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mRNA expression, tumor heterogeneity, and response to therapy in patients with advanced renal cell carcinoma treated with immune-based combinations (ARON-1α)

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ABSTRACT

Background: Renal Cell Carcinoma (RCC) represents a spectrum of tumors, characterized by heterogeneous growth patterns, histology and response to immune-based combinations.

Objectives: The aim of the present retrospective analysis was to investigate the mRNA expression of 32 genes associated with RCC carcinogenesis and their potential involvement in patients treated with first-line immune-based combination therapies. Additionally, we examined the role of tumor heterogeneity by comparing mRNA expression levels between primary renal tumors and metastatic sites in a group of patients included in the ARON-1 study.

Patients and methods: The study included patients with advanced RCC treated with first-line immune-based therapies. Total RNA was extracted from fixed paraffin-embedded tissue slices using the RNeasy FFPE Mini Kit. Quantitative RT-PCR was performed using the IQ5 Multicolor real-time PCR detection system. Coefficient of variations were calculated for each gene and compared between primary and metastatic samples.

Results: 17 patients were included in this analysis; 9 of them had both primary and metastatic samples available. Three of the 4 patients showing the highest mRNA expression levels of the 32 analyzed genes reported complete remissions, while 2 of the 3 patients with the lowest expression levels were primary refractory to first-line therapy. As for tumor heterogeneity, *VEGFA* was the only gene significantly deregulated in the paired comparison.

Conclusions: We showed differences in mRNA expression between primary and metastatic sites, and proposed a possible link to the response to first-line immune combination therapies. Additional research is required to clarify their potential as prognostic or predictive biomarkers.

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1. Introduction

Kidney cancer is one of the ten most frequently occurring cancers in the Western world, with clear cell renal cell carcinoma (ccRCC) being the most prevalent histological subtype [1]. Metastatic spread of RCC may involve several districts and organs such as lung, bone, liver, lymph node, brain and soft tissues. Synchronous metastatic lesions occur in 25 %–30 % of patients, whereas approximately 30 % of patients after curative kidney surgery develop metastases [2,3].

Recently, the first-line treatment of metastatic RCC (mRCC) has been fundamentally transformed by the introduction of immunotherapy-based combinations, which have enhanced both survival, as well as quality of life, of mRCC patients [4]. Heterogeneous responses to immune-based combinations result from the distinct mechanisms of action of immune-checkpoint inhibitors (ICIs) and tyrosine kinase inhibitors (TKIs) and the interplay between tumor cells and the different microenvironments in the primary and metastatic sites.

Although RCC molecular profiling has revealed a variety of genetic alterations and identified recurrent loss-of-function mutations in several genes (e.g., *VHL*, *PBRM1*, *BAP1*, and *SETD2*), only *VHL* has sufficient evidence to be considered a driver for RCC. The role of other alterations remains to be clarified [5–7].

In 2012, Marco Gerlinger et al. [8] examined intratumor heterogeneity by exome sequencing, chromosome aberration analysis and ploidy profiling on samples from RCC primary tumors and matched metastatic sites. They showed the complexity of RCC genomic landscape, characterized by mutational intratumor heterogeneity and by different gene signatures associated with good or poor prognosis within the same tumor, supporting Darwinian selection as a key player in tumor adaptation and resistance to therapy. At present, how intratumor heterogeneity may influence the response to immunotherapy in RCC is still unclear.

The research for predictive and prognostic factors in RCC patients receiving immunotherapy is one of the major challenges. The possibility to investigate for the presence of predictive and prognostic factors in this context is one of the aim of the ARON-1 real world study. ARON-1 (NCT05287464) is a part of the ARON Project (www.aronwg.com) and involves analyses of real-world data of patients with metastatic RCC from multiple Centers around the world [9–17]. In this sub-analysis, we led an exploratory analysis of the mRNA expression of 32 genes involved in RCC carcinogenesis and their potential role in patients treated with first-line immune combinations. Furthermore, we analyzed the potential role of tumor heterogeneity in terms of mRNA expression between primary renal tumors and metastatic sites in a series of patients included in the ARON-1 dataset.

