCNA after cellular stress, e.g. after treatment with chemotherapeutics, this will mainly inhibit cellular stress processes. Here we show that impairing the cellular stress responses regulated by PCNA-protein interactions alters cellular signaling and DNA repair, resulting in reduced cancer growth. Thus, our data supports further development of the APIM-peptide as a cancer drug.

T1 bladder cancer in Norway: treatment and survival

A Blindheim, S Fosså, R Babigumira, T Å Myklebust, E Haug, C J Arum, B K Andreassen

Treatment of bladder cancer (BC) tumor stage T1 is challenging because of the risk of understaging the tumor at diagnose and the risk of progression into muscle invasive BC (MIBC). Most T1 patients have conservative early treatment which according to the European association of urology (EAU) guidelines includes transurethral resection of the bladder (TURB) combined with Bacille Calmette Guerin (BCG) bladder instillations and cystoscopy surveillance. A repeated TURB (reTURB) is recommended for all T1 patients within 4-6 weeks to increase the diagnostic accuracy. Patients at a special high risk of progression such as those with large and multiple tumors, concomitant cis and WHO high grade (HG) tumors should be assessed for early radical cystectomy (RC). A challenge for clinicians is to decide who can safely be followed conservatively and who needs RC as early treatment. In this register- based retrospective study we present data on early and delayed treatment such as BCG instillation and RC as well as estimated survival data of Norwegian T1 patients in 2008-2012.

The T1 study population includes 1,008 patients of which 76 % were men, median age at diagnose was 75 years, women were 2 years older than men (74 vs 76). More men than women had HG tumor, concomitant cis and muscle in the specimen at TURB. In total 180 (16 %) had RC of which 96 (9 %) as early treatment, defined as RC within 6 months after T1 diagnosis. T1 patients having MIBC or metastasis within 4 months after the T1 diagnosis were excluded from the study population. BCG was given to 42 % of the patients but only 15 % as early treatment within 8 weeks after T1 diagnosis. More men than women received both BCG and RC. Of all 1,008 patients, 52 % had reTURB. The probability of having RC or BCG as early treatment decreased significantly with age. Gender did not impact treatment choice. For the study population, 5-year cause specific survival (CSS) was 84 % and overall survival (OS) 64 %. Patients with early conservative treatment and delayed RC, defined as RC after 6 months, had the lowest 5-year CSS: 73 % for those treated early with BCG and 61 % for those treated early without BCG. Patients with early RC had a 5-year survival rate of 85 %. The best 5-year survival rate were observed for the group receiving only BCG early treatment, 91 %, and for the group initially followed conservative without BCG but given BCG treatment later during follow up, 87 %.

The number of patients treated with RC is higher and BCG treatment lower than comparable population-based Swedish studies. The low early BCG treatment rates could be influenced by underreporting and/or registration failure. Other reasons could be patients age and comorbidity

making them less fit for treatment although higher adherence to guidelines is reported in comparable populations.

Conclusion: We report patients with delayed RC to have the lowest survival rates and the management of this group needs follow up. The low adherence to recommended use of early BCG treatment needs further investigation. A closer focus both regarding treatment and surveillance of T1 patients could increase treatment quality of T1 patients in Norway.

Errata Fig 1: The total number of conservative early treatment should be 1012 and not 1019.

The use of reTURB T1 bladder cancer, a Norwegian population-based study.

A Blindheim*, Sofie D Fosså*, R Babigumira, B K Andreassen

*equal contribution

International guidelines recommend a repeat transurethral resection of the bladder (reTURB), within 4-6 weeks after the primary TURB for stage T1 bladder cancer (BC), to increase the diagnostic accuracy and specifically to avoid overlooking muscle invasive bladder cancer (MIBC) at the primary TURB (primTURB).

In this paper we report the use of reTURB in a Norwegian T1 population of 1,130 eligible patients diagnosed between 2008-2012, and the impact on early treatment and survival. Patients with immediate cystectomy without reTURB, patients with cancer within 5 years of the T1 diagnosis and patients whom died within 3 months after T1 diagnosis were excluded.

In total 648 (57 %) of the study population had a reTURB. The reTURB patients were 6 years younger and were more frequently diagnosed with WHO high grade (HG) tumors. 275 (24%) patients did not have detrusor muscle (DM) in the specimen at primTURB of which 114 (41%) did not have a reTURB. Histology reports were available for 323 (50 %). Pathology laboratories are required to submit all cancer histologies to the CRN; as well as histology reports without cancer in known cancer patients. However, we assigned cases without histology reports to stage T0. It is reasonable to assume that reporting of specimen without tumor easily is forgotten specially in cases with no information about earlier cancer given by the surgeon.

We report muscle invasive bladder cancer (MIBC) in 7 %, T1 in 17 % and Ta/cis/T0 in 76 % at reTURB. Early radical cystectomy (RC) within 6 months was performed in 81 (13 %) mainly because of MIBC or T1 in the reTURB specimen. Only half of the patients upstaged to MIBC and one third of those with T1 at reTURB had early RC. Of the patients with detrusor muscle (DM) in the primTURB specimen 24 (5 %) was found to have MIBC at reTURB as compared to 21 (13 %) for those without DM.

No difference in CSS was seen between patients who had a reTURB compared to those without when adjusting for age, sex, grade, concomitant cis and early treatment. In contrast, the adjusted OS was higher in patients with reTURB probably because of a younger and healthier patient group. However, we showed that patients with T1 at reTURB had decreased survival compared to patients with lower stage at reTURB, thus representing a group that should be extra closely followed. The reTURB led to an increase in diagnostic accuracy in our study which hopefully was beneficial for some of our patients, although this was not confirmed by survival data.

This is a retrospective study with several limitations, the reTURB patients were not randomly chosen and had probably features making the reTURB more likely e.g. big and multiple tumores making the comparison between the groups with or without reTURB difficult.

Our conclusion is that both the choice of early treatment and survival depend on the reTURB histology. We further conclude that our report demonstrates a need for a closer adherence to guidelines since only about half of the patients had a reTURB and only half of the patients without DM at primTURB had a reTURB. Our study also underpins the need for a national bladder cancer register to monitor treatment of bladder cancer in Norway.

4. Discussion

4.1 Increased efficacy of combination treatments

Attacking several targets simultaneously has been a leading research strategy in clinical studies in recent years. However, the strategy of combining drugs with different modes of action in order to reduce resistance and increase treatment efficacy started in the sixties and lead to different combination regimens of chemotherapeutics [203]. This can be combinations of multiple cytostatic drugs and/or chemotherapeutics in combination with radiation e.g. as in NAC and TMT for BC. Different combinations of CHIs and kinase inhibitors in combination with traditional treatments such as radiation and chemotherapy are also under clinical trials in effort to increase treatment efficacy [204].

In paper 1 we combined cisplatin and cisplatin-based treatment with the APIM-peptide. Because PCNA localizes both in the nucleus and in cytoplasm, inhibition of the PCNA-protein interaction by the APIM-peptide might influence both DNA repair, DNA tolerance mechanisms and cellular signaling pathways simultaneously. For example, we report that the DDR proteins ATM, a protein kinase activated by DNA double strand break, tumor suppressor Rb1, a TLS polymerase REV1 and HERC2 involved in regulation of NER and essential for recognizing DNA damage are all downregulated by the cisplatin-APIM-peptide treatment. Reduced expression of glycolytic enzymes and the glucose consumption/lactate production ratio were downregulated by cisplatin-APIM-peptide treatment. Several glycolytic enzymes are putative PCNA partners [159, 205] and thus impairing PCNA scaffold function in cytosol could potentially impair cancer growth via impaired glycolysis and reduced Warburg effect. Signaling EGFR/AKT/MAPK are often upregulated in cancer. We found downregulation of EGFR and ERBB2, both receptors important for MAPK and PI3K/Akt signaling by cisplatin-APIM-peptide treatment. Furthermore, genes encoding proteins in DNA repair were downregulated, e.g. HERC2 involved in regulation of NER and essential for recognizing DNA damage and the APIM containing REV1 involved in TLS supporting our hypothesis that the cisplatin-APIM-peptide treatment impacts DNA repair and tolerance mechanisms. We also showed that the cisplatin- APIM-peptide treatment increased the number of DNA lesions e.g. DNA single and double strand breaks in comet assay supporting that DNA-repair was decreased in the combination treated cells. Which of these pathways are more important for the inhibition of tumor growth observed in the BC model is difficult, if not impossible to determine.

4.2 Toxic effect of the APIM-peptide

Cisplatin is targeting DNA, and thus both replication and transcription. Cisplatin is in addition modifying proteins and lipids and is therefore toxic to both normal rapidly proliferating and non-proliferating cells, and consequently entire organs. A major question is whether the cisplatin-APIM-peptide combination will give additional toxic effects also in normal cells. Most of the other PCNA targeting drug candidates (see 1.6.5), are targeting the replicative function of PCNA. The APIM- peptide on the other hand does not affect replication, and induces apoptosis in all phases of cell cycle, not only in S-phase [165]. Normal cells are less sensitive to the APIM-peptide, and the sensitivity of different cancer cell lines, including the BC cell panel tested, varies. This difference in sensitivity is not dependent upon growth rate, PCNA level nor distribution of PCNA in the cytosol versus the nucleus [171].

Initially it was shown that overexpressed APIM-peptide did not inhibit growth in the absence of DNA damage, but strongly enhanced the growth inhibitory effects of chemotherapy. Further analysis of PCNA pull down by APIM-peptides showed multiple forms of PCNA (likely PTMs) with lower isoelectric points compared to the total PCNA in the cells [164]. This led to the hypothesis that APIM-PCNA interactions had a special role after cellular stress and that the affinity to PCNA was increased by specific PTMs. Later data has shown that APIM has increased affinity to poly-ubiquitinated PCNA [168], supporting that PTMs of PCNA is important for the APIM-PCNA affinity. Cancer cells experience high levels of endogenous stress due to impaired signaling and regulations, thus cancer cells likely contain increased amounts of modified PCNA which can explain their increased sensitivity.

Good tolerability of the lead APIM-peptide drug, ATX-101, in humans is observed in the currently ongoing dose escalating clinical Phase 1 studies in cancer patients (all-comers) (ANZCTR:12618001070224). Prolonged weekly administration in several patients have not revealed any myelosuppressive properties, while several patients have obtained prolonged stabilization of their disease (data shared by APIM Therapeutic AS). Therefore, the APIM-peptide as a single agent has shown low toxicity. However, in multiple cancer cells tested an increased efficacy of various chemotherapeutics are seen when combined with the APIM-peptide, and this is seen also for cell's with low sensitivity to the APIM-peptide as single agent. Therefor increased cytotoxicity in normal cells is also expected, thus a lower than maximum tolerated dose of the chemotherapeutic drug might be needed in the combination treatments. Thus, whether the reduction in dose of the chemotherapeutics in order to avoid increased toxicity is favorable regarding increased anticancer efficacy must be determined. The enhanced effect of the cisplatin-APIM-peptide treatment could also enable the use of lower dosage of cisplatin for elderly with comorbidities.

4.3 Resistance

Resistance to DNA damaging chemotherapy could partly be due to increased repair of the DNA lesions induced. Inhibiting the repair mechanisms is therefore interesting as it can increase sensitivity to treatment. To explore the effect of cisplatin-APIM-peptide treatment on cisplatin resistant cells, we developed a cisplatin resistant cell line by prolonged incubation with increasing doses of cisplatin, starting at low doses. We found that the APIM-peptide re-sensitized the resistant cells to cisplatin treatment. The reason for this can at least partly be explained by inhibition of DNA repair as the levels of DNA lesions increased. However, treatment with APIM-peptide-cisplatin combinations also reduced expression of genes reported to be important for cisplatin resistance, thus, this could also contribute. These data could indicate that tumors resistant to cisplatin could benefit from the combination treatment.

Genes in multiple repair pathways are often mutated in cancer, for example the gene Excision repair cross-complementation group 2 (*ERCC2*), belonging to the NER pathway is mutated in 9-15 % of BC cases [115, 186]. Cancer cells with such and similar mutations in DNA repair pathways may potentially be even more sensitive to the cisplatin-APIM-peptide combination treatment as these cells are reliant on an already damaged repair system and therefore easier can be pushed towards cell death when additional inhibition is given. This "synthetic lethality" approach is explored in the case of the inhibition of PARP1 in cancers deficient in homologous recombination (HR). PARP inhibitors reduce repair of single strand DNA breaks, thereby, leading to increased levels of DNA double strand breaks which is especially difficult to repair in proliferating HR deficient cells. PARP inhibitors are already approved for clinical use [206]. In addition, because the APIM-peptide is shown to reduce the mutation frequency via reducing TLS, cisplatin resistance development will likely be slowed down in combination treatments [188]

4.4 The diagnosis of T1 BC

The outcome of T1BC treatment is highly dependent on the quality of the daily work done by surgeons and pathologists. The result from the initial TURB is important both for diagnosis and for treatment of the T1 tumor, and the quality of the surgery is critical for the diagnosis. TURB, has been in use nearly 60 years; however, some technical improvements have been added such as fiber optics, the possibility of having the operation on screen, and visualization enhancements such as NBI and PDD. Another

strategy to improve treatment quality includes en block removal of the tumor, but unfortunately, the quality of the TURB is still often not good enough, leading to residual tumor tissue and understaging of the tumor [207, 208].

How can the quality of the TURB procedure be improved? It is accepted that documenting the results of a procedure generally will increase the quality of a procedure. In Norway no quality registry of the TURB procedure has been established. It is well documented that the experience of the urologist is important for the outcome of the operation such as rate of recurrences and presence of DM in the specimen [20, 21]. Until now a certain number of TURB-procedures were required to become a specialist of urology in Norway but there has not been any systematic teaching program in performing the procedure. Trainees therefore often experience a steep learning curve with limited supervision, mainly because of the high workload at urological departments. Furthermore, the procedure is often performed by several surgeons leading to limited volume per surgeon. Studies have showed that high volume hospitals as well as high volume surgeons are associated with better treatment outcome [209]. Centralization of urological cancer surgery has generally been accepted for major surgery such as RC, but centralization of TURB has never been considered. For most BC patients the quality of the treatment given is adequate; however, it should be questioned whether some of the T1 patients could benefit from treatment at high-volume hospitals or at least high-volume surgeons, especially patients with large tumors and patients scheduled for reTURB.

The histology report gives stage and grade of the tumors, but studies are reporting a substantial variation in assessment of T1 tumors between pathologists [30]. In Norway, the histology report is not given in a standardized way neither by surgeons giving the tumor information nor by pathologists, for example there is no depth of infiltration in lamina propria classification. Histology reports should always include both size and number of tumors, presence of DM and depth/extension of infiltration. A national standard of the histology report could increase the diagnostic quality of the T1 tumors and should be possible given the data technology available. A close collaboration between urologists and pathologists is important to clarify critical tumor information at diagnosis. For pathologists at local hospitals with few T1 cases, it could be discussed whether a second opinion at regional or university hospitals should be undertaken to secure the diagnosis.

Better diagnostics is expected from improvement of imaging modalities, with MRI being the most promising tool so far [210]. When large tumors are found, MRI can help in assessing depth of invasion and it could be discussed whether MRI should be standard work-up of large or multiple tumors.

Cooperation in diagnostics and treatment of T1 BC is crucial and T1 cases should be discussed at the multiple disciplinary team (MDT) meetings.

4.4.1 BCG treatment

Bladder instillation therapy is suitable for T1 tumors since the tumor can be directly targeted by intravesical administration of the drug. More efficacious intravesical treatment regimens are however needed. BCG-studies report diverging treatment effects and a recent metaanalysis published by the Cochrane group questions both the effect on relapses and on progression in treatment of T1BC [90]. It is also questioned whether elderly patients are responding adequately to BCG treatment since the effect relies on an immune response that might be impaired by age, e.g. at age above 80 a decreased effect of BCG treatment measured as a higher rate of relapses are seen [211]. In our data, BCG treatment did not impact survival, even when the BCG treated patients were younger and with a possibly better immune response. To increase treatment efficacy of BCG, combination treatments are reported, but so far without impact on clinical practice.

The APIM-peptide (ATX-101) is reported to increase the effect of MMC on tumor growth when given as a combination treatment with the DNA damaging chemotherapeutics MMC and Bleomycin intravesically to rats in two different rat models of NMIBC [171]. This may suggest a possible use of the APIM-peptide in intravesical combination treatments. With the APIM-peptide's possible link to the immune system, combination treatment with BCG might increase the immune response.

