

DOTTORATO DI RICERCA IN

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Deciphering the Renal tumor microenvironment to Personalize immunE-targeted Treatment (DROPLET):

transcriptomic profiling of primary and secondary lesions identified a potential crosstalk between MARCO+ macrophages and deregulated cancer-cell proliferation in mediating progression and therapy response of advanced renal cell carcinoma.

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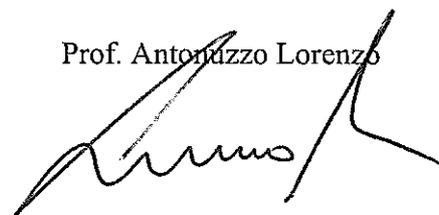
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“Il mio maestro m’insegnò com’è difficile trovare l’alba dentro l’imbrunire”
Prospettiva Nevski, F. Battiato

A Lorenzo e Serena, per aver creduto in me

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1. Background

Kidney cancer is one of the most common urological cancers, accounting for 2.2% of new cancer diagnoses and 1.8% of cancer deaths worldwide [1], with a higher burden falling on North American and Western European populations [1]. Renal cell carcinoma (RCC) is the most common type of kidney cancer, with the clear cell subtype (ccRCC) accounting for about 70% of all RCC cases [2]. 30% of patients are metastatic at diagnosis and almost 30% of the remaining patients will develop metastases detected during the follow-up. The 5-year relative survival rate across all risk groups for metastatic RCC in the SEER database from 2011 to 2017 was approximately 14% [3]. ccRCC tumors have nearly always sustained inactivating mutations (or, less commonly, hypermethylation) of the maternal and paternal copies of the gene *VHL* (von Hippel–Lindau). The *VHL* gene product, pVHL, is part of an E3 ubiquitin ligase that has a fundamental role in oxygen sensing by targeting the α -subunit of the heterodimeric transcription factor HIF (hypoxia-inducible factor) for degradation under normoxic conditions. However, when *VHL* is lost or silenced, the accumulated HIF α transcriptionally upregulates tumorigenic hypoxia-responsive genes, including *VEGFA*, which encodes VEGF (vascular endothelial growth factor). VEGF, originally known as ‘vascular permeability factor’, is an extracellular signalling protein produced by cells that stimulates the formation of blood vessels. HIF2 α , one of three HIF α subunits, and the main driver of ccRCC, is upstream of multiple critical oncogenic pathways, and is therefore considered to be an ideal target for the treatment of ccRCC [4]. Notably, RCC is one of the most immunogenic tumor with a very high T cell infiltration score. The inhibition of vessel formation by anti-angiogenic tyrosine kinase inhibitors (TKIs), sunitinib, pazopanib, cabozantinib and lenvatinib [5] and more recently the blockade of HIF2 α by HIF2 α inhibitors (i.e. belzutifan) [4, 6], as well as the stimulation of the immune system firstly by immune checkpoint(s) inhibitors (ICIs) monoclonal antibodies (mAb), mainly represented by those targeting the programmed cell death 1(PD-1)/PD ligand-1 (PD-L1), have revolutionized the therapeutic landscape of advanced RCC in recent years [3]. Combination

trials of anti-angiogenic TKIs plus ICIs or different types of ICIs have been fruitful [7-14]. In 2015, nivolumab was the first therapy belonging to the class of ICIs to demonstrate efficacy in the treatment of advanced RCC. In the phase III CheckMate 025 trial, anti-PD1 mAb nivolumab improved overall survival (OS) compared with everolimus in 821 pre-treated patients (median OS: 25.8 versus 19.7, HR 0.73, 95% confidence interval (CI), 0.62–0.85; $p < 0.0001$) [15]. ICIs had considerable benefits compared to the previous generation of immunotherapies, with less toxicity, higher and more durable responses, and, as it would be later discovered, the possibility of combining them with TKIs without a substantial increase in toxicity. In recent years, four phase 3 trials in advanced RCC in the first-line setting demonstrated an improved OS with ICI-based combination (ICI-ICI and ICI-TKI) compared to TKI monotherapy [3]. Thus, ICI-ICI and ICI-TKI combinations have become the standard of care in the frontline setting [3]. The addition of PD-(L)1 inhibitors to target therapies provides a higher objective response rate (ORR) and durable responses, with complete response rates of up to 8–16% in pivotal trials [3]. The Checkmate 214 was the first phase III trial evaluating an ICI-ICI combination; the experimental arm received nivolumab 3 mg/kg combined with ipilimumab 1 mg/kg, anti- cytotoxic T lymphocyte antigen 4 (CTLA-4) [3, 7] mAb, every 3 weeks for 4 doses, followed by nivolumab 3 mg/kg every 2 weeks, compared with sunitinib 50 mg daily for 4 weeks then 2 weeks off (ClinicalTrials.gov identifier: NCT02231749) [7]. The KEYNOTE-426 evaluated in the experimental arm pembrolizumab 200 mg every 3 weeks with axitinib 5 mg twice daily versus sunitinib (ClinicalTrials.gov identifier: NCT02853331) [3, 8-11]. The CLEAR evaluated in the experimental arm pembrolizumab 200 mg every 3 weeks with lenvatinib 20 mg daily versus sunitinib. (ClinicalTrials.gov identifier: NCT02811861) [3, 12, 13]. The Checkmate 9ER evaluated in the experimental arm nivolumab 240 mg every 2 weeks plus cabozantinib 40 mg daily or sunitinib (ClinicalTrials.gov identifier: NCT03141177) [3, 14]. These trials led to the four combination therapies currently approved by the FDA and EMA in the first-line setting, supported by an advantage in OS over sunitinib in phase III clinical trials: axitinib plus pembrolizumab, cabozantinib plus nivolumab, lenvatinib plus pembrolizumab, and nivolumab plus ipilimumab [3].

Unfortunately, ORR is yet limited and only a minority of advanced RCC subjects achieves long-term disease control [16], with a significant portion of patients that fails to respond (primary resistance) or develops secondary resistance [16, 17] after initial response. Thus, a research priority for RCC patients is to overcome resistance to combinatorial approaches of ICI and TKIs or ICI and ICI. The TME is emerging as a novel potential subject involved in

resistance to ICI-based combinatorial treatment, since its dynamic nature modulates many aspects of tumor development and therapy resistance [17, 18]. Dynamic interactions of cancer cells with their microenvironment consisting of cellular and non-cellular parts are essential to stimulate the heterogeneity of cancer cells, clonal evolution and to increase the multidrug resistance ending in cancer cell progression and metastasis. Understanding the interactions of tumor cells with TME can help to implement better therapeutic regimes for cancer management, however, a combination of strategies appears to be more effective than single modalities, since the tumor heterogeneity arises from a variety of signalling pathways/cross-talks existing in the network of communicating cancer cells [17]. Along this line, exploring new combinatorial approaches could represent areas of active investigation that could eventually lead to enhanced efficacy as well as overcoming resistance remodelling the RCC microenvironment and improve antitumour T cell responses [17-19]. Therefore, identifying optimal therapeutic combinations and sequences could facilitate the integration of novel and more effective immunotherapy-based combinations into the clinical practice. Current clinical and biological markers, such as MSKCC (Memorial Sloan–Kettering Cancer) [20] score, PD-L1 expression and tumor mutational burden (TMB) failed to predict ICIs efficacy in advanced RCC [21]. Predictive biomarkers for immunotherapy differ from the traditional ones used for targeted therapies: the complexity of the immune response and tumor biology requires a more holistic approach than the use of a single analyte biomarker [20, 21]. In light of this evidence, novel clinical-grade biomarkers are needed to guide the choice of treatment to improve clinical outcome. Innovative technologies can be used to explore the immune contexts of tumors and to find predictive and prognostic biomarkers. As ICIs execute their antitumor effects by activating the patient's own immune system, the TME is known to be highly involved in tumor progression and treatment response. Hence, it is essential to deeply investigate the TME to understand tumor heterogeneity better and to individuate potential novel biomarkers with the aim of improvement the effectiveness of current therapeutic strategies.

2. Tumor microenvironment (TME)

TME is a multifaceted and interconnected network containing various cells and components, including extracellular matrix (ECM), lymphocytes, tumor-associated macrophages (TAMs), dendritic cells (DCs), growth factors, cytokines, chemokines, natural killer cells, myeloid-derived suppressor cells (MDSCs). TME affects not only the therapeutic effect of primary tumor treatments but also the evolution and advancement of tumor metastasis. The

concept of TME has evolved to recognize that cancer behaviour is influenced not only by the genetic characteristics of tumor cells but also by the surrounding environment crucial to carcinogenesis and the response to immunotherapies [22] RCC are highly infiltrated with leukocytes, especially T cells of various phenotypes [22]. Studies have shown that the nature and degree of the tumor infiltrate has prognostic significance, with worse outcomes being associated with the burden of accumulated T cells and the presence of specific subpopulations of TAMs [23- 25]. Additionally, RCC has an increased expression of immune checkpoint molecules, such as PD-L1, which can inhibit T cell activation and promote tumor immune evasion [26, 27]. These cells interact with one another and tumor cells through a complex signalling pathway network, leading to a dynamic and ever-changing microenvironment [26].

The TME with its highly complex tumor-promoting features is a critical component contributing to cancer metastasis and response to therapy. Several genetic and epigenetic events are emerging as a novel hallmark contributing to cancer cell survival and metastasis through their regulation of different mechanisms of cancer progression, acting also on TME [28- 31]. Furthermore, diverse components of the TME (e.g., cytokines, hypoxia, cellular stress) have been identified to modulate the cancer epigenome and immune cell trafficking at tumor sites [32, 33]. Hence the identification of treatment strategies to reprogram the immunosuppressive landscape of RCC and enhance ICIs is strongly related to a better comprehension of cancer cell-intrinsic mechanisms linked to the establishment of an immunosuppressive TME in RCC [19].

