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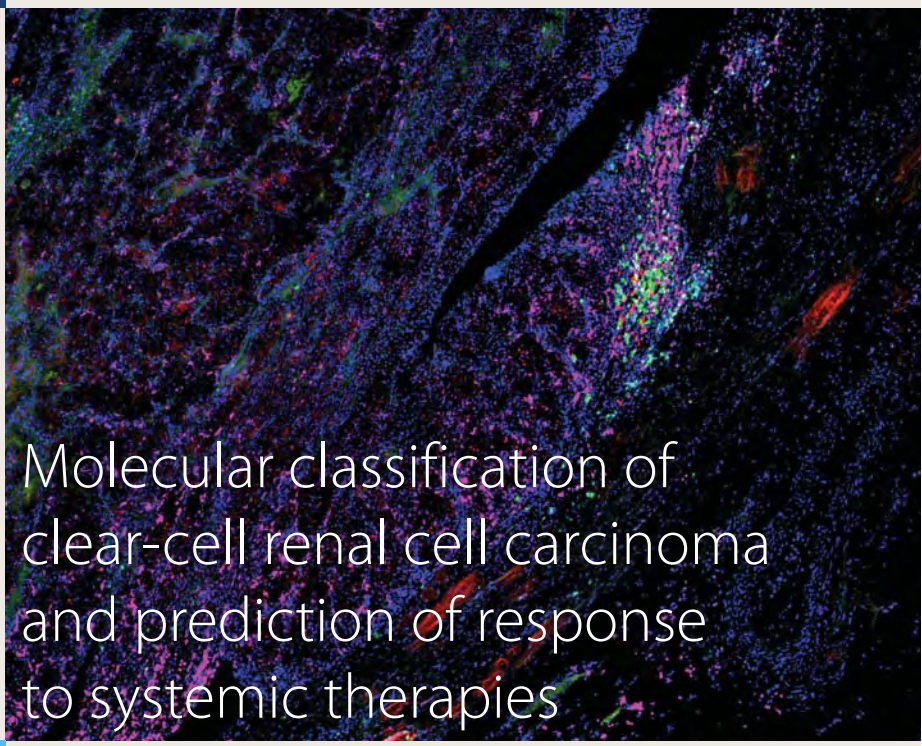
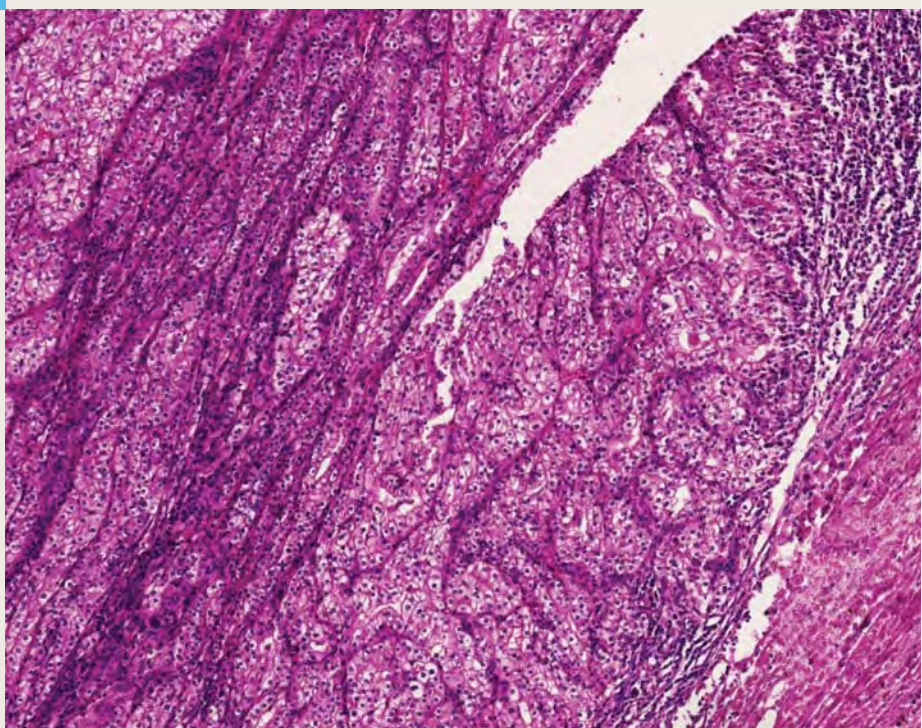


Molecular classification of clear-cell renal cell carcinoma and prediction of response to systemic therapies

Annelies Verbiest



Supervisor:
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Co-supervisor:
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June 2020

KU Leuven
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Department of Oncology

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Molecular classification of clear-cell renal cell carcinoma and prediction of response to systemic therapies

Annelies Verbiest

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in the Biomedical Sciences

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Gratitude

"Och kijk! Nu is niets anders nog belangrijk!"

Ik citeer Benoits eerste reactie op het beeldje van de 12-weken echo, dat flikkert op het scherm van mijn gsm. Een omhelzing is op dat moment nog toegelaten: dat is zijn tweede reactie. Het is 28 februari 2020, minder dan vier maanden voor mijn thesisverdediging. Het zal dan ook niemand verbazen dat ik van alle mensen Benoit als eerste ontzettend wil bedanken. Het was een voorrecht om deze vier jaar je eerste thesisstudent te zijn. De vanzelfsprekendheid waarmee je je op hetzelfde niveau plaatst tijdens discussies, en het vertrouwen waarmee je mij het onderzoek mee laat vormgeven, zijn ontnuchterend en tegelijk de best mogelijke motivatie om te denken alvorens te doen. Bij jou vervloeien mens, wetenschap, familie en zingeving naadloos tot één geheel. Die bijzondere drive, tesamen met de nauwgezetheid en de focus waarmee je over de jaren aan je projecten blijft werken, zijn een inspiratie.