2. Patients and methods

2.1. Study population

We retrospectively collected data from patients aged ≥18 years with a histologically confirmed diagnosis of RCC and histologically or radiologically confirmed metastatic disease. In this study, patients from 6 RCC referral centers located in Italy, Finland and Spain, treated with first-line immune-oncology (IO) + IO or IO + TKI immune combinations from January 1st' 2016 to September 1st' 2024 were included. We considered patients from all three International mRCC Database Consortium (IMDC) risk groups (favorable, intermediate and poor).

We retrospectively analyzed data about age, gender, histology, IMDC risk group, nephrectomy status, sites of metastases, immune-combinations received and response to treatment from patients' electronic and paper charts. Patients with inadequate data on tumor assessment or response to therapy were excluded from the analysis.

First-line therapy was continued until the evidence of clinical and/or radiological tumor progression, unacceptable toxicities, or death. Computed tomography (CT) or magnetic resonance imaging (MRI) scans were carried out following standard local procedures every 8 or 12 weeks, while physical examination and laboratory tests were performed every 4- or 6-weeks during patients' follow-up, depending on the treatment received.

2.2. Study endpoints

The primary objective of this exploratory analysis was to assess the relationship between the mRNA expression of a series of 32 selected genes and the real-world effectiveness of first-line immune-combinations. Tumor radiological assessment was conducted according to RECIST 1.1 [18] and designated as complete [CR] or partial responses [PR], stable [SD] or progressive disease [PD].

2.3. Ethical considerations

The "ARON-1" project was approved by the ethics committee of the Marche Region (2021-492) and was performed in accordance with the Declaration of Helsinki. Due to the retrospective nature of the study, informed consent was not required.

2.4. Statistical analysis

The statistical analysis was performed by MedCalc version 19.6.4 (MedCalc Software, Broekstraat 52, 9030 Mariakerke, Belgium).

Fisher's exact test was employed for comparing binary categorical variables, while chi-square tests were used for multiple group comparisons. Pearson's correlation coefficient was used to assess associations between variables. Significance levels were set at a value of 0.05, and all *p* values were two-sided.

To perform the analysis of heterogeneity of expression the coefficient of variation for each gene was calculated through the following formula:

$$CV = \frac{standard\ deviation}{mean} * 100$$

CVs of primary and metastatic gene expression were statistically compared.

A linear correlation between gene expression values from primary and metastatic tumor samples was analyzed by the Pearson correlation coefficient. The heatmap, after scaling data as z-score by row, was built up through "ComplexHeatmap" R package. Wilcoxon test, chi-square test and correlation analyses were performed with "ggpubbr" R package. All the analyses were performed in R version 4.4.1.

2.5. Analysis of mRNA expression

Total RNA was extracted from fixed paraffin-embedded tissue slices (5–7 μm thick) using the RNeasy® FFPE Mini Kit (Qiagen, Milan, Italy). All RNA samples were eluted in the appropriate buffer, and their concentration and purity were assessed through $A_{260/280}$ nm measurements. RNA was subjected to reverse transcription in a total volume of 20 μL, employing the High-capacity cDNA reverse transcription kit (Applied Biosystem, Whaltham, Massachusetts, USA) according to the manufacturer's guidelines. Subsequently, cDNA was utilized as a template for quantitative real-time polymerase chain reaction (qRT-PCR).

Quantitative RT-PCR was conducted using the IQ5 Multicolor real-time PCR detection system (Bio-Rad Labs., Hercules, CA, USA). The reaction mixture included the TaqMan™ Universal PCR Master Mix (Applied Biosystem). The PCR conditions consisted of an initial step at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All samples were analyzed in triplicate on the same plate. The relative quantity of target mRNA was determined using the $2^{-\Delta\Delta Ct}$ method, with 18S serving as the housekeeping gene.