4.4.2 The use of RC

The main challenge for urologists in treatment of T1BC is to decide who can safely be treated conservatively and who should be offered early RC. Clinically T1 tumors at RC are reported to be upstaged in up to 50 % of the cases and up to 20 % of these patients are reported to have lymph node metastasis [76, 78]. These reports highlight the inaccuracy in diagnosis of T1BC, and since patients with MIBC might benefit from neoadjuvant treatment some of the patients will not be given the opportunity of receiving the best possible treatment. The RC operations are centralized in Norway and not performed at local hospitals. This gives all T1 patients referred for RC a second opinion. However, in local hospitals with few urologists and with more generalized radiologists not specifically dedicated to uro-radiology as well as general pathologist as opposed to dedicated uro-pathologists, diagnostic workups can be suboptimal. MDT discussions might become limited with the risk of suboptimal treatment for some of the T1 patients. Therefore, it should be discussed if referral to central MDT meetings for all T1 cases could be a strategy to increase the quality of therapy for these patients.

Patients having delayed RC, i.e. during follow up, have the lowest survival rates (reported in paper II). A closer look at this group could be interesting in future studies in order to identify risk factors leading to their inferior survival rate. Our data suggest that the number of RC in Norway is higher than in comparable population-based studies in Sweden, and in future studies it would also be interesting to see whether there are any regional differences in Norway and how the RC rates develops over time.

4.4.3 reTURB

There are discussions whether a reTURB is needed if DM is present in the primary TURB specimen and one study have reported impact on recurrence rates, progression rates and CSS only when DM was not present in the primary TURB specimen [71]. We report an increase in MIBC at reTURB in patients without DM in the reTURB specimen compared to those with. However, DM in the primary TURB specimen did not exclude the possibility of overlooking MIBC since 5 % of the patients with DM showed MIBC at reTURB

Histology reports were available for only 50 % of the reTURB cases. We assume that this low rate was caused by a tradition of not reporting specimen without tumor tissue especially if pathologists were unaware of the earlier cancer diagnosis of the patients. In paper III we report 58 % to be tumor free at reTURB and although our result is better than several other reports, the fact that 42 % showed remaining tumor tissue illustrates the difficulty in performing the TURB operation properly, and that the quality of the TURB is not dependent on DM in the specimen only. In our study, patients selected for reTURB most likely represent the most severe cases. Comparison of survival between those with vs those without reTURB is therefore difficult. The fact that the reTURB group showed similarity in survival compared to the no reTURB group might be interpreted as an actual increased survival for the reTURB group if these patients initially had a more severe tumor than the no reTURB patients. Furthermore, since TURB is a procedure with risk of incomplete resection even when DM is found in the specimen, reTURB will secure the best possible oncological result, at least until a good quality primary TURB can be documented.

4.5 Limitations

Using a rat model

Bladder tumor models that resembles human disease both histologically and in carcinogenesis are necessary for evaluating effects of new therapeutic agents. Dr Lamm has summed up the ideal characteristics of an animal bladder tumor model and stresses that i) the tumor should grow intravesically (orthotopically), so the tumor can be directly exposed to antitumor drugs in its natural environment. ii) The tumor should be of transitional carcinoma origin, iii) animal host should be immunocompetent and reasonable large. iv) The tumor should be technically easy to develop within a reasonable period of time, and highly reproducible with respect to its natural history[212].

The rat model used in our study meets these requirements; however, as the tumor in our case is monoclonal (from a rat bladder cancer cell clone) it differs from most human BC tumors which are heterogenic. The tumor growth rate is for example very high compared to human tumor growth, so despite the model being in accordance to best practice there are differences between animal and human cancer cell growth. Therefore, the conclusion on treatment effect cannot be drawn until human studies have been completed. However, in support for an effect of the APIM-peptide in BC, the APIM-peptide was shown to increase the effect of MMC in an endogenously BBN induced NMIBC rat model [171].

One of the main limitations with the rat model used is the lack of determination of the tumor size at treatment start, making accurate measurement of growth reduction in the individual tumors impossible.

Cell line studies

We used the MTT assay for quantification of cell viability and proliferation. MTT is a yellow tetrazolium salt that is reduced to purple formazan crystals by mitochondrial succinate dehydrogenase[213]. The observed purple color is therefore representative for mitochondrial activity and not directly cell growth.

Register studies

Register based studies highly rely upon the quality both in enrollment of information from hospitals and the register work done in the registers. Norway in known for the high quality of its cancer registry [214]. We are also using the NPR where all treatments given at the hospitals should be reported. When merging two registers NPR and CRN the numbers of surgery are highly reliable. The histological reports were however given for only about 50 % of the reTURB patients and revealed a pronounced lack of reports to the CRN for this patient group, probably due to underreporting of T0 cases.

4.6 Conclusion

Cisplatin based chemotherapy is the treatment of choice for metastatic bladder cancer; however, it only marginally improves the survival rate. We found that cisplatin given in combination with the experimental drug APIM-peptide, reduced the bladder cancer growth *in vivo* in an immunocompetent MIBC rat model. In accordance with this, we found that the APIM-peptide enhanced the efficacy of cisplatin *in vitro*. Gene expression analysis on bladder cancer cell lines indicated that the combination of cisplatin and the APIM-peptide alters expression of multiple genes involved in cellular signaling, apoptosis, and bladder cancer development. These changes were not detected in single agent treated cells. Our results suggest that targeting PCNA and thereby affecting multiple cellular pathways simultaneously has the potential to improve cisplatin-based therapy of advanced bladder cancer. It could also be interesting to see whether a combination of for example MMC and the APIM-peptide as bladder instillation treatment could impact relapses and/or progression of T1 tumors in a future clinical study.

T1 bladder cancer is challenging to treat properly. We report low rates of BCG treatment as well as low rates of reTURB. Elderly were less frequently treated both with BCG and RC and had a lower rate of reTURB compared to younger patients. The treatment quality of elderly therefore needs closer follow up. Patients with delayed RC had the lowest survival rates suggesting both inferior early and follow-up treatment for a group of patients. We further report T1 at reTURB with decreased survival compared to lower stages at reTURB thus identifying a group with higher risk of progression. Discussions of T1 cases at MDT meetings and closer surveillance of patients in accordance with EAU guidelines is needed. Our report hopefully can open for increased focus on the treatment of BC patients, and supports the work with establishing a national bladder cancer registry.

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PAPER I

"Two hits - one stone"; increased efficacy of cisplatin-based therapies by targeting PCNA's role in both DNA repair and cellular signaling

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ABSTRACT

Low response rate and rapid development of resistance against commonly used chemotherapeutic regimes demand new multi-targeting anti-cancer strategies. In this study, we target the stress-related roles of the scaffold protein PCNA with a cell-penetrating peptide containing the PCNA-interacting motif APIM. The APIM-peptide increased the efficacy of cisplatin-based therapies in a muscle-invasive bladder cancer (MIBC) solid tumor model in rat and in bladder cancer (BC) cell lines. By combining multiple omics-levels, from gene expression to proteome/kinome and metabolome, we revealed a unique downregulation of the EGFR/ERBB2 and PI3K/Akt/mTOR pathways in the APIM-peptide-cisplatin combination treated cells. Additionally, the combination treatment reduced the expression of anti-apoptotic proteins and proteins involved in development of resistance to cisplatin. Concurrently, we observed increased levels of DNA breaks in combination treated cells, suggesting that the APIM-peptide impaired PCNA - DNA repair protein interactions and reduced the efficacy of repair. This was also seen in cisplatin-resistant cells, which notably was re-sensitized to cisplatin by the APIM-peptide. Our data indicate that the increased efficacy of cisplatin treatment is mediated both via downregulation of known oncogenic signaling pathways and inhibition of DNA repair/translesion synthesis (TLS), thus the APIM-peptide hits both nuclear and cytosolic functions of PCNA. The novel multi-targeting strategy of the APIM-peptide could potentially improve the efficacy of chemotherapeutic regiments for treatment of MIBC, and likely other solid tumors.

INTRODUCTION

BC is the ninth most common cancer worldwide, with an expected increase in incidence [1]. MIBC contributes to 30% of BC patients, and the 5-year survival rate after cystectomy is only 50% [2]. There have been few improvements in therapy since the advent of cisplatin. Immunotherapy via PD-1 inhibition is the only novel treatment recently accepted for MIBC, but the improvement in survival is so far modest [3]. Recent advances in genomic research have identified several therapeutic targets, however, their efficacy in therapy remains to be tested [4].

The gold standard in MIBC therapy is neoadjuvant cisplatin-containing treatment and cystectomy. Cisplatin-based chemotherapy is also the first line treatment for patients with metastatic disease, where gemcitabine/ cisplatin (GC) and methotrexate/vinblastine/adriamycin/ cisplatin (MVAC) are the main chemotherapeutic alternatives [2]. Formation of DNA interstrand crosslinks are responsible for the major cytotoxicity of cisplatin, but increased DNA repair, overexpression of ERBB2 and activation of the PI3K/Akt pathway often contributes to development of cisplatin resistance [5]. Cisplatin may offer longer survival, nonetheless, long term survival is uncommon in metastatic disease [6]. Cisplatin sensitization via strategies that can reduce cisplatin resistance can potentially improve metastatic as well as non-metastatic MIBC therapy.

PCNA acts as scaffold protein in several essential processes such as DNA replication, DNA repair and epigenetics [7, 8]. More recently, cytosolic scaffold roles of PCNA in apoptosis, glycolysis and signaling have been demonstrated [8–11]. The essential roles of PCNA during cellular stress and replication makes it a potential drug target, and a few PCNA-targeting drugs are under pre-clinical development [12].

The two known, and highly conserved, PCNA-interacting motifs, the PCNA-interacting peptide (PIP)-box and AlkB homologue 2 PCNA-interacting motif (APIM), are present in more than 600 proteins, and share the same binding site on PCNA [13–16]. Peptides and/or small molecules that bind with high affinity to this binding site will inhibit the majority of PCNA-protein interactions, and thereby inhibit essential cellular functions. Thus, such drugs will be cytotoxic to all cells. Accordingly, overexpression of a high affinity (canonical) PIP-box peptide is cytotoxic. On the other hand, overexpression of an APIM-peptide is well tolerated in the same cells in the absence of exogenous stress, but it strongly reduces cell growth and induces apoptosis in cells stressed with DNA damaging agents [10, 14, 17]. This is in line with the presence of APIM in many proteins involved in cellular stress responses, including the nucleotide excision repair (NER) protein XPA, the TLS polymerase POL ζ and proteins such as RAD51B, Topo IIa, TFII-I, ZRANB3 and FBH1, all which are important during replication stress

and involved in repair of cisplatin-induced DNA lesions [14, 18–22]. Furthermore, the APIM-peptide is shown to enhance the efficacy of various chemotherapeutic drugs in multiple cancer cells both *in vitro* and *in vivo*, i.e. i) in a multiple myeloma xenograft model and an endogenous orthotopic prostate cancer model after intraperitoneal administration in combination with melphalan and docetaxel [10, 23], ii) in both syngeneic and endogenous orthotopic non-MIBC models in rats after intravesical administrations in combination with mitomycin C [24]. Several lines of evidence indicate that the chemo-sensitizing effect of the APIM-peptide is caused by the direct binding of the APIM-peptide to PCNA and that APIM-PCNA interactions are stronger under cellular stress and at least partly mediated by posttranslational modifications on PCNA [8, 10, 14, 18, 19, 22, 25].

Here we show that the APIM-peptide enhances the anti-cancer efficacy of cisplatin in a syngeneic orthotopic MIBC model in rats and increases the efficacy of GC and MVAC in a panel of human BC cell lines. The APIM-peptide-cisplatin combination reduces the expression of multiple proteins and oncogenic pathways, often upregulated in BC as well as in other solid tumors. We detect increased levels of DNA strand breaks after APIM-peptide-cisplatin treatment, suggesting that the APIM-peptide inhibits repair of cisplatin-induced lesions. Notably, the APIM-peptide re-sensitizes cisplatin-resistant BC cells and elevates the levels of DNA strand breaks in these cells to the same level as in cisplatin-sensitive cells.

RESULTS

APIM-peptide increased the anti-cancer efficacy of cisplatin *in vivo*

The anti-cancer effect of the APIM-peptide in combination with cisplatin was first examined in a MIBC model in rat. Inoculated cells were left to grow for three weeks before three rats were terminated to establish that the instilled cells had progressed to MIBC (untreated, Figure 1). Histopathological evaluation confirmed that two of these bladders had muscle invasive high grade (T2G3) tumors at this time point, while the last was classified as non-muscle invasive high grade (T1G3) (Table 1A). We therefore treated the remaining rats at this time point and evaluated treatment efficacy one week later.

Effect of the treatment was defined as bladder weight lower than the average bladder weight of the vehicle group (broken line in Figure 1). Effect of treatment was found in 100% of the combination group, compared to 81% of the cisplatin group and 43% of the APIM-peptide group (29% in vehicle group). Importantly, the combination group had a significantly lower tumor weight (p=0.04) and a more uniform response to treatment than the cisplatin group (Table 1B and Figure 1). Of notice, no acute toxicity was observed in rats treated with the APIM-peptide.

Histopathological evaluation of the bladders confirmed fewer invasive tumors (T2/3G3) in the combination group (47%) than in the cisplatin group (63%) (Table 1B). Because the initial tumor volume in individual rats prior to treatments is unknown in this model, it was difficult to establish whether bladders classified as histopathological "normal" were cured, or if they were non-takes (one in cisplatin and two in combination group, see Table 1B). However, the bladder weights were significantly lower in the combination group than in the cisplatin group even if the cured/potential non-takes were excluded (p=0.05). Our results suggest that the APIM-peptide can potentiate the anti-cancer efficacy of cisplatin.

To explore the biodistribution of APIM-peptide after *i.v.* infusion, we harvested tissue from thigh, heart, kidney, brain, liver and bladder immediately after infusion of fluorescently tagged APIM-peptides. Positive fluorescence was detected by confocal imaging in frozen sections from all organs evaluated, including the bladder, supporting that the increased anti-cancer activity of cisplatin on bladder tumors was due to the presence of the APIM-peptide (Supplementary Figure 1).

Efficacy of cisplatin-containing treatments were enhanced by the APIM-peptide *in vitro*

Next, we examined if the APIM-peptide could increase the sensitivity of several cisplatin-containing treatments using a panel of human BC cells. Previously, we found that the sensitivity towards the APIM-peptide as a single agent varied in these cell lines, but that this was not linked to their PCNA levels [24]. However, their sensitivities towards cisplatin were similar and, importantly, the efficacies of cisplatin, MVAC and GC were enhanced by the APIM-peptide in all cell lines (Figure 2). Our results suggest that the APIM-peptide increases the efficacies of several chemotherapeutics used for MIBC therapy.

APIM-peptide-cisplatin treatment increased the number of differentially expressed (DE) genes

We selected the Um-Uc-3 and T-24 cell lines for gene expression analysis because they represent one APIM-peptide single agent sensitive (Um-Uc-3) and one insensitive (T-24) cell line. Still, APIM-peptide treatment increased the efficacy of cisplatin in both cell lines. We only included DE genes similarly changed in all three biological replicas of both cell lines. The APIM-peptide as a single agent did not have any similar effects on gene expression in the two cell lines (Figure 3A). Cisplatin as a single agent altered gene expression of multiple genes similarly in the two cell lines, and 75% of these DE genes overlapped with those in the APIM-peptide-cisplatin treated group. However, the combination treatment resulted
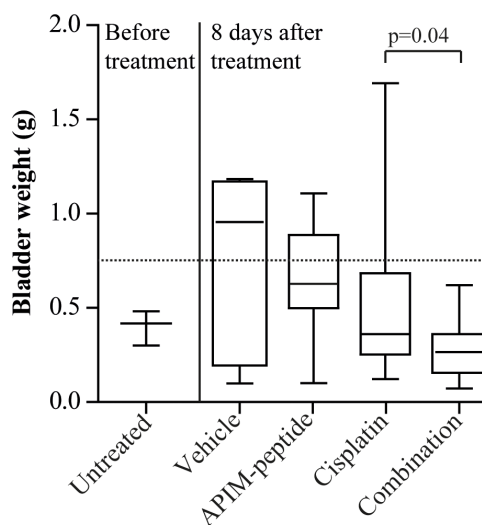

Figure 1: Combination of APIM-peptide and cisplatin therapy inhibits tumor growth in an orthotopic MIBC solid tumor model. Box-and-whisker plot of rat bladder weights harvested before treatment (n=3) or eight days after intravenous treatment with vehicle (NaCl, 0.9%, n=7), APIM-peptide (8.5 and 12.5 mg APIM-peptide peptide/kg, n=7) and cisplatin (2 mg/kg, n=16) alone or in combination (n=19). Data from three biological replicas is included in the figure. P-values were calculated using unpaired, two-tailed student *t*-test. The line in the box is the median, the box extends from the lower to the upper quartile, and the whiskers represent the lowest and highest bladder weight in each group.