Het gezegde luidt dat je kinderen sterke wortels moet geven voordat ze vleugels krijgen. Mijn familie heeft gezorgd voor een rijke voedingsbodem waarin de liefde voor mens en wetenschap konden wortelen. Ook na het uitvliegen ben ik hen veel verschuldigd: voor praktische hulp en voor hun unieke combinatie van onvoorwaardelijke bewondering en nuchterheid ("Dus ASO, dat is dan dokter in niks?"). Kathleen, jou moet ik als zus expliciet bedanken om na vele jaren toch te aanvaarden dat je een goede arts kan zijn als je geen verkoudheid kan genezen. Tom, jij bent mijn grootste supporter al vanaf het moment waarop ik je acht jaar geleden verklaarde dat ik oncoloog zou worden: "Dat is fantastisch! Normale mensen moeten op het werk hun beste glimlach opzetten en reageren dan thuis hun frustraties af. Maar jij zal zo ernstig moeten zijn op je werk, dat je alle vrolijkheid voor thuis kan sparen!" En hoewel het er op oncologie een stuk vrolijker aan toe gaat dan men zou vermoeden, is het onmogelijk om niet spontaan gelukkig te worden wanneer ik bij jou en Elise thuis mag komen. Dank voor een warme thuis!

Gesproken over een thuishaven: dank aan de hele ploeg van oncologie om mij deel te doen voelen van een team waar ik af en toe kon aanmeren. Het is een plezier om opgeleid te worden in een omgeving die vertrouwen ademt. De manier waarop de stafleden de haast onmogelijke verwachtingen inlossen die aan hen worden gesteld als clinici, wetenschappers en opleiders, is een voorbeeld voor mij. Aan verpleging, logistiek, secretariaat en alle andere paramedici kan ik maar één ding zeggen: jullie zijn mijn voornaamste argument om jonge collega's te overtuigen van medische oncologie! Wie kiest voor zorg in de oncologie doet dat met volle overtuiging, en het is dan ook elke dag een plezier om in zo'n team te werken! Maar het plezier beperkt zich niet collega's alleen. We hebben allemaal ontdekt dat de zorg voor mensen met kanker niet de Griekse tragedie is die men zich daarbij voorstelt. Veel van onze patiënten ontdekken in zichzelf een kracht en een sereniteit die elke dag opnieuw bewondering oproept. Velen geven zelf meer zorg dan ze ontvangen – aan hun familie, en ook aan hun zorgverleners. Wij mogen niet enkel de uitdaging aangaan om kanker te onderzoeken en te behandelen, maar ook om met deze mensen op pad te gaan. Dankzij hen is dit veelal geen opgave, maar een voorrecht.

Bij de persoonlijke bedankingen is mijn voornaamste bezorgdheid die om mensen te vergeten: er hebben er zovelen deze vier jaar een onmisbare steen bijgedragen. Bedankt aan het team van LEO om een vaste uitvalsbasis te creëren, en in het bijzonder aan Aga voor de oprechte interesse en immer snelle en nuttige feedback op elk manuscript. Dank aan Jessica's team op INSERM, met onder andere Gabrielle en Stefano, om me de weg te wijzen in onze weefselcollectie en naar jullie befaamde bakker, aan wiens baguettes en frambozentaart ik nog steeds krokante herinneringen koester. Bedankt aan Marcella Baldewijns voor de enthousiaste pathologiereview, aan Thomas op VIB voor de RNA sequencing, aan Isabelle, Sara en Frederik op CME voor de samenwerking en heel in het bijzonder dank aan het team van pathologie dat ons toegang heeft verschaft tot de schier oneindige weefselbank van Gasthuisberg: Tom, Magda, Geert, Kathleen, Eef, Wilfried, Sabrina en co. Kevin en Ineke, bedankt voor de gezelligheid op bureau. Kom op tegen Kanker en FWO wil ik bedanken voor hun geloof in dit onderzoek. En tot slot aan Eduard: merci om ons project met zoveel enthousiasme voort te zetten! Kwaliteitsvol onderzoek is maar

mogelijk als het wordt warm gehouden, en ik ben dan ook ontzettend blij dat ons project nu bij jou in goede handen is.

Als bovenstaande paragrafen iets leren, dan is het wel dat solovliegen niet bestaat. Dat maken we nu zelf mee. De coronapandemie heeft op dit moment niet zijn voorspelde apocalyptische koers gevolgd, omdat we daar allemaal samen voor zorgen. Niemand gelooft dat ze het grote verschil maakt door een barbecue te vervangen door een videocall – maar het resultaat is overduidelijk. In onze snel veranderende wereld is het bijna onmogelijk geworden om het grote plaatje te zien, laat staan de impact van je eigen werk daarop. Maar we zien wel hoe onze samenwerking en nieuwsgierigheid ons razendsnel voortstuwen. Niemand weet hoe ons werk en ons leven er over twintig jaar zullen uitzien.

En dat maakt het net zo spannend.

Chapter 1

Introduction

Chapter 1: Contents

The thesis manuscript is a scientific publication and as such should consist of objective facts and validated findings. In this first paragraph however, I am taking the liberty of adding an opinion: oncology is one of the most exciting fields to be working in right now. On top of that, renal cell carcinoma is one of the most thrilling tumors to be working on. A few decades ago, it morphed from a dreaded chemo- and radioresistant disease into a first beacon of hope for cytokine-based immunotherapy. It then quickly spearheaded the anti-angiogenic revolution, becoming a prime target for the new wave of precision molecules that were poured into the clinic. After the start of this thesis, in the heyday of angiogenesis inhibitors, renal cell carcinoma went on to prove itself an excellent target for the novel immune checkpoint inhibitors and recently even more so for combinations of these molecules. Along with the entire field, this biomarker-focused thesis has reinvented itself a couple of times. The field of renal cell carcinoma is a wonderful micro-example of our rapidly evolving world: our combined efforts are changing it so quickly, that no single person can fully grasp where we are heading. It is humbling to be part of, and exciting every time we catch a glimpse of what is still to come.

– Er gaat meer boven mijn pet dan er onder –

Toon Hermans

Epidemiology and distinguishing features of renal cell carcinoma

– Key message –

Clear-cell RCC (ccRCC) make up >80% of kidney carcinomas. They are hallmarked by ubiquitous loss of the Von Hippel Lindau (VHL) gene, which leads to accumulation of Hypoxia Inducible Factor (HIF) proteins despite normoxic conditions. This in turn results in increased angiogenesis, metabolic alterations and apoptosis resistance. Besides ubiquitous VHL loss, ccRCC display notorious intra- and intertumor heterogeneity on genetic, histological and clinical levels. For reasons that remain incompletely understood, they are also immunogenic tumors with high levels T-cell infiltration. Their signature hypervascularity and immunogenicity have made them preferred targets for treatment with angiogenesis inhibitors and immunotherapy.

