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Deciphering immune response to checkpoint inhibitors and finding novel biomarkers in metastatic renal-cell carcinoma

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GLOSSARY

ASCO	American Society of Clinical Oncology
ASCO-GU	American Society of Clinical Oncology – Genitourinary Congress
BMI	Body Mass Index
ccRCC	Clear-Cell Renal Cell Carcinoma
CEA	Carcino-Embryonic Antigen
CI	Confident Interval
CIMP-RCC	Cpg Island Methylator Phenotype
CPIs	Checkpoint Inhibitors
CRC	Colorectal Cancer
CTAs	Cancer/Testis Antigens
DCR	Disease Control Rate
dMMR	Mismatch Repair Deficient
DTT	time from Diagnosis To systemic Treatment
eCRF	Electronic Case Report Form
EDTA	Ethylenediaminetetraacetic Acid
EMA	European Medicines Agency
ESMO	European Society of Medical Oncology
FCM	Flow Cytometry
FCS	Flow Cytometry Standard
FDA	Food And Drugs Administration
FH	Fumarate Hydratase
fRCC	Fast Progression Renal Cell Carcinoma
GEP	Gene Expression Profile
HPV	Human Papilloma Virus
HR	Hazard Ratio
IDO1	Indoleamine 2,3-Dioxygenase
IDO1-expressing DCs	IDO Expression By Dendritic Cells
IFN-γ	Interferon-γ
IHC	Immunohistochemical
IL-10	Interleukin-10
IL-2	Interleukin-2
IMDC	International Metastatic renal cell carcinoma Database Consortium
iMFI	Integrated Median Fluorescence Intensity
iNOS	Inducible Nitric Oxide Synthase
irAE	Immune-Related Adverse Event

iRECIST	Immune-Response Evaluation Criteria In Solid Tumours
KPS	Karnofsky Performance Status
LNL	Low Normal Level
lnNLR	Natural Log-Transformed Neutrophils/Lymphocytes Ratio
MAGE	Melanoma-Associated Antigen
Mb	Mega-Basepairs
MDSCs	Myeloid-Derived Suppressor Cells
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
mRCC	Metastatic Renal Cell Carcinoma
MSI-H	Microsatellite Instability-High
NCI	National Cancer Institute
NGS	Next-Generation Sequencing
NLR	Neutrophils/Lymphocytes Ratio
NR	Not Reached
NSCLC	Non-Small Cell Lung Cancer
ORR	Objective Response Rate
OS	Overall Survival
PD-L1	Programmed Death 1 Ligand
PD1	Programmed Death 1
PFS	Progression-Free Survival
PLR	Platelet/Lymphocyte Ratio
PSA	Prostatic Specific Antigen
RCC	Renal Cell Carcinoma
RCT	Randomized Clinical Trial
RECIST 1.1	Response Evaluation Criteria In Solid Tumours 1.1
RNA-seq	RNA Sequencing
SEER	Surveillance Epidemiology and End Results
SII	Systemic Inflammatory Index
sarcoRCC	Sarcomatoid Renal Cell Carcinoma
sRCC	Slow Progression Renal Cell Carcinoma
TAAs	Tumour-Associated Antigens
TAMs	Tumour-Associated Macrophages
TCGA	The Cancer Genome Atlas
Teff	Effector T Cells
TGFb	Transforming Growth Factor beta
Th	T Helper

TILs	Tumour Infiltration Lymphocytes
TMB	Tumour Mutational Burden
TME	Tumour Microenvironment
TNF	Tumour Necrosis Factors
TRAE	Treatment-Related Adverse Events
Treg	Regulatory T Cell
TSAs	Tumour-Specific Antigens
tSNE	T-Distributed Stochastic Neighbour Embedding
TTF	Time to Treatment Failure
UNL	Upper Normal Level
VEGF/VEGFR	Vascular Endothelial Growth Factors/Vascular Endothelial Growth Factors Receptor
WES	Whole Exome Sequencing

ABSTRACT (English version)

Nivolumab represents the new second-line treatment for metastatic renal cell carcinoma (mRCC). This drug inhibits programmed death-1 (PD1)/PD1 ligand 1 (PD-L1) immune checkpoint. In the majority of patients, nivolumab is able to restore the patient's tumour-specific T-cell-mediated response thus improving both overall survival and objective response rate. However, a lack of clinical response occurs in a number of patients, raising questions about how to predict and increase the number of patients who receive long-term clinical benefit from immune checkpoint therapy or not. Unlike traditional cancer therapies, checkpoint inhibitors act primarily on cells of the immune system. High-dimensional flow cytometry was used to analysis peripheral blood immune cells in an effort to identify a responsiveness-associated predictive signature in cancer therapy.

The aim of this project is to identify immune biomarkers that are modulated in patients with mRCC treated with nivolumab and that can discriminate patients who most likely benefit from such therapy.

This is a prospective study on patients with mRCC who will receive nivolumab in standard clinical practice. The project investigates changes in main immune parameters in patients with mRCC treated with nivolumab by analysing peripheral blood mononuclear cells (PBMCs) at baseline and after 1, 2, 3, 5 and eventually further cycles of therapy. Two groups of patients were identified and compared: slow progression RCC (sRCC) progression-free survival (PFS) ≥ than 6 months or PFS < 6 month and overall survival (OS) ≥ 12 months from nivolumab start; fast progression RCC (fRCC) are patients achieving PFS < 6 months and OS < 12 months from nivolumab starting.

From January 2016 until October 2019 25 patients were enrolled. The majority of them (64%) received nivolumab as second-line therapy. With a median follow up of 20 months (range 2 - 43), 11 (44%) deaths observed. Six-months survival rate and 12-months survival rate were 79% (95% CI, 0.57 - 0.91) and 65% (95% CI, 0.42 - 0.81). Partial response was registered in 5 patients (20%), while stable disease was obtained in 3 (12%). Progression disease occurred in 17 subjects (68%). Eleven patients (44%) were identified as fRCC and 14 (56%) as sRCC. High-dimensional flow cytometry was performed in 7 patients (3 sRCC and 4 fRCC). sRCC PBMCs resulted enriched in effector memory-like CD8+ T cells at baseline and after treatment, while fRCC displayed higher levels of naive-like T cells and senescent-like phenotype.

In this research two subsets of population defined as sRCC and fRCC display differences in CD8+ T cell compartment before starting therapy. This difference further increased after treatment, suggesting that cancer patients undergoing anti-PD1 therapy could be selected according to their CD8 signature.

ABSTRACT (Italian version)

La nuova seconda linea di trattamento del carcinoma renale metastatico è rappresentata dal nivolumab. Questo farmaco agisce bloccando il checkpoint immunitario PD1/PDL1. Nella maggior parte dei pazienti nivolumab è in grado di ripristinare la risposta immunitaria antitumorale mediata dai linfociti T, migliorando la sopravvivenza dei pazienti e il tasso di risposte obiettive osservate. Tuttavia, in un certo numero di casi si registra una mancata risposta antitumorale, sollecitando la necessità di poter predire le risposte cliniche e incrementare il numero di pazienti che presetano un beneficio clinico a lungo termine. Contrariamente ai farmaci convenzionali, gli inibitori dei checkpoint agiscono sul sistema immunitario. La citofluorimetria a flusso multiparametrica per l'analisi delle cellule mononucleate periferiche è utilizzata con lo scopo di individuare un sottotipo cellulare potenzialmente predittivo di una mancata risposta alla terapia.

L'obiettivo dello studio è identificare biomarcatori immunitari in grado di discriminare i pazienti che possono beneficiare maggiormente dell'immunoterapia.

E' uno studio prospettico che coinvolge pazienti con carcinoma renale metastatico che ricevono nivolumab nella normale pratica clinica. Il progetto esamina le variazioni nei principali parametri immunitari in pazienti con carcinoma renale metastatico trattati con nivolumab, attraverso l'analisi delle cellule mononucleate periferiche al basale e dopo 1, 2, 3, 5 ed eventualmente ulteriori cicli di terapia. Sono stati individuati e confrontati due gruppi di pazienti: slow progression (sRCC) con sovravvivenza libera da progressione (PFS) ≥ di 6 mesi oppure PFS < di 6 mesi e sopravvivenza globale ≥ di 12 mesi; fast progression (fRCC) con PFS < di 6 mesi e sopravvivenza globale minore di 12 mesi.

Da gennaio 2016 a ottobre 2019 sono stati arruolati 25 pazienti. La maggior parte dei pazienti (64%) ha ricevuto nivolumab come seconda linea di trattamento. Con un follow up mediano di 20 mesi (range 2 – 43) sono state osservate 11 morti (44%). I tassi di sopravvivenza globale a 6 e 12 mesi sono stati rispettivamente del 79% (95% CI, 0.57 - 0.91) e del 65% (95% CI, 0.42 - 0.81). Una risposta parziale è stata osservata in 5 pazienti (20%) e stabilità di malattia in 3 pazienti (12%). Una progressione di malattia è stata registrata in 17 pazienti (68%). Sono stati identificati 11 pazienti sRCC (44%) e 14 sRCC (56%). In sette pazienti (3 sRCC e 4 fRCC) è stata effettuata l'analisi con citofluorimetria a flusso multiparametrica. Nei pazienti con sRCC tra le cellule mononucleate periferiche è presente in maggior numero il sottotipo di cellule T effettrici memory-like CD 8+, sia al baseline sia dopo l'inizio della terapia; mentre nei pazienti fRCC è stato osservato un maggior numero di cellule T naïve e cellule T senescenti.

Nello studio due sottotipi di popolazioni, sRCC e fRCC, mostrano due diversi pattern di cellule T prima dell'inizio della terapia. Questa differenza inoltre si accentua dopo il trattamento, suggerendo che i pazienti trattati con immunoterapia potrebbero essere selezionati in base al sottotipo di popolazione CD8+ espressa.

BACKGROUND

Renal cell carcinoma: an overview

Renal cell carcinoma (RCC) is responsible for 90-95% of neoplasms arising from kidney and renal pelvis, accounting approximately 3% of adult malignancies. Worldwide, RCC represents the 14th most frequent neoplasm with 403,262 new cases in 2018[1]. According to the 2011 to 2016 National Cancer Institute (NCI) Surveillance Epidemiology and End Results (SEER), RCC predominantly occurs in the sixty to eighty decade of life[2].

Currently, RCC constitutes the 16th cause leading to cancer death, with 5-year overall survival (OS) rates of about 75%[1]. However, the 5-year relative survival rate varies depending on extension disease at presentation. From retrospective SEER data analysis, at diagnosis the majority of patients (66%) have localized disease, 16% regional metastatic disease and 14% metastatic disease (4% unstaged)[2]. Surgical resection or local ablative treatments are curative approaches for localized and sometimes for advanced disease; unfortunately, about 20-30% of patients treated with curative intent develop local or distant recurrences[3,4].

The most common histological type is clear-cell carcinoma, which represents about 80% of RCC. Papillary and chromophobe are the two most frequent subtypes of non-clear cell RCC[5].

Metastatic renal cell carcinoma (mRCC) cannot be cured and 5-year survival rate is about 12%, with a decreasing trend correlated to improvement of OS after the introduction of new drugs[2,6].

Six negative prognostic factors were identified to predict survival in mRCC: Karnofsky Performance Status (KPS) < 80, haemoglobin level < low normal level (LNL), time from diagnosis to systemic treatment (DTT) < 1 year, corrected serum calcium > upper normal level (UNL), neutrophil count > UNL and platelet count > UNL. These factors are included in the International Metastatic renal cell carcinoma Database Consortium (IMDC) score, currently used as prognostic model to stratify patients with mRCC in three subgroups: favourable, intermediate and poor-risk group. Patients lacking these factors have the best prognosis and may reach a longer survival (43.2 months [95% confident interval CI, 31.4 to 50.1]); patients presenting 1 or 2 factors have an intermediate risk of death with a median OS of about 23 months (95% CI, 18.7 to 25.1); patients with 3 or more factors have an expected poor-risk outcome with median survival about 8 months (95% CI, 6.5 to 9.7; $p < 0.001$)[7,8].

Two major revolutions markedly changed management of mRCC: 1) in 2005 molecular-targeted therapy introduction has led to a prolonged median progression-free survival (PFS) and to an increased control of disease in one third of cases; moreover, drugs availability for subsequent lines of therapy improved overall patients' survival; 2) from 2015 onward immunotherapy regimes continue to increase survival benefit and to achieve durable response for patients with mRCC.

During the last fifteen years, inhibition of vascular endothelial growth factors/vascular endothelial growth factors receptor (VEGF/VEGFR) system represented a cornerstone of mRCC treatment.

In the first-line setting, anti-VEGF treatment represented the best strategy, with median OS ranging from 19.3 to 29.3 months[9-13].

In second-line setting, anti-programmed death 1 (PD1) antibody (nivolumab) has shown better median OS compared with everolimus (25 months [95% CI, 21.8 to not estimable] versus 19.6 months [95% CI, 17.6 to 23.1]; hazard ratio [HR] 0.73; p = 0.002) and 20% higher objective response rate (ORR) (p < 0.001)[14]. In the same setting, the new generation tyrosine-kinase inhibitor cabozantinib has shown longer PFS (9.1 months [95% CI, 5.6 to 11.2] versus 3.7 months [95% CI, 1.9 to 4.2]; HR 0.41; p < 0.0001), longer OS (21.4 months [95% CI, 18.7 to not estimable] versus 16.5 months [95 CI, 14.7 to 18.8]; HR 0.66; p = 0.0003) and higher ORR than everolimus (22% [95% CI, 14 to 33] versus 3% [95% CI, 0 to 9]; p < 0.0001)[15,16].

Since 2017, the scenario substantially changed, basically due to two main factors: 1) immunotherapy using checkpoint inhibitors (CPIs), alone or in combination, has now integrated all guidelines based on OS benefit both in first and second-line setting; 2) treatment strategy and approval of first-line treatment was delineated by IMDC risk group classification[17].

In the phase III randomized clinical trial (RCT) Checkmate-214 nivolumab plus ipilimumab immunotherapy combination significantly prolonged OS versus sunitinib (median OS not reached [95% CI, 28 months to not reached] versus 28 months [95% CI, 26 months to not reached] respectively; HR 0.63 [99.8% CI, 0.44 to 0.89]; p < 0.001) in intermediate and poor-risk untreated patients with mRCC[18].