Table 1: Histopathological classification of bladders before and after therapy

A

Bladder weight (g)	Histological classification	Clinical classification
0.30	T2G3	MIBC
0.48	T2G3	MIBC
0.42	T1G3	NMIBC

B

Cisplatin		APIM-peptide-cisplatin	
Bladder weight (g)	**Histological classification**	**Bladder weight (g)**	**Histological classification**
0.12	Normal	0.07	TaG2
0.22	T1G3	0.08	Normal
0.23	T1G2	0.09	Normal
0.23	T1G2	0.12	TaG2
0.27	T2G3	0.16	TaG1
0.29	T1G2	0.21	T2G3
0.33	T2G3	0.23	TaG1
0.36	T2G3	0.24	T2G3
0.36	T2G3	0.27	T1G3
0.39	T2G3	0.27	T1G3
0.43	T3G3	0.27	T1G2
0.57	T3G3	0.31	TaG2
0.61	TaG1	0.32	T3G3
0.76	T3G3	0.36	T2G3
0.86	T3G3	0.36	T2G3
1.69	T3G3	0.41	T2G3
Average: 0.48		0.47	T2G3
		0.48	T3G3
		0.62	T3G3
		Average: 0.28	

Histopathological evaluation of rat bladders inoculated with AY-27 cells for three weeks at **(A)** treatment start and **(B)** 8 days after treatment with cisplatin (2 mg/kg) as single agent or in combination with APIM-peptide (8.5 mg/kg or 12.5 mg/kg APIM-peptide/kg). Normal bladder weight is approximately 0.1 g, while tumor-containing bladders are heavier corresponding to size and grade of tumor [24].

in more than 1200 additional DE genes not affected by cisplatin treatment alone (combination minus shaded area, Figure 3A). The majority of these genes were downregulated (73%). Similar trends were seen after 4 hours of treatment, but the number of DE genes were strongly increased from 4 to 24 hours (Supplementary Figure 2).

Combination treatment downregulated genes frequently overexpressed in cancer

The DE genes identified only in the combination group (orange area in Figure 3A, gene lists in Supplementary Table 1) were subjected to gene enrichment analysis. Alterations in several pathways
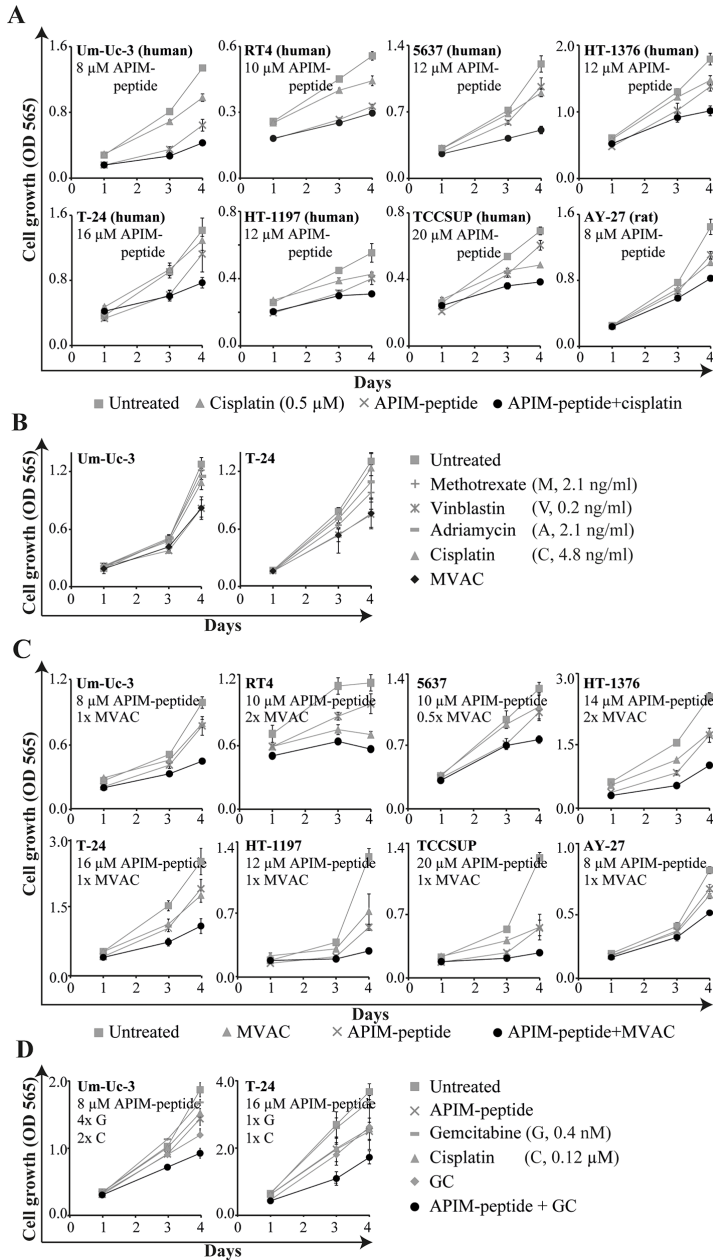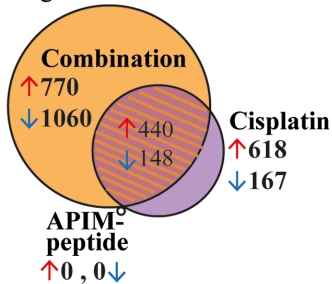
Figure 2: APIM-peptide in combination with clinically relevant cisplatin-containing combinations inhibits cell growth *in vitro*. Cell growth (MTT assay) of BC cell lines after continuous exposure to the APIM-peptide and chemotherapeutic agents as single agents or in combination (added on day 0). Each graph is from one representative replica of at least three biological replicas. Data is displayed as average ± SD (4-6 technical replicas). **(A)** APIM-peptide and cisplatin as single agents and in combination. **(B)** Methotrexate, vinblastine, adriamycin and cisplatin as single agents and in combination (MVAC). The sensitivity to vinblastine as a single agent are similar as to MVAC. **(C)** APIM-peptide and MVAC (0.5-2x of the doses in (B)) alone and in combination. **(D)** APIM-peptide, gemcitabine and cisplatin as single agents and in combinations (GC).
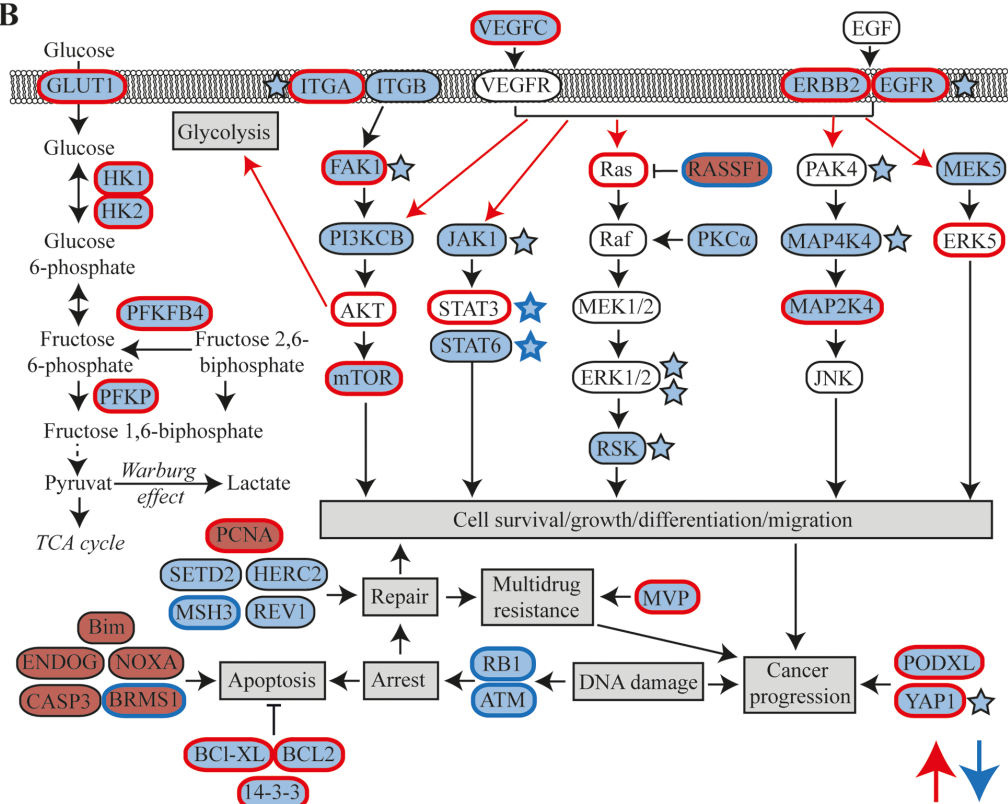
Figure 3: APIM-peptide in combination with cisplatin downregulates expression of frequently overexpressed genes in MIBC. Microarray analysis on Um-Uc-3 and T-24 cells treated for 24h with APIM-peptide (8 and 16 µM, respectively) and cisplatin (10 µM) alone or in combination (n=6). **(A)** Venn diagram illustrating number of differentially expressed (DE) genes in each treatment group (relative to untreated control, FC>1.25). Orange area marks the part unique for the combination group. **(B)** Schematic overview highlighting the most interesting upregulated (red background) and downregulated (blue background) DE genes detected only in the combination group with relevance to MIBC. Red edge = often overexpressed in MIBC, blue edge = often inactivated in MIBC, red arrows = often upregulated pathways in MIBC [4, 26-30, 32, 33, 37, 49-54]. Stars denote downregulated proteins detected by the MIB-assay; i) only in the combination group (blue stars with blue edges) or ii) more downregulated in the combination group than the cisplatin group (blue stars black edges).

including cell cycle, DNA damage, EGFR/VEGF signaling, transcription and apoptosis were identified (Table 2). A simplified schematic overview highlighting DE genes after combination treatment in relation to the most relevant pathways for MIBC are shown in Figure 3B. Expression of *VEGFC, EGFR, ERBB2* and several genes encoding proteins in downstream MAPK and PI3K/Akt signaling pathways were downregulated. Interestingly, these are commonly overexpressed in MIBC, as well as other solid cancers [26, 27]. Furthermore, downregulation of several genes encoding proteins involved in the DNA damage response, e.g. RB1, ATM, HERC2 (NER), REV1 (TLS), MSH3 (mismatch repair) and SETD2 (homologues recombination) were detected. Downregulation of glycolysis was indicated by the reduced expression of *GLUT1, HK1/2* and other glycolytic enzymes often overexpressed in BC [28]. Moreover, pro-apoptotic factors such as Bim and caspase 3 were upregulated, while anti-apoptotic factors such as BCL2 and BCL-XL, commonly overexpressed in BC [26], were downregulated. Our results demonstrate that combination treatment alters key genes in MIBC that are supportive of the inhibited BC growth observed both *in vivo* (Figure 1) and *in vitro* (Figure 2).

APIM-peptide enhanced cisplatin-induced changes in cellular signaling

To confirm the alterations in cellular signaling indicated by gene expression analysis on protein level, we enriched the cell extracts from Um-Uc-3 and T-24 for kinases and other dNTP/NTP interacting proteins prior to mass spectrometry (MS) analysis using the multiplexed inhibitor bead (MIB)-assay. We detected significant changes in 522 proteins after APIM-peptide-cisplatin treatment compared to untreated control (Figure 4A). This included 4 phosphatases, 15 ubiquitin ligases and other proteasome/chaperone proteins as well as 32 signaling kinases. Of these proteins, 148 were unique for the combination group (orange area in Figure 4A, protein lists in Supplementary Table 2). Many of the same proteins were pulled down in all treatment groups, however, 67% of the proteins pulled down in both cisplatin and combination groups (shaded area Figure 4A) were more increased/reduced by the combination treatment (Figure 4B). Reduced pull-down of multiple proteins in the combination group supported downregulation of the EGFR/ERBB2, MAPK and PI3K/Akt pathways as suggested by the gene expression analysis (stars in Figure 3B).

APIM-peptide-cisplatin combination increased glucose and glutamine consumption and affected central carbon metabolism

Gene expression analysis indicated that the APIM-peptide-cisplatin combination downregulates genes encoding glycolytic enzymes. To investigate whether these changes were reflected in the metabolome we next measured glucose and glutamine consumption, lactate production and applied targeted metabolic profiling of central carbon metabolism. We detected low residual glucose in Um-Uc-3 cell cultures, and even though addition of glucose in control experiments did not affect cell growth or sensitivity to treatment (Supplementary Figure 3), it could cause altered carbon metabolism. Therefore, emphasis was placed on metabolic responses in T-24 cells, although most trends were reproduced in Um-Uc-3 cells (Supplementary Figure 4B, and bolded in 4C). APIM-peptide-cisplatin treatment significantly increased glucose and glutamine consumption compared to cisplatin as a single agent. Lactate excretion was increased in both cisplatin and combination treated cells, yet the lactate/glucose ratio was decreased in combination treated cells only (Figure 5A–5B). The reduced ratio, although not significant, suggests that the APIM-peptide reduces the Warburg effect in cisplatin treated cells.

The altered glucose and glutamine consumption of cisplatin and APIM-peptide-cisplatin treated cells was reflected intracellularly by several significantly changed metabolite pool sizes (Supplementary Figure 4). Common to both treatments was increased levels of essential amino acids and deoxynucleosides, likely attributed to growth arrest and inhibition of replication. The combination treatment evoked larger changes in more metabolite pools than cisplatin as a single agent (Figure 5C, "+" in Supplementary Figure 4C). The most prominent changes were a buildup of metabolites after the rate-limiting conversion of fructose-6 phosphate to fructose 1,6-bisphosphate in glycolysis, a reduction of the 6-phospoglyconate pool in the entry to pentose phosphate pathway (PPP) and a reduction in the α-ketoglutarate pool of tricarboxylic acid (TCA) cycle (Supplementary Figure 4C). Altogether, the upregulated glucose and glutamine consumption, reduced lactate/glucose ratio and altered metabolite pool sizes at important metabolic branch points shows that BC cells undergo considerable changes in central carbon metabolism as a response to the APIM-peptide-cisplatin combination therapy. However, an exact explanation for the anti-cancer activity observed requires further studies.

APIM-peptide re-sensitized cisplatin resistant cells

Development of resistance is a major problem in cancer therapy and the mechanisms are multifactorial, including enhanced DNA repair, impaired signaling and reduced intracellular cisplatin accumulation [5]. Gene expression analysis indicated that the APIM-peptide-cisplatin treatment downregulated expression of *PODXL, YAP1* and *MVP* (Figure 3B); genes that are commonly overexpressed in MIBC and associated with multidrug

Table 2: Gene enrichment indicates altered cell cycle regulation and signaling by the APIM-peptide-cisplatin combination at 24h

GeneGo pathway map		Genes in pathway	False discovery Rate (FDR)
Upregulated:			
Cell cycle			
1.	Role of APC in cell cycle regulation	6/32	5E-5
3.	Transition and termination of DNA replication	4/28	5E-3
7.	Role of SCF complex in cell cycle regulation	3/29	5E-2
10.	Start of DNA replication in early S phase	3/32	5E-2
Transcription			
2.	Assembly of RNA Polymerase II preinitiation complex on TATA-less promoters	4/18	1.4E-3
5.	Huntington-depended transcription deregulation in Huntington's Disease	3/24	4E-2
Apoptosis and survival			
4.	p53-dependent apoptosis	4/29	5E-3
DNA damage			
6.	ATM / ATR regulation of G2 / M checkpoint	3/26	5E-2
9.	ATM/ATR regulation of G1/S checkpoint	3/32	5E-2
Metabolism			
8.	CTP/UTP metabolism	5/108	5E-2
Downregulated:			
Cytoskeleton remodeling			
1.	TGF, WNT and cytoskeletal remodeling	16/111	4E-4
5.	Cytoskeleton remodeling	14/102	9E-4
Signaling			
2.	HBV signaling via protein kinases leading to HCC	9/36	4E-4
9.	Regulation of p38 and JNK signaling mediated by G-proteins	8/39	3E-3
Development			
3.	Growth factors in regulation of oligodendrocyte precursor cell survival	9/37	4E-4
4.	PIP3 signaling in cardiac myocytes	10/47	4E-4
14.	EGFR signaling via small GTPases	7/33	3E-3
16.	VEGF signaling via VEGFR2 - generic cascades	11/84	3E-3
17.	Cytokine-mediated regulation of megakaryopoiesis	9/57	3E-3
Transport			
6.	Clathrin-coated vesicle cycle	11/71	2E-3
Cell adhesion			
7.	Chemokines and adhesion	13/100	2E-3
19.	Histamine H1 receptor signaling in the interruption of cell barrier integrity	8/45	3E-3
20.	Ephrin signaling	8/45	3E-3

(Continued)

GeneGo pathway map	Genes in pathway	False discovery Rate (FDR)
Neurophysiological process		
8. Main pathways of Schwann cells transformation in neurofibromatosis type 1	10/62	2E-3
18. Receptor-mediated axon growth repulsion	8/45	3E-3
Muscle-contraction		
10. S1P2 receptor-mediated smooth muscle contraction	7/30	3E-3
Translation		
11. Translation regulation by Alpha-1 adrenergic receptors	9/53	3E-3
Apoptosis and survival		3E-3
12. BAD phosphorylation	8/42	3E-3
13. Anti-apoptotic action of Gastrin	8/43	3E-3
Cell cycle		
15. ESR1 regulation of G1/S transition	7/33	3E-3

List of all significant upregulated and top 20 significant downregulated GeneGo pathway maps. The gene enrichment analysis were done on the differentially expressed genes (fold change > 1.25 relative to control, and found in all six biological replica of Um-Uc-3 and T-24 cells) unique for the APIM-peptide-cisplatin combination group, and not detected in cisplatin or APIM-peptide single agent groups (lists of genes in Supplementary Table 1). The GeneGo pathway maps are grouped by their main category.

resistance [4, 29, 30]. We therefore developed a cisplatin resistant Um-Uc-3 cell line (Um-Uc-3-R) and investigated the effect of the APIM-peptide on cisplatin sensitivity in this cell line. Um-Uc-3-R, cells were more resistant to cisplatin compared to original Um-Uc-3 cells at all doses tested and importantly, the APIM-peptide increased the sensitivity of both Um-Uc-3 and Um-Uc-3-R cells (Figure 6A, viability after 48 hours exposure). For instance, the viability of Um-Uc-3-R cells was not reduced by 2 μM cisplatin, while the viability of Um-Uc-3 cells was reduced with 20% at this time point. However, when combined with the APIM-peptide, the Um-Uc-3-R cells were re-sensitized to this dose of cisplatin (Figure 6A).