Epidemiology

RCC are tumors originating from the renal epithelium, that account for >90% of kidney carcinomas. They rank in the top ten of most frequent cancers and are responsible every year for 295.000 new diagnoses and 134.000 deaths worldwide. (1,2) In Belgium, about 1700 people per year are diagnosed with RCC. (3) The median age at diagnosis is 64 years, with a male to female ratio of 2:1. In recent decades, both the incidence and survival of RCC have steadily increased until recently reaching a plateau. This evolution can be largely attributed to increased imaging, which has led to more and earlier incidental detection of RCCs. A little under one third of patients are metastatic at diagnosis, with another half of those with initially localized disease developing metachronous metastases later on. (4) RCCs can be divided into several histological subtypes that display different clinical behavior. Clear-cell RCC (ccRCC) make up the largest majority, accounting for over 80% of RCC. Among the other subtypes, which are typically grouped together as non-clear-cell RCCs (non-ccRCC), papillary and chromophobe RCC are the most frequent histologies, whereas other types of non-ccRCC have incidences of less than five percent (Figure 1.1, Table 1.1). (5)

Genetic alterations

ccRCC are hallmarked by ubiquitous loss of the *VHL* gene, through mutation, deletion, arm level loss of chromosome 3p or promotor methylation. (4) Under

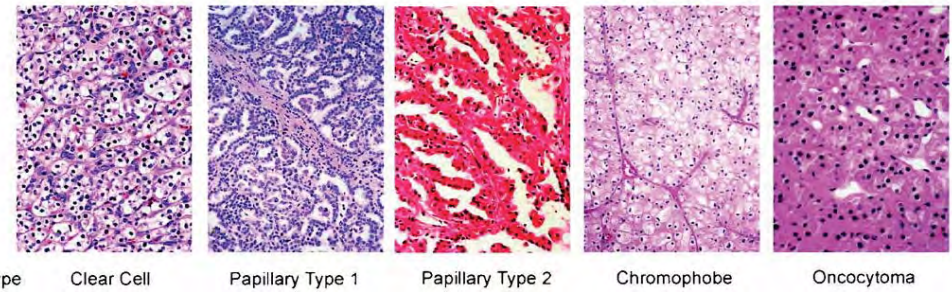

Figure 1.1. *Major histological subtypes of RCC.*
Adapted from Bottaro et al, Clinical Cancer Research 2005. (6) Copyright held by AACR

Table 1.1. *World Health Organization (WHO) classification of tumors of the kidney.*

Renal cell tumours	
Clear cell renal cell carcinoma	8310/3
Multilocular cystic renal neoplasm of low malignant potential	8316/1*
Papillary renal cell carcinoma	8260/3
Hereditary leiomyomatosis and renal cell carcinoma–associated renal cell carcinoma	8311/3*
Chromophobe renal cell carcinoma	8317/3
Collecting duct carcinoma	8319/3
Renal medullary carcinoma	8510/3*
MiT family translocation renal cell carcinomas	8311/3*
Succinate dehydrogenase–deficient renal carcinoma	8311/3
Mucinous tubular and spindle cell carcinoma	8480/3*
Tubulocystic renal cell carcinoma	8316/3*
Acquired cystic disease–associated renal cell carcinoma	8316/3
Clear cell papillary renal cell carcinoma	8323/1
Renal cell carcinoma, unclassified	8312/3
Papillary adenoma	8260/0
Oncocytoma	8290/0
Metanephric tumours	
Metanephric adenoma	8325/0
Metanephric adenofibroma	9013/0
Metanephric stromal tumour	8935/1
Nephroblastic and cystic tumours occurring mainly in children	
Nephrogenic rests	
Nephroblastoma	8960/3
Cystic partially differentiated nephroblastoma	8959/1
Paediatric cystic nephroma	8959/0
Mesenchymal tumours	
Mesenchymal tumours occurring mainly in children	
Clear cell sarcoma	8964/3
Rhabdoid tumour	8963/3
Congenital mesoblastic nephroma	8960/1
Ossifying renal tumour of infancy	8967/0
Mesenchymal tumours occurring mainly in adults	
Leiomyosarcoma	8890/3
Angiosarcoma	9120/3
Rhabdomyosarcoma	8900/3
Osteosarcoma	9180/3
Synovial sarcoma	9040/3
Ewing sarcoma	9364/3
Angiomyolipoma	8860/0
Epithelioid angiomyolipoma	8860/1*
Leiomyoma	8890/0
Haemangioma	9120/0
Lymphangioma	9170/0
Haemangioblastoma	9161/1
Juxtaglomerular cell tumour	8361/0
Renomedullary interstitial cell tumour	8966/0
Schwannoma	9560/0
Solitary fibrous tumour	8815/1
Mixed epithelial and stromal tumour family	
Cystic nephroma	8959/0
Mixed epithelial and stromal tumour	8959/0
Neuroendocrine tumours	
Well-differentiated neuroendocrine tumour	8240/3
Large cell neuroendocrine carcinoma	8013/3
Small cell neuroendocrine carcinoma	8041/3
Phaeochromocytoma	8700/0
Miscellaneous tumours	
Renal haematopoietic neoplasms	
Germ cell tumours	
Metastatic tumours	

The morphology codes are from the International Classification of Diseases for Oncology (ICD-O) (917A). Behaviour is coded /0 for benign tumours; /1 for unspecified, borderline, or uncertain behaviour; /2 for carcinoma in situ and grade III intraepithelial neoplasia; and /3 for malignant tumours. The classification is modified from the previous WHO classification (756A), taking into account changes in our understanding of these lesions.
*New code approved by the IARC/WHO Committee for ICD-O.