A total of 32 genes were analyzed for mRNA expression, the list of genes is available on supplementary materials (Table S1). The functional classification of the 32 genes in resported in Table S2. The choice of the

32 genes came from the results obtained by Motzer et al. [19] and Rini et al. [20] who tested the correlation between gene expression and the response to axitinib plus avelumab and atezolizumab plus bevacizumab, respectively, in mRCC patients.

The potential interactions among the 32 selected genes was realized by genemania network (www.genemania.org) as was illustrated in Fig. S1, while Fig. S2 showed the Venn diagram of genes by functional category.

3. Results

3.1. Study population

This exploratory analysis included 17 matched cases. Nine patients had both primary and metastatic samples available. Patients' selection process from the ARON-1 study is reported in Fig. S3.

Eleven males and 6 females were included. Six patients presented with metastatic disease at diagnosis, with clear cell histology being the most prevalent subtype (16/17, Table 1); the most common metastatic site was the lung (77 %). Good, intermediate and poor IMDC features were present in 2, 13 and 2 cases, respectively. The clinicopathological characteristics of included patients are summarized in Table 1.

Sixteen patients (94 %) underwent nephrectomy, with no successive adjuvant therapy. Eight patients received the combination of nivolumab plus ipilimumab as first-line therapy, 7 pembrolizumab plus axitinib, 1 pembrolizumab plus Lenvatinib and 1 nivolumab plus cabozantinib.

3.2. Intratumor heterogeneity and response to therapy

We firstly compared the CVs of primary and metastatic gene expression in the 9 patients with matched samples. It can be easily observed that there is no variation in gene expression between metastasis and primary samples (Fig. 1A and B). This result was confirmed by performing correlation analysis in terms of statistical significance. However, it can be further observed that there are genes with strong Pearson correlation values (Fig. S4).

Of note, we found that *VEGFA* was the only gene significantly deregulated in the paired comparison (Fig. 2).

Results on the potential correlation between *VEGFA* heterogeneity and CR + PR vs PD responses are shown in Fig. 3. It can be observed that 5 out 8 patients with high expression level of *VEGFA* in primary tumor showed CR + PR. The association between response and *VEGFA* expression is inverted in metastatic sample with 4 out 8 patients with low *VEGFA* expression showing CR + PR.

Table 1
Characteristics of patients.

Variables	Patient (N = 17)
Gender, N (%)	
Male	11 (65)
Female	6 (35)
Current or former Smoker	
Yes	7 (41)
Not	10 (59)
Surgery	
Yes	16 (94)
Not	1 (6)
Histology	
clear cell	16 (94)
Papillary	1 (6)
Sarcomatoid de-differentiation	
Yes	4 (24)
No	13 (76)
IMDC, N (%)	
Favorable	2 (12)
Intermediate	13 (77)
Poor	2 (11)
Metastatic at diagnosis	
Yes	6 (35)
Not	11 (65)
Metastatic sites, N (%)	
Lung	13 (77)
Lymph node	6 (35)
Liver	3 (18)
Bone	4 (24)
Brain	0 (0)
Tissue	
Only primary tumor	8 (47)
Primary tumor and metastasis	9 (53)

3.3. mRNA expression from primary tumor samples and response to therapy

The mRNA expression of the 32 selected genes in the 17 samples from RCC primary tumors was illustrated in Fig. 1B.

We further investigated the correlation between mRNA expression levels and tumor response to first-line immune combination therapy according to RECIST 1.1. Patients were divided into two groups according to whether the mRNA expression value of each gene was higher or lower than the median expression value (high expression group in green, low expression group in red). In each group, we used the lightest colors to identify the CR subgroup and progressively darker colors to indicate PR, SD and PD subgroups (Fig. 4). We also reported in the yellow column the type of first-line therapy. Three of the 4 patients showing the highest mRNA expression levels reported CR, while we registered SD in patient 14. Otherwise, 2 (n. 4 and 8) of the 3 patients with the lowest expression levels were primary refractory to first-line therapy (Fig. 4).