To explore the molecular mechanism behind this sensitizing effect, we examined if the APIM-peptide increased the levels of DNA lesions by impairing DNA repair in cisplatin treated cells. All treatments significantly increased the level of DNA damage relative to untreated control in both original Um-Uc-3 and cisplatin-resistant Um-Uc-3-R cells. In accordance with lower cisplatin sensitivity, Um-Uc-3-R cells had lower levels of DNA damage than Um-Uc-3 cells treated with the same dose of cisplatin after 24 hours (Figure 6B). However, the combination of cisplatin and APIM-peptide increased the amount of DNA damage in both these two cell lines and leveled out the differences between them. This indicates that at least part of the APIM-peptide re-sensitizing effect is mediated via inhibition of DNA repair. Multiple APIM-containing proteins, such as XPA and polymerase

ζ, are directly involved in bypass or repair of cisplatin-induced DNA lesions and could be inhibited by APIM-peptide treatment, in support for this finding. Furthermore, expression of HERC2 and REV1, also important for NER and TLS, were downregulated in combination treated cells (Figure 3B) and could also contribute to the increased level of DNA lesions observed.

Next we analyzed Um-Uc-3 and Um-Uc-3-R cells for cell cycle effects and fraction of apoptotic cells upon treatment with cisplatin and the cisplatin-APIM-peptide combination. Both cell lines were arrested to the same extent in S-phase and no significant changes could be detected between the cell lines after 24 hours (Supplementary Figure 5A). The APIM-peptide increased the fraction of apoptotic cells after cisplatin treatment in Um-Uc-3 while apoptosis was not affected by any of the treatments in Um-Uc-3-R cells (Supplementary Figure 5B). Thus, there is no direct link between increased level of DNA damage induced by the combination treatment and an increase in apoptosis in the Um-Uc-3-R cells at 24 hours. Both the cisplatin alone and the combination treatment did cause a small reduction in viability for both cell lines at this time point, and in accordance with the apoptosis data it was greater for Um-Uc-3 than for the Um-Uc-3-R cells (Supplementary Figure 5C). The reduction in viability and difference between the cell lines was further enhanced after 48 hours (Figure 6A, 10 μM cisplatin), suggesting a delayed and/or reduced DDR response in the Um-Uc-3-R cells.

DISCUSSION

Our results demonstrate that the PCNA-interacting APIM-peptide increases the anti-cancer efficacy of cisplatin *in vivo* by reducing tumor load and down staging BC, and thus has the potential to improve MIBC therapy. This is supported by previous work showing that the APIM-peptide is able to increase the efficacy of mitomycin C on non-MIBC [24]. Furthermore, this study reveals DE of apoptotic genes, changes in glycolytic enzymes and metabolites, and alterations in several signaling pathways often involved in oncogenic transformation when cisplatin is combined with the APIM-peptide. The exact same changes were not identified on all omics levels, however,
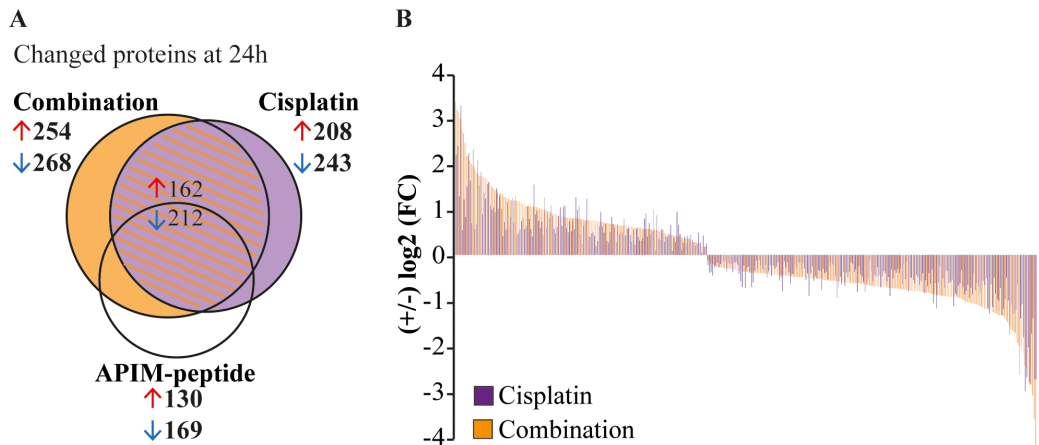

Figure 4: APIM-peptide enhances protein changes induced by cisplatin. Significantly changed proteins measured using the MIB-assay (Wilcoxon Sign Rank test, p<0.25) in Um-Uc-3 and T-24 cells treated for 24h with APIM-peptide (8 and 16 μM, respectively) and cisplatin (10 μM) (relative to untreated control). **(A)** Venn diagram illustrating the number of changed proteins in each treatment group. **(B)** Log2 fold change (FC) of proteins detected in both cisplatin and the combination group. Each protein presented by one bar, only proteins with >5% difference in relative values of combination (orange bars) vs cisplatin (purple bars) are shown.
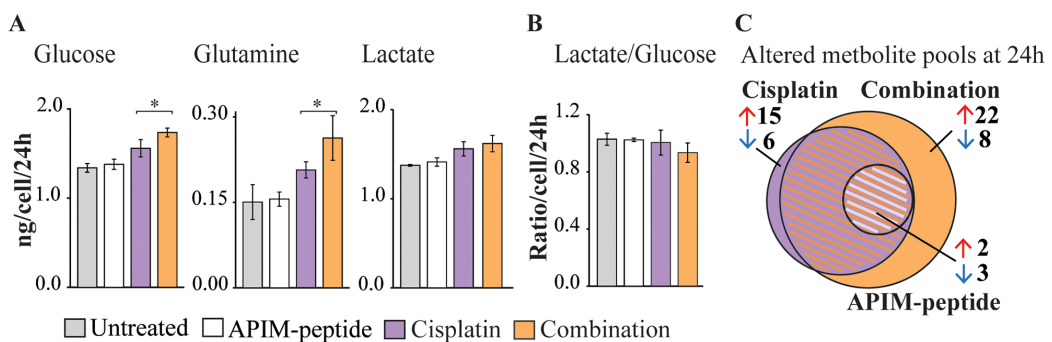

Figure 5: APIM-peptide-cisplatin combination increases energy source consumption and affects central carbon metabolism. Consumption/excretion of extracellular metabolites and targeted metabolic profiling of T-24 cells treated for 24 hours with APIM-peptide (16 μM), cisplatin (10 μM) and the combination (n=4). **(A)** Glucose and glutamine consumption and lactate excretion per live cell per 24 hours in each treatment group ± SD. Significant (*p<0.05) and non-significant (ANOVA and post hoc Tukey's range test) differences between cisplatin and APIM-peptide-cisplatin treated cells are indicated. Combination and cisplatin treated cells were significantly different from untreated control in all, while APIM-peptide single agent treatment was not (not marked in figure). **(B)** Lactate/glucose ratio per live cell per 24 hours in each treatment group ± SD. **(C)** Venn diagram illustrating the number of significantly (ANOVA and post hoc Tukey's range test, p<0.05) changed intracellular central carbon metabolite pools relative to control) in each treatment group.

this was expected because the genome, proteome and metabolome are highly dynamic, and not necessarily in phase at a given time point. Further, we discuss only a subset of the altered genes, proteins and metabolites in our data sets because exploring the complete system-biological effects of treatment via integrating all omics levels requires dedicated computational tools. Time series and further computational analysis will be the focus of future work. Yet, we demonstrate that many predicted therapeutic targets in BC are affected by the APIM-peptide-cisplatin treatment on more than one omic level, and that the changes observed are in in accordance with the observed *in vivo* and *in vitro* anti-cancer effects.

In this study, we show that APIM-peptide-cisplatin treatment leads to changed expression of multiple proteins implicated in cancer cell growth and development of cisplatin resistance. Combination treated cells reduced the expression of genes encoding proteins in the DNA damage response, both in cellular signaling, NER and TLS, and had increased levels of DNA damage. In addition to the changes in gene expression, the APIM-peptide likely directly inhibits NER and TLS as APIM-containing protein in these pathways are dependent on interaction with PCNA for optimal function [18, 22]. EGFR, ERBB2 and

members of the downstream PI3K/Akt and Ras pathways are potential therapeutic cancer targets, and they were all downregulated by the combination treatment. Mutations in these pathways are found in over 40% of BC tumors and inhibitors of these are suggested to restore cisplatin sensitivity [4, 31]. It is difficult to determine whether it is the direct inhibition of NER or TLS, or the effects on the EGFR/ERBB2 and downstream pathways or both that is responsible for the re-sensitization of cisplatin resistant cells observed after APIM-peptide-cisplatin treatment. Most likely the re-sensitizing effect is a combination of multiple factors.

The APIM-peptide-cisplatin combination also reduced the levels of *JAK, STAT* and *FAK1* expression. STAT3 and FAK1 activation are reported to be important in multiple cancer types, including BC [32, 33]. Although several inhibitors targeting EGFR, MAPK, FAK1 or PI3K/Akt pathways are undergoing clinical trials for MIBC therapy, no drug has yet been approved for BC treatment [32, 34–36]. RASSF1 is suggested to have tumor suppressor functions through inhibition of the Ras pathway. Reduced expression due to hypermethylation is frequently observed in BC (~80%), and is associated with progression and shorter overall survival [37]. Interestingly,
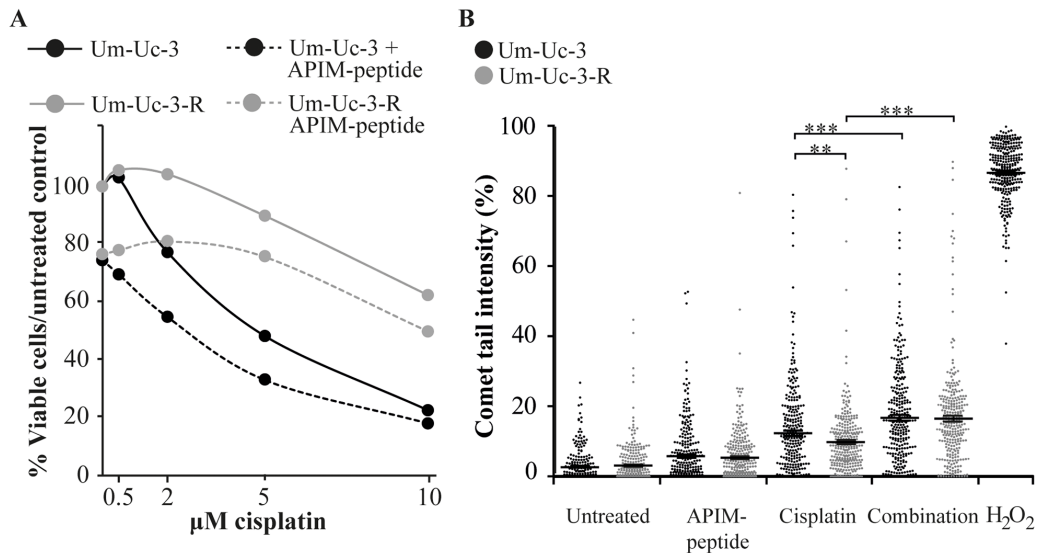

Figure 6: APIM-peptide re-sensitizes cisplatin-resistant cells. Original Um-Uc-3 and cisplatin-resistant Um-Uc-3-R cells treated with the APIM-peptide (8 μM) and cisplatin (**A:** 0.5-10 μM, **B:** 10 μM). **(A)** Dose-response of treated cells relative to untreated cells measured by the MTT assay after 48 hours of continuous exposure to treatments. Data presented is one representative experiment out of at least three biological replicas. **(B)** Percentage tail intensity of comets from alkaline comet assay analysis after 24h exposure to treatments. H_2O_2 (100 mM) was used as positive control. Data is merged from three biological replica in which 100 comets were randomly selected from each experiment (n=300), and presented as scatter plot with mean ± SEM. **p<0.01, ***p<0.0001 (student-*t* test, two tailed). All treatments were significantly (***) different from untreated control, and all single treatments were significantly (***) different from combination treatments (not marked in Figure).

the APIM-peptide-cisplatin treatment increased RASSF1 expression. This effect could be mediated via inhibition of PCNA´s role in signaling, however, many proteins involved in regulation of DNA methylations e.g. the TET-proteins and DNA methyl transferases contain PCNA interacting motifs [14]. Therefore, increased RASSF1 could also be due to APIM-peptide mediated inhibition of DNA methylation.

It is not straightforward to predict the most prominent effects of the APIM-peptide in specific cancer cells because more than 300 proteins involved in multiple signaling and DNA damage pathways contain APIM. All of these potential PCNA interactions might be more or less impaired, although not identically in cells of different

origin. The dependence on, and regulation of, different cellular pathways varies between cells of different tissue origins, as well as between normal and cancer cells. In any case, targeting PCNA with the APIM-peptide has the potential to affect, i.e. partly impair, but not completely inhibit, multiple pathways important in cellular stress responses simultaneously. Because cancer cells are more dysregulated and often lack normal check point regulation, this stress-confined treatment strategy is shown to have larger impact on cancer cells than normal cells across a range of cancer subtypes [8, 10]. This treatment strategy is less likely to be circumvented by development of resistance because it targets multiple pathways, and by itself targets TLS and therefore reduces mutagenicity [22].
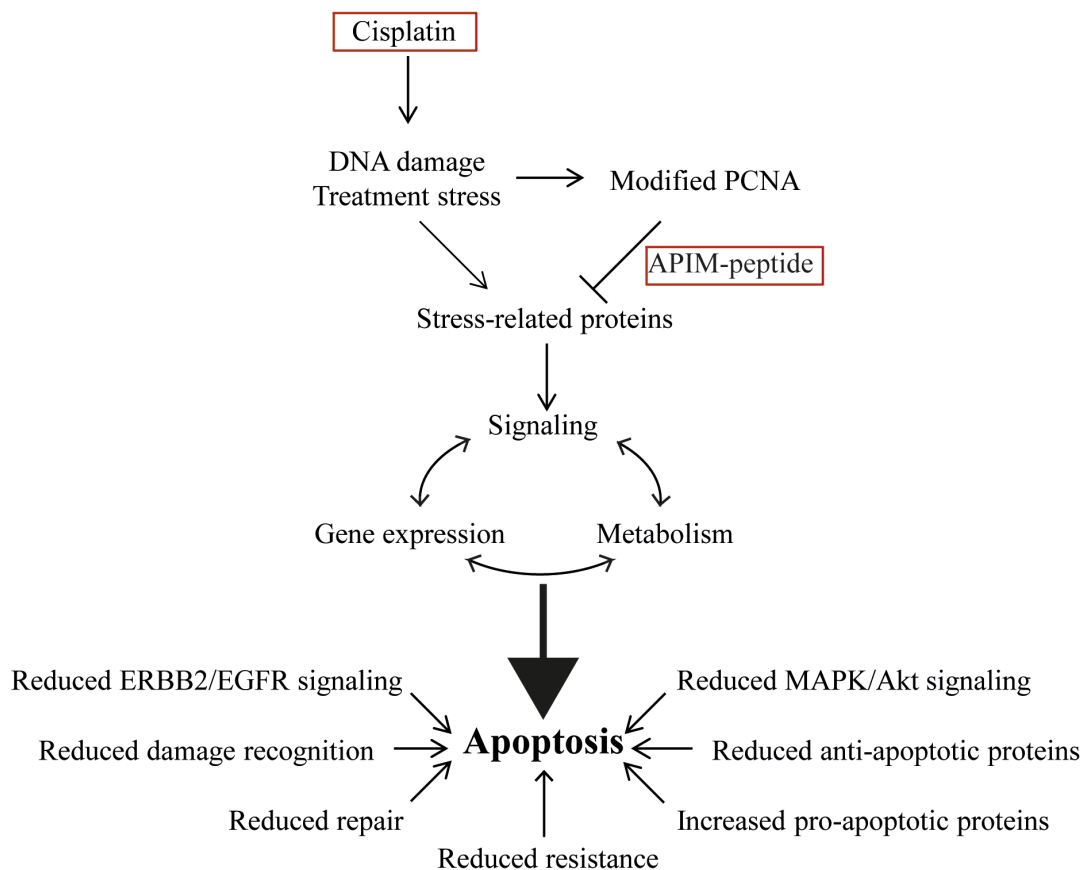

Figure 7: Combination therapy of cisplatin and APIM-peptide produce multiple effects driving the cells towards apoptosis. Cisplatin introduces DNA damage and treatment stress that increases the affinity of APIM-containing proteins for PCNA. The APIM-peptide inhibits these interactions, producing alterations in the cells signaling, gene expression profile and metabolism that ultimately pushes the cells towards apoptosis. Reduced EGFR/ERBB2, MAPK and AKT signaling, reduced damage recognition and DNA repair, reduced cisplatin resistance, reduced energy charge and increased expression of pro-apoptotic factors are all contributing to the APIM-peptide-cisplatin combinations mode of action in bladder cancer cells.