Reproduced from the WHO International Agency for Research on Cancer
(WHO classification of tumors of the urinary system and male genital organs, 4th edition 2016). (5)

normoxic conditions, the VHL protein is responsible for ubiquitylation of HIF1α and HIF2α through the E3 ligase complex, thereby inducing their proteasome-mediated degradation. Loss of *VHL* mimics cellular hypoxia, resulting in the aberrant accumulation of HIF proteins that activate pathways leading to increased angiogenesis, metabolic alterations and apoptosis resistance. This cascade is responsible for the typical histological features of ccRCC: clear cells filled with lipid vesicles, surrounded by an extensive vascular network. The HIF-induced aberrant angiogenesis is mediated by Vascular Endothelial Growth Factor (VEGF). For this reason, the last 15 years have seen the establishment of several tyrosine kinase inhibitors of the VEGF-receptor (VEGFR-TKIs) as a solid backbone of ccRCC treatment: sunitinib, pazopanib, cabozantinib, axitinib, sorafenib, tivozanib and lenvatinib. (7)

ccRCC vary widely in their clinical behavior, ranging from very indolent to highly aggressive diseases. They also display a marked intra- and intertumor genetic heterogeneity. Genetic driver events besides *VHL* loss are frequent, but usually subclonal: they include mutations in *PBRM1* (30-40%), *SETD2* (10%), *BAP1* (5-10%), *KDM5C* (5%), *MTOR* (5%), *PTEN* (4%) and other genes. (8) Interestingly, *PBRM1, SETD2* and *BAP1* are all involved in chromatin and histone regulation and are situated on the short arm of chromosome 3p, in the vicinity of *VHL*. Arm level losses of chr3p are frequent in ccRCC and result in haploinsufficiency of all four tumor suppressor genes. Mutations in these chromatin-regulating genes on the other hand, are typically mutually exclusive and carry different prognostic implications. (9–11)

Immune microenvironment

It has long been known that ccRCC are immunogenic tumors. Complete regression of metastases, due to an abscopal effect after cytoreductive nephrectomy, can be observed rarely but consistently. In the cytokine era, high dose interleukin 2 and interferon alpha could induce durable responses in a small fraction of patients. (12) Moreover, ccRCC have the highest cytolytic scores among the 18 tumor types from The Cancer Genome Atlas (TCGA). (13) The reasons for this particular immunogenicity are however not completely understood. In contrast to other immunogenic tumors such as melanoma, smoking-related lung carcinoma or mismatch repair deficient colon carcinoma, ccRCC carry only a modest tumor mutational burden. (14) One theory poses that they are relatively rich in indel mutations, which are more likely to create recognizable neoantigens compared to point mutations. But in a randomized phase II trial there was no link between indel load and $T_{effector}$ (T_{eff}) cell signature.

(15,16) Some studies have suggested a role of aberrantly expressed retroviruses in eliciting an immune response. (13,17,18)

Although immune responses are clearly present in the majority of metastatic ccRCC, these responses seem often poorly functional. (19) Higher infiltration rates by cytotoxic CD8+ T-cells signal a poor prognosis in metastatic ccRCC, in contrast towith most other tumor types and in contrast to early ccRCC, were CD8+ T-cell infiltration is favorable. (20–23) ccRCC often seem to fail to organize effective priming and maturation of cytotoxic T-cells in tertiary lymphoid structures or antigen-presenting intratumoral niches. Indeed, one ccRCC often lack functional tertiary lymphoid structures or other antigen-presenting intratumoral niches, that can effectively prime and mature cytotoxic T-cells. When present, these niches are associated with good prognosis. One study showed that in a small subset of ccRCC, that contained tertiary lymphoid structures with antigen-presenting dendritic cells, CD8+ T-cell infiltration was indeed correlated with better prognosis instead of worse. (21) Another recent study showed that intratumoral lymphoid aggregates, in which antigen-presenting cells interacted with CD8+ T-cells, were frequent in localized ccRCC that did not relapse, but absent in tumors that relapsed early. (23)

The contemporary therapeutic landscape of metastatic renal cell carcinoma

– Key message –

Treatment strategies for metastatic RCC have been turned upside down quite a few times over the past decades. When it comes to systemic therapies, cytokine-base immune therapies have been largely replaced by angiogenesis inhibitors, which are now again challenged by immune checkpoint inhibitors (ICI). In 1st line, combination regimens using an ICI backbone with another ICI or angiogenesis inhibitor are now the standard of care for all patients. In later lines, angiogenesis inhibitors remain active and can be used sequentially both after ICI and after previous angiogenesis inhibitors. In patients who have become resistant, the mTOR inhibitor everolimus remains an option. Local treatment can be appropriate in patients with favorable features for whom immediate start of systemic therapy is not necessary. Options are cytoreductive nephrectomy, or even, in highly selected patients, radical ablative treatment of all disease localizations. A proposed treatment algorithm is illustrated in Figure 1.2.

Role of surgery

In the setting of localized RCC, for which (partial) nephrectomy is the gold standard, there is no place for (neo-)adjuvant systemic therapy. Several trials of adjuvant sunitinib, sorafenib and pazopanib have failed to demonstrate benefit. (24,25) Only the S-TRAC trial, testing adjuvant sunitinib in high risk ccRCC, showed some benefit in disease-free survival, but at the cost of considerable toxicity and without effect on overall survival (OS). (26) The neo-adjuvant use of VEGFR-TKIs in order to downstage locally advanced tumors is not recommended as standard practice, but can be considered in selected cases that are primarily inoperable. Trials testing (neo-)adjuvant ICI are ongoing.

In metastatic disease, the role of cytoreductive nephrectomy has recently been redefined. Where cytoreductive nephrectomy could prolong OS in the cytokine era, the CARMENA trial has now shown that patients who need to start sunitinib immediately at time of diagnosis, derive no OS benefit from cytoreductive nephrectomy: it is therefore no longer recommended in this setting. (27) In contrast, patients for whom start of systemic therapy can be deferred, or those with symptomatic tumors, were not included in this trial: for them, cytoreductive nephrectomy remains the standard of care. Of note, both the CARMENA and SURTIME trials have shown that deferred nephrectomy, after start of sunitinib, is feasible and safe. (27,28) Trials investigating the place of cytoreductive nephrectomy in the context of ICI are ongoing.

For selected patients with oligometastatic RCC, radical local treatment by metastasectomy or stereotactic body radiotherapy can be offered after multidisciplinary review. (29)metastasectomy still remains the only potentially curable intervention and plays an important role both in disease control, cancer-specific survival (CSS Afterwards, patients should be offered active surveillance without systemic therapy, as two trials testing pazopanib and sorafenib after complete metastasectomy did not show any benefit. (30,31) Trials testing ICI combined with local treatment are ongoing. Some emerging treatment strategies include local treatment of oligoprogressive metastases while continuing systemic therapy for responding lesions, but these approaches should still be considered experimental.