3. The role of tumor associated macrophages (TAMs)

In the TME, TAMs are highlighted as the most crucial element, making up around 50% of the weight of the tumor tissue. TAMs come from two distinct lineages, including tissue-resident macrophage-derived and monocyte-derived [34, 35]. In the early stages of tumor growth, tissue-resident macrophages gather and surround the tumor tissue to stimulate regulatory T-cell reactions, facilitate tumor cell evasion of the immune system, trigger epithelial-mesenchymal transition (EMT), and boost tumor cell invasion and dissemination [34- 36]. Many cytokines and chemotactic proteins, produced by hematopoietic stem cells in the bloodstream, draw monocyte-derived macrophages to the TME. Afterward, various stimuli encourage the transformation of these monocyte-derived macrophages into TAMs. IL-1 β , CCL2, VEGF, and SDF-1 α secreted by tumors attract proangiogenic macrophages to the tumor sites [34- 26]. Although the specific timing of when recruited monocytes transform

into TAMs is unknown, there is strong evidence that tissue-induced changes influence the transcriptional profiles of these monocytes. Different stimuli can polarize macrophages into two basic types, M1 and M2 [34]. TAMs have high plasticity and polarization regulation. While there is still more to learn about TAM polarization in terms of its nature, mechanism, and naming conventions, the concept of M1-like TAMs and M2-like TAMs existing at opposite ends of the TAM polarization axis has been unanimously validated based on in vitro experiments [34- 36]. The M1-like TAMs and M2-like TAMs are situated at opposite ends of the continuous dynamic TAM polarization spectrum, each having distinct cell surface markers and functional factors, and serving diverse functions in the TME [34- 37]. The M1-like TAMs exhibit pro-inflammatory characteristics. M1-type macrophages are activated by cytokines such as interferon (IFN), colony-stimulating factor (CSF), tumor necrosis factor (TNF). In addition, lipopolysaccharides (LPS) can bind to toll-like receptor (TLR)4 on macrophages and induce M1-like macrophage activation by influencing NF- κ B and IRF3 [38]. Two characteristics of M1-like macrophages are effectively displaying antigens and producing a large amount of pro-inflammatory cytokines. M1-type macrophages release numerous co-stimulatory molecules like CD86, CD60, and CD80, in addition to inflammatory markers such as TNF- α , IL-1 β , IL-6, IL-12, IL-23 [38, 39, 40]. They also greatly indicate molecules of major histocompatibility complex (MHC) II. They do demonstrate IL-10 expression, albeit to a lesser extent. M1-like macrophages secrete matrix metalloproteinases (MMPs) such as MMP1, MMP2, MMP7, MMP9, and MMP12. These enzymes have a specific purpose of breaking down components of the extracellular matrix (ECM). M1-type macrophages produce chemokines such as CCL2, CCL3, CCL5, CXCL8, CXCL9, CXCL10, CXCL11, and CXCL16 [38, 39, 40, 40]. Additionally, they secrete IFN- γ , iNOS, and ROS. M1-like macrophages trigger a potent T-helper1(Th1)-type immune reaction through the release of inflammatory mediators, which hinder cell growth and eliminate pathogens and tumor cells in the body, leading to antitumor outcomes [38, 39]. M2-like TAMs, known for their poor antigen presentation, have anti-inflammatory properties in contrast to the M1 phenotype. There is no prescribed method required to begin the activation of M2-like macrophages [40, 41]. M2-like macrophages are predominantly activated by certain cytokines such as TGF- β , IL-4, IL-13, IL-10, and M-CSF. M2-type macrophages show high levels of CD163, CD206, CD200R, CD209, CD301, and chemokines such as CCL1, CCL17, CCL18, CCL22, and CCL24 [40, 41]. They secrete many substances that reduce inflammation, such as TGF- β , IL-4, IL-13, IL-10, and IL1RA [40]. Moreover, M2-like TAMs produce lower levels of inflammatory cytokines IL-6, IL-12, IL-

23, and TNF- α [40]. M2-like macrophages support the expression of Arginase (Arg)-1 and VEGF, which play a role in the production of proline and polyamines [40]. Proline aids in ECM formation, while polyamines play a role in cell growth [40]. Multiple factors produced by M2-like macrophages, like PDGF and IGF, play a role in promoting cell proliferation and are also related to angiogenesis [42]. Macrophages with characteristics like M2 cells secrete substances that inhibit Th1 immune responses and enhance Th2 immune responses. This activity limits inflammatory reactions while enhancing tumor cell growth, drug resistance, angiogenesis, and tissue healing. Thus, it was hypothesised that in the initial phase of tumor development, TAMs were mainly M1 phenotype. As the tumor grew, the tumor microenvironment experienced changes that caused TAMs to shift from the M1 to the M2 phenotype. Hence most tumors have TAMs that are predominantly of the M2 phenotype, with a weak immune response, impacting the growth of the tumor and resistance to chemotherapy [38, 40, 41].

It must be pointed out that the hypothesis of tumor suppressor M1 and tumor promoter M2 could represent an oversimplification of the true TAM phenotype. Extensive research on macrophages has shown noticeable variations in structural layout, functioning abilities, and surface marker expression among tissue-resident macrophages (TRMs) in different organs. Moreover, genes associated with M1-like and M2-like TAM characteristics are commonly expressed together in various cancer macrophage subgroups.

Considering their role within TME, TAMs have emerged as therapeutic targets in cancer therapy. Macrophage-targeting strategies include inhibitors of cytokines and chemokines involved in the recruitment and polarization of tumour-promoting myeloid cells as well as activators of their antitumorigenic and immunostimulating functions [40]. For example, early clinical trials suggest that targeting negative regulators (checkpoints) of myeloid cell function indeed has antitumor potential. These include the receptor/ligand pairs signal regulatory protein- α (SIRP α)–CD47, LILRB1–HLA1, sialic acid-binding immunoglobulin-like lectin 10 (SIGLEC10)–CD24, and PD1–PDL1, which inhibit phagocytosis, and macrophage receptor with collagenous structure (MARCO), CD169 and mannose receptor scavenger receptors [40, 43]. Clever 1, triggering receptors expressed on myeloid cells 2 (TREM2) and P-selectin glycoprotein ligand 1 (PSGL1) are also depicted [40, 43]. Targeting of Clever 1 and TREM2 does not specifically interfere with phagocytosis but with immunosuppressive activation [40, 43]. In strategies featuring the use of therapeutic antibodies, such as anti-CD20 or anti-epithelial growth factor receptor (EGFR), combinatorial use of anti-CD47 enhances antibody-dependent cellular phagocytosis (ADCP)

and increases antigen presentation to T cells [40, 43]. Bispecific CD47 antibodies are designed to recognize CD47 and tumour-associated antigens (such as CD20 or PDL1), which enhances selective blocking of CD47 on tumour cells, avoiding on-target toxicity due to recognition of CD47 on red blood cells and platelets [40, 43]. Thus, macrophage-centred therapeutic strategies will hopefully have the potential to complement, and synergize with, currently available tools in the oncology armamentarium (**Table 1**).

Finally, there is growing evidence that macrophages lead to inflammation and autoimmunity with potential increasing in the incidence of immune-related adverse events (ir- AEs) due to an increased pro-inflammatory M1/anti-inflammatory M2 ratio [44]. For example, it was recently reported that macrophages could contribute to the development of ICI-related myocarditis. Immune checkpoint inhibition might indeed contribute to atherosclerotic cardiovascular disease (ASCVD) by promoting plaque progression or by increasing dyslipidemia [45]. Overall, an increase in major adverse cardiovascular events is observed after ICI therapy. Potential mechanisms include crosstalk between T cells and macrophages leading to macrophage activation, and the release of proinflammatory cytokines [45]. Activated M1 macrophages promote the accumulation of intracellular lipids, initiate and sustain inflammation, and secrete proinflammatory factors such as IL-1, IL-6, and TNF- α [45, 46]. In contrast, activated M2 macrophages work toward resolving inflammation, promote the clearance of lipids, and secrete anti-inflammatory factors such as IL-10 and collagen [45, 46, 47]. Inflammation in the plaque results in foam cell formation, intimal thickening, increased necrotic core and decreased collagen content [45, 46, 47].

4. Aim of the study

The aim of DROPLET was to identify prognostic and therapy-specific predictive biomarkers to determine the optimal personalized therapy for advanced RCC patients, with a focus on genes related to TAMs - and their role in shaping the TME during immunotherapy. By employing advanced techniques such as transcriptomics, this research aimed to uncover immune signatures that predict response or resistance to ICIs, offering insights that could lead to more tailored and effective therapeutic approaches for RCC patients. Finally, DROPLET investigated the correlation between genes related to cancer-cell intrinsic mechanisms potentially linked to the establishment of an immunosuppressive TME with the aim to overcome resistance to ICI-based therapy.