The CABOSUN phase II RCT reported PFS benefit of cabozantinib over sunitinib (median OS 26.6 months [95% CI, 14.6 to not estimable] and 21.2 months [95% CI, 16.3 to 27.4] respectively; HR 0.80 [95% CI, 0.53 to 1.21]) in intermediate or poor-risk patients in first-line setting[19].

Unlike the Checkmate-214, two trials using a combination of VEGFR-tyrosine kinase inhibitor axitinib plus pembrolizumab demonstrated benefit over sunitinib in an unselected population in first line.

In detail, the KEYNOTE-426 phase III RCT demonstrated longer OS (HR 0.53 [95% CI, 0.38 to 0.74]; $p < 0.0001$), longer PFS (median 15.1 [95% CI, 12.6 to 17.7] versus 11.1 months [95% CI, 8.7 to 12.5]; HR 0.69 [95% CI, 0.57 to 0.84]; $p < 0.0001$) and higher ORR (59.3% [95% CI, 54.5 to 63.9] versus 35.7% [95% CI, 31.1 to 40.4]; $p < 0.0001$) than sunitinib[20].

Axitinib plus avelumab in the JAVELIN-101 trial reported longer PFS (median 13.8 [95% CI, 11.1 to not estimated] versus 8.4 months [95% CI, 6.9 to 11.1]; HR 0.69 [95% CI 0.56 to 0.84]; $p < 0.0001$) and higher ORR (51.4% [95% CI, 46.6 to 56.1] versus 25.7% [96% CI, 21.7 to 30.0]) over sunitinib[21].

Table 1 summarize comparison among phase III RCT in mRCC.

All these new treatment strategies were FDA (Food and Drugs Administration) and EMA (European Medicines Agency) approved.

Table 1: Comparison among phase III RCT in metastatic-renal cell carcinoma.

Study	**N°**	**Median PFS (months)**	**Median PFS Δ**	**HR (95%CI)**	**Median OS (months)**	**Median OS Δ**	**HR (95%CI)**	**ORR %**	**ORR Δ**	**p**
CheckMate025*	821	4.6 vs 4.4	0.2	0.88 (0.75-1.03)	25 vs 19.6	5.4	0.74 (0.57-0.93)	25 vs 5	20	-
CheckMate214	1096	9.7 vs 9.7	-	0.87 (0.73-0.98)	NR vs 32.9	-	0.71 (0.59-0.86)	42 vs 27 9 CR	15	<0.0001§
KEYNOTE-426	861	15 vs 11	4	0.69 (0.57-0.84)	NR vs NR	-	0.53 (0.38-0.74)	59.3 vs 35.7 5.8 CR	23.6	<0.0001#
JAVELIN-101	886	13.8 vs 8.4	5.4	0.69 (0.56-0.84)	NR vs NR	-	0.78 (0.55-1.08)	51.4 vs 25.7	25.3	-
IMmotion-151	915	11.2 vs 8.4	2.8	0.83 (0.70-0.97)	33.6 vs 34.9	-1.3	0.93 (0.76-1.14)	37 vs 33 5 CR	4	-

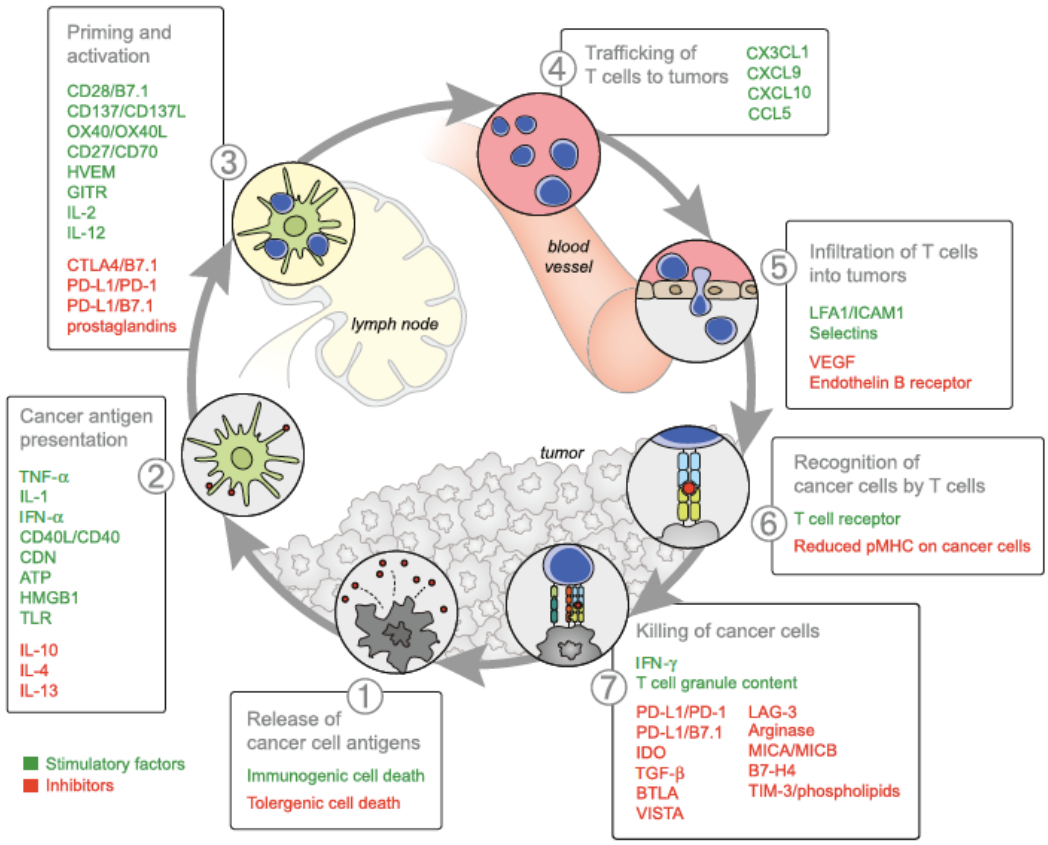
*Second line trial;
§Clopper–Pearson method;
#Miettinen and Nurminen method
Abbreviation: NR: Not Reached; PFS: progression-free survival; HR: hazard ratio; OS: overall survival; ORR: objective response rate; Δ: difference; p: p-value; CR: complete response

Checkpoint Immunotherapy

Tumour immunology

In the last decades, the role of immune system in cancer development has been better clarified, but the underlying mechanisms of this relationship remains widely unsolved. The activation of immunity to cancer has been described as a cyclic process that can be self-sustained through stimulatory and inhibitory factors (Figure 1)[22].

Figure 1: Stimulatory and Inhibitory Factors in the Cancer-Immunity Cycle[22]. *The generation of anticancer immunity is a cyclic process. It has been described the division of the cycle into seven major steps, starting with the release of antigens from the cancer cell and ending with the killing of cancer cells. In each steps, both inhibitory (shown in red) and activator (shown in green) factors are required.*



The early phases of cancer-immunity cycle involve the crucial actors of this scene: the antigens. Indeed, processed and presented antigens thought major histocompatibility complex (MHC) is the key for priming and activation of effector T cells (Teff).

There are three wide categories of antigens:

1) Tumour-associated antigens (TAAs)
2) Cancer/Testis antigens (CTAs)
3) Tumour-specific antigens (TSAs)

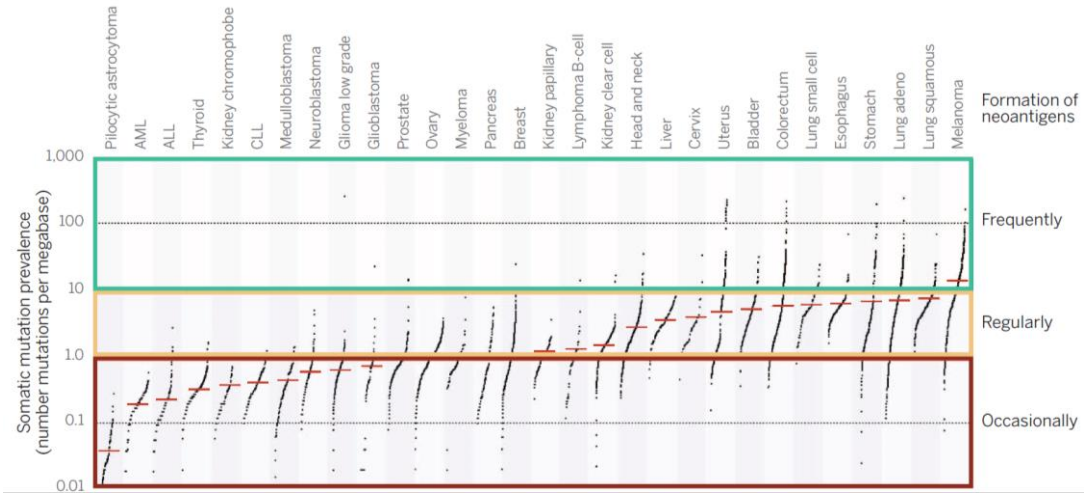
TAAs are normally present in human cells, but they are over-expressed on cancer cells. Because of this greater production, TAAs can be detected into the blood stream as markers of disease (e.g. Carcino-Embryonic Antigen [CEA] or prostatic specific antigen [PSA]). Alterations of TAAs may result from genetic amplification or post-translational modifications and can be selectively expressed by the cell lineage from which the cancer evolved.

CTAs are usually expressed only in male germ cells and absent in normal cells, but these antigens are re-expressed in various tumour types, correlating with cancer progression (e.g. melanoma-associated antigen – MAGE).

TSAs are selective expressed by cancer cells, arising from non-synonymous mutations and other genetic mutations; they are also called neoantigens (e.g. Human Papilloma Virus [HPV] oncoproteins E6 and E7 in HPV-associated cancers)[23].

All these three categories of antigens have been explored as target for immunotherapy. TSAs are considered more attractive because of their specificity, which makes them clearly recognized as "non-self" by immune system. The new genome analysis and advances in neoantigen identification technologies allowed to describe the relation between somatic mutation prevalence and neoantigen formations (Figure 2)[24].

Figure 2: Estimate of the neoantigen repertoire in human cancer[24]. *The figure shows number of somatic mutations in solid tumours. On the right the categories indicate the estimate of neoantigen formation in each tumour. Notably, melanoma and lung cancer are related to mutagens exposure (ultraviolet light in the case of melanoma and tobacco respectively), resulting in the highest somatic mutation burden of any cancer type (right side).*



Moreover, it has been demonstrated that sensitivity to CPIs is broadly related to frequency of tumour somatic mutations, supporting the crucial role of neoantigens for antitumor action of immunity. Indeed, tumours expressing higher number of neoantigens are more likely to induce immune-mediate tumour elimination[23].

Increasing evidences demonstrate that malignancies evolution needs to overcome immune surveillance and that cancer becomes capable to inactivate immune resistance by itself (immune tolerance). This process is called "immunoediting" and includes three phases: elimination, equilibrium and escape[23].

Briefly, in the "elimination" phase, innate and adaptive immune responses induce the eradication of cancer lesions. Some lesions are able to survive the elimination phase, switching to the "equilibrium" phase, where less immunogenic clones were selected. Subsequently, "escape" phase refers to evasion from immune surveillance, through several mechanisms:

- Cancer promotes physical barriers to hide through alteration of angiogenesis, induction of hypoxia and modification of stromal component;
- Cancer cells produce or induce secretion of immunosuppressive factors (VEGF, IDO1, adenosine, IL-10, TGFb, galectins);
- Upregulation of immune checkpoints, playing a role in immunosuppressive pathways (PD1/PD-L1);
- Tumour modulation of metabolism (activation of arginase, iNOS and glucose consumption);
- Recruitment of immune cells that actively mediate tolerance (IDO1-expressing DCs, Treg cells, MDSCs);

Each of these phases represents a potential approach for cancer treatment and for advances in integrated strategies of care for malignancies.

Notably, immune checkpoints inhibition, blocking PD1/PD-L1 pathways, was one of the most expensively explored, revolutionizing cancer treatment in the last years.

PD1/PD-L1 blockade

Ordinarily, the checkpoint inhibitor PD1 protein (also known as CD279), expressed by immune cells, is involved in self-tolerance by controlling T cell activities. PD1 binding with his major ligand PD-L1 (also known as CD274), expressed by a variety of human cells, delivers inhibitory signalling limiting self-reactive T cell proliferation and cytokine production. This mechanism is

crucial for maintaining self-tolerance and protects tissues from damage when the immune system is responding to pathogenic infections.

In the tumour microenvironment, PD-L1 may be expressed by surface of tumour cells, tumour-associated macrophages (TAMs), and T lymphocytes. Induced by cytokines such as interferon-γ (IFN-γ), alternatively, PD-L1 can be expressed constitutionally.

Cancer cells become able to express PD-L1 on their surface and when PD1 is met, the pathway of immune inactivation takes place. Tumour grows and spreads further as mechanism of immune resistance.

The PD1 pathway has also an important role in T cell exhaustion process. Prolonged or high expression of inhibitory factors is a key feature of CD8+ T cells dysfunction, leading to loss of some main functions such as interleukin-2 (IL-2) production, proliferative capacity, ability to produce tumour necrosis factors (TNFs), IFN- γ or chemokines[25].

PD1 is also expressed on regulatory T cells (Treg) and regulates their proliferation. Because of high infiltration of many tumours by Treg, suppressing Teff, blockade of PD1 pathway may have a dual role in enhancing anti-tumour immune response, by preventing the interaction between Teff and tumour cells expressing PD-L1 or by diminishing the number and/or suppressive activity of intratumoural Treg.

Preclinical experience reports as the inhibition of PD1/PD-L1 interaction promote antitumour activity of immune system[26,27]. These evidences support the design and the carrying out of clinical trials to assess efficacy of therapeutic immune checkpoint inhibition in several malignancies, including lung cancer, melanoma, renal cancer, gastro intestinal cancer, urothelial cancers.

Nivolumab in mRCC

In 2010, the first phase I trial was published reporting the promising outcome in 39 patients affected by heavily treated solid tumours, including non-small cell lung cancer (NSCLC), melanoma, prostate cancer, colorectal cancer (CRC) and mRCC treated with anti-PD1 nivolumab[28].