First-line systemic treatment: ICI combinations

In 2017, the ICI + ICI combination nivolumab + ipilimumab demonstrated a clear OS benefit over sunitinib in 1st line, in patients with Intermediate or Poor risk according to the International Metastatic ccRCC Database Consortium (IMDC)

criteria (hazard ratio, HR, 0.63). (32,33) Responses rates (RR) and progression-free survival (PFS) were also increased (42% *vs* 27% and 11.6mo *vs* 8.4mo, HR 0.82). Interestingly, sunitinib yielded higher RR (52%) and PFS (25mo) in IMDC Good risk tumors, whereas the effects of nivolumab + ipilimumab were similar across IMDC risk groups.

In 2018, the ICI + VEGFR-TKI combination pembrolizumab + axitinib has proven itself superior over sunitinib in all IMDC risk groups (RR 59% vs 36%, HR PFS 0.69, HR OS 0.53). (34) Another combination, avelumab + axitinib, showed improved PFS over sunitinib, but OS data were immature and lacked a signal towards OS benefit: recent guidelines therefore do not currently recommend it as 1st line option. (7,35,36) Other phase III trials testing ICI + VEGFR-TKI combinations are ongoing. Of note, the combination of ICI with a VEGFR-TKI is supported by a strong scientific rationale. VEGF exerts well-known immune suppressive effects, which can dampen the response to ICI. In preclinical models, antiangiogenic therapy can decrease immunosuppressive cells (myeloid derived suppressor cells, regulatory T-cells), decrease immunosuppressive cytokines (IL-10, TGF-b), activate expression of immune checkpoints by tumor cells, facilitate homing of lymphocytes and increase expansion of tumor infiltrating lymphocytes. (37,38) Therefore, the addition of a TKI does not only provide an additive anti-angiogenic effect, but acts synergistically to boost the immune invigorating effects of ICIs.

Despite these tremendous advances, several open questions remain in 1st line. Most importantly, it is currently not possible to judge whether nivolumab + ipilimumab or pembrolizumab + axitinib are preferred in IMDC Intermediate/ Poor risk patients. Response rates to pembrolizumab + axitinib seem higher based on the registration trials, but this might be expected as it also targets the VEGF-pathway, and follow-up is too short to assess the OS plateau. In PD-L1 positive tumors, complete response rates to nivolumab + ipilimumab reached an impressive 16% in the Checkmate214 trial, but the definition of PD-L1 positivity was stricter compared to the Keynote426 (pembro + axi) trial and PD-L1 positivity is not used for patient selection in the clinic. A first real-world retrospective comparison of 188 patients receiving either nivolumab + ipilimumab or an ICI + VEGFR-TKI combination did not signal improved benefit of either strategy, but consisted of very heterogeneous populations. (39) Long term survival data, real world experience with toxicity and emerging molecular biomarkers will guide treatment decisions in the future. The optimal duration of treatment in case of long-lasting remission is currently unknown.

Further line systemic treatment: TKI monotherapy

ccRCC is known first and foremost as an angiogenic disease, with data from the TKI era indicating continued benefit of VEGFR-TKIs in early and later treatment lines. Emerging evidence is now supporting the benefit of VEGFR-TKIs after previous ICI. Several small prospective trials and retrospective series have reported response rates of 18 to 47% and PFS of 6 to 9 months on TKI after previous ICI or ICI combinations. (40–46)

There is insufficient evidence to recommend any specific TKI from 2nd line on. In Belgium, the recently revised reimbursement criteria put forward cabozantinib as the preferred 2nd line treatment after ICI, in patients with good performance status (KPS ≥ 70%). Indeed, cabozantinib is a TKI with pleiotropic effects that extend beyond VEGFR-inhibition (such as MET inhibition), which has proven its efficacy in RCC. A small phase II trial in Intermediate/Poor risk patients in 1st line, showed improved PFS and a trend towards OS benefit compared with sunitinib. (47) After previous TKI treatment, OS was also improved with cabozantinib compared with everolimus: a finding that also held true in the subgroup of patients who had also received previous ICI. (42,48) A recent large real-world retrospective series has suggested continued efficacy of cabozantinib from 2nd to 4th line, with response rates of about 25% both after VEGFR-TKI and ICI. (49) In patients with poor performance status or other contra-indications, TKIs with a more attractive safety profile can be used. Importantly, every TKI should be administered at the highest tolerable dose, as this clearly improves outcomes. (50–52)

From 3rd line on, any VEGFR-TKI can be used. Abundant data support the continued efficacy of TKI after previous TKI, mostly if a patient has experienced long-lasting remission on earlier therapy. (48,53,54) In TKI-resistant patients, the mTOR inhibitor everolimus remains a valid option. Although responses are rare and PFS usually short, some patients can still achieve durable responses.

There is no evidence that supports the use of ICI after previous failure of ICI. In a very small retrospective series, 3 of 5 patients immediately progressed upon rechallenge with ICI. Importantly, the other two (1 partial response and 1 stable disease) had discontinued 1st line ICI combination after less than three months, for other reasons than disease progression. (43)

Non-clear-cell RCC

Non-clear-cell histologies make up <20% of RCC, with papillary and chromophobe accounting for 80% of these. As these subtypes are usually

excluded from clinical trials, data to guide treatment decisions are scarce. In general, the same strategy is recommended as in ccRCC, but it is encouraged to include patients in clinical trials if possible. (7) The sensitivity of non-ccRCC to ICI in 1st line was demonstrated prospectively in the Keynote427 trial (26% RR to pembrolizumab) and in a small retrospective study (28% RR to nivolumab + ipilimumab). (55,56) VEGFR-TKIs have shown efficacy in various non-clear-cell histologies, both in trial and real world settings, in first and later lines.

Of note, papillary RCC often harbor *MET* mutations or *MET* amplification, making them intuitive candidates for treatment with MET inhibitors. Several MET inhibitors have shown activity in papillary RCC, of which cabozantinib is the only one available in Belgium. (57) It is therefore first choice after 1st line ICI combinations.

Collecting duct carcinoma (Bellini duct carcinoma) are highly aggressive tumors that arise from renal collecting tubules and are notoriously TKI-resistant. Limited data suggest activity of ipilimumab + nivolumab as 1st line treatment for these tumors. (56) In case of progression, they should preferentially be treated with cisplatinum-based chemotherapy.