5. Study Design and Methods

5.1. Collection of candidate biomarkers from online data repositories and observational retro-prospective study

5.1.1. *Patients' recruitment and clinical follow up:* patients with histologically confirmed diagnosis of unresectable locally advanced or metastatic RCC who were treated with a first line immunotherapy-based combination treatment at Careggi University Hospital between October 2021 and February 2024 were retrospectively selected. All enrolled patients were treated according to current guidelines of European Society of Medical Oncology (ESMO) [48, 49]. Archival formalin-fixed, paraffin-embedded (FFPE) samples of primary tumor or metastatic site obtained at diagnosis were retrieved from the pathology department. The main inclusion criteria were histological diagnosis of RCC; presence of advanced or metastatic (synchronous or metachronous) disease; the availability of sufficient histological material to perform an adequate RNA extraction evaluation of TME; at least one cycle of a first-line combination treatment (including dual ICIs or ICI in combination with a TKI); at least one disease assessment to evaluate response to first-line therapy. Patients without clear cell components were also included. For each patient, pathological data (stage at diagnosis, histology of RCC, nucleolar grading, presence of sarcomatoid features) and clinical data (sex, age, IMDC risk score, sites of metastasis) have been collected. In addition, data related to first-line immunotherapy-based treatment (type of combination therapy, adverse events, best overall response (BOR), disease control rate disease control rate ([DCR], percentage of patients with a complete or partial response or a stable disease using response evaluation criteria (RECIST) version 1.1 [50, 51], overall response rate (ORR), (percentage of patients with a complete (CR) or partial response (PR) using iRECIST), PFS (defined by time from treatment initiation until death, radiographic or clinical progression), and OS (defined as time from treatment initiation until death) have been recorded. Patients without progression or death at the study cutoff date or date of last follow up were censored for analysis. Non-responder patients were defined as patients with SD as best overall response according to RECIST 1.1 and/or iRECIST. Responders were patients with CR or PR as best overall response according to RECIST 1.1 and/or iRECIST criteria. Every information

regarding human material was managed using anonymous codes and all samples were handled in compliance with the Declaration of Helsinki. All patients have signed an informed consent for therapy. This study has been approved by the Local Ethics Committee for Clinical Trials.

5.1.2. Sampling: surgical samples of primary tumor and secondary lesions (if any) have been collected at baseline.

5.2. Tumor microenvironment characterization and identification of prognostic and predictive biomarkers

5.2.1. RNA extraction and nCounter analysis. The NanoString nCounter analysis platform was used to perform the transcriptomic profiling of primary and secondary lesions harvested prior treatment with ICI treatment from patients with advanced ccRCC. Total RNA was extracted from 1-up-to-4 10µm FFPE tissue sections by The RNeasy FFPE Kit (by Qiagen) and quantified by Nanodrop. 100 ng total RNA were hybridized to nCounter probes for the 770 predefined genes of the PanCancer IO360 Panel (by NanoString) and incubated overnight at 65°C. After incubation, samples were run on the nCounter NanoString platform. nCounter raw data were processed using the R/Bioconductor package ‘NanoStringQCPro’. Counts were log₂-transformed and normalized by housekeeping gene expression, after removing the unwanted variations with RUV method and R NanoNormIter package. Normalization efficiency was determined observing RLE plots. Differential Gene Expression Analysis (DEGA) was carried out using the R software to calculate the fold-differences and p-values between the primary and secondary samples, and between secondary samples obtained from responder (PR and CR patients according to RECIST 1.1 [50] and iRECIST criteria [51]) and non-responder (SD patients according to RECIST 1.1 [50] and iRECIST criteria [51]) patients.

5.2.2. Validation of data. Data obtained in our independent cohort were validated by retrieving public RNA-seq and clinical data for n=509 ccRCC patients from the TCGA-KIRC cohort. The Kaplan–Meier plots for overall survival were generated with the R software and statistical significance on overall survival of patients from the TCGA-KIRC dataset were assessed by Logrank test.

6. Results

We have retrospectively identified 12 advanced RCC patients (8 male and 4 female) treated with first line ICI- based combination according to ESMO guidelines [48, 49] from October 2021 to February 2024. Additionally, they were selected to perform RNA extraction from FFPE. Most patients were male and had a clear cell component (83.3%); sarcomatoid features were reported in 16.7% of patients. According to the IMDC score, most patients had intermediate risk (58.3%) (**Table 2**). The most common site of metastasis was lung (58.3%), followed by lymph nodes (50%), renal lodge (41.7%) and peritoneum (16.7%) (**Table 3**). Overall, 9 patients received ICI in combination with TKI as first-line treatment (pembrolizumab plus axitinib), whereas 2 received dual-ICI (nivolumab plus ipilimumab) and 1 patient nivolumab monotherapy as second-line treatment (**Table 4**).

We have performed the transcriptomic profiling of primary (P; n=12) and secondary (S; n=6) lesions harvested prior treatment. All samples were processed for RNA extraction but only 16 out of 18 passed quality control. Thus, of 12P lesions, 10 were suitable for transcriptomic analysis; all of 6S lesions could be used. Total RNA was hybridized to nCounter probes for the 770 predefined genes of the PanCancer IO360 Panel.

Differential gene expression analysis (DGEA) between primary (P, n=10) vs secondary (S, n=6) RCC lesions DGEA identified 67 deregulated genes. Genes that were up-regulated in S lesions compared to P lesions were a total of 37 and included TAM-related genes, such as *APOE*, *MARCO* and *TREM2*, and *CD274* (encoding PD-L1) [7] (**Figure 1**).

APOE (*Apolipoprotein (Apo) E*) is recognized for its ability to exert pleiotropic properties to maintain tissue homeostasis, including a capacity to regulate immune cell activity and inflammation [52]. Evidence indicating the involvement of *APOE* in tumor progression and metastasis. For example, in gastric cancer, TAMs promote tumor cell metastasis by releasing exosomes that secrete *APOE*, thereby activating the PI3K-Akt signalling pathway. Moreover, *APOE* has been shown to exert an essential role in the immune TME. In triple negative breast cancer (TNBC), the difference in score of *APOE*⁺ macrophages between responders and non-responders to immunotherapy was particularly notable. Therefore, it is hypothesised that *APOE*⁺ macrophages may play a significant role in the immunotherapy's failure [53].

MARCO is a scavenger receptor involved in macrophage-mediated phagocytosis responsible for tumor cells clearance by regulating cytoskeletal rearrangement in macrophages and binding to $\beta 5$ integrin on tumor cells via PI3K-Rac pathway [54]. Single cell analysis

performed for prostate cancer revealed a specific enrichment of *MARCO* positive (*MARCO*⁺) clusters in tumor versus normal tissue [55]. In non-small cell lung cancer (NSCLC) patients, a distinct subpopulation of TAMs expressing *MARCO* aggregated near tumor cell nests was found [55]. Multiplex immunofluorescence staining confirmed the co-expression of *CD68*, *CD163* and *MARCO*. Co-staining of *PD-L1*, *MARCO* and *CD68* revealed *MARCO*⁺ macrophages in direct contact with PD-L1-positive tumor cells. On the transcriptomic level, *MARCO* gene expression is positively correlated to immunosuppressive TAMs, T-cell infiltration and immune checkpoint molecules [55]. The subpopulation of *MARCO*⁺ macrophages was exclusively found in *IDH1-wild-type* (Isocitrate dehydrogenase 1 (NADP⁺), soluble) glioblastoma (GBM), rather than *IDH1-mutated* or lower-grade counterparts [55]. The transcriptome of *MARCO*⁺ macrophages was enriched with signatures of epithelial-mesenchymal transition, angiogenesis, glycolysis, and hypoxia. The expression of pro-inflammatory molecules, including HLA class II genes (*HLA-DRB1*, *DRA*, *DPA1*, and *DPB1*) and *CD74* (MHC class II invariant chain) was highly downregulated in *MARCO*⁺ macrophages. NSCLC patients with tumors characterized by high *MARCO* expression had lower OS and disease-free survival (DFS) rates compared to *MARCO-low* patients [55]. Experimental models in several tumor type (NSCLC, breast, pancreatic, melanoma, prostate, GBM, and ovarian cancer) demonstrated that blocking of *MARCO* induced repolarization of TAMs into pro-inflammatory phenotype, activated anti-tumoral capacity of natural killer (NK) cells and T cells, and inhibited T regulatory (Treg) activities, as well as decreased tumor growth in murine models, indicating that *MARCO* can be a target for immunotherapy [55, 56].

TREM2 (Triggering-Receptor-Expressed on Myeloid cells 2) are key regulators of antitumor immunity, reporting to mark TAMs with potent immunosuppressive activity. *TREM2* blockade dramatically alters the immune microenvironment, leading to better antitumor immunity [57]. In RCC, *TREM2*⁺ *APOE*⁺ macrophages were reported to be associated with early post-surgical disease recurrence, as well as a potential target for therapeutic intervention. The role of the *TREM2-ApoE* pathway in RCC tumor biology has not been fully explored. A recent study profiling a murine ccRCC model using scRNA-seq in conjunction with intracellular proteomic staining identified a population of *TREM2*⁺ tumor-infiltrating macrophages, which appears to be phenotypically similar to the population we discovered in patients [24, 57]. Functional studies showed that co-culture of CD8 T⁺ cells with these macrophages significantly impeded T cell proliferation, and that *TREM2* knockdown led to favourable pre-clinical outcomes. Previous studies based on RNA-seq and

transcriptomics methods have suggested a relationship between TAMs and CD8⁺ T exhausted (ex) cells in multiple cancers, and CD8⁺ T cells are preferentially located in the TAM-rich region in the TME. This finding reveals that TAMs inhibit antitumor T-cell immunity in solid tumors, which is related to poor prognosis and anticancer treatment failure. APOE⁺ macrophages, which are closely connected to CD8⁺ Tex cells, may be one of the causes of the failure in immunotherapy [24, 58]. Finally, it is possible that *TREM2*, together with *MARCO*, contributes to the functional maturation of TAMs by sensing various ligands released in the TME, such as lipids, lipoproteins, and apoptotic debris [24, 58].