MATERIALS AND METHODS

Cell lines

The syngeneic rat urothelial carcinoma cell line AY-27 used in the *in vivo* studies was kindly provided by Professor S. Selman, Department of Urology, Medical College of Ohio and grown as described [38]. A panel consisting of the human urothelial carcinoma cell lines TCCSUP, HT-1197, Um-Uc-3, HT-1376, RT4, T-24 and 5637 (ATCC No. TCP-1020) were used for the *in vitro* studies. All cells were grown as recommended and cultivated in a humidified atmosphere (5% CO_2, 37°C). Additionally, a cisplatin resistant Um-Uc-3 cell line (Um-Uc-3-R) were established by continuously exposing the cells to increasing doses of cisplatin over one year (0.0625-1 μM cisplatin, added twice a week).

Treatment agents

APIM-peptide (ATX-101, MD-RWLVK-W-KKKRK-I-RRRRRRRRRRR) (APIM Therapeutics, Bachem) [10], cisplatin (Hospira), methotrexate (Pfizer), vinblastine (Velbe), adriamycin (Pfizer), gemcitabine (Santa Cruz Biotechnology) and hydrogen peroxide (Sigma-Aldrich).

Animals and ethics

The study was approved by the Norwegian National Animal Research Authority (Forsøksdyrutvalget, FDU) (FOTS applications 5502 and 6842) and in accordance with Norwegian and EU guidelines for care and use of laboratory animal.

Female CDF344 rats (Harlan Laboratories, Blackthorn) were kept in a standardized environment. Rats were anesthetized (subcutaneously) with a mixture (0.35-0.40 mL/100 g body weight (BW)) consisting of haloperidol (5 mg/mL, Janssen) (17% v/v), fentanyl (50 μg/mL, Actavis) (25% v/v) and midazolam (5 mg/mL, Actavis) (25% v/v) before orthotopic implantation. After implantation, the rats received NaCl (0.9%, 5-10 mL) and temgesic (0.3 mg/mL, 0.33 mL/200 g BW, RB Pharmaceuticals Ltd.) subcutaneously if needed, as judged by their condition. Intravenous (i.v.) treatment was performed under general anesthesia with isoflurane (4% induction, 1.5-2% maintenance). Anaesthetized rats were kept on a heat blanket to maintain body temperature. The rats were monitored for general health status and BW throughout the duration of the experiments.

In vivo MIBC model

The *in vivo* studies were performed with an immunocompetent rat orthotopic BC model previously described with the instillation of $4x10^5$ AY-27 rat BC cells [38, 39]. The rats were kept for three weeks to establish muscle-invasive tumors before treatment [40]. The rats were randomly distributed into treatment groups; i) vehicle (NaCl, 0.9%), ii) APIM-peptide (8.5 or 12.5 mg net APIM-peptide/kg), iii) cisplatin (2 mg/kg) and iv) APIM-peptide-cisplatin combination. First, cisplatin was given intravenously with a syringe (0.4 mL over 2 min), and the APIM-peptide was given subsequently via i.v. infusions using a pump (Aleris Guardrails Rolle) to ensure accuracy (2.4 mL/h, 12.5 mg/kg BW/mL) (rats in vehicle and cisplatin group were given saline infusions). The rats were treated once and the bladders were harvested after eight days. The bladders were macroscopically evaluated, weighed and stored in buffered formaldehyde solution (4%) until processing for histopathological evaluation. Statistical significance between the cisplatin and APIM-peptide-cisplatin groups was calculated using student *t*-test (unpaired, two-tailed, $p<0.05$).

In total, 57 rats from three independent biological replicas were used in this study. Of these, 5 rats are not included in Figure 1: i) three rats died before treatment, ii) one NaCl-treated rat died due to large tumor, iii) one rat was terminated before treatment due to reduced health status. The APIM-peptide and cisplatin combination treated groups with 8.5 or 12.5 mg APIM-peptide/kg were combined as there were no difference between these two groups.

Histopathological assessment

Paraffin embedding followed by slicing of formalin-fixed bladders and hematoxylin-erythrosine (HE) staining were done using standard procedures at Cellular & Molecular Imaging Core Facility NTNU. HE stained tissues were examined for morphological changes by an uropathologist using a light microscope (Nikon Eclipse 80i).

Cell viability assay

Cell viability (MTT-assay) was measured as previously described [14]. Data is reported as average ± SD of at least four technical replicas. Data is from one representative experiment out of at least three with similar results.

In vitro cell treatments for microarray, MIB-assay, mass spectrometric metabolic profiling, quantification of extracellular metabolites and comet assay

Um-Uc-3 and T-24 cells were seeded ($3-4x10^6$ cells/15 cm plate) and treated with APIM-peptide (8 μM (Um-Uc-3) and 16 μM (T-24)) and cisplatin (10 μM) alone or in combination the next day (three treatment groups and one untreated control per cell line). Extracts from three

individual biological replicas (done on different days) were prepared after 24 hours (h) for all conditions of each cell line. The doses were chosen based on the MTT data and the doses given intravenously to rats in the *in vivo* studies (~1/10 of this dose).

Microarray- analysis

Samples were prepared as previously described [23]. The microarray experiments have been deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5644. Gene expression data was normalized and analyzed using GeneSpring 12.6-GX (Agilent Technologies). DE genes were selected by comparing treated samples to untreated controls, and filtered by flags and fold change ≥1.25. Lists of up- and downregulated genes identified in all three biological replicas of both Um-Uc-3 and T-24 cell lines (n=3+3), and unique for the combination group (not in common with cisplatin group) were extracted. The GeneGo database (MetaCore) was used to annotate these lists of DE genes to gene ontology (GO) pathways.

MIB-assay

Total cell extracts were prepared as previously described [8]. Kinase enrichment was performed and eluted peptides were analyzed by Orbitrap MS as previously described [41]. The MS proteomics data has been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE [42] partner repository with the data set identifier (PXD008724). Label-free quantification values were log-transformed with the base 2 and the transformed control values were subtracted. The resulting values reflecting the change relative to control for each condition were subjected to two-sided Wilcoxon Sign Rank Test [43] as implemented in MATLAB R2015a (Mathworks Inc.). Proteins with p-value <0.25 were considered significantly changed. Three biological replicas were analyzed for each of the treatments. Proteins exhibiting the same trends in both T-24 and Um-Uc-3 cells, and significantly changed in at least one of the cell lines, were selected.

Quantification of extracellular metabolites

Supernatants were collected, lyophilized and up-concentrated four times in deuterium oxide (Sigma-Aldrich). 1D proton spectra were recorded at 25°C on a Bruker Ascend 400 MHz Avance III HD equipped with a 5 mm Z-gradient SmartProbe (Bruker). The anomeric proton of α-glucose (5.2 ppm), methyl Hβ of lactate (1.3 ppm) and methylene Hγ of glutamine (2.4 ppm) were integrated and quantified by electronic reference to access *in vivo* concentrations (ERETIC2, Topspin 3.5, Bruker). The methylamine H of a creatine (3.0 ppm) external standard (Sigma-Aldrich) was defined as the ERETIC reference. Consumption/production

was normalized to average number of live cells (average of live cell density when treatment was initiated and live cell density at time of harvest) within the 24h time interval examined to obtain consumption/production /cell/24h. Four independent cultures of Um-Uc-3 and T-24 cells were analyzed for each condition.

Targeted mass spectrometric metabolic profiling

Cells were sampled as described in [44], transferred directly to liquid nitrogen and extracted and up-concentrated as described in [45]. Phosphorylated metabolites were prepared for and analyzed by capillary ion chromatography (capIC)-MS/MS as described in [44]. Organic acids were derivatized as described in [46] prior to analysis by liquid chromatography (LC)-MS/MS. Derivatized samples (5 μl) were injected onto a Waters Aquity BEH C_{18} 2.1 x 100 mm column, maintained at 40°C and eluted with mobile phases (A) water added 0,1% formic acid and (B) methanol. The following gradient (v/v%) was applied with a flow rate of 0.25 ml/min: 0-0.5 min; 50% B, 0.5-6 min: 50-99% B, 6-7 min: 99% B, 7-7.1 min: 100-50% B, 8 min: end. Amino acids were derivatized by a protocol adapted from [47], making use of propyl chloroformate and n-propanol, and analyzed by LC-MS/MS. Derivatized samples (1 μl) were injected onto a Phenomenex EZ faast AAA-MS 250 x 0.2 mm column maintained at 25°C and eluted with mobile phases (A) water and (B) methanol, both added 10 mM ammonium formate. The following gradient (v/v %) was applied with a flow rate of 0.25ml/min: 0-1min: 68% B, 1-11min: 68-85% B, 11-11.5min: 85-68% B, 15 min: end. Both LC-MS/MS analyses were performed on a Waters AQUITY UPLC/Xevo TQ-S MS system operated in positive electrospray mode. Absolute quantification from a dilution series of external standards (organic and amino acids, Sigma-Aldrich) was performed in MassLynx V4.1 (Waters). LC-MS/MS analysis was performed for four independent cultures per condition from three biological replicas, capIC-MS/MS analysis was performed for four independent cultures per condition. Metabolome concentrations/abundances were normalized to total ion intensity and tested for significant differences between treatment groups by ANOVA and post hoc Tukey's range test (p<0.05).

Alkaline comet assay

Single-cell gel electrophoresis (comet assay) detecting DNA single and double strand breaks, alkali-labile sites, interstrand crosslinks and incomplete excision repair sites, were performed as previously described [48] with minor modifications: Harvested cells were suspended in low melting agarose (1%, 10^5 cells/mL) and spread on CometAssay® HT slides (Trevigen) (40 μL) in technical duplicates for each condition. Samples were incubated in lysis buffer overnight (4°C) and in alkaline solution (pH>13, 60 min) before gel electrophoresis (0.3A, 30 min). The slides were washed in neutralization buffer

(0.4M Tris-HCl), fixed in ethanol and stained with SYBR® Green I (Sigma-Aldrich) before analysis using the Comet Assay IV software (Perceptive Instruments). Cells treated with hydrogen peroxide (100 mM, 20 min, 4°C) were used as a positive control. Fifty comets from each technical duplicate were randomly selected and analyzed for each condition (100 comets) in each biological experiment. Data for all three biological replica is presented (300 comets), and average ± SEM is given. Statistical significance between groups were calculated by student *t*-test (unpaired, two-tailed, **p<0.01, ***p<0.0001).

CONCLUSIONS

In this study we demonstrate an increased anti-cancer efficacy of cisplatin when combined with the PCNA-targeting APIM-peptide, both *in vitro* in human BC cell lines and *in vivo* in the MIBC model. Our results suggest that several key genes and pathways relevant for multiple solid tumors, including MIBC, are affected after treatment with the APIM-peptide-cisplatin combination. In particular, reduced EGFR/ERBB2 signaling, reduced repair of cisplatin-induced DNA damage, re-sensitization of cisplatin-resistant cells and increased apoptosis were features of the combination treatment (summarized in Figure 7). All these changes contribute to the increased anti-cancer efficacy observed for the combination treatment. In conclusion, our results suggest that the APIM-peptide has the potential to improve cisplatin-therapy in the clinical setting and cause an increased anti-cancer response less likely to be circumvented by resistance.

Abbreviations

APIM- AlkB homologue 2 PCNA interacting motif; BC- Bladder cancer; DE- Differentially expressed; GC- Gemcitabine and cisplatin; MIB-assay- Multiplexed inhibitor bead assays; MIBC- Muscle invasive bladder cancer; MVAC- Methotrexate, vinblastine, adriamycin and cisplatin; NER- Nucleotide excision repair; PCNA- Proliferating cell nuclear antigen; TLS- Translesion synthesis.

Author contributions

Study design: CKS, AB, LMR, CJA, PB and MO; Data collection: CKS, AB, LMR, VP, AN, SB, NBL, OAG, TV, MO; Writing of manuscript: CKS, AB, LMR, CJA and MO.

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CONFLICTS OF INTEREST

APIM Therapeutics is a spin-off company of the Norwegian University of Science and Technology, and has co-funded this study. Professor Marit Otterlei is an inventor, minority shareholder and CSO of this company. Patent application no: PCT/GB2009/000489 "New PCNA interacting motif", filed on February 20, 2009. There are no further patents, products or development or marked products to declare. The other authors declare no conflict of interest.

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PAPER II

T1 bladder cancer in Norway: treatment and survival

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T1 bladder cancer in Norway: treatment and survival

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ABSTRACT

Aim: Evaluation of treatment and survival of pT1 stage (T1) bladder cancer (BC) patients diagnosed with transitional cell carcinoma of the urinary bladder in Norway.

Material and Methods: According to the Cancer Registry of Norway, 1,108 patients were diagnosed with T1 BC between 2008-2012. Information on surgical and medical procedures was provided by the Norwegian Patients Registry. Regression and survival models were applied to characterize patients receiving bacillus Calmette-Guerin (BCG) and radical cystectomy (RC) as early and delayed treatment and to estimate overall and cause specific survival rates (OS; CSS). Adjustments for sex, age, WHO grade and concomitant cis were made.

Results: In total, 449 (41%) patients received BCG treatment, 162 (15%) as early treatment. RC represented the early treatment in 96 (9%) patients and the delayed treatment in 84 (8%). Overall, 850 (77%) patients received neither BCG nor RC as early treatment, of whom 287 (26%) were treated with BCG and 66 (6%) with RC during follow-up. Patients <75 years and patients with high grade tumors or concomitant cis were more likely to receive BCG and RC as early treatment. 5-year survival rates for all T1 BC patients were 84% (CSS) and 65% (OS). Delayed RC was associated with the lowest 5-year CSS (70%). After adjustment, gender did not impact treatment choice and CSS.

Conclusions: The use of BCG as early treatment indicates low adherence to existing guidelines. Delayed RC was associated with low survival rates. An increased focus on the management of T1 patients is needed in Norway.

Abbreviations: BC: Bladder Cancer; CI: Confidence Interval; CRN: Cancer Registry of Norway; MIBC: Muscle-Invasive Bladder Cancer; BCG: Bacille Calmette Guérin; cis: Carcinoma in Situ

1. Introduction

Worldwide, 550,000 new bladder cancer (BC) cases were diagnosed in 2018, making BC the sixth most common cancer type for men and the 17th for women [1]. In Norway 1,516 new BC cases were diagnosed in 2018 [2], of which 25% in women. Known risk factors include age, smoking, arsenics in drinking water, radiation exposure and occupational exposure to carcinogens [3].

BC tumor stage T1 is infiltrating lamina propria and comprises 15-20% of all BC tumors [4]. The cancer specific survival (CSS) is reported to be 87% in a metaanalysis [5]. T1 BC represents a tumor stage with several challenges and controversies. The major concerns in the management are the risk of understaging the tumor at the primary diagnostic transurethral resection of the tumor (TURB) and the risk of tumor progression into muscle invasive BC (MIBC) [6]. The 5-year

progression rate in T1 tumors is about 20% [5,7], and the risk of understaging the tumor at diagnose is 8%, although with high variation across studies (0–32%) [8]. The routine use of repeated TURB (reTURB) within the first 4–6 weeks after diagnosis has therefore been recommended in the European association of Urology (EAU) guidelines since 2008 [9].

For most T1 patient conservative treatment after the initial TURB and reTURB represents the early treatment, which according to EAU guidelines comprises repeated intravesical Bacillus Calmette–Guérin (BCG) instillations and cystoscopy surveillance. BCG treatment reduces both progression rates and tumor relapses [4,10]. However, conservative treatment is not the optimal early management for all T1 patients, and the challenge is to identify the sub-group of T1 patients who should be offered radical cystectomy (RC) as early treatment in order to prevent progression and thereby hopefully obtain

Table 1. Characteristics of T1 BC patients diagnosed from 2008-2012 in Norway.