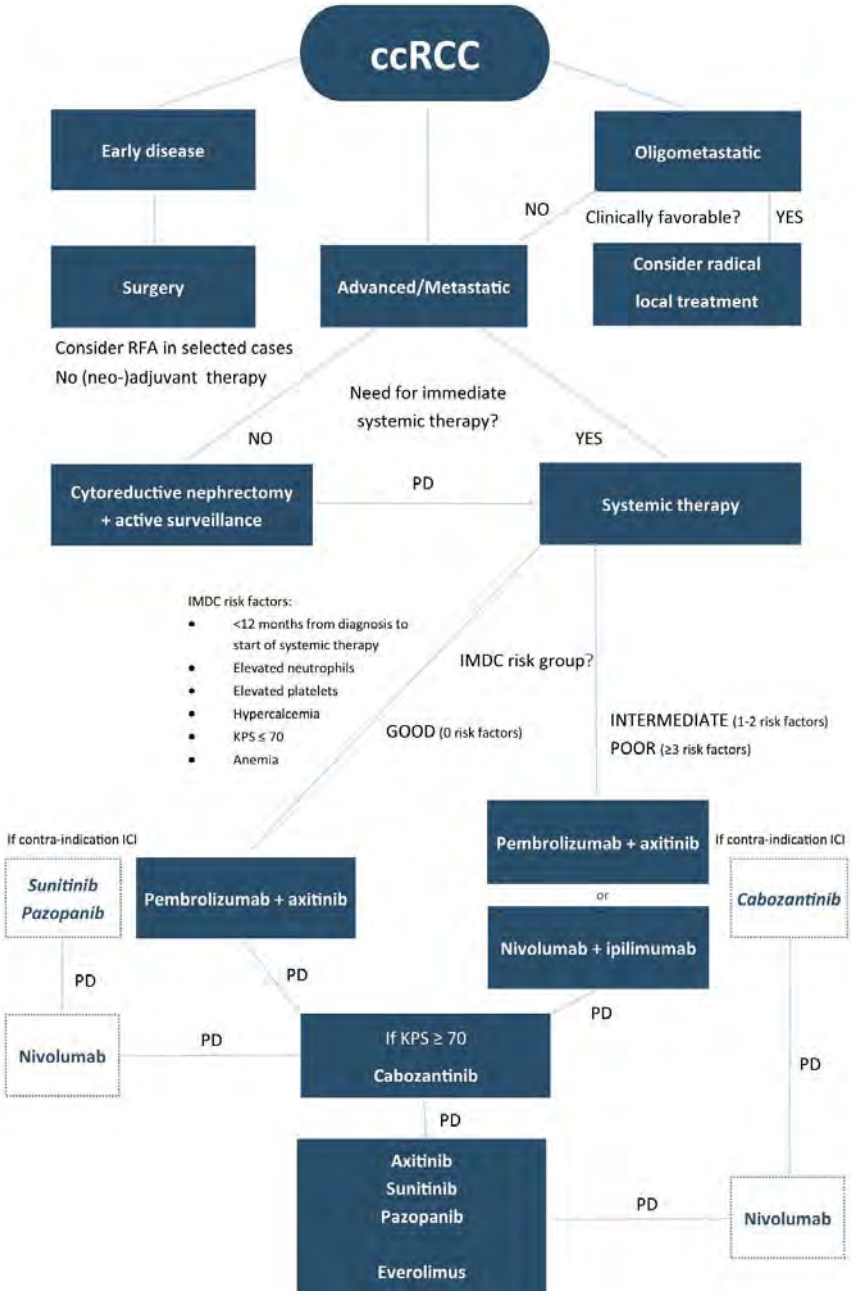

Figure 1.2. *Simplified treatment algorithm for ccRCC*
This figure also features in "An update on the management of metastatic clear-cell renal cell carcinoma: the BSMO expert panel recommendations". (58)

The urgent need for predictive biomarkers

– Key message –

At this moment, no reliable biomarkers are clinically available to guide treatment decisions. A favorable risk according to the clinical IMDC criteria and increased expression of angiogenic genes have been associated with response to VEGFR-TKIs. Sarcomatoid features, a $T_{effector}$-cell gene expression signature and PD-L1 positivity are associated with response to ICI.

Emerging biomarkers

Despite the current abundance of therapeutic molecules with different modes of action, only a subset of patients responds to any given treatment and we are not currently able to adequately identify them. For example, in unselected populations in 1st line, response rates to sunitinib reach about 30-35%, to ipilimumab + nivolumab 39% and to pembrolizumab + axitinib 59%. For this last combination however, it is unknown which and how many patients benefit only from the VEGFR-TKI or the ICI, and who needs the synergy of the combination to achieve a durable response.

In the TKI era, it became clear that patients who experience toxicity from VEGFR-TKIs such as arterial hypertension, or need dose reductions, have an increased chance of response. However, such on-target biomarkers merely reflect adequate drug exposure and are useful for dose optimization but not primary patient selection. Only since 2018, well after the start of this PhD, some predictive biomarkers have been proposed for VEGFR-TKIs and ICI.

Angiogenesis inhibitors

The IMDC risk score was developed as a prognostic model during the TKI era, to estimate the prognosis of patients treated with 1st line VEGFR-TKIs. (33) The score consists of six clinical risk factors: anemia, elevated platelets, elevated neutrophils, hypercalcemia, Karnofsky performance status ≤70, <1 year between diagnosis and systemic treatment. Patients with zero risk factors are considered Favorable risk (±15%, OS 43mo), those with 1-2 risk factors Intermediate risk (±60%, OS 23mo) and those with ≥3 risk factors Poor risk (±25%, OS 8mo). Beside a solely prognostic value, the Checkmate214 trial showed in 2018 that response rates to sunitinib are higher in Favorable compared to Intermediate/Poor risk patients (52% *vs* 22%). IMDC is however lacking as a predictive biomarker for

VEGFR-TKIs, as the Favorable risk group selects only 15% of patients whereas about 35% responds to sunitinib. Moreover, IMDC is not associated with response to ICI: responses across risk groups are similar, both for the combinations nivolumab + ipilimumab and pembrolizumab + axitinib. This is despite the fact that at least four of the risk factors (anemia, neutrophilia, thrombocytosis, poor performance status) reflected an inflammatory tumor subtype in a recent xenograft model. (59)we developed an empirical approach, DisHet, to dissect the tumor microenvironment (eTME Furthermore, the prognostic value of the IMDC score is less defined in the ICI era: even though survival still decreases with increasing IMDC risk factors, the outcomes of Intermediate/Poor risk patients have improved relatively more than those of Good risk patients. (60) Therefore, a new prognostic model for patient counselling is needed.