CD274, better known as *PD-L1*, is widely expressed in most tumor cells, macrophages, activated T cells, B cells, monocytes, and endothelial cells. The binding of PD-1 to PDL1 recruits SH2 domain-containing protein-tyrosine phosphatase-2 (SHP-2) via the immunoreceptor tyrosine-based switch motif. This event subsequently triggers the dephosphorylation of spleen tyrosine kinase and PI3K, which then results in the inhibition of downstream *AKT*, *ERK*, and other signalling pathways; thereby suppressing the function of initial and effector T cells, inducing the production and activity of regulatory T cells, promoting tumor progression, and ultimately leading to a poor prognosis. *PD-L1*⁺ Macrophage modulates the immunosuppressive nature of the TME through its function or by affecting T cells. PD-L1⁺ TAMs expressed both the pro-tumor markers CD204, CD206, and CD163, as well as the anti-tumor marker CD169, indicating that PD-L1⁺ TAMs have a dualistic nature, which primarily manifests as immunosuppressive functions. In line with this evidence, it was demonstrated that macrophages with high PD-L1 expression were significantly associated with M2 polarization and secreted the typical chemokines, TGF- β and IL10 [59]. Then, we performed DGEA between responder patients, that were those who achieved PR or CR (n=2) vs non responder patients who achieved SD (n=4), considering only S samples. The analysis of DGEA showed 70 genes significantly deregulated in S lesions of responder patients vs non responder patients. Genes that were up regulated in S lesions from responders were a total of 27 and included *CD3D*, *CD79A*, and *CD8A* (**Figure 2**).

CD79 is a heterodimeric protein comprised of two transmembrane subunits CD79A and CD79B which are expressed on B cells and in B cell neoplasms [60]. B cells are the dominant cell population inside tertiary lymphoid structures (TLS) and, in contrast to T cells, are mostly confined to TLS in the TME [61]. TLS are organized lymphoid aggregates composed by a network of fibroblasts and comprising a T cell zone, in which mature dendritic cells are in contact with T cells, and a follicular B cell zone. Mature TLSs are defined by the presence

of a germinal center (GC) containing T follicular helper (Tfh) cells and follicular dendritic cells in close contact with B cells. Recent evidence reported that GC-containing mature TLS, rather than early TLS without a GC, are associated with clinical benefits in hepatocellular carcinoma, NSCLC, colorectal cancer, and pancreatic cancer. The principal function of the GC is to produce memory B cells and long-lived plasma cells (PCs) secreting high-affinity antibodies. High expression levels of B cell and PC transcriptomic signatures and high PC densities are related with longer survival in various tumor types. The presence of B cells and mature TLS predict therapeutic responses to ICIs and survival, more accurately than T cells, in several cancers, including RCC [62, 63]. The mechanisms by which B cells and PCs influence clinical outcome and response to ICIs are not yet fully understood [62-64].

CD3D is one of the components of the *TCR/CD3* complex involved in the activation of T-cell-related signal transduction. Previous study suggested that high *CD3D* expression could function as a positive prognostic marker for several types of cancers including colon, breast and head and neck squamous cell cancer. Moreover, *CD3D* was found to be significantly decreased in colon adenocarcinoma. The expression of *CD3D* was downregulated with increasing clinical stage, and a close relationship was observed between *CD3D* and microsatellite status. Functional enrichment analysis revealed that *CD3D* is highly related to immune activation and regulation in colon adenocarcinoma, suggesting the participation of *CD3D* in the immune response of tumor. Coexpression analysis revealed that *CD3D* is strongly correlated with immune score, immune checkpoint and immune-infiltrated cells. Furthermore, patients with higher expression of *CD3D* showed better clinical outcome compared with those with lower *CD3D* expression. The observations collectively demonstrated that *CD3D* may serve as a prognostic marker of colon adenocarcinoma and may guide the development of novel immunotherapy [65- 67]. CD8A molecule (*CD8A*) is a member of T cytotoxic pathway-related genes and encodes the CD8 antigen that is a cell surface glycoprotein found on most cytotoxic T cells. The CD8 antigen acts as a coreceptor with the T-cell receptor on the T cell to recognize antigens displayed by an antigen-presenting cell in the context of class I MHC molecules. *CD8A* expression may be a useful and measurable predictive marker of immunotherapeutic response and immune cell infiltration. Pre-therapy levels of the *CD8A*⁺ tissue-resident was reported to be associated with improved PFS outcome after ICI-based combination regimens or anti-VEGF therapy in RCC [68, 69].

Finally, we report genes that were up regulated in responder patients. There was a total of 43 and included genes involved in cell proliferation and angiogenesis, such as *CCND1*,

CDKN1A, *EGFR* and *ERBB2* and *VEGFA*, respectively. Loss of genes involved in cell cycle arrest such as cyclin-dependent kinase inhibitor 2A (*CDKN2A*) confers a hyperproliferative phenotype to malignant cells. Specifically, amplification of *CDK4* and *CDK6* or their partner cyclin D1 (*CCND1*) has been observed in various human tumours [28]. In line with this notion, at least three different CDK4/6 inhibitors (that is, palbociclib, abemaciclib and ribociclib) are currently approved by regulatory agencies for use as first-line intervention in patients with advanced/metastatic breast cancer [28, 70]. Besides acting as cytostatic agents, CDK4/6 inhibitors promote the recruitment of immune effector cells to the TME and their activation via a multitude of mechanisms [28, 70, 71], thus standing out as promising combinatorial partners for ICIs. For example, CDK4/6 inhibitors are particularly cytostatic for Treg cells as they inhibit DNA methyltransferase 1 (DNMT1) and hence drive cyclin-dependent kinase inhibitor 1A (*CDKN1A*, encoding p21^{CIP1}) expression [28, 72, 73] and inhibiting CDK4/6 with clinically approved agents resulted in multipronged immunostimulatory effects in preclinical models of breast cancer as well as in patients with breast cancer [28, 74].

HER2 (Human Epidermal Growth Factor Receptor 2), also known as *ERBB2*, and *EGFR* (epidermal growth factor receptor) were among the first oncogenic drivers to attract interest as potentially druggable targets for cancer therapy, rapidly translating into an array of TKIs and mAbs for use in patients with *ERBB2* amplifications and activating *EGFR* mutations. Both humanized *HER2*-targeting mAbs (trastuzumab and pertuzumab) and chimeric *EGFR*-specific mAbs (cetuximab and panitumumab) mediate therapeutically relevant immunomodulatory effects. They have been associated with the repolarization of TAMs towards an M2-like PDL1⁺ phenotype that may limit overall ICI therapeutic activity [28, 75]. In support of this notion, accumulation of M2-like TAMs in the TME correlates with reduced sensitivity to trastuzumab in patients with *HER2*⁺ breast cancer, as well as with poor responses to cetuximab in patients with locally advanced head and neck squamous cell carcinoma [28, 76]. Conversely, multiple *EGFR*-targeting TKIs (for example, erlotinib, osimertinib and afatinib) appear to decrease PD-L1 expression by NSCLC cells [28, 77, 78], correlating with reduced amounts of M2-like TAMs and Treg cells plus increased tumour infiltration by DCs and CD8⁺ T cells in vivo [28, 79]. At least in part, these effects reflect the ability of *EGFR*-targeting TKIs to trigger secretion of CXCL10 (a chemoattractant for CD8⁺ T cells) and suppress CCL22 (a Treg cell chemoattractant) release by malignant cells [28, 80, 81]. These observations are in line with the notion that lung adenocarcinomas

bearing oncogenic EGFR mutations exhibit a cold TME characterized by abundant infiltration by Treg cells [28, 81].

VEGFA has been considered an appealing target for the development of anticancer therapeutics for decades, largely reflecting its role in key processes that involve an interaction between malignant cells and their microenvironment, such as neo-angiogenesis [28, 82]. Multiple agents that target *VEGFA* (for example, bevacizumab) and its receptor (for example, sunitinib and pazopanib) are currently licensed for use in various oncological indications [28, 82]. Reflecting the key role of *VEGFA* in immunoevasion [28, 83] agents that target this cytokine mediate therapeutically relevant immunostimulatory effects. Consistent with this notion, both bevacizumab and lenvatinib are currently approved in combination with pembrolizumab or atezolizumab (a PDL1 inhibitor) for the treatment of various solid tumours, including RCC [3]. Indeed, bevacizumab as well as the broad-spectrum TKIs sunitinib and pazopanib promote Teff cell recruitment and activation by preventing Teff cell anergy, promoting Treg cell depletion [28, 84] as well as favouring DC differentiation [28, 85] and limiting the expression of immunosuppressive molecules (including PD-L1) by malignant and myeloid cells [28, 85]. That said, chronic *VEGFA* inhibition may have deleterious consequences downstream of the abundant recruitment of immunosuppressive myeloid cells that foster Teff cell dysfunction, as documented in various tumours [86, 87]. These findings are in line with the notion that *VEGFA* signalling drives the expression of co-inhibitory receptors including PD1 by CD8+ T cells, expands Treg cells and limits DC maturation. Furthermore, the broad-spectrum TKIs regorafenib and lenvatinib inflame the TME of mouse hepatocellular carcinomas established in immunocompetent hosts by promoting the secretion of pro-inflammatory chemokines including CXCL10 [28, 88, 89]. These effects promote robust tumour infiltration by CD8+ T cells coupled to TAM depletion, ultimately enabling therapeutic synergy with PD1 blockers [28, 90]. Finally, we have found a positive correlation only between *MARCO* and cell cycle-related genes (i.e. *CCND3* and *CDK6*) (**Figure 3**), but not between *MARCO* and *VEGF*-, *EGF*- or *HER2*-related genes.