Later, data from 296 patients with treatment-refractory NSCLC, melanoma and RCC were reported: ORR was between 18-28%; interestingly the registered response were durable and the OS was 9.9, 16.8 and 22.4 months for NSCLC, melanoma and mRCC, respectively[29].

A phase II trial was conducted testing nivolumab in patients with mRCC previously treated with VEGF pathway inhibitors. In a population study of 168 patients, ORR was 21% and 40% of patients were responding at 24 months of study treatment[30].

In 2015, the large phase III RCT Checkmate-025 enrolling 821 patients showed superiority of

nivolumab 3mg/Kg compared with the standard treatment everolimus (as scheduled 10mg/die), in mRCC patients previously treated with anti-VEGF therapy. These results lead to drug approval by FDA and then by EMA. In this study, nivolumab achieved 25% of objective response (versus 5% with everolimus), and 25 months of OS (95% CI, 21.8 to not estimable) versus 19.6 months (95% CI, 17.6 to 23.1) with everolimus[14]. Moreover, nivolumab was associated with health-related quality of life improvement compared with everolimus[31].
Currently, nivolumab is the standard of care in second-line setting after first line anti-VEGF treatment failure.

Predictive biomarker for immunotherapy in mRCC

Extended OS and durable responses have led to regulatory approval of nivolumab for treatment of mRCC. Despite the undoubted great benefits of immunotherapy, anti-PD1 agents are not effective in all patients. In the mentioned Checkmate-025 trial, 25% of patients achieved an objective response (complete or partial response) but about one third (35%) had a progression of disease as best response, and one third (34%) had a stable disease. Currently, one of the major challenges is to identify a predictive biomarker of response, useful to select the subset of patients who are likely to respond to CPIs, in order to optimize the choice of treatment and the sequential strategies.

PD-L1 expression

Immunohistochemical (IHC) staining of tumour cells PD-L1 is the most explored biomarker in clinical trials. Topalian et al. provided the first important data from the first-in-human study of nivolumab in several solid tumours. This trial demonstrated safety and activity of anti-PD1 treatment in NSCLC, melanoma and renal cancer[29]. Additionally, it has been reported an immune correlate of nivolumab in treated population. Biopsy samples from 25 of the 42 patients were positive for PD- L1 expression by IHC analysis. Among these 25 patients, 9 (36%) had an objective response, while none of the patients with PD-L1 negative tumours achieved an objective response. PD-L1 positivity was defined per specimen by a 5% expression threshold. These preliminary results suggested that PD-L1 expression in tumours might represent a candidate molecular marker.
So far, PD-L1 tumour expression by IHC is FDA approved biomarker only for NSCLC, driving the selection of first and second-line treatment strategies.

In mRCC, PD-L1 assessment demonstrated more prognostic than predictive value and, therefore, it is unable to become a useful marker of treatment benefit.

Available data derived from exploratory analysis of five phase III randomized controlled trials and one phase II RCT.

In Checkmate-025 study (phase III RCT comparing nivolumab versus everolimus in second-line setting), both PD-L1 positive (PD-L1+) and PD-L1 negative (PD-L1-) patients benefit from CPIs therapy. In detail, among patients with ≥ 1% PD-L1 expression (n = 181), the median OS was 21.8 months (95% CI, 16.5 to 28.1) in the nivolumab group and 18.8 months (95% CI, 11.9 to 19.9) in the everolimus group (HR 0.79 [95% CI, 0.53 to 1.17]). Among patients with < 1% PD-L1 expression (n = 575), the median OS was 27.4 months (95% CI, 21.4 to not estimable) in the nivolumab group and 21.2 months (95% CI, 17.7 to 26.2) in the everolimus group (HR 0.77 [95% CI, 0.60 to 0.97]). In this study, PD-L1 testing confirmed his prognostic but not predictive value, meaning that benefit of nivolumab was irrespective of PD-L1 status[14].

In Checkmate-214 study (phase III RCT comparing first-line combination of nivolumab plus ipilimumab versus sunitinib), OS was longer in nivolumab plus ipilimumab arm than sunitinib, regardless PD-L1 expression levels. The 18-month OS rate in patients with < 1% PD-L1 expression (n = 562) was 74% (95% CI, 69 to 79) in nivolumab plus ipilimumab arm (10% higher than sunitinib arm; HR 0.73 [95% CI, 0.56 to 0.96]). The 18-months OS rate in patients with ≥ 1% PD-L1 expression (n = 214) was 81% (95% CI, 71 to 87) in nivolumab plus ipilimumab group (28% higher than control group; HR 0.45 [95% CI, 0.29 to 0.71]). ORR among patients with < 1% PD-L1 expression was 37% in combination arm (9% more than sunitinib arm; p = 0.0252, Clopper–Pearson method); among patients with ≥ 1% PD-L1 expression ORR was 58% (36% more than control group; p < 0.001). In summary, even in Checkmate-214 study, benefit of immunotherapy over standard treatment was irrespective of PD-L1 status, but only it was more pronounced in PD-L1+ patients[18].

To note, in both Checkmate-025 and Checkmate-214 trials, PD-L1 expression on tumour cells was assessed with Dako IHC staining and cut-off was fixed at both 1% and 5% values (5% data not reported).

Afterward, in KEYNOTE-426 study (phase III RCT comparing pembrolizumab plus axitinib versus sunitinib as first-line therapy) the benefit of combination with respect to OS and PFS was observed in both PD-L1 expression categories fixed at 1% cut-off (assessed with IHC 22C3 pharmDx assay according to the combined score, including tumour cells, lymphocytes and macrophages). In patients with < 1% PD-L1 expression (n = 325), the 18-month OS rate was 82.3% (95% CI, 77.2 to 86.3) in pembrolizumab plus axitinib arm (10.2% higher than sunitinib arm; HR 0.59 [95% CI, 0.34

to 1.03]). Median PFS was 15 months (95% CI, 12.4 to not estimable) in combination arm versus 12.5 months (95% CI, 11 to not estimable) in sunitinib arm (HR 0.87 [95% CI, 0.62 to 1.23]). In patients with ≥ 1% PD-L1 expression (n = 497), the 12-months OS rate was 90.1% (95% CI, 85.5 to 93.3) in pembrozitinib plus axitinib treated group (11.7% higher than control group; HR 0.54 [95% CI, 0.35 to 0.84]). Median PFS was 15.3 months (95% CI, 12.6 to not estimable) in combination arm versus 8.9 months (95% CI, 7.6 to 11.3) in sunitinib arm (HR 0.62 [95% CI, 0.47 to 0.80])[20].

Moreover, in JAVELIN-101 study (phase III RCT comparing avelumab plus axitinib versus sunitinib as first-line therapy) PD-L1 positivity was assessed with Ventana PD-L1 (SP263) assay on immune cells infiltrating the tumour with a 1% cut-off. Among patients with PD-L1–positive tumours (n = 560) PFS was significantly longer when patients received the combination therapy versus sunitinib: 13.8 months (95% CI, 11.1 to not estimated) versus 7.2 months (95% CI, 5.7 to 9.7), respectively (HR 0.61 [95% CI, 0.47 to 0.79]; p < 0.001, Clopper–Pearson method). The ORR was 61.9% (95% CI, 55.8 to 67.7) with avelumab plus axitinib (32.2% higher than sunitinib group; odds ratio 3.98 [95% CI, 2.7 to 5.7])[21].

Furthermore, in IMmotion-150 RCT trial (phase II RCT exploring atezolizumab alone or combined to bevacizumab versus sunitinib as first-line therapy) VentanaSP142 on tumour cells with fixed cut-off 5% was used to evaluate PD-L1 expression. The ORR in the combination arm was 46% in the PD-L1+ population (n = 164), 20% more than comparison arms[32]. Median PFS was 11.7 months compared with 8.4 months for sunitinib (not statistically significant).

Subsequently, in IMmotion-151 study (phase III trial comparing atezolizumab plus bevacizumab versus sunitinib in first-line setting) for the PD-L1+ population (n = 362) median PFS was 11.2 months in the atezolizumab plus bevacizumab group versus 7.7 months in the sunitinib group (HR 0.74 [95% CI, 0.57 to 0.96]; p = 0.0217, chi-squared test); 36% of patients achieved an ORR with combination (3% more than sunitinib)[33]. To note, in IMmotion-151 trial subgroups analysis by PD-L1 expression categories was preplanned.

Finally, during last European Society of Medical Oncology (ESMO) Congress, Carretero-Gonzales et al. presented a metanalysis from five RCTs, in order to assess PD-L1 expression role as biomarker in mRCC. Totally, 3 720 patients were included (CPI arm: 1 913 patients; control arm: 1 807 patients). Higher expression of PD-L1 seems select a subset of patients who could benefit more in terms of PFS (p = 0.003, Mantel-Haenszel method) but it did not seem to impact in OS results (p = 0.29)[34].

To now, PD-L1 expression should not be used as biomarker to predict CPIs outcome in patients with mRCC, according to heterogeneous clinical outcomes and not comparable results.

Moreover, using PD-L1 immunohistochemistry as biomarker is controversial due to a lot of technical variables, listed below from Topalian et al. review[35]:

- Focal PD-L1 expression in some tumours may be missed in small biopsy specimens, such as needle biopsies;
- PD-L1 expression among multiple tumour lesions from individual patients can vary over time and by anatomical site;
- PD-L1 expression in tumour biopsies collected months or years earlier might not accurately reflect PDL1 status at the time of treatment initiation;
- PD-L1 epitopes detected by some antibodies are potentially unstable with prolonged specimen fixation or inadequate tissue handling before fixation;
- Antibodies used for PD-L1 detection have different affinities and specificities;
- PD-L1 protein expression can be membranous and/or cytoplasmic; however, only membranous PD-L1 is functionally relevant, by contacting PD1+ T cells;
- PD-L1 can be expressed by multiple cell types within the tumour microenvironment, which poses challenges for scoring and interpretation.

Additionally, PD-L1 expression may present several clinical interpretations, compared to other associated markers such as tumour infiltrating cells patterns or gene expression profiles.

Table 2: Comparison according to PD-L1 expression among phase III RCT in metastatic-renal cell carcinoma

Study	N°	Median PFS (months)	Median PFS Δ	HR (95%CI)	Median OS (months, 1ysOS)	Median OS Δ	HR (95%CI)	ORR %	ORR Δ	p
PDL1+										
CheckMate025*	181	-	-	-	21.8 vs 18.8	3	0.79 (0.53-1-17)	-	-	-
CheckMate214	214	23 vs 6	17	0.46 (0.31-0.67)	NR vs 19.6 86% vs 66%	28 20%	0.45 (0.29-0.71)	58 vs 22 16 CR	36	<0.0001#
Keynote-426	497	15.3 vs 8.9	6.4	0.62 (0.47-0.80)	90% vs 78%	12%	0.54 (0.35-0.84)	-	-	-
Javelin-101	560	13.8 vs 7.2	6.6	0.61 (0.47-0.79)	-	-	0.82 (0.53-1.28)	62 vs 30 41 CR	32	-
IMmotion-151§	362	11.2 vs 7.7	3.5	0.74 (0.57-0.96)	34 vs 32	2	0.84 (0.62-1.15)	43 vs 35 9 CR	8	-
PDL1-										
CheckMate025*	575	-	-	-	27.4 vs 21.2	6.3	0.72 (0.60-0.97)	-	-	-
CheckMate214	562	11 vs 10.4	0.6	1 (0.80-1.26)	80% vs 75%	15%	0.73 (0.56-0.96)	37 vs 29 9 CR	9	0.0025#
Keynote-426	325	15 vs 12.5	2.5	0.87 (0.62-1.23)	92% vs 78%	14%	0.59 (0.34-1.03)	-	-	-
Javelin-101										
IMmotion-151§	553	11.2 vs 9.5	1.7	0.89 (0.67-1.04)	-	-	-	33 vs 32 3 CR	-	-

*Second line trial
§Preplanned subgroups analysis by PD-L1 status
Clopper–Pearson method
Abbreviation: NR: Not Reached; PFS: progression-free survival; HR: hazard ratio; OS: overall survival; ORR: objective response rate; Δ: difference; p: p-value; CR: complete response

Tumour Mutational Burden

Tumour Mutational Burden (TMB) is defined as a quantitative measure of somatic non-synonymous mutations per coding area of a tumour genome[36]. A value of 10 somatic mutations per mega-basepairs (Mb) of coding DNA corresponds to ~150 non-synonymous mutations within expressed genes[24].

TMB can be assessed using a number of next-generation sequencing (NGS) platforms, including whole-exome sequencing, whole-genome sequencing or targeted panel sequencing. Preclinical studies have identified neoantigens produced by somatic mutations in genes of tumour cells as primary drivers of anti-tumour adaptive immune response. Subsequently, increasing evidences supported that tumours with high mutational burden could be more responsive to immunotherapy[24,37]. This observation was confirmed by clinical data, indeed, the highest overall response rates to immune checkpoint blockade were observed in melanoma and NSCLC. Notably, both these cancers are related to mutagens exposure (ultraviolet light in the case of melanoma and tobacco smoke in the case of NSCLC), resulting in the highest somatic mutation burdens of any cancer type. Namely, a mutational load of more than 100 non-synonymous somatic mutations is associated with clinical benefit in melanoma treated with ipilimumab and in NSCLC treated with pembrolizumab[38,39].

In addition, in 2017 FDA approved pembrolizumab for patients with advanced, microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) solid tumours who have no satisfactory alternative treatment options. Indeed, dMMR cancers are characterized by genomic instability, high mutational burden and potential high number of neoantigens.

RCC has a relatively low non-synonymous mutations single nucleotide variant burden (around ten times lower than melanoma)[40], varying according to different histological subtypes (chromophobe histotype less then clear cell)[24].

The subgroup analysis of Checkmate-025 study (phase III RCT comparing nivolumab versus everolimus in second-line setting) showed enhanced benefit for poor-risk patients treated with nivolumab, with double survival benefit compared with everolimus[41]. In addition, data from Checkmate-214 trial (phase III RCT comparing nivolumab plus ipilimumab versus sunitinib in first-line setting) confirmed the major benefit from immunotherapy combination for patients classified as intermediate/poor risk by IMDC prognostic score. Both these findings raised the question about a potential association between TMB and IMDC score's prognostic factors, probably due to a more inflamed and active microenvironment.