Shortly after the predictive value of the IMDC risk groups was established, several groups have also reported the association of angiogenic gene signatures with susceptibility to VEGFR-TKIs. (16,61,62) Some reports have also suggested that *PBRM1* mutations are associated with increased angiogenic gene expression and response to VEGFR-TKIs, which is in line with preclinical studies showing that *PBRM1* inactivation further upregulates *HIF1*. (16,22)

Immune checkpoint inhibitors

Typical ICI biomarkers that are well known in other tumor types, such as PD-L1 positivity and tumor mutational burden, are not useful in RCC. Across ICI trials, PD-L1 positivity (measured with different assays and cutoffs) is consistently associated with higher RR, but PD-L1 negativity was never sufficient to exclude patients from treatment. (32,34,35) Tumor mutational burden is lower in RCC compared to other tumors that are responsive to ICI, such as melanoma or non-small cell lung carcinoma, and is not associated with response. (15,16)

On the contrary, histological features are important: RCC with sarcomatoid dedifferentiation are rare but have a very poor prognosis, and have long been known to be resistant to VEGFR-TKIs. They are however surprisingly sensitive to ICI, with response rates that seem to even surpass those of RCC without sarcomatoid features. (16,34,62,63)

Perhaps the most promising ICI biomarker results were reported by the phase 2 IMmotion150 trial, that compared 1st line atezolizumab + bevacizumab with sunitinib. (64) The PD-L1 antibody atezolizumab was superior over sunitinib in tumors with a T_{eff} cell signature. However, the addition of the VEGF-antibody bevacizumab improved outcomes only in tumors that also exhibited a myeloid cell signature. (16) These findings demonstrate the value of bevacizumab as an ICI booster in tumors with an immune suppressive microenvironment, but

also suggest that combination strategies act synergistically in a specific subset of patients, which has yet to be defined. Unfortunately, these signatures were developed specifically for the atezolizumab + bevacizumab combination, and this regimen never filed for FDA approval as it seemed less promising than concurrent ICI + VEGFR-TKI combos. Of note, the predictive impact of these signatures was not fully replicated in the JAVELIN Renal 101 trial, which compared the PD-L1 inhibitor avelumab + axitinib with sunitinib: avelumab + axitinib performed better than sunitinib in T_{eff} high tumors, but not in T_{eff} + Myeloid high tumors. (62)

Open questions

For the majority of patients, the clinically most important question at this moment, is the position of ICI + VEGFR-TKI combinations against nivolumab + ipilimumab in IMDC Intermediate and Poor risk patients. Both VEGFR-TKIs and ipilimumab are added to anti-PD1 as an immune booster, but they have an entirely different mechanism of action which is likely to be relevant in different tumors (e.g. those with a myeloid high signature will probably benefit more from VEGFR-TKI, but several other immune cell populations and pathways are involved as well).

Apart from models for patients selection, we also need new models to counsel patients on their prognosis. The current IMDC model held true for 1st line treatment with VEGFR-TKIs, but the prognosis of Intermediate/Poor risk patients and of those with sarcomatoid tumors, has improved relatively more with ICI combination therapies than the prognosis of Favorable risk patients.

Furthermore, a major problem in the metastatic setting are mixed responses to treatment. We understand little of how metastatic lesions are similar to or different from the primary tumor and how they are influenced by their host organ. As metastases are rarely resected, molecular data are very scarce. These are urgently needed to gain deeper insights in the dynamics of metastases and organ-specific metastatic niches.

In conclusion, the current crowded guidelines demonstrate the urgent need for biomarkers for patient selection. Moreover, as the standard of care is changing so rapidly, an ideal biomarker would be generic, reflecting intrinsic tumor biology, rather than be developed as a companion for a specific therapy. And most of all, a deeper molecular understanding of ccRCC and their immune environment is crucial to guide future research and trial design. After all, throughout the field of oncology there is a need for trials driven by a sound scientific rationale, as potential treatments have become too numerous to test

even a small fraction of them. Gaining these insights is particularly challenging in ccRCC, which are notoriously heterogeneous on a clinical, histological, molecular and immunological level.

ccRCC can be divided into four molecular subtypes

– Key message –

In 2015, our team has proposed four molecular subtypes of advanced ccRCC, ccrcc1 to -4, based on unsupervised clustering of whole transcriptome data. These subtypes differ not only in terms of gene expression, but also mutation and methylation profiles, immune cell infiltration, histological features, prognosis and response to sunitinib. The rare ccrcc3 subtype has the best prognosis and a gene expression profile that resembles that of normal kidney. Ccrcc2 tumors, accounting for almost half of ccRCC, have a good prognosis and are sensitive to sunitinib. Ccrcc1 tumors have an intermediate prognosis and an immune cold phenotype. Finally, ccrcc4 tumors are highly aggressive, often have sarcomatoid features, respond poorly to sunitinib and display a highly inflamed phenotype with an immunosuppressive microenvironment.

The ccrcc1 to -4 molecular subtypes

This thesis is built on earlier work by our team, which in 2015 has described four molecular subtypes of ccRCC. (65) These subtypes were discovered through unsupervised cluster analysis of microarray data of 53 fresh-frozen untreated primary ccRCC, which metastasized and were treated with 1st line sunitinib. The subtypes were subsequently validated on another 47 ccRCC and on the TCGA ccRCC cohort. These four transcriptomic groups, named ccrcc1 to -4, reflect intrinsic ccRCC tumor subtypes with different tumor biology and clinical behavior: they not only differed in terms of gene expression, but also mutation and methylation profiles, immune cell infiltration, histological features, prognosis and response to sunitinib. Their main differences are summarized in Figure 1.3 and Table 1.2.