6.1. Results' validation on The Cancer Genome Atlas Kidney Renal Clear Cell Carcinoma (TCGA-KIRC)

To validate our findings in a larger patient cohort and investigate the prognostic value of *MARCO* in RCC patients, we retrieved RNA-sequencing and clinical data for RCC patients (n = 509) from The Cancer Genome Atlas Kidney Renal Clear Cell Carcinoma (TCGA-

KIRC). In line with our data, *MARCO* is highly expressed in higher stages and even more so in metastases (**Figure 4**), and the stratification of RCC patients based on the median expression of *MARCO* shows a significant correlation between high expression of *MARCO* and reduced overall survival (**Figure 5**), thus confirming its negative prognostic value in RCC. In addition, in *MARCO*+ samples we found a positive enrichment of a cell-cycle signature (KEGG cell cycle pathway: hsa04110; **Figure 6**), thus confirming a potential correlation between the deregulation of tumor cell proliferation and the presence of an immunosuppressive TME with an abundance of *MARCO*+ M2-macrophages.

7. Discussion

We have analyzed tumor samples of advanced RCC patients, with the aim to provide a characterization of TME by RNA-seq and to profile the immunophenotypes within the TME to guide prognosis and to predict and improve antitumor response mediated by ICIs. In line with previous literature, we observed the overrepresentation of TAM-related genes (i.e., *APOE*, *TREM2*, *MARCO*) and *CD274* (better known as *PD-L1*) in secondary lesions compared to primary lesions in our dataset (**Figure 1**). Interestingly, by harnessing public RNAseq and clinical data from the ccRCC cohort KIRC of TCGA among these, we confirmed that *MARCO* expression is higher in metastatic samples compared to non-metastatic samples, (**Figure 4**) and its expression is correlated to worse prognosis in advanced RCC patients (**Figure 5**). This evidence suggests a potential role of *MARCO* to predict RCC relapse. Expression of *MARCO* on TAMs indicates an anti-inflammatory pro-tumor M2-like subtype, which can be able to promote an immunosuppressive environment that inhibits the activity of cytotoxic CD8+ T cells, reducing the effectiveness of ICIs [91, 92]. Consequently, inhibition of *MARCO* is expected to remodel TAMs toward the M1-like subtype. In a mouse model of melanoma, targeting *MARCO* relieved the inhibitory effect of TAMs on NK cells, moreover, the combination of anti-*MARCO* antibody and PD-1/PD-L1 enhanced the efficacy of ICIs. In NSCLC studies, inhibiting *MARCO* or blocking IL37 to suppress *MARCO* expression restores the anti-tumor activity of NK cells and T cells [91, 93]. In prostate cancer studies, *MARCO* blockade impaired lipid accumulation in TAMs, diminished TAMs aggregation at tumor sites, and increased MHC II expression in TAMs, thereby inhibiting tumor growth and metastasis [38, 91, 92]. Anti-*MARCO* antibody also improves the antitumor effect of docetaxel in advanced prostate cancer [38, 91- 93]. Anti-*MARCO* treatment also limited the growth and metastasis of mouse breast cancer and melanoma, enhanced the immunogenicity of TME, and improved the therapeutic efficacy of

anti-CTLA4 mAbs [38, 92]. The available studies thus conclude that targeting *MARCO* can act as a potential effective approach to inhibiting TAMs' pro-tumor activity [38, 91- 93]. Regarding RCC, even if the role of TAMs in pathogenesis is well established, more research is needed to develop novel and effective diagnostic or therapeutic approaches targeting macrophages. To date, mostly traditional M2 markers (CD206, CD163) or pan-macrophage marker CD68 are used to quantify macrophages in RCC. However, usage of these markers for simple quantification of macrophages is not sufficient for prediction of clinical outcome of the disease or can be even misleading since CD68 was reported to be expressed by ccRCC cells [94, 95]. More TAM markers should be studied, selected from the spectrum of known M2 markers. Thus, investigation about the role of *MARCO* even in advanced RCC could be useful to allow the development of a novel diagnostic approach of high predictive capacity [93- 95].

Intriguingly, we found that *MARCO* expression correlates with the expression of cell cycle-related genes (**Figure 3** and **Figure 6**), and a downregulation of cell cycle genes correlates to the upregulation of genes discriminating immune effector cells (i.e., *CD79A*, *CD8A*, *CD3D*) in specimens from responder patients to ICI-based therapy (**Figure 2**). Overall, these preliminary data, revealing a potential crosstalk between deregulated cancer cell proliferation and *MARCO*⁺ macrophages in mediating disease progression and ICI resistance of advanced RCC, could lay the groundwork for novel clinical trials testing potential synergic effect of combination of CDK inhibitors and ICIs to revert primary and/or acquired resistance to immunotherapy. Indeed, the cyclin-dependent kinases CDK4 and CDK6 are involved in cell-cycle regulation with additional roles in immunogenicity and antitumour immune response, both on malignant and non-malignant components of the TME [71]. For example, in breast cancer mouse that were treated with CDK-inhibitors, the levels of M1 macrophages and cytotoxic CD8⁺ T lymphocytes infiltrating the tumor after treatment with CDK inhibitors were found to be significantly increased; these cells directly kill tumor cells during immunotherapy [96]. This finding could be the rationale to combine CDK inhibitor with ICI and macrophage target agents, with the aim to reprogram from the tumor-supporting M2 phenotype to the tumor-inhibitory M1 phenotype and improve the effectiveness of anticancer treatment. Overall, several mechanisms contribute to the good response to combination therapy with CDK4/6 inhibitors and ICIs, and much more remains to be discovered, including whether these mechanisms are cell-cycle dependent or independent. Numerous clinical trials examining the efficacy of combination therapy with CDK4/6 inhibitors and ICIs are ongoing in various cancers, including prostate, breast, and

head and neck cancer [97]. Additionally, even in RCC, CDK4/6 inhibitor-based combinations could prove very effective in RCC. A phase 2 study that are testing the combination of NKT2152 (a novel anti- HIF-2 α) with palbociclib and anti-PD1 sasanlimab in ccRCC is ongoing and recruiting (NCT05935748).

Our study has several limitations that should be underlined. Particularly, the retrospective nature and the small sample size remarkably reduce the power of statistical analysis and do not allow to draw definitive conclusions. Due to the limited number of patients, we did not distinguish patients based on the type of combination therapy (dual ICI or ICI in combination with TKI) and did not perform separate statistical analyses. A small proportion of patients had nccRCC: due to the small overall sample size, this has contributed to reducing the homogeneity of our study population. Despite its limitations, our study confirms the need for a better understanding of cancer cell-intrinsic mechanisms affecting TAM polarization in RCC that might help the identification novel predictive and druggable biomarkers of response to ICIs, as well as the design of innovative combinatorial therapeutic strategies to improve ICI efficacy and the management of certain life-threatening ir-AEs, such cardiovascular ir-AEs. Our pilot study will certainly need further investigation on a larger cohort of patients and prospectively correlate with oncological efficacy and safety outcomes. Additionally, such advanced technological approaches are being actively incorporated into RCC clinical trial designs. It is hoped that by applying high-dimensional approaches in small exploratory cohorts, potential biomarkers will be discovered, and the most promising ones will be validated in validation cohorts using more conventional (and economical) approaches. Though, given the complexity of RCC, it is likely that many biomarkers will need to be integrated to be truly useful as a predictive tool. Therefore, the field should focus systemically on discovering, validating, and integrating potential biomarkers to ultimately aid in determining treatment strategy and understanding treatment resistance. Furthermore, it is worth noting that most of what has been identified in RCC has focused on the clear cell histology, and there is much work to be done in atypical histology (or nonclear cell [nccRCC]). Similarly, multiplex immunofluorescence characterization of tumor-infiltrating CD8 T cells revealed a similar association between immunophenotype and outcome in ICI-based therapy for nccRCC. The future of biomarkers will combine a variety of multi-omics techniques, incorporating both tumor-associated and host-associated factors. In the face of ever-increasing data, deep learning models may facilitate the extraction of relevant features that can better predict clinical outcomes and safety [98].

Table 1. Selected macrophage-targeting trials*.

Trial	ClinicalTrials.gov ID	Phase		Drug	Status
A Trial of BI 765063 Monotherapy and in Combination with BI 754091 in Patients with Advanced Solid Tumours	NCT03990233	1	Solid tumors	BI 765063, a mAb Antagonist of SIRP α , as Single Agent and in Combination with BI 754091, a PD-1 mAb	Active, not recruiting
Phase 1 Study of Anti-Macrophage Migration Inhibitory Factor (Anti-MIF) Antibody in Solid Tumors	NCT01765790	1	Solid tumors	Anti-MIF Antibody	Completed
Study of Magrolimab (Hu5F9-G4) in Combination with Cetuximab in Participants with Solid Tumors and Advanced Colorectal Cancer	NCT02953782	1/ 2	Solid tumors	Magrolimab, a mAb that blocks the inhibitory CD47- SIRP α interaction. Cetuximab, a mAb against EGFR	Completed
A Study of PY314 in Subjects with Advanced Solid Tumors	NCT04691375	1	Solid tumors	PY314, a TREM2 targeting mAb	Completed
PRE-I-SPY Phase I/Ib Oncology Platform Program (PRE-I-SPY-PI)	NCT05868226	1/ 1b	Solid tumors	ALX148, CD47 Inhibitor: a fusion protein containing a high affinity engineered D1 domain of human SIRP α variant 1 (v1) genetically linked to a modified and inactive Fc domain of human Ig G1. Trastuzumab Deruxtecan, ADC: A recombinant humanized anti-human HER2 IgG1 mAb, conjugated with linker to a Topoisomerase I inhibitor	Recruiting
A Study to Evaluate Safety, Tolerability and Preliminary Efficacy of FP-1305 in Cancer Patients (MATINS)	NCT03733990	1/ 2	Solid tumors	FP-1305 (bexmarilimab), a potent humanized anti-CLEVER-1 IgG4-Ab, capable of inducing a phenotypic M2 to M1 immune switch of TAMs	Completed
"Don't Eat Me" Signal in Haematological Malignancies: CD24 as New Target to Improve Anti-cancer Immunity.	NCT05888701	Observational	MCL or B-cell CLL	anti-CD24 antibody	Recruiting
A Study of AMG 820 in Subjects with Advanced Solid tumors	NCT01444404	1	Solid tumors	AMG 830, a fully human IgG2 mAb against the CSF-1 or M-CSF receptor c-fms (or CSFR1), AMG 820 binds to and blocks c-fms, thereby blocking CSF-1 binding to its receptor and suppressing CSF-1-induced c-fms signaling. This results in the suppression of recruitment and activation of TAM within the TME.	Completed

*As of October 26, 2024. Source: clinicaltrials.gov.