4. Discussion

Renal cell carcinoma (RCC) is a heterogeneous group of malignancies of the kidney characterized by distinct genetic and epigenetic alterations [3,6]. Advances in genomic technologies, such as next-generation sequencing (NGS), have significantly enhanced our understanding of the molecular landscape of RCC. Ongoing research continues to uncover new biomarkers and therapeutic targets, paving the way for advancements in RCC management [21].

Key genetic mutations associated with RCC include alterations in the *VHL* gene. The loss of *VHL* function leads to the stabilization of hypoxia-inducible factors (HIFs), promoting angiogenesis and tumor growth. Other notable mutations involve genes such as *PBRM1*, *SETD2*, and *BAP1*, which play roles in chromatin remodeling and DNA repair. Notably enough, all the above genes are located on the short arm of chromosome 3, so that RCC has been also called the "disease of chromosome 3" [22]. The presence of these mutations can influence prognosis and response to therapy, underscoring the importance of personalized medicine in RCC management [5,6,23].

Recent studies have also explored the role of the tumor microenvironment and immune evasion mechanisms, providing insights into potential therapeutic targets [24,25]. The use of immune checkpoint inhibitors has revolutionized the treatment landscape, highlighting the relevance of genomic profiling in identifying patients who may benefit from these therapies.

Cancer originates from a single precursor somatic cell that accumulates cancer-causing mutations over time, making it a clonal disease. Despite this common origin, the cells within a tumor often display a range of genetic and non-genetic differences—a phenomenon known as intratumor heterogeneity [26]. Tumor heterogeneity impacts therapy by either directly altering therapeutic targets or modifying the tumor microenvironment (TME) through changes in gene expression and cell characteristics, which can drive drug resistance. As tumors grow and progress, this heterogeneity shifts over time and across different regions, continually reshaping the TME [27]. In our study, we observed no significant differences in terms of CV in the gene expression levels of primary tumors and matched metastases from patients treated with