	Men	Women	All
Sex	851 (77%)	257 (23%)	1,108
Median Age (IQR)	74 (65–81)	76 (66–83)	75 (65-81)
No previous cancer*	774 (91%)	222 (86%)	996 (90%)
Concomitant cis	124 (15%)	18 (7%)	142 (13%)
WHO High Grade (HG) at T1 diagnosis**	701 (91%)	202 (87%)	903 (90%)
Muscle in specimen at T1 diagnosis**	756 (89%)	207 (81%)	963 (87%)
ReTURB (<12 weeks)	440 (52%)	135 (53%)	575 (52%)
Early treatment			
Conservative	645 (76%)	205 (80%)	850 (77%)
Without BCG			
With BCG (<8 weeks)	127 (15%)	35 (14%)	162 (15%)
Cystectomy (<6 months)	79 (9%)	17 (7%)	96 (9%)
Delayed treatment			
Conservative			
Without BCG	370 (43%)	127 (49%)	497 (45%)
With BCG	221 (26%)	66 (26%)	287 (26%)
Cystectomy	68 (8%)	16 (6%)	84 (8%)
Death			
BC-related Death	132 (16%)	43 (17%)	175 (16%)
Death of other causes	240 (28%)	59 (23%)	299 (27%)

*no other cancer within 5 years before BC diagnosis.
**in any TURB within 12 weeks since first T1 BC diagnosis.

favorable survival [6]. To help identifying the T1 patients with high risk of progression EAU guidelines give a set of risk factors highly associated to progression. In the 2008 guideline risk factors included were high grade (HG) tumors, large and multiple tumors and concomitant carcinoma *in situ* (cis) [4,9]. Additional parameters such as the age and comorbidities of the patients along with morbidity and mortality associated with RC are considered when clinicians make treatment decisions. Therefore, the management of T1 patients is complicated and requires close collaboration with several medical specialists involved.

To our knowledge, no population-based studies are available addressing treatment and survival outcome in T1 patients diagnosed in Norway. Thus, with focus on early treatment we describe management and survival in a population-based cohort of T1 BC patients in order to identify diagnostic or therapeutic tasks of future improvement.

2. Material and methods

2.1. Material

2.1.2. Data sources

The Cancer Registry of Norway (CRN) has since 1953 registered new cancer diagnoses in Norway. The registry receives information from several independent sources (clinicians, pathology laboratories, radiation machines, Norwegian Patient Registry and the Cause of Death Registry), thus ensuring high completeness of data [11]. Patients were identified through the personal identification number assigned to all newborns and residents in Norway since 1960. Cases were selected based on morphological snomed CT codes for transitional cell carcinoma of the urinary bladder. All histological reports for BC patients diagnosed between 2008 and 2012 and subsequent reports until the 31st of December 2016, registered in the CRN, were quality insured by the research team. Type of surgery, stage, WHO grade ([12]), concomitant

cis and the presence of muscle in the tissue specimen were determined.

The Norwegian Patient Registry includes administrative data on all patients from publicly financed hospitals as well as private hospitals and specialists, as a supplement to services at the public hospitals. For every study patient, we obtained dates for medical and surgical procedures related to the BC diagnosis such as TURB, reTURB, BCG treatment and RC. ReTURB was defined as a TURB within 12 weeks after the initial T1 diagnosis. As the coverage for the ATC code for BCG treatment was low, we defined BCG treatment either by the ATC code (L03AX03) or as at least three subsequent intravesical treatments with not more than 15 days in between two subsequent intravesical treatments. In order to differentiate BCG from intravesical treatment with Mitomycin, we excluded intravesical treatment given the day of TURB. First-line BCG treatment was defined as BCG treatment within 8 weeks after T1 diagnosis and first-line RC as RC within 6 months after the T1 diagnosis.

2.1.3. Study population

We identified 1,506 patients with an initial T1 urothelial carcinoma of the urinary bladder diagnosed by TURB between 2008 and 2012 in the CRN. We excluded 144 patients with another cancer diagnosis 5 years prior to the BC diagnosis and 254 patients because of upstaging of the T1 tumor within 4 months. Upstaging was defined as MIBC diagnosis by reTURB or metastatic disease within 4 months after the initial T1 diagnosis. In total, the study population comprised 1,108 T1 BC patients which were followed for treatment and outcome until death, migration or end of follow up on the 30th of June 2017, whichever came first. The total follow-up time was 15,260 person-years with a median follow-up time of 5.8 years. The last update of *cause* of death was on December 31st of 2016.

2.2. Statistics

Descriptive statistics and survival models were used to evaluate management and survival of the study population. Risk factors potentially influencing the early treatment were evaluated by (logistic) regression models, CSS and overall survival (OS) by applying flexible parametric models [13,14]. When evaluating risk factors for early treatment choice, we included sex, age, WHO grade, concomitant cis, reTURB, presence of muscle in the diagnostic histological and whether the patients had another cancer diagnosis more than 5 year before BC diagnosis into the multivariate model. Age, sex and all relevant risk factors ($p < 0.20$ in the risk factor analysis Table 1) was adjusted for in the survival analyses. The baseline hazard was modeled using 4 degrees of freedom (df) for the spline variables using the Stata command stpm2 [15]. After fitting the model, excess mortality rates could be estimated for any covariate pattern. The quantities reported are the hazard ratios (HRs) including 95% confidence intervals (CIs) and p-values.

3. Results

3.1. Patient, tumour and treatment characteristics

Patient characteristics of the 1,108 T1 BC patients are provided in Table 1. Median age at diagnosis was 75 years with women being 2 years older (76 vs. 74 years). More men than women were diagnosed with HG tumors, concomitant cis, and with higher rate of muscle tissue in the specimen. In total 180 patients (16%) had RC of which 96 (9%) as early treatment. 42% of the patients received BCG but only 15% as early treatment. Cis was reported in 13% of the histologies. In total 52% of the patients had a reTURB. During the study follow-up, 175 (16%) patients died of BC and 299 (27%) of other causes.

Figure 1 illustrates the numbers of patients treated with early conservative treatment (with and without BCG) or RC in addition to the numbers of patients receiving delayed RC and BCG treatment . The figure is also reflecting the timeframe of subsequent delayed RC or BCG treatments with majority of treatments given the first year after the early treatment. The tumor stage at RC revealed T0 for 14, Tis for 13, Ta for 5, T1 for 7 and MIBC for 31 patients. In Table 2, we present patient characteristics which are associated with early treatment. Older patients were significantly less likely to receive RC or BCG treatment (when compared to conservative treatment without BCG). The presence of a HG tumor and concomitant cis increased the probability of both RC and BCG treatment.

3.2. Survival analyses

CSS and OS estimates including confidence intervals are shown in Figure 2. The 1-, 2- and 5-years survival estimates for CSS were 95%, 92% and 84% respectively and the corresponding estimates for OS were 91%, 83% and 64%. Results from the survival analyses are presented in Table 3. After adjustment for clinical parameters, BCG treatment was significantly associated with better OS ($p = 5.3 \cdot 10^{-3}$) and to less extent ($p = 0.094$) with better CSS when compared to conservative treatment without BCG. Early RC was not associated with CSS ($p = 0.47$) or OS ($p = 0.16$). HG tumors (vs. LG tumors) at diagnosis were directly related to worse outcome (CSS: $p = 0.034$; OS: $p = 3.6 \cdot 10^{-5}$). Concomitant cis did not impact survival. Women had a significantly better OS than men.

Figure 3 shows CSS dependent on combined information on early and delayed treatment. Patients receiving conservative early treatment and a delayed RC had the lowest 5-year CSS: 73% (CI: 56–94%), for those treated early conservative with BCG and 61% (CI: 51–74%) for those early conservative without BCG. The best 5-year survival rates were observed for the group receiving only early BCG treatment (91%; CI: 86–96%) and the group with conservative early treatment without BCG and delayed BCG(87%; CI: 83–91%). 5-year CSS estimates for early RC were 85% (CI: 76–94%) and for the group both with neither early nor delayed (BCG or RC) treatment: 83% (CI: 79–86%).

4. Discussion

To our knowledge, this is the first Norwegian retrospective register-based study examining treatment and survival of patients with T1 transitional cell carcinoma of the urinary bladder. All patients were diagnosed between 2008 and 2012 at a median age of 75 years. The main findings were a relatively low rate of early BCG treatments (15%) and an overall high rate of RC (16%). 5-year CSS and OS rates were 84% (CI: 82–86%) and 64% (CI: 62–67%). The lowest CSS rates were found in patients undergoing RC more than 6 month after diagnosis. Patients older than 75 years were less likely to receive both BCG and RC as early treatment.

T1 BC is a heterogenic tumor demanding high quality in diagnostic work-up and therapeutic decisions to secure the correct diagnosis and optimal management [16]. The quality of the diagnostic procedures is generally assessed by the presence of muscle tissue in the TURB specimen and the rate of patients receiving reTURB. We report a reTURB rate of 52%, and 87% of the patients had muscle in the histological specimen, obtained by the primary or at the reTURB histology, which means that 13% had their T1 diagnose without any muscle in the tissue.

BCG is the internationally recommended conservative treatment for T1 BC patients [4] and is the only bladder instillation treatment shown to reduce progression rates of T1 tumors, but with modest effect, reducing progression rate from 13 to 9%, a risk reduction of 27% [10]. Although controversies regarding impact of progression [17,18] and survival [4] BCG has remained essential in T1BC treatment for decades [19]. Our total rate of T1 patients having BCG either as early or as delayed treatment is 41%. However, only 15% received BCG as early treatment (within 8 weeks after final diagnosis), as recommended in the EAU guidelines. The age distribution presented in a Swedish T1 nation-wide population-based study was similar to ours, but the BCG rate was higher, about 50% [20]. The reason for the overall low adherence to guidelines in our study could be caused by high age and comorbidities of the patients as well as the urologist's disbelief with respect to effectiveness of BCG treatment and fear of severe side effects.

In total, 180 (16%) of the study population had RC. The SEER (The Surveillance, Epidemiology, and End Results) database with 8,476 T1 patients diagnosed from 2004 to 2007, reported a RC rate of 4.7% within the first year after diagnosis [21]. A Swedish cohort reported 6.8% cystectomies for T1 WHO Grade (G) 2/3 cases, 11% when including only T1G3 [22]. The latest number of RC in the Swedish National Registry for Urothelial Bladder cancer (SNRUBC) was 12% for T1G2/3, 14% for only T1G3, in 2017 [23]. Compared to the referred studies our percentage of cystectomies is higher. We do not expect the low number of LG cases (10%) to impact the overall results as most RC cases are HG tumors.

T1 patients at a particularly high risk of tumor progression into MIBC should, according to EAU guidelines, be assessed for and offered RC when fit for it. Correct timing of the RC is crucial for optimal treatment results. In our study, 31 (44%) out of 70 patients with delayed RC and available histology had a MIBC at RC. Delayed RC at tumor relapse is reported
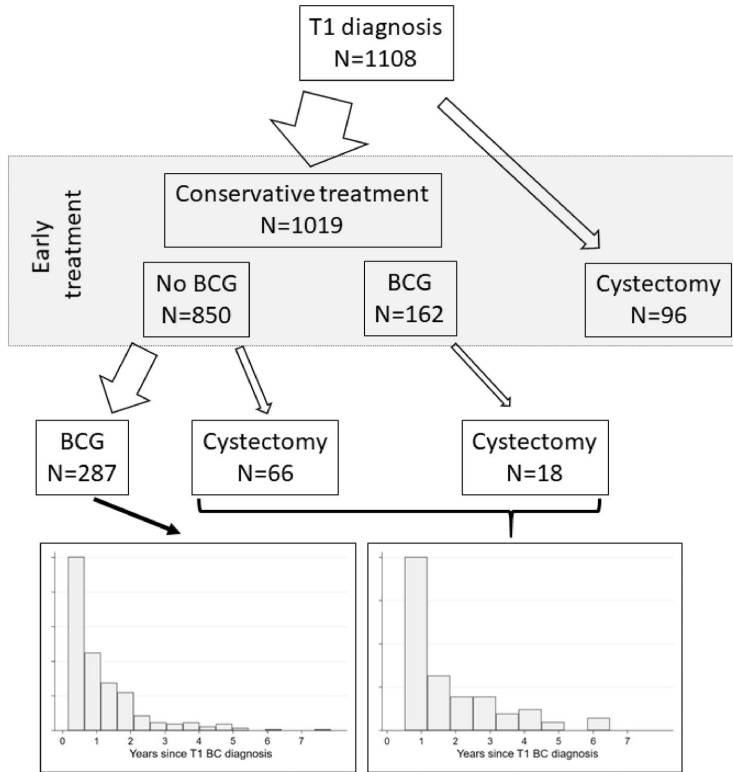
Figure 1. Early and delayed treatment for T1 BC patients. Treatments considered are conservative treatments with and without BCG and RC. The histograms show the distribution of time since diagnosis of the respective delayed treatments.

Table 2. Patient characteristics for T1 BC patients receiving conservative treatment or cystectomy as early treatment. P-values indicate the effect of the respective patient characteristics on early BCG and RC treatment choice when compared to conservative treatment without BCG in a multivariable model.

		Early treatment				
		Conservative			Cystectomy	
	All	No BCG	BCG	p Value		p Value
	1,108	850	162		96	
Men	851 (77%)	645 (76%)	127 (78%)	0.86	79 (82%)	0.88
Median Age (Interquartile Range)	75 (65-81)	76 (67-82)	71 (64-78)	$1.8 \cdot 10^{-5}$	66 (60-71)	$2.3 \cdot 10^{-5}$
No previous cancer*	996 (90%)	756 (89%)	151 (93%)	0.24	89 (92%)	0.92
Concomitant cis	142 (13%)	80 (9%)	36 (22%)	$2.2 \cdot 10^{-4}$	26 (27%)	$1.1 \cdot 10^{-3}$
WHO High Grade (HG) at T1 diagnosis**	903 (81%)	663 (78%)	152 (94%)	$1.1 \cdot 10^{-5}$	88 (92%)	$6.4 \cdot 10^{-3}$
Muscle in specimen at T1 diagnosis**	963 (87%)	491 (85%)	286 (88%)	0.63	91 (91%)	0.29

*no other cancer within 5 years before BC diagnosis.
**in any TURB within 3 month since first T1 diagnosis.

to decrease 10 years CSS from 78% to 51% compared to early RC [24]. Delayed RC after progression to MIBC is followed by lower survival rates than RC for primary MIBC [25,26]. In agreement with these published survival rates, we report the lowest CSS rate for patients receiving delayed among our treatment groups. Delayed RC in our study comprised about half of the total number of RCs. It could be questioned whether both the early and the follow-up management of these patients have been optimal. Many of these patients have probably undergone RC too late. But to determine the most appropriate date of RC is difficult, since both urologists and the patients want to avoid overtreatment with

the possible morbidities as well as mortality associated to RC. At the same time, RC may for some patients be the only chance for cure [6]. Survival of T1 study patients with early RC was close to that of the two groups with the best CSS; i.e. patients with early BCG only and those with delayed BCG. Whether any of these patients with RC as early treatment were over-treated is impossible to evaluate from this study.

Survival estimates for T1 patients vary considerably in the literature, reflecting differences in study populations and methods from modeling survival. In the Norwegian population, 16% of the T1 patients died of BC during the study

period with a median follow up time of 5.8 years. Sjostrom et al reported BC-related death of 18% in a T1 cohort, but patients with primary RC were excluded [20]. Another Swedish study reported 5-year relative survival of 76% for T1 patients in the period 2007–2011 [27]. In our study, we observed a 5-year relative survival of 81% (CI: 77–84%) (results not shown).

Both a tumor with HG (compared to LG) and concomitant cis led to significantly more early BCG and RC treatment and were thus, as expected, identified as risk factors considered in treatment decisions for T1 cases [4]. We could not confirm any impact of concomitant cis on survival. Our rate of concomitant cis is rather low (13%) when compared to about 25% in other reports [28,29].

It has been reported, that women are at risk to undergo sub-optimal treatment of BC in general more often than men, and that they have a less favourable prognosis [30]. Another study also showed inferior treatment of women with T1 BC diagnosis as they had less BCG treatment and a lower relative survival when compared to men [20]. In agreement with these observations, we found that a higher proportion of men received both RC and BCG treatment. This could be caused by a higher rate of HG tumors and more

concomitant cis in men. Less muscle in the TURB specimens in women (78 vs. 71%) could also potentially lead to more understaging in women leading to lower rates of BCG and RC treatment. However, in contrast to other studies, we did not find any gender differences related to treatment choice or CSS, after adjustment for clinical parameters.