Monoclonal Antibody (mAb); Programmed Death-1 (PD-1) mAb; Signal regulatory protein (SIRP α); Macrophage Migration Inhibitory Factor (MIF); epidermal growth factor receptor (EGFR); TREM2 (Triggering Receptor Expressed on Macrophages 2); immunoglobulin (Ig); Antibody-drug conjugate (ADC); Tumor-associated macrophages (TAMs); Mantle-cell Lymphoma (MCL) or B-cell Chronic Lymphocytic Leukemia (CLL); cColony-stimulating factor-1 (CSF-1 or M-CSF) receptor

Table 2. Patients' characteristics.

Patient_ID	Age	Sex	Histological report primary lesion	Nucleolar grade	TNM stage according to AJCC 8th ed.	IMDC score	IHC	Availability of primary tumor tissue sample
RCC_01	70	M	ccRCC	3	pT3a Nx	Good		Y
RCC_02	49	F	ccRCC with chromophobe features		pT1a Nx	Intermediate	CD117+, CK7+	Y
RCC_03	63	M	ccRCC	4	pT3a	Intermediate		Y
RCC_04	59	M	ccRCC	3	pT3a Nx	Good		Y
RCC_05	56	F	ccRCC with sarcomatoid features	4	pT3a	Intermediate		Y
RCC_06	70	M	ccRCC	3	pT1a	Intermediate		Y
RCC_07	83	M	ccRCC with sarcomatoid features	4	pT3a	Intermediate		Y
RCC_08	62	M	ccRCC	3	pT3a	Intermediate		Y
RCC_09	62	F	ccRCC	3	pT3a Nx	Good	CD10+, CAIX+, CK7-, 34betaE12-, Racemase-	Y
RCC_10	68	M	ccRCC	3	pT3a Nx	Good		Y
RCC_11	57	M	ccRCC	3	pT3a pN0	Intermediate	CD10+, CAIX+, K CATHEPSIN -, HMB45-, TFE3-conservation of FH	Y
RCC_12	47	F	ccRCC	3	pT3a Nx	Poor	PAX8+,K CATHEPSIN -, MART1-, HMB45-, TFE3-	Y

M: male; F: female; ccRCC: clear cell renal cell carcinoma; American Joint Committee on Cancer: AJCC; IMDC: International Metastatic RCC Database Consortium; Immunohistochemistry: IHC; Fumarate hydratase: FH; Y: Yes; NA: not available

Table 3: Metastatic disease's characteristics.

Patient_ID	Site of metastasis	Timing	Number of metastases	Histologically confirmed stage IV	Site of biopsy	Availability of FFPE
RCC_01	Peritoneum, renal lodge	Metachronous	Multiple	Y	Peritoneum	Y
RCC_02	Liver	Metachronous	Multiple	Y	Liver	Y
RCC_03	Lung, thoracic lymph nodes	Synchronous	Multiple	N		N
RCC_04	Lung	Metachronous	Multiple	N		N
RCC_05	Bone, lung	Synchronous	Multiple	Y	Bone	Y
RCC_06	Soft tissue, renal lodge, thoracic lymph nodes	Metachronous	Multiple	N		N
RCC_07	Lung, renal lodge, abdominal lymph nodes, brain	Metachronous	Multiple	N		N
RCC_08	Lung, thoracic and abdominal lymph nodes	Synchronous	Multiple	N		N
RCC_09	Lung, renal lodge, thoracic lymph node, pancreas	Metachronous	Multiple	N		N
RCC_10	Soft tissue, lung	Metachronous	Multiple	Y	Soft tissue	Y
RCC_11	Abdominal lymph nodes	Synchronous	Multiple	Y	Lymph node	Y
RCC_12	Renal lodge, peritoneum, soft tissue	Synchronous	Multiple	Y	Peritoneum	Y

Y: yes; N: no

Table 4. Treatments' characteristics.

Patient_ID	1st line- treatment	Ir-AEs	IR-AEs' grade according to CTCAE V.5.0	BOR according to RECIST 1.1 criteria
RCC_01	Pembrolizumab 200 mg every 3 weeks with axitinib 5 mg twice daily	Arthralgias	Grade 2	PR
RCC_02	Pembrolizumab 200 mg every 3 weeks with axitinib 5 mg twice daily	Hypothyroidism	Grade 2	SD
RCC_03	Pembrolizumab 200 mg every 3 weeks with axitinib 5 mg twice daily	Diarrhoea	Grade 3	SD
		Transaminitis	Grade 4	
RCC_04	Pembrolizumab 200 mg every 3 weeks with axitinib 5 mg twice daily			SD
RCC_05	Pembrolizumab 200 mg every 3 weeks with axitinib 5 mg twice daily	Arthralgias	Grade 2	SD
RCC_06	Pembrolizumab 200 mg every 3 weeks with axitinib 5 mg twice daily			CR
RCC_07	Nivolumab 3 mg/Kg every 2 weeks			SD
RCC_08	Pembrolizumab 200 mg every 3 weeks with axitinib 5 mg twice daily	Diarrhoea	Grade 3	SD
RCC_09	Pembrolizumab 200 mg every 3 weeks with axitinib 5 mg twice daily	Cutaneous Toxicity	Grade 2	SD
		Hypothyroidism	Grade 2	
RCC_10	Pembrolizumab 200 mg every 3 weeks with axitinib 5 mg twice daily			SD
RCC_11	Nivolumab 3 mg/kg combined with ipilimumab 1 mg/kg every 3 weeks for 4 doses, followed by nivolumab 3 mg/kg every 2 weeks	Hypothyroidism	Grade 2	CR
		Diarrhoea	Grade 3	
RCC_12	Nivolumab 3 mg/kg combined with ipilimumab 1 mg/kg every 3 weeks for 4 doses, followed by nivolumab 3 mg/kg every 2 weeks			PD

IrAEs: immune-related adverse events; BOR: best overall response; CTCAE: Common Terminology Criteria for Adverse Events; RECIST v 1.1: Response Evaluation Criteria in Solid Tumors; SD: stable disease; PR: partial response; CR: complete response; PD: progressive disease