Data supporting TMB as potential predictive biomarker to immunotherapy response in mRCC are limited. Exploratory analyses from two RCTs (IMmotion-150 and JAVELIN-101) and two academic experiences provide the main available evidences in this field.

IMmotion-150 phase II RCT (atezolizumab alone or in combination with bevacizumab versus sunitinib in first-line setting) analysis revealed that tumour mutation load and tumour neoantigen burden were not associated with clinical benefit in all the three arms of treatment[42]. Similarly, in JAVELIN-101 trial TMB did not distinguish patient with respect to PFS[43].

In a cohort of 54 mRCC patients included in The Cancer Genome Atlas (TCGA) database, correlation between tumour mutational load and prognostic factors was explored. Whole exome sequencing (WES) provided a median non-synonymous mutational load of 1.42 mutations/Mb (range, 0.035–2.77). Moreover, IMDC score prognostic groups did not significantly differ in non-synonymous mutational load, suggesting a more complex interaction between tumour, microenvironment and immune system[44].

In addition, de Velasco et al. presented the results from WES of 9 patients with mRCC receiving nivolumab. They reported that mRCC had relatively few non-synonymous mutations and neoantigens. Surprisingly, they found that neoantigens' load was significantly higher in non-responder patients (n = 6) compared to responder patients (n = 3) (p = 0.048, bootstrap-t), but non-synonymous mutation load was not. These data are limited, primarily by small number of patients; however it is probable that TMB and non-synonymous mutations play a different role in mRCC than in other neoplasms[45].

A phase II trial, NIVES (NCT03469713), currently recruiting, is evaluating nivolumab plus radiotherapy and will assess the TMB in mRCC and its impact on the response to therapy.

Although no final conclusion could be resumed from these studies, mainly due to contradictory data and small number of patients explored. TMB and non-synonymous mutations roles as predictive biomarkers in mRCC are still not well explored and should be better investigated in further clinical trials.

However, variable clinical outcome data suggest that the presence of multiple factors may influence CPIs action; moreover, the heterogeneity in TMB definition and assessment methods still represents an additional issue.

Gene expression profile (GEP)

WES and RNA sequencing (RNA-seq) are helpful tools for evaluating the genomic landscape of a tumour. WES captures the exonic gene regions, identifying mutations in coding regions and it is useful in measuring of mutational load. RNA-seq can provide the entire transcriptome of a sample and it is powerful for establishing gene signatures for specific cohorts within a patient group.
Both WES and RNA-seq have been widely explored to discover mutational load and gene expression profiling utilities as predictive biomarkers to both chemotherapy and immunotherapy, mainly in melanoma and NSCLC. Overall, a tumour gene expression signature that reflects a series of gamma interferon–inducible genes may define a "hot," inflamed tumour, and it is associated in several studies with a good outcome with checkpoint inhibition across several cancer types[46].

In mRCC setting the major evidences were provided by three RCTs and few academic experiences.
Rini and colleagues, in IMmotion-150 study (phase II RCT evaluating first-line atezolizumab alone or combined to bevacizumab versus sunitinib), identified 3 subgroups of patients based on gene signature. Angiogenesis subgroup (high angiogenesis gene signature - VEGFA, KDR, ESM1, PECAM1, CD34, ANGPTL4) had better outcome in terms of ORR and PFS than patients with low angiogenesis gene expression in sunitinib arm. High T-effector subgroup (expressing T-effector presence and function, IFN-γ response, checkpoint inhibitors, and antigen presentation genes - CD8, IFNG, PRF1, EOMES, CD274) achieved longer PFS and higher ORR from atezolizumab plus bevacizumab compared to sunitinib. Myeloid inflammation gene signature, associated to immune suppression, characterizes the third subgroup; patients with myeloid signature had shorter PFS with atezolizumab monotherapy versus sunitinib, data not observed between atezolizumab plus bevacizumab versus sunitinib[42].
These results have been validated in IMmotion-151 study (phase III RCT comparing bevacizumab plus atezolizumab versus sunitinib as first-line therapy), showing that differential gene signatures are associated with different outcome in atezolizumab plus bevacizumab arm versus sunitinib arm. Indeed, the biomarkers exploring analysis revealed that high T-effector GEP patients had longer PFS when received combination compared to sunitinib (12.5 versus 8.3 months; HR 0.76 [95% CI, 0.59 to 0.99]); this profile was also associated with IHC PD-L1 expression. Patients with high angiogenesis profile had longer PFS in sunitinib arm (10.1 versus 5.9 months; HR 0.59 [95% CI, 0.47 to 0.75]) compared to low angiogenesis GEP patients, but not differences emerged when compared with atezolizumab plus bevacizumab arm (HR 0.95 [95% CI, 0.75 to 1.19]). In low angiogenesis profile, combination therapy improved PFS versus sunitinib (HR 0.68 [95% CI, 0.52

to 0.89]). Moreover, angiogenesis GEP was found to be higher in favourable versus intermediate/poor MSKCC risk groups and was lower in sarcomatoid RCC compared to non-sarcomatoid tumours[33].

Recently, biomarkers analysis from JAVELIN-101 phase III trial (comparing avelumab plus axitinib versus sunitinib in first-line setting) was also reported. High angiogenesis profile was associated with significantly improved PFS in the sunitinib arm but not in the combination arm. In the low angiogenesis GEP, avelumab plus axitinib improved PFS versus sunitinib. Patients with high T-effector and T cell inflamed had longer PFS when treated with combination over sunitinib. Moreover, PFS increased in patients with immune GES–positive tumours versus those in the negative group when treated with avelumab plus axitinib (HR 0.63; 95% CI, 0.46 to 0.86; p = 0.004, log-rank test)[43].

In a cohort of 54 mRCC patients included in TGCA database, RNA-seq revealed no differences in expression of cytolytic genes granzyme A (GZMA) and perforin (PRF1) or in selected immune checkpoint molecules (PD-1, PD-L1, PD-L2, and CTLA-4) across MSKCC risk groups ($p > 0.05$)[44].

The same team, headed by de Velasco, performed a RNA-seq in a cohort of 9 RCC patients treated with nivolumab in TCGA database. They found a significantly higher expression of PD-1, PD-L1, GZMA, LAG3, IDO1, ICOS, and BTLA in responder versus non responder patients ($p < 0.05$, bootstrapping analysis)[45].

Despite both WSE and RNA-seq gene signatures were not validated as predictive biomarkers, the mentioned data support the use of these technologies to explore immunomodulatory action of CPIs and to clarify our knowledge of immune-oncology.

Intrinsic molecular subtype

Currently, there are few evidences on correlations between clinical response to immune checkpoint blockade and specific oncogene, tumour suppressor gene or overall oncogenic pathway-associated mutations.

The commonest event involved in clear-cell RCC (ccRCC) is the loss of chromosome 3p, which is associated with the development of VHL, PBRM1, BAP1 and SETD2 alterations in about 90% of cases; in addition with KDM5C, PTEN, MTOR and TP5 they are the eight most common altered

genes in ccRCC[47]. These groups of genes and others were explored in order to identify prognostic and predictive biomarkers in several therapeutic setting.

Chen et al. analysed 894 RCC cases including gene expression analysis, founding 9 genomic subtypes according to specific molecular pathways. Namely, they explored genes involved in immune checkpoint pathways, including PD1 and PDL1 genes, showing that ccRCC subtypes had relatively high expression of several genes representing targets for immunotherapy, including PDCD1 (PD1), CD247 (CD3), PDCD1LG2 (PDL2), CTLA4 (CD152), TNFRSF9 (CD137), and TNFRSF4 (CD134); moreover, these subtypes express distinct patterns of checkpoint inhibitors-related genes, representing different aggressive phenotypes. It has been described that PTEN plays a role in intrinsic cellular control of PD-L1 expression[48]. Aggressive subtypes showed increased promoter methylation of miR-21 (MIR21) with relating decrease in miR-21 levels on PTEN control[49].

More recently, TCGA research network described different immune gene signatures corresponding to different histological subtypes. Notably, it has been defined a distinct papillary renal cell carcinoma subtype characterized by a CpG island methylator phenotype (CIMP-RCC) and associated with early-onset disease, poor survival, and germline or somatic mutation of the fumarate hydratase (FH) gene. Moreover, TCGA group also found that CIMP-RCC subtype has an increased immune signature expression for selected immune gene signatures, including the T helper (Th) 2 gene signature like that seen in ccRCC. This suggests that the most aggressive type of RCC, CIMP-RCC, may benefit from checkpoint inhibitor therapy in a similar manner to ccRCC[50].

In the previously mentioned JAVELIN-101 study (phase III RCT comparing avelumab plus axitinib versus sunitinib as first-line therapy), significant treatment arm–specific differences in PFS relative to wild type were observed when mutations in genes such as CD1631L, PTEN, or DNMT1 were present[43].

Miao et al. performed WES of 35 patients with ccRCC, finding that loss-of-function mutations in the PBRM1 gene, involved in chromatin remodelling, were associated to major clinical benefit ($p = 0.012$, Fisher's exact test). These findings were also validated in a second cohort of patients treated with immunotherapy[51].

Currently, the only one ongoing trial, the BIONIKK randomized phase II trial, stratified patients according to the four transcriptomic signatures of clear cell RCC according to Beuselinck et al.[52].

Tumour infiltrates lymphocytes

The presence of T cell inflamed tumour microenvironment (TME) has been associated with clinical benefit from immunotherapy. TME is a highly heterogeneous and dynamic milieu consisting of several components, including immune cells (tumour-associated macrophages, myeloid-derived suppressor and lymphocytes), fibroblasts, vessels, and a wide spectrum of signalling molecules. All these elements tightly interact each other and with tumour cells and continuously crosstalk. Tumour promotes its growth, survival and immune escape by releasing factors, while TME components contribute to all phases of tumour genesis by controlling or promoting pathways.

IHC analysis of TME in a variety of cancer revealed that the majority of tumours present T cell infiltration. Tumour infiltration lymphocytes (TILs) include a wide spectrum of white cells implicated in cancer immunoediting and in anti-tumour immune response. Interestingly, the presence of a T cell inflamed tumour microenvironment has been associated with clinical benefit from immunotherapies[53]. In particular, CD8+ cytotoxic lymphocyte infiltration in tumour biopsy samples has been associated with improved survival in retrospective studies in melanoma and NSCLC[54,55]. In a phase II study of ipilimumab in patients with metastatic melanoma, baseline tumour-infiltrating lymphocyte status was not associated with clinical activity. However, increases in tumour-infiltrating lymphocyte density in tumour biopsy samples collected after the second dose of ipilimumab were associated with significantly greater clinical activity with ipilimumab than samples without increases in lymphocyte density[56]. On the other hand, partially exhausted cytotoxic T lymphocytes infiltrates strongly correlate with response to anti-PD1 therapy in melanoma[57].

Conflicting evidences about prognostic role of TILs in RCC and few data about their predictive role in immunotherapy era are available. The most consistently data derived from two RCTs and few academic experiences.

Choueiri et al. reported data of pharmacodinamic immunomodulatory activity of nivolumab, analysing tumour-associated lymphocytes from baseline metastatic biopsies of 36 patients. IHC analysis showed enrichment in CD3+, CD4+ and CD8+ cells from baseline to further cycles, measured as median percent change of 69%, 180% and 117%, respectively. The authors concluded supporting the hypothesis that nivolumab increased cells trafficking and T cells infiltration, consistently with median increase of serum levels of chemokines (101% for CXCL9 and 37% for CXCL10) and IFN-γ genes expression upregulation[58].

Exploratory analysis from IMmotion-150 study (phase II RCT comparing atezolizumab plus bevacizumab versus sunitinib in first-line setting) described three different gene signatures, angiogenesis profile, T-effector profile and myeloid inflammatory profile and their association with

outcome. Moreover, the authors also reported association between T effector GEP and both PD-L1 and CD8+ T cell infiltration[42].

Biomarkers analysis of JAVELIN-101 (phase III RCT comparing avelumab plus axitinib versus sunitinib as first-line therapy) explored CD8+ TILs expression at lesion invasive margin examining samples from 795 patients. The researchers found that in avelumab plus axitinib arm median PFS was significantly longer when CD8+ T cell expression was above than below the median (non-estimable versus 9.8 months; HR 0.59)[43]. On the contrary, among sunitinib-treated patients, when CD8 expression was above the median, shorter median PFS was described (7.1 months versus 11.1 months for those with expression below the median; HR 1.42, not significant)[43].

Recently, Steindl et al. presented at ESMO Congress data from 10 patients affected by mRCC and brain metastases. No significant correlation of CD3+ and/or CD8+ TIL density and TMB was observed in the cohort. However, the authors concluded that TMB and TILs density were numerically higher in brain metastases patients compared to the matched extra-cranial samples in the study cohort.

Microbioma

Microbioma is defined as the collection of genomes from all the microorganisms found in a particular environment. In the last years, correlations with microbioma, metabolism and immune system in cancer development have been explored. The potential relation between microbioma and response immunotherapy is particular interesting.

Recently, Routy et al. demonstrated that abnormal gut microbiome composition could have an influence on primary resistance to PD-1blockade in mice xenografts and patients with cancer[59]. Moreover, the association between antibiotic use within 30 days before therapy and CPIs resistance was described in mRCC and NSCLC cohorts. In patients with mRCC, recent antibiotic therapy was associated with increased rate of PD (75% versus 22%, $p < 0.01$, Fisher's exact test). In addition, PFS and OS were shorter in those patients received antibiotic therapy than in those did not: median PFS 1.9 months versus 7.4 months (HR 3.1 [95% CI, 1.4 to 6.9]; $p < 0.01$) and median OS 17.3 months versus 30.6 months (HR 3.5 [95% CI, 1.1 to 10.8]; $p = 0.03$). In multivariate analysis antibiotic therapy and tumour burden remained independently associated with worse PFS (HR 2.0; $p < 0.01$). No statistically significant association was found for OS (HR 2.1; $p = 0.11$)[60].

Peripheral blood

Traditionally, testing of peripheral blood biomarkers is a non-invasive source of potential biomarkers in oncology. In a wide spectrum of malignancies, blood biomarkers association with clinical benefit has been noted.