The rare ccrcc3 subtype (11%) showed a gene expression profile that resembles that of normal kidney and an indolent clinical behavior (OS 50mo after 1st line sunitinib). It upregulated mainly metabolic pathways, but hardly expressed immune signatures and showed little infiltration by CD8+ cytotoxic

Table 1.2. *Discriminatory features of the ccrcc1 to -4 molecular subtypes as discovered on fresh-frozen ccRCC.*

Subgroup (frequency)			**ccrcc1 (33%)**	**ccrcc2 (41%)**	**ccrcc3 (11%)**	**ccrcc4 (15%)**
Outcome under sunitinib						
Early progressive disease			22%	3%	0%	27%
Partial response			41%	53%	70%	20%
Median OS (mo)			24	35	50	14
Median PFS (mo)			13	19	24	8
Clinical characteristics						
IMDC	Good		6%	21%	18%	7%
	Intermediate		69%	60%	64%	60%
	Poor		25%	18%	18%	33%
Molecular characteristics						
Pathology characteristics	Mean inflammation intensity (scale 0-3)		1.3	1.2	0.8	2.2
	Mean sarcomatoid differentiation		7.5%	3.7%	1.7%	24.6%
Mutations		VHL	47%	63%	20%	20%
		PBRM1	47%	38%	20%	0%
Upregulated pathways			MYC targets Glycolysis Hypoxia	Glycolysis Hypoxia		Immunity Apoptosis Chemotaxis MYC targets Glycolysis Hypoxia
MYC expression level			++	+	--	++
Methylation status			Hyper-methylated+			Hyper-methylated ++
Polycomb stem cell phenotype			++		--	+++
Copy number amplification						2p12 / 2p22.3/8q21.13
Proposed name			**MYC.UP**	**Classical**	**Normal like**	**Immun.UP/MYC.UP**

Adapted from Beuselinck et al, Clinical Cancer Research 2015. (65) Copyright held by AACR.

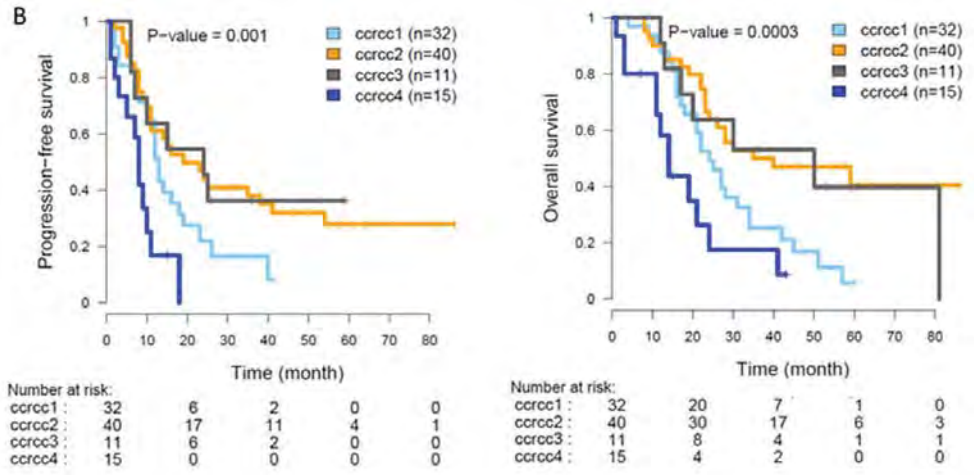

Figure 1.3. *PFS and OS on 1st line treatment with sunitinib according to molecular subtype. Adapted from Beuselinck et al, Clinical Cancer Research 2015. (65) Copyright held by AACR.*

T-cells. The ccrcc2 subtype was the most frequent one (41%), but no particular upregulated pathways were established at that time. It also showed a favorable baseline prognosis (OS 35mo) and high response rates to sunitinib (53%). Ccrcc2 tumors had intermediate infiltration by CD8+ T-cells and expression of immune signatures.

The ccrcc1 and -4 subtypes displayed a more aggressive gene expression pattern, with upregulation of *MYC* and MYC-targets. Both subtypes also showed a more undifferentiated phenotype, with hypermethylation and consequent downregulation of polycomb targets, as well as higher histological grades. The ccrcc1 subtype (33%) had an intermediate prognosis and response to sunitinib (OS 24mo, RR 41%) and showed little infiltration by CD8+ T-cells or expression of immune signatures. The ccrcc4 subtype (15%) on the other hand, had the shortest OS (8mo) and lowest RR (20%) to sunitinib. This subtype was enriched for tumors with sarcomatoid features. It showed high infiltration by CD8+ T-cells and high expression of immune signatures. These latter however also included high expression of checkpoints and immune suppressive cells such as myeloid cells, indicating an inflamed but suppressed immune response.

Importantly, a 35-gene classifier algorithm was constructed that was able to classify independent ccRCC samples into the four groups, without the need to cluster them against reference samples as is the case with the other molecular classifiers discussed below.

Other transcriptome-based ccRCC molecular subtypes

Three other teams have performed unsupervised cluster analysis of whole transcriptome ccRCC data and reported very similar results, which confirms the existence of four robust transcriptomic subtypes with different clinical behavior. (8,22,66)and to aid in predicting clinical outcomes. However, there are no current signatures for kidney cancer that are applicable in a clinical setting. Objective To generate a signature biomarker for the clear cell renal cell carcinoma (ccRCC In the adjuvant setting, the TCGA research programme identified four clusters (m1 to m4), of which m1 was the most frequent (35%) and had a favorable prognosis, whereas m2 and m3 (together 44%) had a dismall prognosis. The group of Brannon et al identified three clusters in the adjuvant setting: ccA with a favorable prognosis, ccB with a poor prognosis and cluster_3 that was rare. There is significant overlap between Brannon's ccA, TCGA's m1 and our ccrcc2 cluster, between Brannon's cluster_3 and our ccrcc3 and between Brannon's ccB, TCGA's m2+m3 and our ccrcc1+ccrcc4 clusters.

Very recently, Hakimi et al performed cluster analysis on a large cohort of primary ccRCCs that developed metastatic disease and received 1st line sunitinib or pazopanib: a clinical setting that is almost identical to the one in which the ccrcc1 to -4 subtypes were discovered. They found four clusters with a relative frequency and outcomes on VEGFR-TKIs that were very similar to the ccrcc1 to -4 clusters, and also validated their findings on our original ccrcc1 to -4 dataset. Their cluster 3 shared many characteristics with ccrcc2 tumors, whereas their cluster 4 clearly stood out as the most dismall subtype and displayed upregulation of MYC targets, proliferation markers and several immune signatures, as does our ccrcc4 subtype.

References to Chapter 1 Introduction: see Chapter 12 Concluding discussion