A limitation in addressing management of T1 BC in a retrospective manner is the lack of essential information about comorbidity, imaging information and important risk factors for progression such as size and number of tumors, which influence both treatment choice and survival. Another limitation is the lack of a systematic depth categorization of tumor infiltration in the histological report. Information given by the histological report were tumor stage, grade, concomitant cis and presence of muscle in the specimen. The findings of this study must be interpreted on the background, that the first Norwegian national guidelines for BC management were published in 2018 with international guidelines available, like the EAU guidelines since 2000.

In conclusion, we report a low adherence to guideline recommendations regarding early BCG treatment for T1BC. The
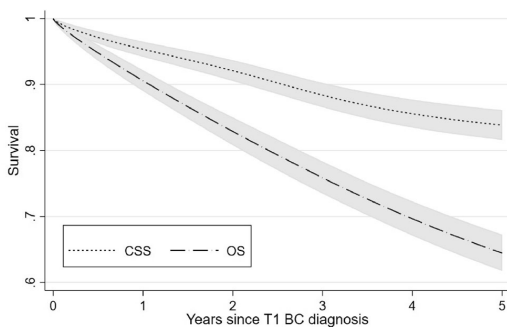

Figure 2. Cause-specific survival (CSS) and overall survival (OS) of T1 BC patients in Norway.
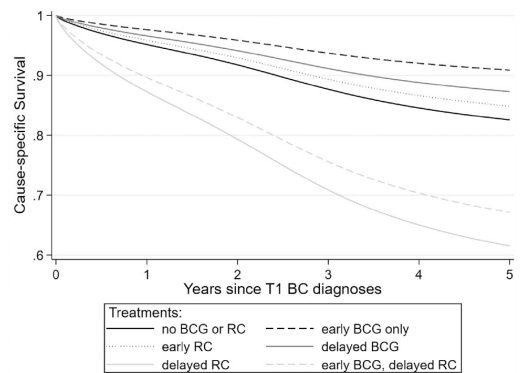

Figure 3. Cancer-specific survival for T1 BC patients in Norway dependent on combined early and delayed treatment.

Table 3. Results from the cause-specific and overall survival analysis. A hazard ratio HR > 1 indicates a higher mortality risk compared to the reference group, HR < 1 a lower risk respectively. We also provide confidence intervals (CI) and p-values.

		Cause-specific survival			Overall survival		
		HR	CI	p Value	HR	CI	p Value
Age	<65	1			1		
	65-74	1.53	0.85-2.76	0.16	2.06	1.23-2.99	$1.5 \cdot 10^{-4}$
	75-80	2.60	1.48-4.58	$9.2 \cdot 10^{-4}$	3.76	2.63-5.37	$4.2 \cdot 10^{-13}$
	>80	5.64	3.31-9.59	$1.9 \cdot 10^{-10}$	7.18	5.07-10.2	$1.1 \cdot 10^{-28}$
Sex	Men	1			1		
	Women	0.89	0.63-1.26	0.50	0.74	0.59-0.92	$7.0 \cdot 10^{-3}$
WHO	LG	1			1		
	HG	1.58	1.03-2.42	0.034	1.73	1.33-2.24	$3.6 \cdot 10^{-5}$
Concomitant cis	No	1			1		
	Yes	0.96	0.59-1.59	0.88	1.02	0.77-1.37	0.88
Early treatment	Conservative Without BCG*	1			1		
	Conservative With BCG**	0.64	0.38-1.08	0.094	0.63	0.46-0.87	$5.3 \cdot 10^{-3}$
	Cystectomy***	0.77	0.38-1.56	0.47	0.74	0.49-1.13	0.16

*conservative treatment without BCG within 8 weeks.
**conservative treatment with BCG within 8 weeks.
***cystectomy within 6 month.

rate of RC was higher than comparable Swedish numbers. About half of the RC patients had RC as early treatment with good survival results, while patients with delayed RC had the worst CSS suggesting inferior early and/or follow-up management. Our results suggest an increased focus on both early and follow-up management of T1 patients.

Author contributions

BKA conceived, coordinated and designed the study, performed the statistical analysis and drafted the manuscript. RB and TAM participated in the statistical analysis and interpretation of the analysis. AB was responsible for the clinical assessment of the patients included and contributed in the interpretation of the results, background knowledge on bladder cancer and writing the manuscript. All authors participated in writing of the manuscript and revised it critically. All authors have read and approved the final version of the manuscript.

Disclosure statement

The authors declare that they have no competing interests.

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PAPER III

The use of reTURB in T1 bladder cancer: a Norwegian population-based study

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Abstract

Aim: To evaluate the use of repeat transurethral resection of the bladder (reTURB) in stage T1 bladder cancer (BC) and its impact on treatment and survival in a Norwegian population-based cohort.

Material and Methods: In total, 1,130 patients registered at the Cancer Registry of Norway between 2008 and 2012 with a primary urothelial T1BC were included. Information on surgical and medical procedures was provided by the Norwegian Patients Registry. Descriptive statistics were used to evaluate characteristics of patients receiving reTURB or not within 12 weeks from primary TURB (primTURB). Survival models identified risk factors and estimated cause-specific survival rates (CSS) adjusted for sex, age, WHO grade, concomitant cis and detrusor muscle at primTURB and treatment.

Results: Compared to patients without reTURB, the 648 (57%) T1BC patients with reTURB were younger and had more WHO high-grade (HG) tumors. Of 275 patients without muscle at primTURB 114 (41%) had no reTURB. Of 648 reTURB patients, 45 (7%) had muscle invasive BC (MIBC), 110 (17%) T1 and 378 (58%) were tumor-free. Two-thirds of 81 patients receiving cystectomy after reTURB had T1 or MIBC at reTURB. ReTURB did not have an impact on adjusted CSS, but patients with T1 at reTURB had significantly lower CSS than those with tumor stage <T1.

Conclusions: Treatment choices and survival of T1BC patients depended on histology results, which became more reliable after reTURB. However, many Norwegian T1BC patients did not undergo reTURB as recommended in EAU guidelines, which supports the establishment of a national bladder cancer quality registry.

KEYWORDS

1. Introduction

Bladder cancer (BC) stage T1is an invasive tumor, infiltrating the lamina propria, but not the detrusor muscle (DM) of the bladder wall. T1 tumors account for about 15-20% of BC cases at diagnosis and are mainly WHO high grade (HG) tumors [1]. The tumor is removed by a transurethral resection of the bladder (TURB) [2]. In the initial diagnosis of T1 tumors, it is of great importance to resect deeply enough to include DM in the specimen. Lack of DM has been shown to increase the risk of overlooking an existing muscle invasive BC (MIBC) and is associated with an increased number of recurrences [3-5]. Tumor tissue remaining after the primary TURB (primTURB) has been reported in 33-78% of the histology reports obtained by a repeat TURB (reTURB) with upstaging to muscle invasive bladder cancer (MIBC) in about 8% of cases [6,7]. In order to increase the diagnostic accuracy in patients with T1BC and to ensure an early diagnosis of MIBC, the European Association of Urology (EAU) guidelines have since 2008 recommended a reTURB after primTURB in patients with a newly diagnosed T1BC and not eligible for immediate radical cystectomy (RC) [8]. Further, the demonstration of DM in the TURB specimen is viewed an obligate quality indicator in the management of T1 tumors [1,9,10].

To our knowledge, this is the first Norwegian population-based study describing the use of reTURB and its consequences for the treatment of T1BC patients. We focus on the differences between the reTURB vs the no reTURB group as well as reTURB histology result and its consequences for early treatment and survival.

2. Material and methods

2.1. Material

2.1.1. Data sources

Both the Cancer Registry of Norway (CRN) and the National Patient Registry (NPR) provided data for the current study. The CRN has since 1953, compulsory by law, registered virtually all new cancer diagnoses in Norway and receives information from three independent sources (clinicians, pathology laboratories, and from the Cause of Death Registry) [11]. The NPR includes administrative data on all treatments patients have received at publicly financed hospitals as well as private hospitals. Patients were identified through the personal identification number assigned to all citizens of Norway.

2.1.2. Study population

Based on morphological *Snomed CT* for transitional cell carcinoma of the urinary bladder, we identified 1,389 patients with a first lifetime T1 urothelial carcinoma of the urinary bladder diagnosed by TURB between 2008 and 2012 (and no MIBC diagnosis prior to the T1 diagnosis). We then excluded 144 patients with another cancer diagnosis within 5 years prior to BC diagnosis. We also excluded 115 patients who were not eligible for reTURB, i.e. 76 patients undergoing immediate RC (within 6 months after primTURB and without reTURB) and 39 patients who died within 3 months after T1 BC diagnosis (Supplementary Figure 1).

For all, 1,130 patients included, we obtained NPR dates for medical and surgical procedures related to BC diagnosis such as dates of primTURB, reTURB, as well as type and date of early treatment provided within 6 months after reTURB. A reTURB was defined as a second TURB within 12 weeks after the primTURB (without BCG instillation in between). The CRN received histology reports at reTURB for 323(50%) out of 648 patients. Missing histology reports were most likely the result of no report being mailed to CRN when the specimen did not reveal any

malignant tumor tissue or when no specimen was taken at reTURB e.g. only coagulation. We therefore assigned T0 as stage for the remaining 325 patients without a reTURB histology report. Early RC treatment was defined as RC performed within 6 months after reTURB. Early BCG treatment was defined as a BCG treatment which started within 8 weeks after primTURB or reTURB if performed. As the coverage for the ATC code for BCG treatment was low, we defined BCG treatment either by the ATC code (L03AX03) or as the application of at least three subsequent intravesical treatments with not more than 15 days in between two instillations. Intravesical treatment given the same day of TURB was not registered as BCG. Our variable "early treatment" comprises information on whether the patient had received early RC, early BCG or none of these treatments.

All histology reports related to primTURB and reTURB, as available at the CRN, were reviewed by the first author (AB) recording stage, WHO grade (WHO 2004) [12,13], the presence of concomitant cis and of DM. In survival analyses, we defined the grade as the highest grade reported among TURB and reTURB histologies. Likewise, we defined concomitant cis as reported if it was described in at least one of these histology reports.

2.2. Statistics

The patients were followed from BC diagnosis until death, migration or end of follow up on the 30th of June 2017, whichever came first. The median follow-up time was 6.9 years. The last update of *cause of death* was on December 31st of 2016.

Descriptive statistics were used to described patient characteristics, the use of reTURB, early treatment and survival of the study population, overall and stratified for reTURB. Unadjusted cause-specific survival (CSS) and overall survival (OS) were calculated by the Kaplan-Meier approach and survival differences were assessed by applying the Log-rank test. Flexible parametric models [14] were applied in order to quantify risk factors for BC-related death and

to estimate adjusted survival curves (CSS). In addition to reTURB (yes/no), factors included in these models, were age, sex, concomitant cis, grade, DM in the specimen and early treatment. We also estimated adjusted CSS stratified for reTURB histology results (no reTURB, T0/Ta/cis, T1, T2-4) within the same framework, thereby adjusting for age, sex, concomitant cis and grade. The baseline hazard in these flexible parametric models was modeled using 4 degrees of freedom (df) for the spline variables using the Stata command stpm2 [15]. The quantities reported are the hazard ratios (HRs) including 95% confidence intervals (CIs) and corresponding p-values.

3. Results

3.1. Use of reTURB

Our study population comprised 1,130 T1BC patients evaluable for reTURB and treatment of their T1 tumor of whom 648 (57%) underwent a reTURB. The use of reTURB remained stable across the years (2008: 56%; 2009: 54%; 2010: 60%; 2011: 62%; 2012: 56%). Out of 275 patients with no muscle in the primTURB specimen, 114 (41%) did not receive reTURB (Table 1). Compared to patients without, those with reTURB were younger (median 72 vs 78 years) and more likely to be diagnosed with high-grade (HG) tumors (96% vs 83%). The distribution of gender and the presence of concomitant cis or DM in the specimen was similar irrespective of the performance of reTURB.

Table 1: Characteristics for T1 BC patients at first T1 TURB diagnosis, stratified for whether the patients received reTURB or not

	reTURB	No reTURB	All
total	648 (57%)	482 (43%)	1,130
Men (%)	499 (77%)	370 (77%)	869 (77%)
Median age (IQR)	72 (64-79)	78 (69-84)	75 (66-82)
No previous cancer[*]	584(90%)	427 (89%)	1,011 (89%)
Concomitant cis	81 (13%)	48 (10%)	129 (11%)
WHO High Grade (HG) at primTURB[**]	562 (96%)	345 (83%)	907 (90%)
No muscle in specimen at primTURB	161 (25%)	114 (24%)	275 (24%)

[*]no other cancer within 5 years before BC diagnosis.
[**]9%/13% missing in reTURB/no reTURB group

3.2. Histology at reTURB

MIBC was identified at reTURB in 45 (7%) out of 648 patients with reTURB, T1 tumor in 110 (17%), and non-invasive or no malignancy (Ta, cis, T0) in 493 cases (76%) (Table 2).

Table 2: Characteristics for T1 BC patients at reTURB dependent on tumor characteristics at primary TURB

		reTURB histology					
		MIBC	**T1**	**Ta**	**cis**	**T0**	**total**
	total	45 (7%)	110 (17%)	74 (11%)	41 (6%)	378 (58%)	648 (100%)
Grade at primTURB	HG	39 (7%)	95 (17%)	63 (11%)	38 (7%)	327 (58%)	562 (100%)
	LG	0	2 (8%)	2 (8%)	0	21 (84%)	25 (100%)
	missing	6	13	9	3	30	61
Muscle in specimen at primTURB	Yes	24 (5%)	88 (18%)	58 (12%)	32 (7%)	285 (59%)	487 (100%)
	No	21 (13%)	22 (14%)	16 (10%)	9 (11%)	93 (58%)	161 (100%)
concomitant cis at primTURB	Yes	3 (4%)	17 (21%)	8 (10%)	16 (20%)	37 (46%)	81 (100%)
	No	42 (7%)	93 (16%)	66 (12%)	25 (4%)	341 (60%)	567 (100%)

Out of 487 patients with reTURB and DM in the primTURB specimen, 24 (5%) reTURB histologies showed MIBC. In contrast, 21 cases of MIBC (13%) were found in the 161 patients without DM in the primTURB specimen. Further, 59% of the 487 patients with DM in the primTURB specimen and 58% of the 161 patients without had T0 at reTURB.

3.3. Early treatment after reTURB

Among the 648 patients with reTURB, 81 (13%) underwent early RC (Table 3), and more were treated with early BCG (20% vs 7%) and fewer had no early treatment (67% vs 93%) compared to patients without reTURB. Half of the patients (22 out of 45) with MIBC at reTURB underwent early RC. MIBC patients with early RC were 8 years younger compared to those without. Twenty-eight percent of the patients with T1 and 6% of patients with tumor stage <T1 at reTURB had early RC.

*Table 3: Early treatment choices for patients with or without reTURB*

	Early RC (within 6 months)	Early BCG (within 8 weeks)	No early BCG/RC treatment	total
All patients n=1130				
no reTURB	0 ***	33 (7%)	449 (93%)	482 (100%)
reTURB	81 (13%)	131 (20%)	436 (67%)	648 (100%)
All patients with reTURB n= 648				
MIBC	22 (49%)	0	23 (51%)	45 (100%)
T1	31 (28%)	13 (12%)	66 (60%)	110 (100%)
Ta	2	16	56	74
cis (<T1)	5 (6%)	21 (24%)	15 (70%)	41 (100%)
T0	21	81	276	378

*** excluded from study

3.4. Survival and the impact of reTURB

Unadjusted 5-year OS was 69% for the reTURB group and 53% for patients without reTURB. The corresponding CSS was 83% (reTURB) and 79% (no reTURB) (Figure 1 A+B). This difference was significant both for OS ($p=8.3\cdot10^{-5}$) and CSS ($p=0.019$).

Figure 1: Cause-specific (A) and overall (B) survival including number of patients at risk for all T1BC patients and stratified by reTURB
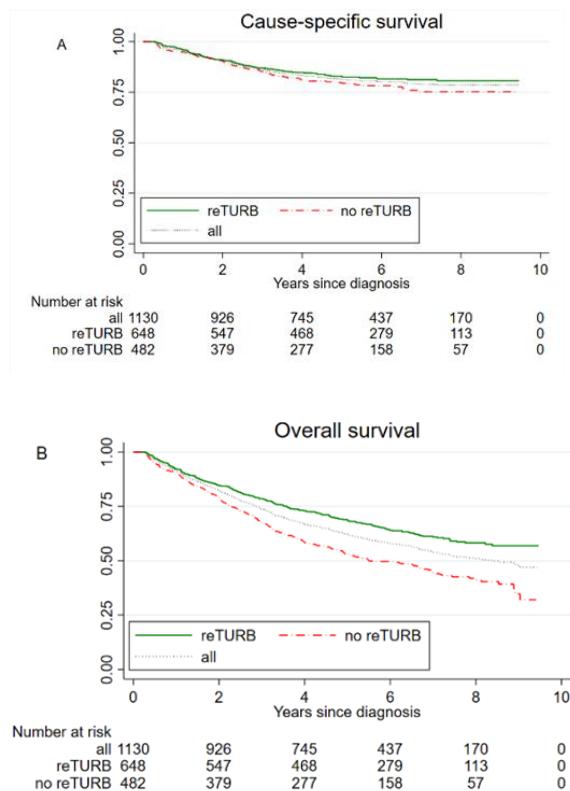
Overall, no difference in CSS was seen between patients with reTURB compared to those without (HR=1.02; CI: 0.75-1.37; p=0.933) when adjusting for age, sex, grade, concomitant cis and early treatment. In contrast, the adjusted OS was higher in patients with reTURB (vs no reTURB) (HR=0.79; CI: 0.66-0.96; p=0.017).