A wide range of serum factors and peripheral blood features have been associated with CPIs response in a variety of tumours: low absolute neutrophil count, low neutrophil to lymphocyte ratio, low absolute monocyte count, high frequency of FoxP3+ regulatory T cell, high frequency of lymphocytes, high eosinophil count, high platelet to lymphocytes ratio, high systemic inflammatory index, lactate dehydrogenase, circulating Treg cell levels, cytokine levels, PDL1+ tumour cells and others[61,62].

In mRCC prognostic role of baseline value and change in neutrophils/lymphocytes ratio (NLR) during treatment were described; moreover, prognostic and predictive value of NLR at 6 weeks of therapy also emerged.

Lalani et al. reported data from a 142 mRCC patients' cohort, investigating NLR role during CPIs administration. Early change (decrease ≥ 25%) in NLR after immunotherapy initiation was associated with an improved PFS (HR 0.55 [95% CI, 0.26 to 1.18]; $p < 0.001$, log-rank test) and significant better OS (HR 0.33 [95% CI, 0.12 to 0.88]; $p = 0.004$). Additionally, higher NLR at 6 weeks was a significantly stronger predictor of all three outcomes than baseline NLR. Higher 6 week NLR was independently associated with a lower ORR (odds ratio per 1 unit increase in natural log-transformed NLR [lnNLR] = 0.22 [95% CI, 0.10 to 0.52]; $p = 0.001$), shorter PFS (HR per 1 unit increase in lnNLR = 3.61 [95% CI, 2.21 to 5.88]; $p < 0.001$), and shorter OS (HR per 1 unit increase in lnNLR = 2.51 [95% CI, 1.71 to 3.69]; $p<0.001$)[63].

De Giorgi et al. reported an exploratory analysis on blood biomarkers from mRCC patients (n = 303) enrolled in Italian Nivolumab Expanded Access Program. Higher Systemic Inflammatory Index (SII), defined as platelet x NLR and platelet/lymphocyte ratio (PLR) were independently associated with worse outcomes in terms of reduced ORR and disease control rate (DCR). The ORR for patients with SII < 1 375 was 29 (versus 13% with SII ≤ 1 375; $p = 0.004$, chi-squared test). The DCR for patients with SII < 1 375 was 68% (versus 41% with SII ≤ 1 375; $p < 0.0001$). Moreover, in multivariate analyses, SII ≤ 1 375 independently predicted OS (HR 2.96; 95% CI, 2.05 to 4.27). The ORR for patients with PLR < 232 was 29% (versus 16% with PLR ≤ 232; $p = 0.02$). The DCR for patients with PLR < 232 was 68% (versus 35% with PLR ≤ 232; $p < 0.0001$).

Recently, Desnoyer et al reported prospective data from fresh blood immune cell monitoring in patients treated with nivolumab in the NIVOREN study. Overall, 44 patients were included in this fresh whole blood immune monitoring. Higher occurrence of grade 3-4 treatment-related adverse

events at 6 months was observed in patients with lower number of CD4+PD1-4.1BB+ T cell (non-exhausted activated CD4 T cells) counts at baseline and with lower number of CD56+ T cells (natural killer T cells) at cycle 3. Higher occurrence of disease progression at 6-months was observed in patients with lower number of CD4+PD1+CD69+ T cells (exhausted activated CD4 T cells) and with higher number of CD4+CD244+ T cells (exhausted CD4 T cells) at baseline. No immune cell populations were associated with the occurrence of a 6-months progression during the course of nivolumab cycle 3.

Although promising in terms of feasibility, clinical implementation of these blood-based biomarkers will require large-scale prospective validation.

Clinical features

So far, in mRCC clinical patients' features seem to represent the most important predictive markers of response to checkpoint inhibition. Major evidences are reported with respect of both IMDC and MSKCC categories, presence of sarcomatoid component, body mass index (BMI) and immune-related adverse events (irAE).

Prognostic scores

Firstly, Checkmate-214 phase III trial (comparing nivolumab plus ipilimumab versus sunitinib in first-line setting) demonstrated significantly longer OS and higher ORR with nivolumab plus ipilimumab than with sunitinib among intermediate and poor-risk patients. In this trial prognostic stratification according to IMDC score strongly predicted response to immunotherapy (prespecified analysis). Indeed, in intermediate/poor-risk patients 12-months and 18-months OS rates were 80% and 75% versus 72% and 60% (HR 0.63; $p < 0.001$, log-rank test), respectively. The ORR was 42% versus 27% ($p < 0.001$), with a complete response in 9% vs 1% of patients. Among favourable-risk patients alone, 12-months OS rate was 94% versus 96% and 18-months OS rate was 88% versus 93% ($p = 0.27$)[18]. These findings supported FDA and EMA approval of immunotherapy combination in intermediate-poor risk patients with untreated mRCC.
More recently, updated data were reported at 2019 American Society of Clinical Oncology – Genitourinary Congress (ASCO-GU) showing that 30-months OS rate was 64% in the nivolumab plus ipilimumab arm and 56% in the sunitinib arm. Improved OS, PFS and ORR were maintained in combination arm versus sunitinib in both intention-to-treat population and intermediate/poor-risk

population. Based on this data, nivolumab plus ipilimumab indication could be extended regardless risk classes[64].

In exploratory analysis from IMmotion-151 (phase III RCT comparing bevacizumab plus atezolizumab versus sunitinib as first-line therapy) which identified 3 different potentially predictive gene expression profile, angiogenesis GEP was higher in favourable versus intermediate/poor MSKCC risk groups, explaining the better outcome from sunitinib in this subgroup [65].

In the phase III trials above mentioned, benefit of immunotherapy (alone or in combination) over control arm was observed across all prognostic subgroups of patients (Table 3).

Table 3: Comparison according to prognostic scores among phase III RCT in metastatic-renal cell carcinoma

Study	N°	Median PFS (months)	Median PFS Δ	HR (95%CI)	Median OS (months, 1ysOS)	Median OS Δ	HR (95%CI)	ORR %	ORR Δ	p
Good-risk										
Checkmate025*	249	15 vs 25	10	2.18 (1.29-3.68)	NR vs 32.9	-	1.45 (0.51-1.42)	29 vs 52 11 CR	-23	<0.0001#
Checkmate214§										
Keynote-426										
Javelin-101										
IMmotion-151										
Intermediate/Poor-risk										
Checkmate025*	847	11.6 vs 8.4	3.2	0.82 (0.64-1.05)	NR vs 26	-	0.63 (0.44-0.89)	42 vs 27 9 CR	15	<0.0001#
Checkmate214§										
Keynote-426	592	12.6 vs 8.2	4.4	0.67 (0.53-0.85)	87% vs 71%	16%	0.52 (0.37-0.74)	56 vs 30 5 CR	26	-
Javelin-101										
IMmotion-151										

*Second line trial

Cochran–Mantel–Haenszel method

Abbreviation: NR: Not Reached; PFS: progression-free survival; HR: hazard ratio; OS: overall survival; ORR: objective response rate; Δ: difference; p: p-value; CR: complete response

Sarcomatoid features

Sarcomatoid differentiation (sarcoRCC) occurs in approximately 1- 8% of all RCC, with overall poor prognosis. It has been described that mRCC with sarcomatoid differentiation shows higher PD-L1 expression and higher density of PD1- and CD8+ cells compared with RCC without sarcomatoid component, potentially explaining the better outcome reported in immunotherapy clinical trials for this subset of patients (Table 3)[66].

At American Society of Clinical Oncology (ASCO) Annual Meeting 2018, a small retrospective study was presented as an abstract by Ross et al. investigating response to CPIs in mRCC patients with sarcomatoid differentiation. This study showed promising outcomes, with durable complete response in up to 15% of patients, and ORR of 62%[67].

The genomic biomarker analyses from the mentioned IMmotion-151 study (phase III RCT exploring bevacizumab plus atezolizumab versus sunitinib as first line therapy) also focus on sarcomatoid histology. Interestingly, the PD-L1 prevalence was higher in sarcoRCC (63%), compared to non-sarcoRCC (39%), whereas angiogenesis gene signature was lower in sarcoRCC (34% versus 65%)[65]. Indeed, sarcoRCC patients (n = 86) in the PD-L1+ study group showed the greatest therapeutic benefit with atezolizumab plus bevacizumab (PFS HR 0.56 [95% CI, 0.38 to 0.83]) compared to sunitinib. Moreover, T effector gene expression was increased in sarcoRCC versus non-sarcoRCC tumours: 54% versus 40%[65].

In addition, a retrospective subgroup analysis from the Checkmate-214 phase III study (comparing nivolumab plus ipilimumab versus sunitinib in first-line setting) evaluating 112 sarcoRCC intermediate or poor-risk patients, confirmed a higher rate of PD-L1 expression (≥1%) in sarcoRCC than in non-sarcoRCC (47–53% versus 26–29%). More importantly, immunotherapy with nivolumab plus ipilimumab achieved a remarkable high ORR of 57%, with a CR rate of 18% and a median OS of 31 months compared to sunitinib (19%, 0% and 14 months for ORR, CR, median OS, respectively)[18].

More recently, a post-hot analysis from JAVELIN-101 (phase III RCT comparing avelumab plus axitinib versus sunitinib as first-line therapy) and KEYNOTE-426 studies (phase III RCT comparing pembrolizumab plus axitinib versus sunitinib as first-line therapy) reported similar findings. In JAVELIN-101 trial 108 sarcoRCC patients were described. Avelumab plus axitinib achieved a clinically significant PFS improvement: 7 versus 4 months (HR 0.57; 95% CI, 0.33 to 1.00) and statistically significant higher ORR (47% versus 21%)[68].

In KEYNOTE-426 trial 105 sarcoRCC patients were described. Pembrolizumab plus axitinib improved 12-months OS 83.4% versus 79.5% (HR 0.58; 95% CI, 0.21 to 1.59), median PFS not

reached (NR) versus 8.4 months (HR 0.54; 95% CI, 0.29 to 1.00), and ORR 58.8% versus 31.5% in patients with sarcomatoid features[69].

Based on this data (summarized in Table 4), it is high recommended to propose immunotherapy in mRCC patients with sarcomatoid differentiation.

Table 4: Comparison according to sarcomatoid differentiation among phase III RCT in metastatic-renal cell carcinoma

Study	**N°**	**Median PFS (months)**	**Median PFS Δ**	**HR (95%CI)**	**Median OS (months, 1ysOS)**	**Median OS Δ**	**HR (95%CI)**	**ORR %**	**ORR Δ**	**p**
CheckMate214	251	8.4 vs 4.9	3.5	0.61 (0.38-0.97)	31.2-13.6	17.2	0.55 (0.33-0-90)	57 vs 19 18 CR	38	<0.001#
Javelin-101	108	7 vs 4	3	0.57 (0.3-1)	83% vs 67%	16%		47 vs 21 4 CR	26	
Keynote-426	105	NR – 8.4		0.54 (0.29-1)	83% vs 80%	3%	0.58 (0.21-1.59)	59 vs 32 12 CR	27	
IMmotion-151	142	8.3 vs 5.3		0.53 (0.38-0.83)	21.7 vs 15.4		0.64 (0.41-1.01)	49 vs 14 10 CR	35	

*Second line trial

Clopper–Pearson method

Abbreviation: NR: Not Reached; PFS: progression-free survival; HR: hazard ratio; OS: overall survival; ORR: objective response rate; Δ: difference; p: p-value; CR: complete response

Immuno-related Adverse Events

Immunotherapy causes a particular spectrum of toxicities, derived from an increased activity of the immune system. IrAEs present a high variability in organs involved (skin, colon, endocrine organs, liver and lungs), severity and occurrences[70]. Possible association between irAEs occurrence and outcome from immunotherapy has been explored, without conclusive results.

In mRCC field, nivolumab Italian Early Access Program provide the main data. Verzoni et al reported a significantly longer OS (median not reached versus 16.8 months, p = 0.002, log-rank test) for those patients who experienced an irAE[71].
Moreover, a secondary analysis from CheckMate 214 trial revealed that the 24-month OS in patients who discontinued nivolumab plus ipilimumab for AE was 74% (versus 71% in all patients treated with nivolumab plus ipilimumab without experiencing of AE) and 42% of them were alive and free from second line therapy at 24 months[72].
However, as for TKIs, adverse events may represent a useful predictive factor when treatment is yet ongoing.

Body Mass Index

Recent data suggested a potential correlation between overweight and the efficacy of immune checkpoint inhibitors in cancer patients. The underlying mechanisms are far to be cleared, although several hypotheses have been postulated regarding the potential relation between adipose tissue and immune system.

Cortellini et al reported a large retrospective analysis exploring BMI and immunotherapy outcome in 976 patients with NSCLC (65%), melanoma (19%) and renal cancer (14%). Authors found that ORR was significantly higher in overweight/obese patients compared to non-overweight ($p < 0.0001$, chi-squared test); median time to treatment failure (TTF), PFS and OS were significantly longer for overweight/obese patients in univariate ($p < 0.0001$, for all the survival intervals) and multivariate models ($p = 0.0009$, $p < 0.0001$ and $p < 0.0001$ respectively). BMI could be an easy and useful predictive tool in clinical practice as well as a potential stratification variable for prospective clinical trials with CPIs.

Despite the amount of available data, crossing numerous and wide explored fields in order to find useful and viable predictive factors, we are far from a reliable prediction of outcome in mRCC patients treated with immunotherapy. However, we gain step-by-step knowledge of tumour-induced immune response.

Flow Cytometry

The complexity of T cell subpopulations, as we understand them today, combined with the immunological and functional diversity of these subsets represent significant complications for the study of T cell biology.

However, flow cytometry (FCM) is a multiparameter analytic test for the characterization of single cells and has been widely used in preclinical as well as in clinical cancer immunotherapy studies.