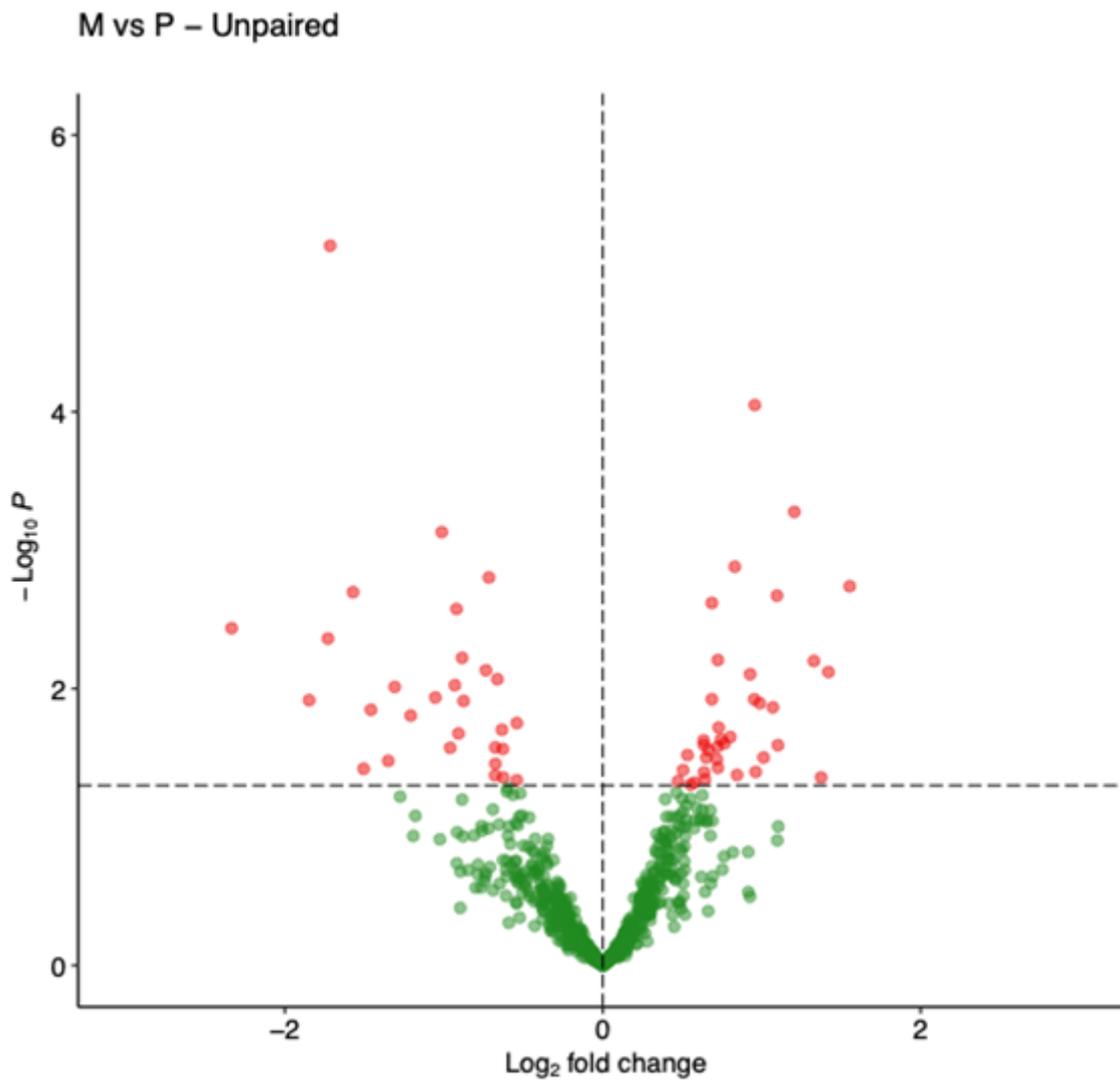


Figure 1. Volcano plot of differentially expressed genes (DEGs) between $n = 6$ secondary (S) and $n = 10$ primary (P) lesions obtained from advanced/metastatic RCC patient treated with first and subsequent line(s) with α PD-1-based combination treatment (i.e., pembrolizumab or nivolumab) \pm VEGF-TKI (i.e., axitinib or cabozantinib) or α CTLA-4 mAb (i.e., ipilimumab) according to ESMO guidelines [ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2021 Mar; ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. Ann Oncol. 2024 Aug]. Patients were enrolled, treated and followed-up at the Oncology Unit (directed by Prof. Antonuzzo) of the Azienda Ospedaliera Universitaria Careggi-Hospital in Florence (AOUC cohort). All samples were collected prior treatment. RNA was extracted from FFPE specimens and hybridized to nCounter probes for the 770 predefined genes of the PanCancer IO360 Panel. The horizontal dotted line represents a Wald test significance level of 0.05. Genes with a $\text{Log}_2(\text{fold-change}) > 0$ were overexpressed in S lesions, and those with values < 0 were under-expressed in S lesions.

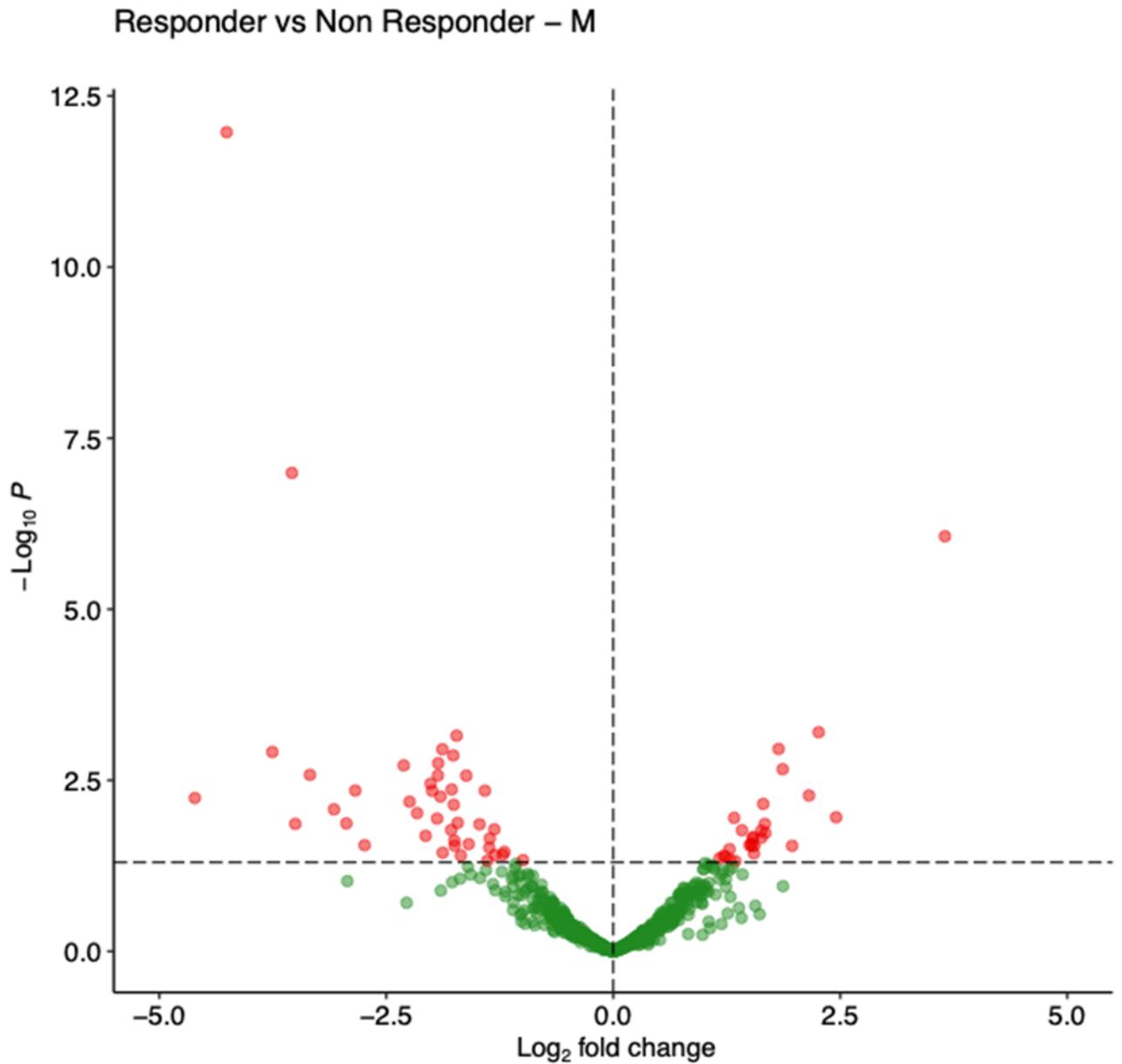


Figure 2. Volcano plot of DEGs between $n = 2$ responder (R) and $n = 4$ non responder (NR) in the S subset of our AOUC cohort. Response was evaluated following RECISTv1.1 criteria after 12 weeks from the start of the treatment. All samples were collected prior ICI treatment. The horizontal dotted line represents a Wald test significance level of 0.05. Genes with a $\text{Log}_2(\text{fold-change}) > 0$ were overexpressed in R samples, and those with values < 0 were under-expressed in R samples.

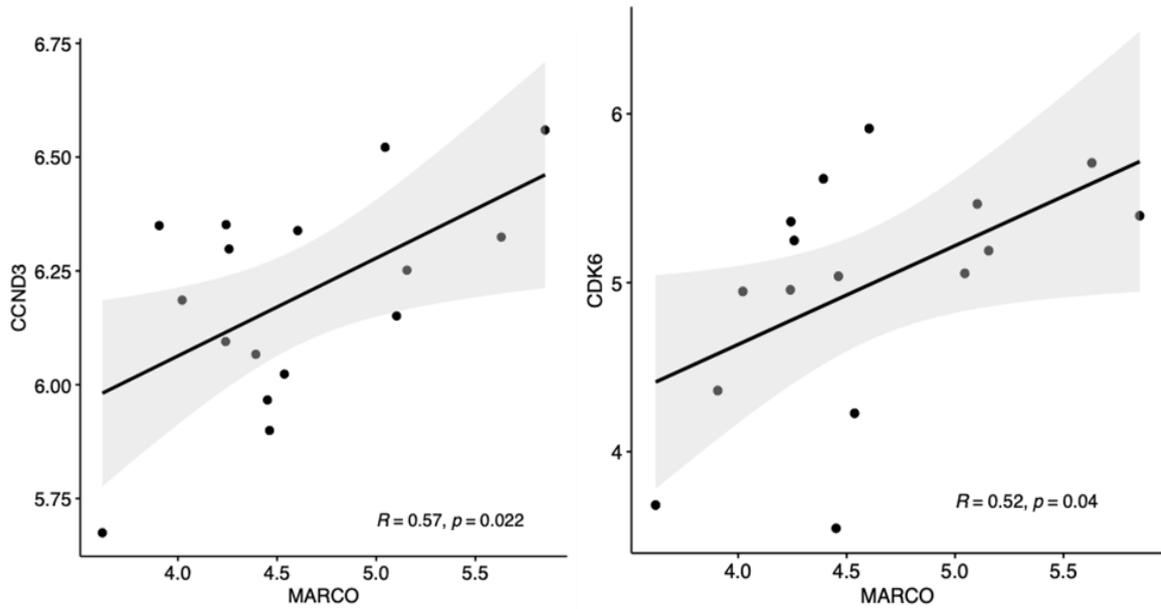


Figure 3. Scatter plots showing the correlation between *MARCO* expression level and *CCND3* (left) and *CDK6* (right) expression levels in S and P samples from our AOUC cohort. Pearson correlation coefficients (R) and p-values are reported (n = 16).