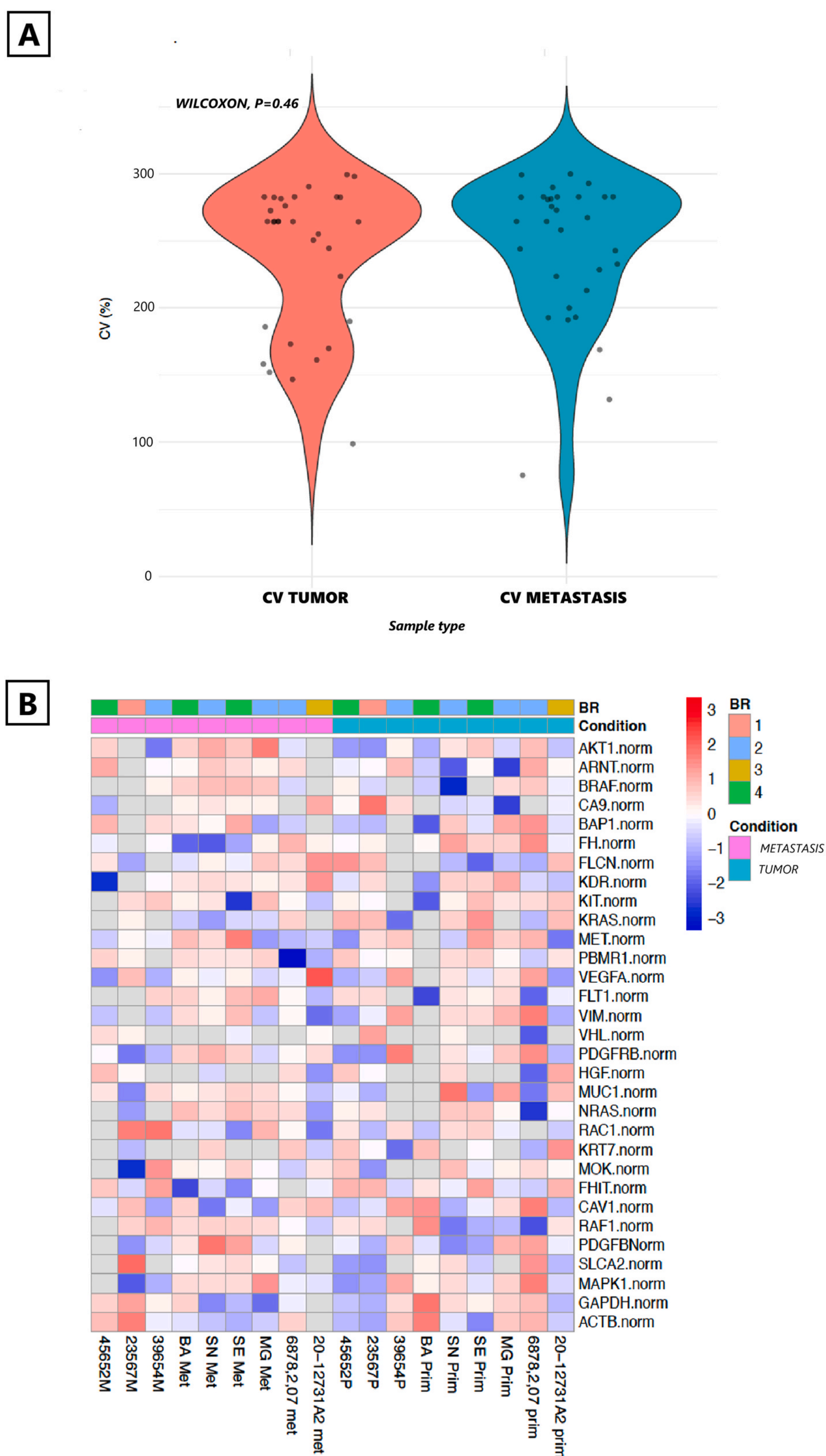

Fig. 1. Coefficient of variation analysis (A) and heatmap depicting gene expression of paired samples (B) showing no significant variations in gene expression between metastasis and primary samples.

first-line immune combinations. We found that *VEGFA* was the only gene significantly deregulated in the paired comparison, being overexpressed in primary tumors. It has been shown that high *VEGFA* expression is associated with shorter OS in RCC patients [28], while it doesn't seem to be correlated with response to anti-VEGFR TKIs [29].

VEGFA overexpression in RCC is largely driven by activation of the hypoxia-inducible factor (HIF) pathway, secondary to VHL loss, a hallmark of clear cell RCC [*Kaelin WG Jr. The von Hippel–Lindau tumour*

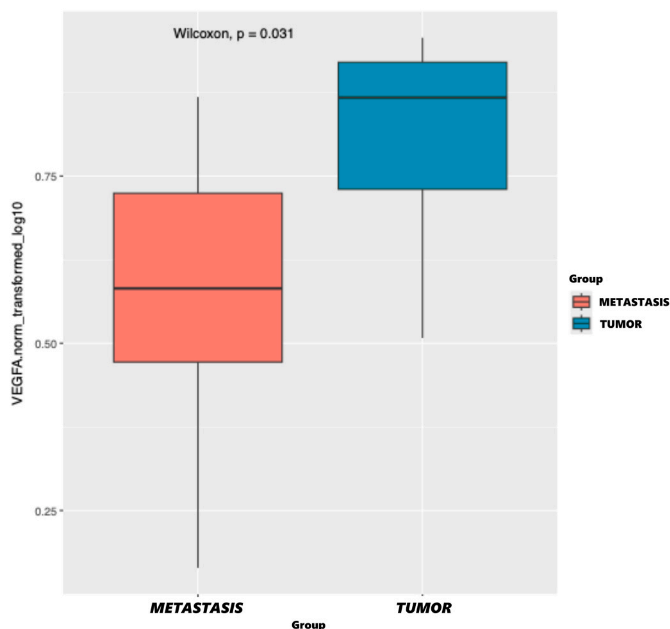

Fig. 2. Comparison of VEGFA expression between primary and paired metastatic samples.