When investigating the risk factors for CSS, it could be seen that older age and the reTURB histology result were the major risk factors (Table 4). Neither grade nor concomitant cis influenced CSS significantly. Compared to patients with tumor stage <T1 (T0, Ta, cis) at reTURB, those with T1 and MIBC at reTURB had a significantly higher risk of BC-related death (T1: HR=2.7; CI: 1.7-4.3; p=2.6·10^{-5} and MIBC: HR=7.2; CI: 4.2-12.4; p=4.5·10^{-13}).

Table 4: Results from the cause-specific survival analysis adjusted for early treatment. A hazard ratio HR>1 indicates a higher risk of BC-related death compared to the reference group, HR<1 a lower risk respectively. We also provide confidence intervals (CI) and p-values.

		Cause-specific survival analysis		
		HR	CI	p-value
Age	< 65	1		
	65-74	1.55	0.90-2.65	0.11
	75-80	2.05	1.21-3.47	7.8·10^{-3}
	>80	4.96	3.02-8.14	2.3·10^{-10}
Sex	Men	1		
	Women	0.91	0.66-1.27	0.59
reTURB histology	<T1	1		
	T1	2.70	1.70-4.30	2.6·10^{-5}
	MIBC	7.23	4.23-12.4	4.5·10^{-13}
	no reTURB	1.46	1.03-2.06	0.032
WHO grade	LG	1		
	HG	1.34	0.91-1.99	0.14
Concomitant cis	No	1		
	Yes	0.92	0.62-1.38	0.70

Moreover, T1 at reTURB significantly worsened CSS compared to T1 only at the primTURB (HR=1.9; CI: 1.2-2.9; p=6.2·10^{-3}). Figure 2 displays the corresponding adjusted CSS curves stratified by reTURB histology. The 5-year CSS was estimated to be 86% (CI: 83%-90%) for patients with T0, Ta or cis at reTURB, 69% (CI: 60%-78%) for those with T1, 41% (CI: 30%-58%) for patients with MIBC and 81% (CI: 78%-84%) for patients without reTURB.

Figure 2: Cause-specific survival for patients with no reTURB as well as different reTURB histology results: non-invasive (T0, Ta, cis), T1 and MIBC
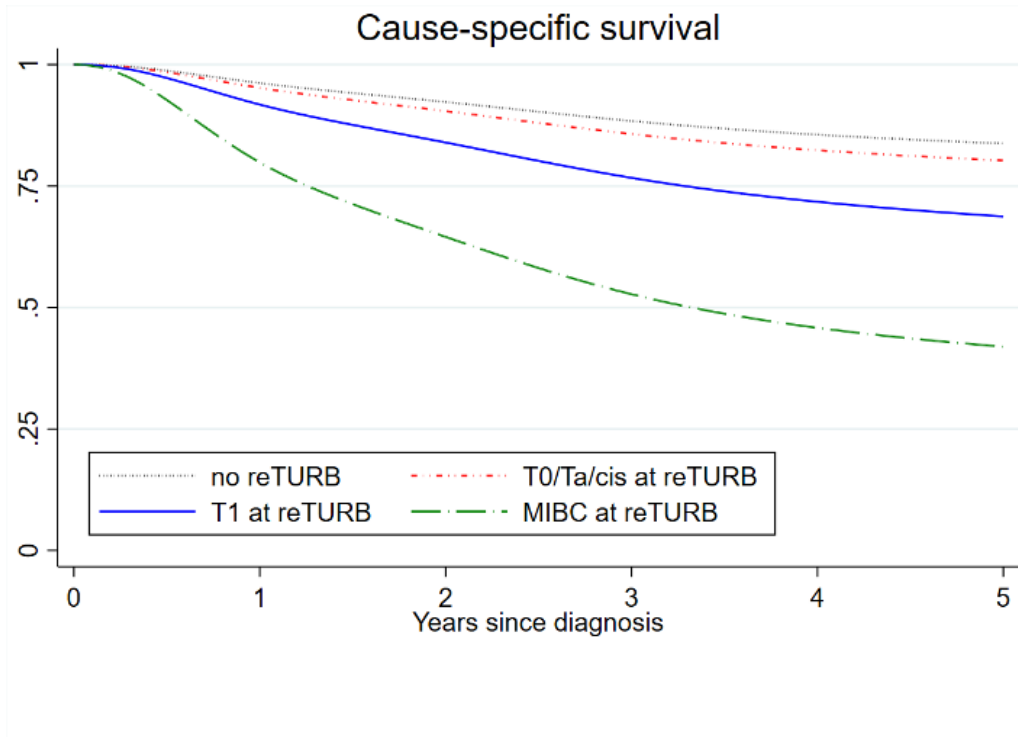
4. Discussion

This retrospective Norwegian population-based study comprised 1,130 patients diagnosed with T1BC in 2008-2012 of which 648 (57%) had a reTURB. Of those without DM in the primTURB specimen 41% had no reTURB. Compared to those without reTURB, patients with reTURB were younger and more had HG tumors. MIBC was diagnosed in 45 (7%) and T1 tumor in 110 (17%) of the reTURB patients. MIBC was found more frequently (13% vs 5%) in patients without vs with DM in the primTURB specimen. Moreover, 22 out of 45 (49%) patients with MIBC and 31 out of 110 (28%) patients with T1 at reTURB, had early RC. A significant

decreased CSS emerged for patients with tumor stage T1 and MIBC at reTURB compared to cases with tumor stage <T1 at reTURB. Further, a T1 tumor at reTURB significantly worsened CSS compared to T1 only at primTURB.

4.1. Frequency of reTURB

TURB represents a diagnostic procedure as well as the only surgical treatment for the majority of T1BC patients. TURB is performed in all Norwegian hospitals with urology service available. In the diagnosis of a T1BC, DM must be verified in the histology report. The role of reTURB is to diagnose an MIBC not detected at primTURB and to identify and completely remove remaining or re-growing tumor tissue. Based on the medical literature before 2008 and the EUA guidelines since 2008 our percentage of reTURB (57%) is surprisingly low, though it is similar to the Swedish study on T1 patients diagnosed in 2008 and 2009 showing a reTURB rate of 55% [16].

In our study, younger patients and patients with a HG tumor at primTURB were more likely to have a reTURB. We did not see any impact of sex or concomitant cis on the performance or not of a reTURB. The lack of DM in the primTURB specimen did not impact the rate of reTURB which is surprising since the impact of DM in the primTURB specimen both on relapses and progression are emphasized by several studies [2,4,17] and by EAU guidelines since 2008 [8]. Several other factors besides age and grade, such as tumor size, tumor multiplicity, and completeness of resection, which were not available to us, may influence the decision to perform or to omit a reTURB. High age alone hardly explains our low percentage of reTURB. A TURB is generally well tolerated even by elderly patients with a complication rate not exceeding 5% [2,18]. Other reasons for not performing reTURB could be unawareness of guideline recommendations or surgeons trusting the primary resection to be complete. Some patients without reTURB might also get an initial TURB for palliative purposes.

4.2 Post reTURB histology

Upstaging to MIBC was found in 7% of 648 patients at reTURB, which is in line with 7-8% reported in other studies [19,20]. The percentage of T1 tumors at re-TURB was 17%, which is less than 31% reported by Gontero et al [17] and 43% reported by Patchan et al in a Swedish cohort study[16]. However, the percentage T1 at reTURB in Gontero et al's study is not directly comparable to our result since patients with MIBC at reTURB were excluded. The tumor stage at reTURB contains important clinical information, e.g. a T1 tumor at reTURB indicates a 25% to 80% risk of progression into MIBC [21-23]. In our study the risk of understaging an existing MIBC increased by a factor of 2.6 (13% vs 5%) if the primTURB specimen lacked DM tissue. However, the demonstration of DM in the primTURB specimen does not exclude MIBC since 24 out of 487 (5%) patients with DM had MIBC at reTURB.

Fifty-eight percent of the patients were tumor-free at reTURB, which is higher than was 35% reported by Patchan et al. and 29% reported by Gontero. A systematic review reported a range of 29-80% tumor-free reTURB histologies [7]. In line with these variations in reported numbers, the Swedish report [16] showed large inter-hospital variations of the number of reTURB. However, it must be emphasized that the histological results from primTURB and reTURB must be viewed on the background of the urologist's experience and technical skills [4,24,25]. Thus, it should be discussed whether at least more challenging TURB procedures e.g. in patients with multiple and/or large tumors and most reTURB procedures should be performed by experienced urologists in high-volume hospitals to secure the best possible diagnostic and therapeutic results.

4.3 Post reTURB treatment

About half of the patients with MIBC and about one third of those with a T1 tumor at reTURB underwent early RC. As published earlier [26], we showed that T1BC patients <75 years and patients with HG tumors or concomitant cis were more likely to undergo RC within 6 months.

Additional factors for not performing or delaying RC could have been severe comorbidity or the patient's refusal. Both clinicians and patients should be aware that survival is unfavorable in some patients undergoing deferred RC [26,27]. Moschini et al even found increased mortality rates for T1 patients with RC after progression to MIBC compared to mortality for primary MIBC [28].

4.4 Survival

There is an ongoing discussion whether there are any survival benefit for patients undergoing reTURB [5,17]. When adjusting for relevant clinical factors, no significant difference of CSS was found for patients with vs without reTURB in our study. Gontero et al showed that reTURB impacted CCS (and OS) but only for patients without detrusor muscle in the primTURB [17] and a Swedish report did not find any significant survival difference when adjusting for clinical factors [16]. Our results do, however, not imply that reTURB is unnecessary but might rather reflect large difference between the reTURB and the no reTURB group not captured by the available clinical factors in the survival model. However, we did find survival differences dependent on the reTURB histology result. As confirmed by other studies, we demonstrated a survival disadvantage (adjusted CSS) for patients who had T1 compared to those with a tumor stage <T1 at reTURB. We also showed a significantly lower adjusted CSS for patients without a reTURB when compared to those patients demonstrating a tumor stage <T1 at reTURB. When it comes to the unfavorable results of stage T1 at reTURB, this might indicate a particularly high biological aggressivity, not reflected by the primTURB histology result alone. Some T1 tumors might also be more challenging to remove completely e.g. because of their location, size and multifocality, thereby already initially representing a patient group with an increased risk of progression. Finally, we consider the early identification of MIBC and of "aggressive" T1 tumors by a reTURB to be clinically important since the reTURB histology increases the scientific basis for consideration of early RC. If RC is not conducted, patients with T1 tumors

at both primTURB and reTURB should at least be considered for frequent controls and/or an additional TURB.

4.5 Limitations and strengths

This retrospective registry-based study has several limitations. We assigned patients with non-available histology report after reTURB to tumor stage T0. All Norwegian pathology laboratories are required to transfer all histology reports showing a cancer diagnosis to the CRN. Such transferals are not required in case of "no malignant tumor" unless the patient had an earlier cancer diagnosis. We are confident that our T0 assignment described above is reasonable since the reporting of "no malignancy" to CRN easily could have been forgotten, especially if information of earlier cancer has not been provided by the surgeon. Thus, the 325 patients without histology report at reTURB most probably represented a positively selected sub-sample, with a higher frequency of T0 after reTURB than the 323 cases with a report available. This is also supported by the more favorable survival of patients without (vs with) a reTURB histology report (Supplementary Figure 2).

Further, we do not have information about size and multiplicity of the T1 tumors or of comorbidities of the T1BC patients. Moreover, comparisons between reTURB and no reTURB patients must be interpreted with caution since these groups are different with respect to many factors, of which not all of them were available for assessment in our analyses.

4.6 Conclusion

In conclusion, both the choice of early treatment after a T1BC diagnosis and survival are dependent on the correct histological evaluation of the tumor, which is improved by the performance of a reTURB. However, in Norway almost half of the T1BC patients diagnosed between 2008 and 2012 and almost half of the patients without DM in the primTURB specimen

did not undergo reTURB as recommended in the EAU guidelines. This supports the establishment of a national clinical bladder cancer registry enabling regular monitoring of diagnostic and treatment management as well as outcome of patients with bladder cancer.

List of abbreviations

BC: bladder cancer; CI: confidence interval; CRN: Cancer Registry of Norway; MIBC: muscle-invasive bladder cancer; BCG: Bacille Calmette Guérin; cis: carcinoma in situ; DM: detrusor muscle

Supplementary

Supplementary Fig 1: Overview over the selection of study samples, exclusions and subgroups
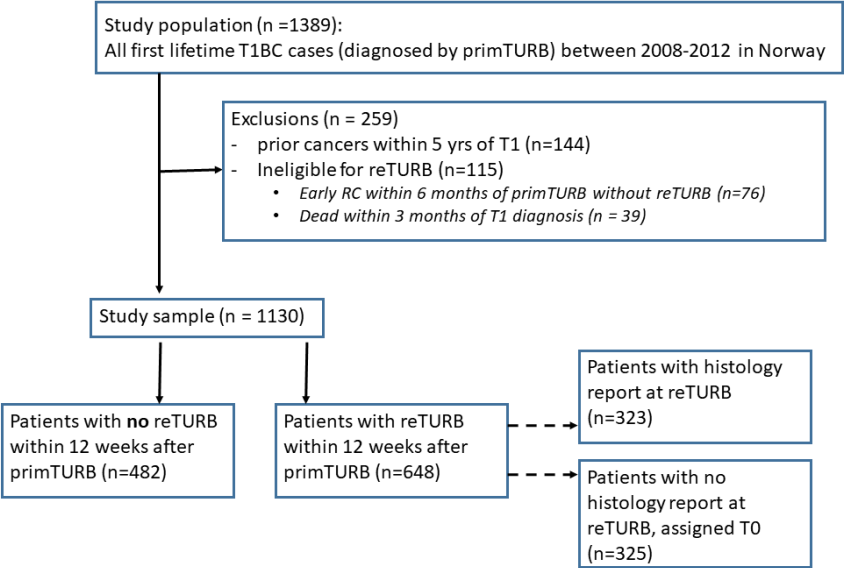
Supplementary Figure 2: Cause-specific survival for patients with reTURB within 12 weeks after initial T1BC diagnosis stratified for the availability of a reTURB histology report
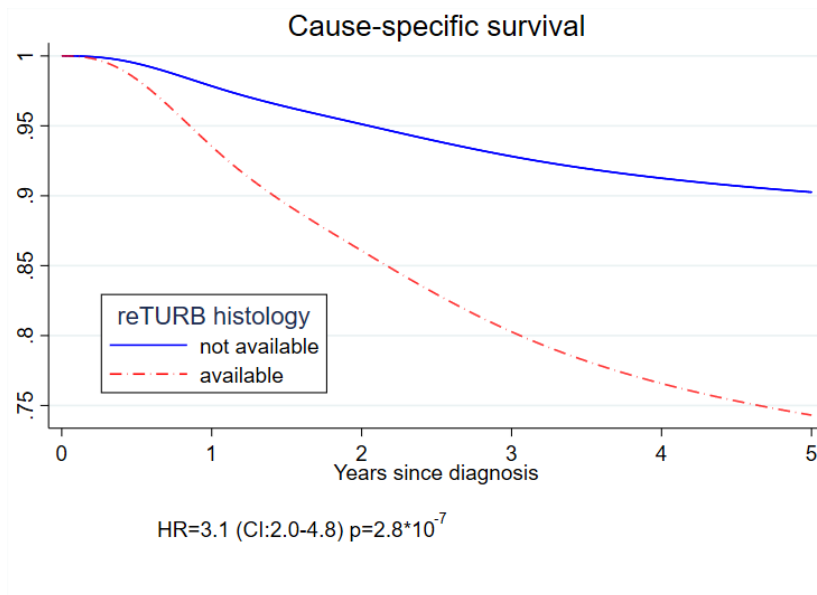
Competing interests

The authors declare that they have no competing interests.

Author´s contributions

BKA conceived, coordinated and designed the study, performed the statistical analysis and drafted the manuscript together with AB and SF. RB contributed to the data management. AB was responsible for the clinical assessment of the patients included and, together with SF, contributed in the interpretation of the results, background knowledge on bladder cancer and writing the manuscript. All authors participated in writing of the manuscript and revised it critically. All authors have read and approved the final version of the manuscript.

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Disclosure statement

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