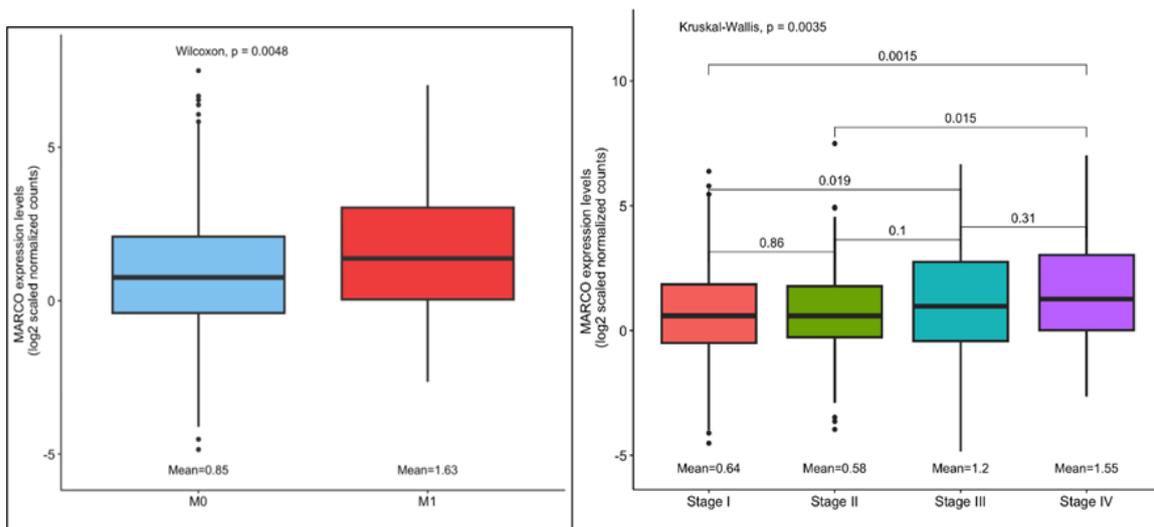


Figure 4. Boxplot showing the Log₂-scaled normalized expression levels of *MARCO* in n=79 metastatic (M1) versus n=430 non metastatic (M0) samples in The Cancer Genome Atlas Kidney Renal Clear Cell Carcinoma, TCGA-KIRC cohort. The comparison between the expression levels in the two groups was carried out with a Wilcoxon test.

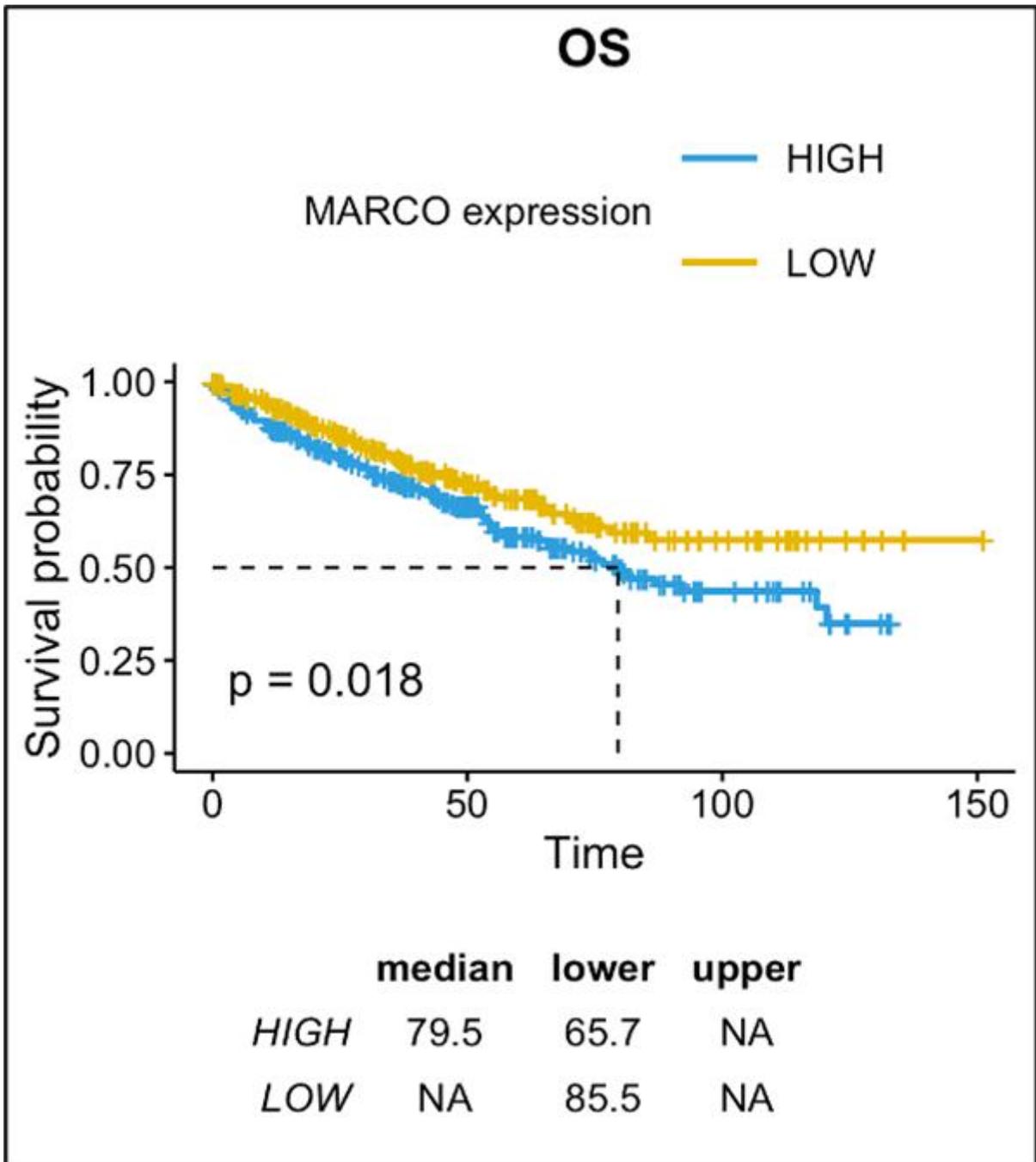


Figure 5. Survival plot showing Kaplan–Meier estimates of Overall Survival (OS) between $n = 260$ $MARCO^{HIGH}$ vs $n = 249$ $MARCO^{LOW}$ TCGA-KIRC samples. OS was calculated as the time from diagnosis to death by any cause or last follow-up. Significance was estimated with a log-rank test.

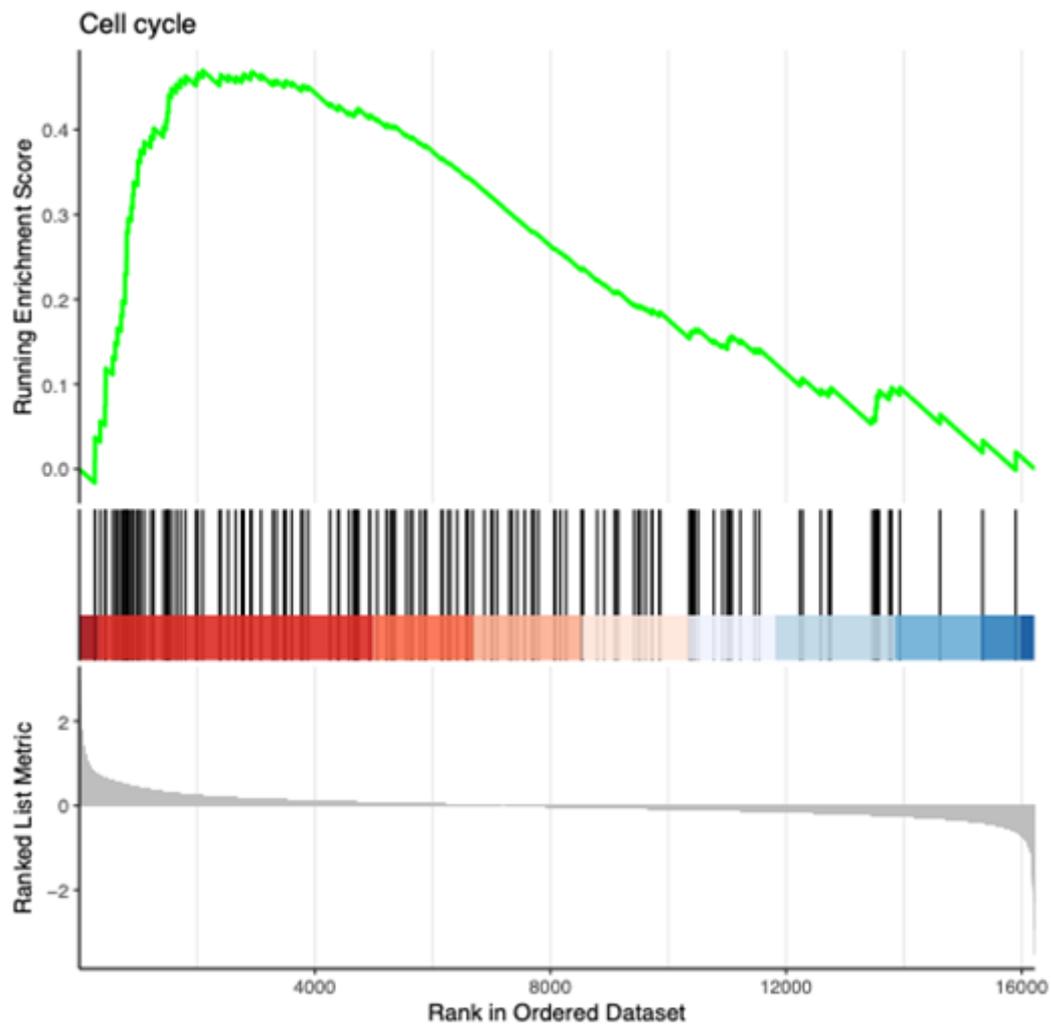


Figure 6. Enrichment plot for the set of KEGG cell cycle pathway (hsa04110) genes in the $n = 260$ $MARCO^{HIGH}$ vs $n = 249$ $MARCO^{LOW}$ TCGA-KIRC samples by GSEA. Adjusted p-values were determined by Bonferroni correction of the GSEA permutation test. The positive Normalized Enrichment Score (NES) indicates the pathway upregulation in $MARCO^{HIGH}$ samples.

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