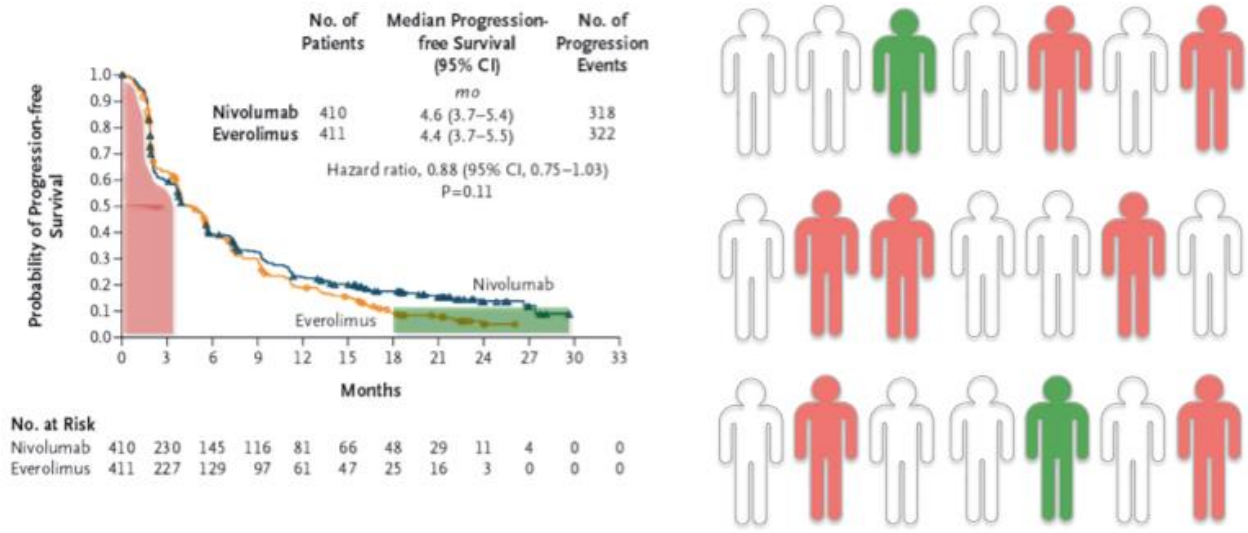
The FCM application in monitoring cancer immune systems includes intracellular staining for effector cytokines, cytotoxicity evaluation, measurement of proliferation and evaluation of cells that regulate the immune system. FCM allows quantification of several parameters in many individual cells that combines various phenotypic and functional markers, and it has the potential to provide information about immune responses in cancer patients.

PBMCs monitoring by FCM is highly applicable to the field of immuno-oncology, where the detection of immune checkpoints is critical to both identifying potential biomarkers and predicting patient response potential.

OBJECTIVE OF THE STUDY

In a number of patients nivolumab restores tumour-specific T-cell mediated response, and improves OS and ORR. However, a lack of clinical response occurs in most treated patients, whose number varies according not only to the stage of the disease, but also to other unknown, but probably immunological factors. This raises crucial questions about how to predict the success of therapy, and to increase the number of patients who benefit from this therapy, eventually improving their selection, or allowing an early change of treatment in case of early failure (Figure 3).

Figure 3: Objective of the study. *Figure modified from Motzer et al, 2015[14]. On the left side progression-free survival curve of nivolumab versus everolimus. In red area fast progression patients and in green area slow progression patients achieving a long term benefit from nivolumab.*



Unlike traditional therapies, drugs that block checkpoints do no target tumour cells directly, but act primarily on immune system cells, enhancing endogenous anti-tumour activity. Accordingly, the aim of the project is to ascertain whether main immunological parameters are predictor biomarkers of a successful therapy or not.

The main aim of this project is to identify immune biomarkers that are modulated in patients with metastatic renal cell carcinoma during treatment with immune checkpoint inhibitors, so to identify patients who most likely benefit from such therapy.

MATERIALS AND METHODS

Ethics Statements

Patients with advanced mRCC treated with nivolumab in routine clinical practice have been recruited at the "Struttura Complessa di Oncologia" – Azienda Ospedaliero-Universitaria Policlinico di Modena (Ethical Committee Prot. n. 3277/CE). All patients provided written informed consent for the collection of blood samples for research in accordance with the declaration of Helsinki.

Patients Data Analysis

This prospective, non-interventional study enrolled consecutive patients with mRCC who received nivolumab at Azienda Ospedaliero-Universitaria Policlinico di Modena from January 2016 until October 2019. The key eligibility criteria included: age ≥ 18 years; histological proved diagnosis of mRCC; radiological proved metastasis of mRCC; signed informed consent form. The patients lacking in the first three assessment time points were excluded.

For each patient demographic data and disease's characteristics were recorded, including pathology, metastatic sites, prognostic factors from IMDC classification. Data about first-line therapy, outcomes and subsequent treatments performed were also collected.

Clinical features at Nivolumab initiation were recorded, including inflammation indexes: NLR defined as neutrophils/lymphocyte; PLR defined as platelet/lymphocytes; SII defined as platelet per neutrophils/lymphocyte. The cut-off values are NLR ≥ 3, PLR ≥ 232 and SII ≥1375, selected from literature data[73]. Body mass index (BMI), defined as weight in Kilogram/height in meters squared, was also considered.

Blood samples at baseline and after 1, 2, 3, 5 and eventually further cycles of therapy were collected.

Nivolumab was administrated intravenously at 3 mg/Kg biweekly, or 240 mg biweekly or 480 mg monthly. Patients were treated until as long as clinical benefit is observed or until unacceptable toxicity.

Two groups of patients were identified and compared:

1) slow progression RCC (sRCC) are patients achieving progression-free survival (PFS) ≥ than 6 months or PFS < 6 month and overall survival (OS) ≥ 12 months from nivolumab start;

2) fast progression RCC (fRCC) are patients achieving PFS < 6 months and OS < 12 months from nivolumab starting.

Polychromatic flow cytometry

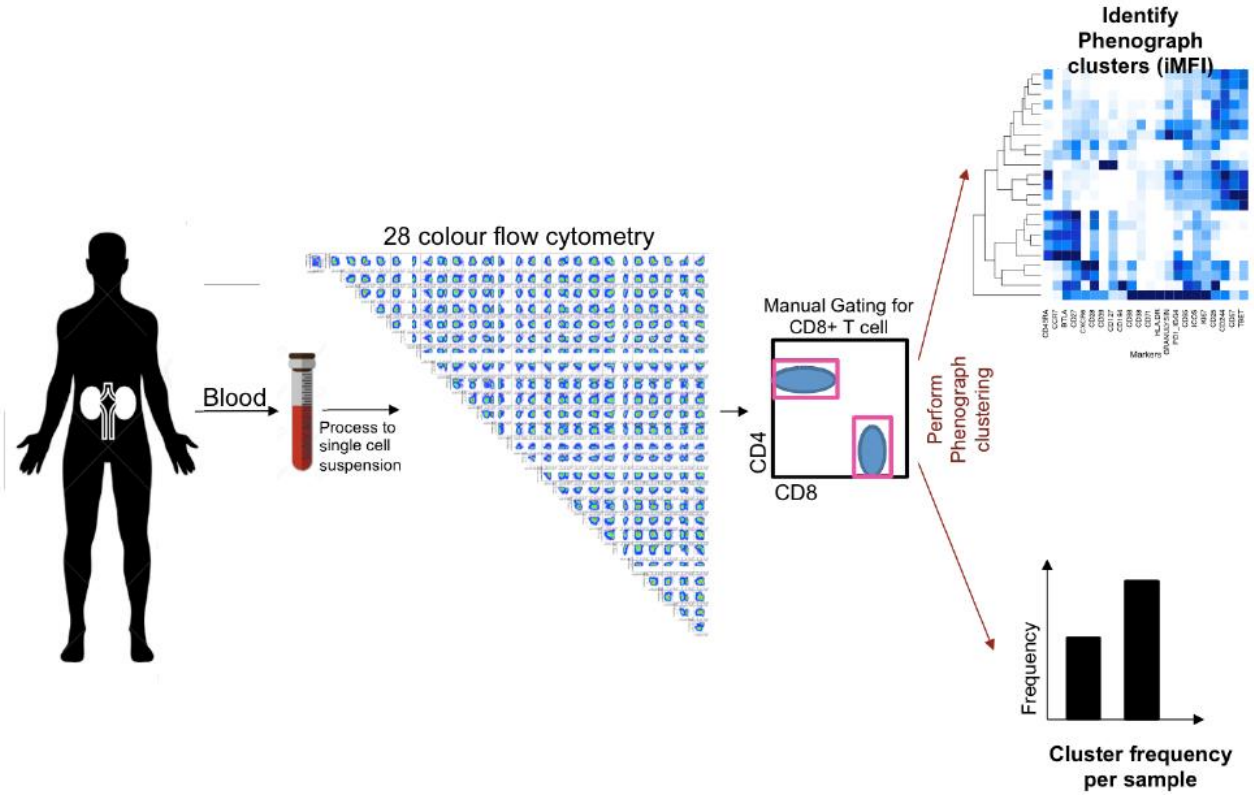
A maximum of 20 ml of venous blood was collected from each patient into ethylenediaminetetraacetic acid (EDTA) tubes. PBMCs were isolated by Ficoll-Hypaque density gradient according to standard procedures and immediately processed for immunophenotypic analysis. The main T cell subsets, i.e. CD4+ and CD8+, were immediately measured. Then, cells were used for functional assays or vitally stored in liquid nitrogen until use, according to well-standardized procedures. Thawed PBMCs were stained with Zombie Dye Viability CD3, CD4, CD8, CD127, CD25, CD38, CD27, CD28, CD45RA, CD197, CD279, CD186, CD194, CD244, CD39, CD95, CD98, CD71, CD278, CD279, CD272, TBET, GRANULYSIN, CD57, HLA-DR, KI67 and analysed by flow cytometry. Manual gating was performed to identify main T cell subpopulations[74]. Either for CD4 and CD8+ T cells, 10 000 events per sample were subsequently exported for further analysis in R by a custom-made script that makes use of Bioconductor libraries and R statistical packages.

High-dimensional flow cytometry data analysis

High-dimensional flow cytometry data analysis was performed as described[75,76]. Briefly, flow Cytometry Standard (FCS) 3.0 files were imported into FlowJo software version 9, and analysed by standard gating to remove aggregates and dead cells, and identify CD3+, CD4+ and CD3+, CD8+ T cells. Then, 5 000 CD4+ T and 5 000 CD8+ T cells per sample were subsequently imported in FlowJo version 10, biexponentially transformed, and exported for further analysis in R by a custom-made script[75,76]. Samples were finally connected in a single matrix. Data were analysed using the Phenograph algorithm (version 1.6.5[77]). Parameter K, indicating the number of nearest neighbours identified in the first iteration of the algorithm, was set at 60. Phenograph clusters were visualized using t-distributed stochastic neighbour embedding (t-SNE)[78]. Clusters representing < 0.5% were not considered in subsequent analysis. The data were reorganized and saved as new FCS files, one for each cluster, that were further analysed in FlowJo to determine the frequency of positive cells for each marker and the corresponding median fluorescence intensity (MFI). These values were multiplied to derive the integrated MFI (iMFI, rescaled to values from 0 to 100). The heat map,

showing the iMFI of each marker per cluster, and the subsequent metaclustering was performed using the gplots R package (Figure 4).

Figure 4: High-dimensional flow cytometry data analysis. *The figure shows the workflow of the analysis: venous blood was collected from each patient and processed for immunophenotypic analysis by flow cytometry; manual gating was performed to identify main T cell subpopulations; data were analysed using the Phenograph algorithm providing heat map (on the right, upper figure); frequency of single Phenograph clusters among different groups of patients (sRCC versus fRCC) was determined.*



Statistical analysis

Outcome in terms of PFS, OS and best tumour response were analysed. PFS was defined as the time from the start of treatment to first documentation of progression or death for any cause. OS was defined as the time from the start of treatment to death for any cause or last contact. Patients alive or not progressed were censored at the date of last follow-up. PFS and OS were estimated by the Kaplan-Meier method and the log-rank test. Best response to therapy was assessed according to Response Evaluation Criteria in Solid Tumours (RECIST) v 1.1 and immune-RECIST (iRECIST). The Chi-Square test and Fisher's exact test or Student's t-test were used to assess difference between the groups as appropriate. A p-value < 0.05 was considered statistically significant. Electronic Case Report Form (eCRF) was created for data collection. Statistical analysis was performed with STATA software (v 14).

For flow cytometry data, statistical analyses were performed using GraphPad Prism version 6. Significance of differences for the frequency of single Phenograph clusters among different groups of patients (sRCC and fRCC as above defined) was determined using two-way ANOVA with Bonferroni post-hoc test. To compare distributions of manually gated subsets significance was determined by paired Wilcoxon T test (non- parametric), unless otherwise specified in the figure legends.

RESULTS

Patients

From January 2016 to October 2019, a total of 25 patients treated at Azienda Ospedaliero-Universitaria Policlinico di Modena received nivolumab for mRCC were enrolled.

Main baseline patient's characteristics are reported in Table 5. The median age of this cohort was 60 years (range: 33 – 79) with 80% of males. The majority of patients had clear-cell histology (88%), with half of them (48%) presenting high Fuhrman grade. Nephrectomy was performed in 85% of patients. One-quarter of patients (25%) were metastatic at diagnosis and the most common involved sites were lung (60%), nodes (20%) and bone (20%).

Sunitinib was the most frequently drug used as front-line therapy (60%); pazopanib was administered in 32% of patients. Median PFS and median OS of first line therapy were 8.6 months (95% CI, 6.8 to 20.1) and 48 months (95% CI, 24.4 to not reached), respectively.