suppressor protein: O2 sensing and cancer. Nat Rev Cancer. 2008;8 (11):865–73]. In addition to hypoxia, epigenetic mechanisms—such as promoter methylation and histone modification—may further regulate VEGFA transcription [*Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med. 2003;9(6):677–84; Zhang J* et al. *Epigenetic regulation of VEGF-A expression in cancer. Epigenomics. 2017;9 (3):363–72*]. The functional classification of the 32 genes analyzed in our study, detailed in Supplementary Table (???)/Figure (???), reflects their involvement in angiogenesis, immune modulation, and RCC development through pathways such as PI3K/AKT and extracellular matrix remodeling. Moreover, VEGFA contributes to shaping the TME by impairing dendritic cell maturation, inhibiting T cell infiltration, and promoting an immunosuppressive milieu that facilitates tumor immune evasion [*Voron T* et al. *VEGF-A modulates immune cell functions in the tumor microenvironment. Front Immunol. 2015;6:427; Gabrilovich DI* et al. *Mechanisms of immune evasion by tumors and role of VEGF in the inhibition of dendritic cell differentiation. Nat Med. 1996;2(10):1096–103*]. These observations support the rationale for therapeutic strategies that target both angiogenesis and immune checkpoints in RCC.

The potential correlation between gene profiling and response to immune combination therapies was further investigated. Data illustrated in Fig. 3 seem to suggest the possibility to identify patients' profiles associated with CR or PD, characterized respectively by a diffuse high and low expression of the genes evaluated in our panel, while patients showing PR or SD as best responses seem to have mixed profiles, suggesting that additional molecular or immune markers should be necessary to characterize and predict these responses to therapy. The possibility to identify patients' profiles associated with response to immune combination therapies would be a major step forward in the management of mRCC patients, thus influencing clinicians' choices and improving the cost-effectiveness of first-line therapies.

This study has some limitations that warrant consideration. First, the relatively small sample size may introduce bias and limit the statistical power of the analyses. The observed association between gene expression and therapeutic efficacy, therefore, should be interpreted with caution. Nevertheless, exploratory studies with similarly small cohorts have been previously conducted to identify molecular predictors of response to VEGF-targeted therapies in RCC, providing methodological support for our approach [*Choueiri TK* et al. *Correlation of gene expression signatures with response to VEGF-targeted therapy in metastatic renal cell carcinoma. J Clin Oncol. 2010;28(13):2292–9*]. Notwithstanding, we acknowledge the need for validation in larger, independent cohorts. Accordingly, future studies with adequately powered sample sizes will be necessary to confirm our findings and further elucidate the clinical significance of VEGFA expression.

In summary, the genomics of renal cancer is crucial for understanding its pathogenesis, improving diagnostic accuracy, and tailoring treatment strategies. Despite several limitations, including the small sample size and the retrospective exploratory nature of the data, we demonstrated differential mRNA expressions between primary and metastatic sites and suggested a potential correlation with the response to first-line immune combinations. Further studies with larger cohorts are needed to elucidate and potentially validate their potential roles as