Table 5: Baseline Patients Characteristics

Characteristics	**N (25) - %**
Age (year, median, range)	60 (33 – 79)
Male	20 (80)
Smoking	
Current Smoker	3 (12)
Former Smoker	5 (29)
Never Smoker	12 (48)
Unknown	5 (20)
Nephrectomy	20 (80)
Histology subtypes	
Clear-cell	22 (88)
Papillary	2 (8)
Unclassified	1 (4)
Histology Fuhrman grading	
I – II	8 (32)
II – IV	12 (48)
Unknown	5 (20)
Metachronous metastases at diagnosis	6 (25)
First Metastatic Sites	
Lung	15 (60)
Bone	5 (20)
Lymphnodes	5 (20)
Liver	2 (8)
Brain	1 (4)
Others	1 (14)
First Line Systemic Treatment	
Sunitinib	15 (60)
Pazopanib	8 (32)
(Other – Clinical Trial)	2 (8)
First Line Treatment Outcomes	
Progression-Free Survival (median, 95%CI)	8.6 (6.8 – 20.1)
Overall Survival (median, 95%CI)	48 (24.4 – Not Reached)

Patients' characteristics at nivolumab initiation

The majority of patients (64%) received Nivolumab as second line therapy, 24% as third line and 12% as fourth line and beyond. Baseline characteristics are shown in table 6. At the time of nivolumab initiation, 60% of patients had BMI ≥ 25. The most common prognostic factor is haemoglobin < than LNL (56%) while 5 patients (20%) have KPS < 80%. According to IMDC prognostic score, 6 patients (24%) were classified as good-risk, 16 (64%) intermediate-risk and 3 (12%) as poor-risk. At time of nivolumab initiation the most common metastatic sites were lung (84%), nodes (52%) and bone (48%), followed by liver (28%), renal bed (24%) and pancreas (20%), while only 1 patient presented brain involvement. One half of patients had 2 or 3 involved sites; 40% had one or more metastatic sites.

Fifteen patients were evaluable for inflammatory indexes: 8 patients (53%) have high NLR ≥ 3; 4 patients (16%) have high PLR and all patients have high SII.

At time of analysis 21 patients discontinued nivolumab and 11 of them received further lines of treatment. The most common reason of discontinuation was progression disease (95%), only one patients stopped nivolumab for intercurrent event, none of the patients discontinued due to adverse events. Seven patients (28%) underwent local treatment during nivolumab therapy.

Table 6: Nivolumab Baseline Patients Characteristics

Characteristics	**N (25) - %**
Line of Treatment	
2	16 (64)
3	6 (24)
4 and beyond	3 (12)
Body Mass Index	
>= 25	15 (60)
Prognostic Factors	
Time to First Line Treatment < 1 year	7 (28)
Karnofsky Performance Status < 80%	5 (20)
Haemoglobin < LNL	14 (56)
Platelet > UNL	3 (12.5)
Corrected Calcium > UNL	2 (8)
Neutrophils > UNL	1 (4)
IMDC score	
Good (0 factor)	6 (24)
Intermediate (1-2 factors)	16 (64)
Poor (≥ 3 factors)	3 (12)
Metastatic Sites	
Lung	21 (84)
Lymph nodes	13 (52)
Bone	12 (48)
Liver	7 (28)
Renal Bed	6 (24)
Pancreas	5 (20)
Kidney	4 (16)
Brain	1 (4)
Others	9 (36)
Number of Metastatic Sites	
1	2 (8)
2 – 3	13 (52)
≥ 4	10 (40)
Inflammation Indexes	
Neutrophils/Lymphocyte Ratio ≥ 3	8/15 (53)
Platelet/Lymphocyte Ratio ≥ 232	4/15 (16)
Systemic Immune Inflammation Index ≥1375	15 (100)
Subsequent treatment after Nivolumab	11 (44)
Local treatment during Nivolumab	7 (28)
Abbreviations: LNL: Low normal level; UNL: Upper normal level; IMDC: International Metastatic renal cell carcinoma Database Consortium	

Nivolumab Outcomes

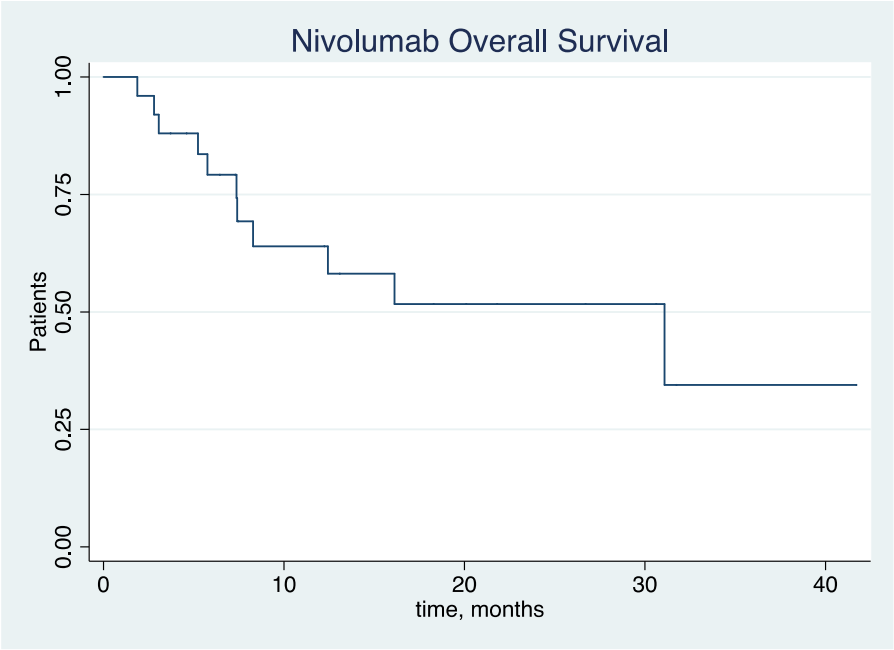
Median follow up was 20.1 months (range 2 - 43) with 11 (44%) deaths observed. Median progression-free survival (PFS) was 4.2 (95% CI, 3 – 8.5) median overall survival (OS) was 31.1 (95% CI, 7.4 – not reached). Figure 5 shows overall survival curve. Six-month survival rate and 12-months survival rate were 79% (95% CI, 0.57 - 0.91) and 65% (95% CI, 0.42 - 0.81).

No complete response was obtained as best RECIST response. Partial response (PR) was achieved in 5 patients (20%); stable disease (SD) was obtained in 3 patients (12%). Progression disease occurred in 17 (68%), while overall disease control rate was 32%. Treatment response using iRECIST criteria compared to RECIST 1.1 were comparable.

Table 7: Nivolumab Outcome

Nivolumab Patients Outcome	**N - %**
Ongoing Treatment at analysis (N, %)	4 (16)
Reasons of Nivolumab Discontinuation Progression (N, %) Intercurrent Event (N, %)	 20 (95) 1 (5)
Patient's deaths (N, %)	11 (44)
Median follow-up (median, range)	20.1 (2 - 43)
Progression-Free Survival (median, 95%CI)	4.2 (3 – 8.5)
Overall Survival (median, 95%CI)	31.1 (7.4 – Not reached)
Landmark Overall Survival (%, 95%CI) 6 months 12 months 18 months	 79 (0.57-0.91) 65 (0.42-0.81) 53 (0.3-0.72)
Disease Control Rate (N, %)	8 (32)
Response Rate (N, %) Partial Response Stable Disease Progression Disease	 5 (20) 3 (12) 17 (68)

Figure 5: Kaplan-Meier analysis of overall survival from nivolumab start.



Analysis by subgroups: fRCC and sRCC

At time of analysis, 11 patients (44%) were identified as fRCC and 14 (56%) as sRCC. The two groups are similar in clinical features, excepting for KPS at nivolumab start. All patients in sRCC group have KPS ≥ 80%, while all patients with KPS < 80 are in fRCC population (p = 0.009, Fisher's exact test).

Both median PFS and OS were longer in sRCC patients: 8.5 months (95% CI, 3 to 21) versus 3.1 months (95% CI, 2.6 to 4.1), 31.1 months (95% CI, 12.5 to non-reached) versus 5.6 months (95% CI, 2.8 to non-reached), respectively (p = 0.003, Kaplan-Meier method and log-rank test).

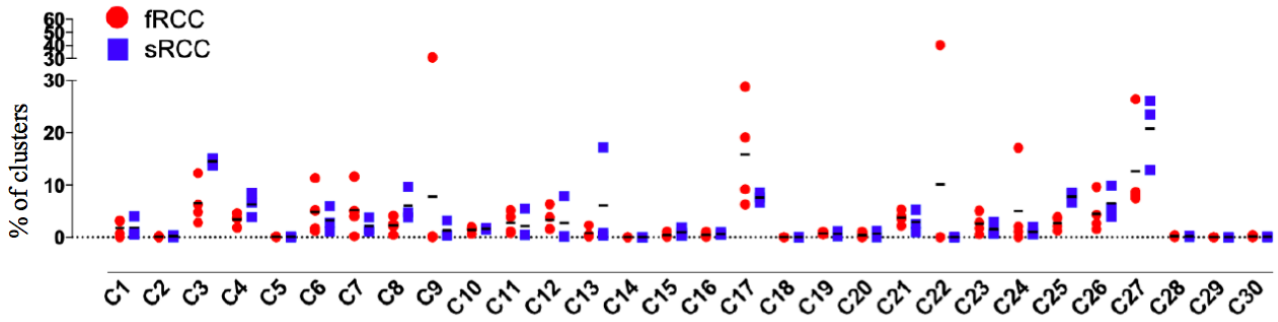
High-dimensional flow cytometry was performed in 7 patients, 3 were sRCC and 4 were fRCC. Similarly, no significant differences were observed between these two small groups of patients, excepting for BMI at nivolumab start. All patients in sRCC cohort have BMI ≥ 25, while all fRCC have BMI < 25 (p = 0.029, Fisher's exact test).

High-dimensional flow cytometry data

Four out of seven patients were fRCC, while three out of seven were sRCC. Two main T cell populations, CD4+ T cell and CD8+ T cell, were identified and measured; in both subgroups t-SNE and Phenograph algorithm found dominant phenotypes.

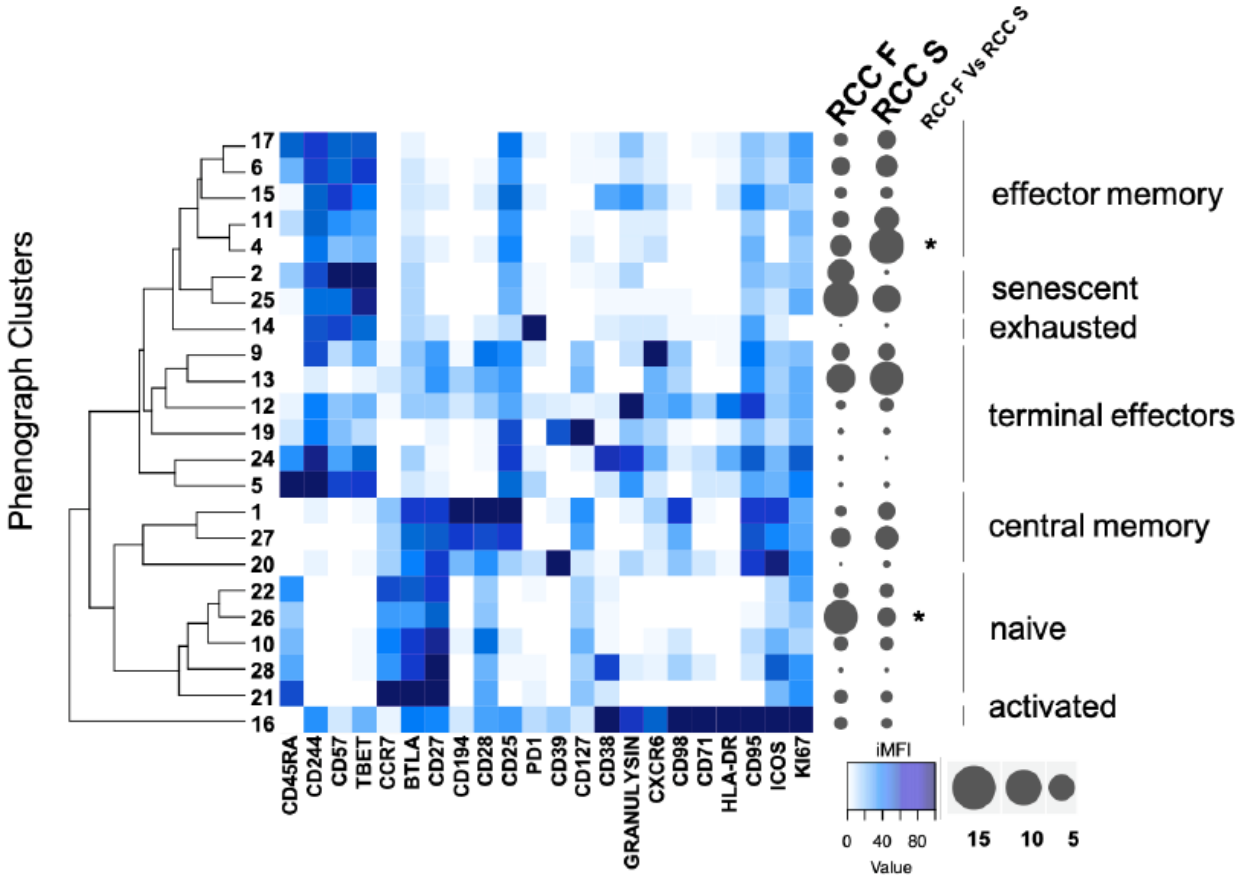
Among CD4+ T cell subset, PhenoGraph allowed the identification of 30 clusters. None of them were represented at different level in both groups of RCC patients before therapy (Figure 6) and after therapy initiation (not shown).

Figure 6: CD4+ T cell clusters frequencies before therapy. *Figure shows the percentage of single clusters in fRCC group (red) and sRCC (blue) in CD4+ T cell population before therapy.*



On the contrary, among CD8+ T cell subset algorithm identified 28 phenotypes. The expression profiles of the T cell PhenoGraph clusters were visualized in a heat map, showing the iMFI of each marker per cluster (Figure 7); frequencies of each cluster were compared between both subpopulation (fRCC and sRCC) as shown in the balloon plot next to the heat map (Figure 7).

Figure 7: Heat map CD8+ T cell clusters; cluster frequencies by fRCC and sRCC. *Heat map shows markers expression of clusters defined by phonograph algorithm. On the right side of the figure the balloon plot shows frequencies of each cluster in both subpopulation (fRCC and sRCC).*



According with markers expression, groups of clusters can be referred to five main CD8+ T cell subsets, although several intermediate subsets may exist reflecting different stage of differentiation (with variable levels of surface markers and co-receptors expression).

Classification is listed below:

- clusters 16, 6, 15, 11 and 4 identified effector memory-phenotype T cells (CD45RA-, CCR7-, CD27+/-, CD28+/-);
- clusters 2 and 25 identified senescent-phenotype T cells (CD57+, TBET+, CD28-, CD27-, CD244+);
- cluster 14 exhausted-phenotype T cells (PD1+, CD57+, TBET+, CD244+);

- clusters 9, 13, 12, 19, 24 and 5 identified terminal effector-phenotype T cells (CD45RA+, CD28-, CD27-, CCR7- CD57+);
- clusters 1, 27, 20 identified central memory-phenotype T cells (CD45RA-, CCR7+, CD27+, CD28+, CD57-, PD1-);
- clusters 22, 26, 10, 28 and 21 identified naïve-phenotype T cells (CD45RA+, CD27+, CD127+, CD28+, CD95-, BTLA+, CCR7+);
- cluster 16 identified activated-phenotype T cells (CD38+, HLD-RA+, CD44+, CCR7-, KI67+)

When the two clinical subgroups were compared we observed that sRCC PBMCs were more rich than fRCC in effector memory-like CD8+ T cells (CD45RA-, CCR7-, CD27-) expressing CD244, CD57, TBET, CD28 and CXCR6dim (Cluster 4, C4, Wilcoxon T-test, $p < 0.05$). Not only, C4 percentage was found higher in sRCC before starting anti-PD1 therapy, but this difference increased after 1 and 2 cycles of therapy ($p < 0.05$).

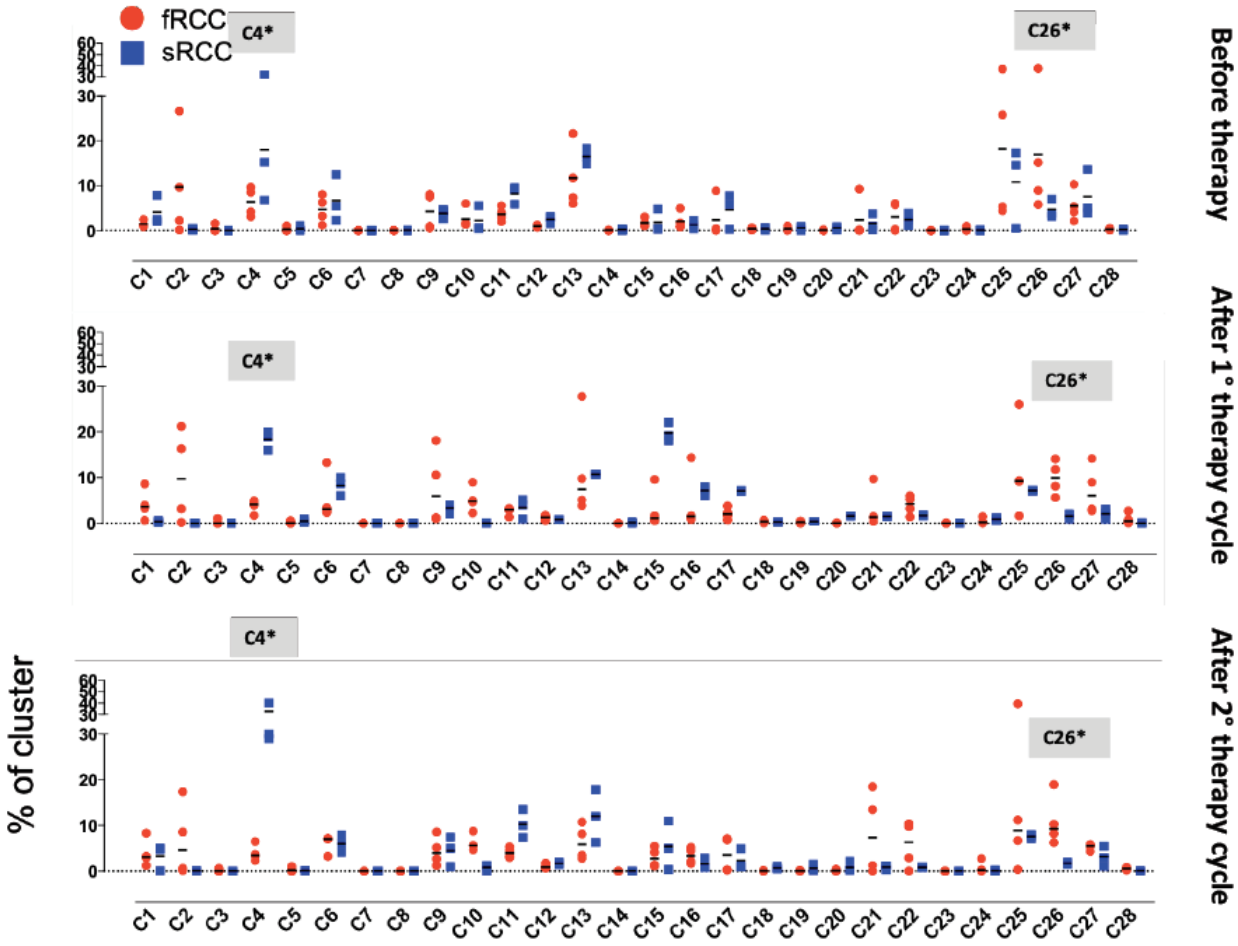
Moreover, as displayed in balloon plot, fRCC displayed higher levels of naive-like T cells identified in cluster 26 (C26, Wilcoxon T-test, $p < 0.05$, Figure 8). This cell subset expresses CD45RA, CD27, CD28, CCR7, BTLA, CD127, ICOSlow, KI67low.

In addition, balloon plot showed higher cluster 2 (C2) frequency in fRCC group than sRCC, despite significant statistically difference was not reached, a relevant disparity between the two groups of patients emerged. C2 identified senescent CD8+ T cells (expressing CD57, CD28 loss).

Cluster 14 cells express highest level of PD1 identifying exhausted-like T cell phenotype.

Globally, non-responder or fast progressed patients (fRCC) are enriched of senescent-like phenotype and naïve-like T cell phenotype; while in responder or slow progressed patients' effector memory-like phenotype is highly represented.

Figure 8: CD8+ T cell clusters frequencies before therapy, after 1 cycle and after 2 cycles. *Figure shows the percentage of single clusters in fRCC group (red) and sRCC (blue) in CD8+ T cell population before therapy and after 1 and 2 cycles of therapy.*



DISCUSSION

The need to identify and validate predictive biomarkers, beyond tumour PD-L1 expression and clinical features, to better select patients who will gain most benefit and avoid unnecessary costs and toxicity in non-responders remains a major challenge.
Given the rapid developments in this setting and the translational potential for some of the biomarkers, we above summarized the current state of biomarker research for immunotherapy focusing on mRCC.

In this study we report the immunomonitoring analysis of peripheral blood via high-dimensional flow cytometry in mRCC patients treated with nivolumab.
In our research two subsets of population defined as sRCC and fRCC display differences in CD8+ T cell compartment before starting therapy. This difference further increased after treatment, suggesting that cancer patients undergoing anti-PD1 therapy could be selected according to their CD8 signature.
CD8+ T cells represent an essential component of the adaptive immune response and it is well known how the expression of a multitude of molecules and functions involved in cytokine signalling, cytotoxicity, migration, proliferation and apoptosis may vary according to T cell differentiation.
On the basis of cluster 4 markers expression (CD244, CD57, TBET, CD28 and CXCR6dim), we could speculate that sRCC are characterized by CD8+ T cells underwent several persistent immune stimulations (higher CD57 expression).
Similar data have been reported in NSCLC by Kunert et al. Blood samples of 71 NSCLC patients treated with second-line nivolumab were stained and analysed with multiplex flow cytometry. In patients with a partial response the number of CD8 T cells at baseline and during treatment was higher than in patients with progressive and stable disease. CD8+ T cell populations in responder patients showed enhanced frequencies of T effector memory[79].
Moreover, C4 expression of higher TBET and CD244 could mean skewed towards Th1 pro-inflammatory profile, in addition to their ability to home the tumour (CXCR6 expression). Indeed, Th1-like phenotype is sustained after prolonged antigen-specific stimulation (as in cancer) and results in multiple CD8+ T cell activities providing a rich set of immune functions.
In melanoma patients, CD8+ T cell-T helper 1 cytokine signature, characterized by IFN-γ expression, was identified in tumour infiltrate of PD-L1+ patients, generally associated to better prognosis. For melanoma and others malignancies it has been clarified that PD-L1 pattern

expression associated to tumour-infiltration pattern may have different immune significance and different outcome[35]. Not for the first time, mRCC seems to have different underlying biology explaining contradictory results compared to other malignancies.

Higher level of naive-like T cells identified in cluster 26 (expressing CD45RA, CD27, CD28, CCR7, BTLA, CD127, ICOSlow, KI67low) was observed in fRCC. After antigen exposure and immune response initiation, naïve T cell are involved in remodelling surface markers acquiring an activated and gradually differentiated phenotype. We could speculate that this subset of naïve-like T cell is not ready or not able to progress in their differentiated descendent, resulting in failure immune response in fRCC patients. Furthermore, after therapy initiation, the percentage of this cluster remained higher in fRCC ($p < 0.05$), suggesting that this cell subset is not involved in cell redistribution after immune system reactivation induced by anti-PD1 therapy. Moreover, naïve T cells do not express PD1, underlining that functional effector-memory cells have a predominant role in boosting immune response after immunotherapy. In addition, despite PD1/PDL1 interaction have a regulatory role; PD1 expression also reflects immune differentiation and activation[80].

Higher frequency in senescent-like phenotype was observed (without statistically significance) in fRCC. Cluster 2 cells express CD57+, TBET+, CD28-, CD27-, CD244+. CD57, such as PD1, is typically recognized as "inhibitory" molecule because of his association with limited responses to proliferative stimulation. Cells expressing these markers may indeed display poor proliferative capacity[81]. Currently, CD57 is considered to be inhibitor of proliferation, while PD1 is primarily associated with immune exhaustion. Blockade of PD1/PDL1 interaction with a specific antibody showed to restore the functionality (mainly cytokine responses) of PD1 cells, suggesting the theoretical reversibility of immune exhaustion mechanism; on the contrary other evidences support that replicative senescence appears to be irreversible. Moreover, in C2 subset we observed the loss of the co-receptors CD27 and CD28, indicating a switch towards replicative senescence[81].
Recently, Ferrara et al identified a senescence immune profile (CD8+ lymphocytes CD28- CD57+ KLRG1+), assessed by flow cytometry on fresh blood in 37 NSCLC patients immunotherapy-treated. Among 12 patients expressing senescence immune profile (SIP), 2 (17%) achieved disease control rate; while 19 patients (76%) achieved disease control in patients negative for senescence immune profile ($p = 0.001$). Moreover, median PFS was significantly lower in SIP+ than in SIP- (1.5 months [95% CI, 1 to 2.2] versus 7.4 months [95% CI, 5.5 to 9.3]; $p = 0.001$, log-rank method)[82].

There are not published data about biomarker analysis from PMBCs in mRCC. To date, only one abstract was presented at last ESMO congress by Desnoyer et al. reported data of fresh whole blood monitoring at baseline and after 2 months of nivolumab in NIVOREN trial. The authors identified immune cell populations associated with grade 3-4 treatment-related events (CD4+ PD-1-4.1BB+ T cells and CD56+ T cells) and disease progression (CD4+PD-1+CD69+ T cells and CD4+CD244+ T cells).

It is important to consider that T cell markers expression and their functional capacities are deeply linked to the differentiation status, in both health individual and cancer patients. Therefore, the broad variability in T cell subsets, function and markers expression may display different immune states. This dynamic status is difficult to capture and to translate in a useful clinical tool.

To date, it is unclear if peripheral blood can be used to detect valid biomarkers to predict response to CPIs. However, high-dimensional flow cytometry overcome the main limitations outlined by other techniques of biomarkers research. Primarily, because it provides an immune portrait just before starting therapy and at the same time allows immunomonitoring during treatment. Advanced technologies on biopsy samples provide precision data, but referred to small lesion of multisite tumour burden, collected months or years before, sometimes methodically not comparable. Moreover, high-dimensional flow cytometry is a rapid quantitative measurement of multiple parameters of cell phenotype simultaneously in a highly sensitive and reproducible manner. Use of PMBCs flow-cytometry as predictive tool in cancer immunotherapy should standardized in assay and instrument set-up. Moreover, because of the massive amount of data generated, flow data analysis can be very complicated and rely almost exclusively on gating by a human expert.

An additional emerging challenge in cancer immunotherapy is interpreting and capturing clinical meaning. RECIST 1.1 criteria continue to be used, recently accompanied by immune-related RECIST and immune-RECIST. However, new patterns of response and progression to immunotherapy emerged with the unmet need to clearly define treatment outcome. PFS has shown to be extraordinarily short during immunotherapy and it is not expression of a real treatment benefit. Overall survival improvement represents the major success of immunotherapy, however long follow up is required to reach it. Objective response rate may represent a reasonable tool to segregate responder versus non-responder patients. However as mentioned RECIST or RECIST-derived may be not the best way to define it. In our study we choose to combine PFS and OS, in

order to clearly identify “true rapid progression” and progression by RECIST combined with a survival benefit from nivolumab, sometimes even achieved in subsequent lines of treatment.

Finally, the major limitation of the study is small sample size, only 7 patients was analysed by high-dimensional flow cytometry. Our data need to be confirmed in a large population and validated in an external cohort.

CONCLUSION

Deciphering of T cell sub-populations is rapidly becoming fundamental in immune-oncology field. The reported findings suggest that cancer patients undergoing anti-PD1 therapy could be selected according to their CD8 signature. Moreover, the results confirm that peripheral T cell distribution may reflect the overall immunological state of patients and can therefore be used to provide key biomarkers of treatment efficacy.

Polychromatic flow cytometry is proving to be a powerful tool for examining changes in immune parameters in the immunotherapy. His implementation of routine application represents a major challenge. In fact, the vast majority of candidate biomarkers never become routine tools.

Immune system biology is far from being simple, because immune response is dynamic and evolves over time. A single biomarker for patient's selection may not be feasible and biomarker development will require integration of multiple biologic components, like checkpoint expression, TILs, mutational load, and probably many others now considered emergent biomarkers.

The ongoing intensive work to establish and understand biomarkers for CPIs response prediction holds great promise for maximizing patient benefit from these transformative therapies.

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