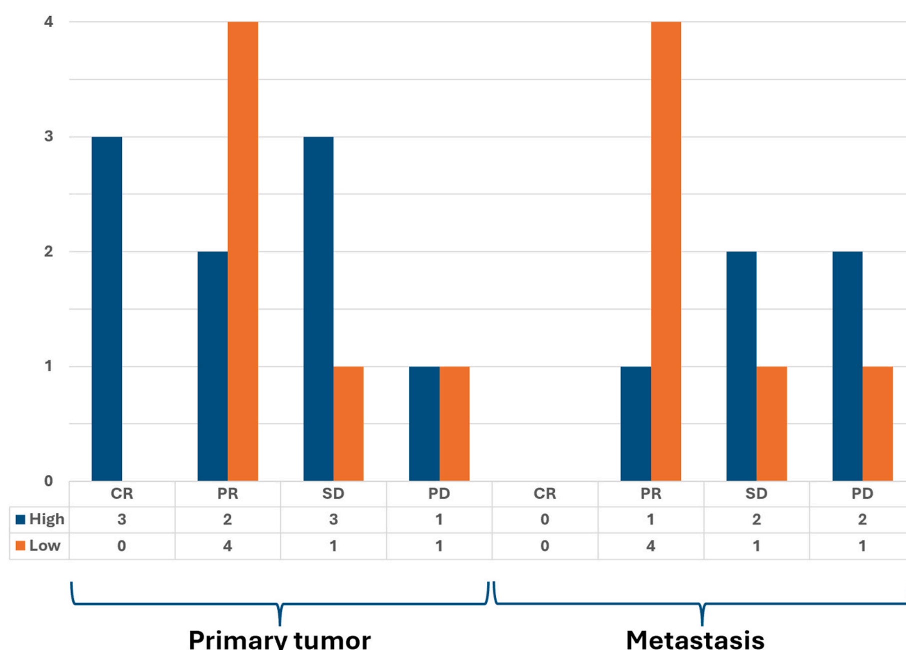

Fig. 3. Barplot showing relationship between VEGFA expression in primary and metastatic samples with respect to response.

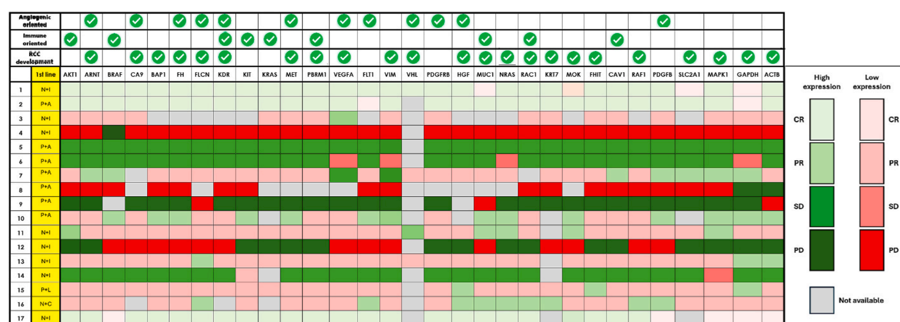

Fig. 4. mRNA expression and response to first-line immune combination therapy according to RECIST 1.1. in patients with advanced RCC from the ARON-1 study. CR = Complete remission; PR = Partial response; SD = stable disease; PD = Progressive disease; A = axitinib; C = cabozantinib; I = ipilimumab; L = Lenvatinib; N = nivolumab; P = pembrolizumab.

prognostic or predictive biomarkers.

Ethics approval

The “ARON-1” project was approved by the ethics committee of the Marche Region (2021-492) and was performed in accordance with the Declaration of Helsinki. Due to the retrospective nature of the study, informed consent was not required.

Author contributions

Cristina Aguzzi: laboratory analysis.
Simona de Summa: biostatistical analysis, manuscript preparation.
Javier Molina-Cerrillo: data and samples collection.
Teresa Alonso-Gordoa: data collection.
Massimo Nabissi: laboratory analysis and final review.
Mimma Rizzo: data and samples collection.
Annalisa Zeppellini: data and samples collection.
Kaisa Sunela: data and samples collection.
Giulia Sorgentoni: study coordinator for datamanagement
Cinzia Ortega: data and samples collection.
Francesco Massari: data and samples collection.
Fernando Sabino Marques Monteiro: manuscript preparation.
Nicola Battelli: final review.
Camillo Porta: final review.
Giorgio Santoni: manuscript preparation and final review.
Matteo Santoni: study design, statistical analysis, manuscript preparation.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2025.102162.

Data availability

Data will be made available on request.

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