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Harnessing Tumor Angiogenesis to Explore Ovarian Cancer Immune Suppression and Address Target-Therapies Outcomes

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To my Mother,
who deeply shaped the blessed woman I am today.

TABLE OF CONTENTS

- **SYNOPSIS……………………………………………………….…..pag.4**
    - **ABSTRACT (ENGLISH)……………………………….…..pag.4**
    - **ABSTRACT (DEUTSCH)…………………………..….pag.5**
    - **INTRODUCTION ………………..……………...….pag.7**
    - **RESEARCH AIMS……………………………………….…..pag.8**
        - **Ruscito et al., Br J Cancer. 2018**
            - **MATTER AND METHODOLOGY………….….pag.9**
            - **RESULTS…………………………..…………...pag.11**
        - **Ruscito et al., J Clin Med. 2019**
            - **MATTER AND METHODOLOGY…………....pag.13**
            - **RESULTS…………………………...……….pag. 15**
        - **Zizzari et al., Cancer Immunol Res. 2018**
            - **MATTER AND METHODOLOGY…………....pag.19**
            - **RESULTS…………………………….……..….pag.21**
    - **INTEGRATED DISCUSSION ……………..…………pag.26**
    - **BIBLIOGRAPHY………………………………….…....pag. 31**
- **STATUTORY DECLARATION…………………....……...pag. 38**
- **COPIES OF THE SELECTED PUBLICATIONS……….....pag. 40**
- **CV………………………………………………...…….....pag. 41**
- **COMPLETE LIST OF PUBLICATIONS…………….….....pag.41**
- **ACKNOWLEDGEMENTS……………………………….…….pag.46**
- **CONFLICT OF INTEREST DISCLOSURE…………..……pag.46**

SYNOPSIS

ABSTRACT (ENGLISH)

In 2020, ovarian cancer still remains the "Biggest Enemy" faced by Gynecology Oncologists, due to the lack of biological and clinical tools for early diagnosis, high recurrence rates despite the recent introduction of targeted-therapies for the management of advanced disease, and 5-year overall survival below 40%.

In particular, high grade serous ovarian cancer (HGSOC), recently classified as a Type II ovarian carcinoma, still accounts alone for the 80% of all ovarian cancer deaths.

In light of these numbers and to the urgent need to reduce mortality from high grade serous ovarian cancer, in 2015 an international group of opinion leaders summarized a comprehensive list of priority issues which are still considered as "unmet needs" in the understanding of high grade serous ovarian cancer. Among these issues, the Authors identify the following biological landscapes to be explored for HGSOC patients's future prognosis change:

1) to exploit HGSOC patients' immune response and interaction between host immune system and tumor microenvironment;

2) to analyse recurrent and end-stage disease samples, in order to shed light on acquired resistance mechanisms;

3) to integrate all –omics data on individual samples with immune and other tumor microenvironment components in primary and recurrent samples.

As a consequence, the research work carried out during this international PhD programme, and here discussed, aimed to add answers to these universally recognized priority issues in ovarian cancer understanding.

In particular, the leading research line developed and carried out aimed to explore the modulation of tumor-derived neoangiogenesis during ovarian cancer progression and the role of anti-angiogenetic agents in reverting tumor immune suppressive circuit thus triggering host's anti-tumoral immunological response.

The 3 research studies here presented* were both carried out in the context of the "OCTIPS Consortium", a 7th Framework Program research Project, funded by the European Commission in 2012, which currently holds in its biobank at Charité Medical University (Berlin, Germany) one of the largest European collection of paired primary and recurrent ovarian cancers samples; and in the context of the "Laboratory of Tumor Immunology and

Cell therapy Unit" directed by Prof. Marianna Nuti, at Sapienza University (Rome, Italy).

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* List of selected publications included into the PhD Thesis:

1) Ruscito I. et al. *Characterisation of tumor microvessel density during progression of high-grade serous ovarian cancer: clinico-pathological impact. An OCTIPS Consortium study.* British Journal of Cancer 2018;119(3):330-338.

IF 2019: 5.791

2) Napoletano C§ & Ruscito I§ et al. *Bevacizumab-Based Chemotherapy Triggers Immunological Effects in Responding Multi-Treated Recurrent Ovarian Cancer Patients by Favoring the Recruitment of Effector T Cell Subsets.* J Clin Med. 2019;8(3). pii: E380. doi: 10.3390/jcm8030380.

§ Equally contributed

IF 2019: 3.303

3) Zizzari IG et al. *TK Inhibitor Pazopanib Primes DCs by Downregulation of the β-Catenin Pathway.* Cancer Immunol Res. 2018 Jun;6(6):711-722.

IF 2019: 8.728

ABSTRACT (DEUTSCH)

Im Jahr 2020, bleibt der Ovarialkarzinom noch der schlimmste der gyneco-onkologische Erkrankungen wegen diesen Gruenden:

1. Fehlen einer wirksahmen screeing Methode
2. hohe Rezidivrate, trozt der neuen "Target therapies" fuer die fortgeschrittenen Stadien dieser Krankheit
3. kurzes 5-Jahren gesamt-Ueberleben (<40%)

Bei dem fortgeschrittenen seroesen Eierstockkrebs (high grade serous ovarian cancer, HGSOC), kuerzlich genauso bekannt wie "Ovarialkarzinoma Typ II", liegt die Sterblichkeit bei 80% unter allen Todesfaelle vom Ovarialkarzinom.

Im Jahr 2015, ist eine Liste von unbeantworteten Schwerpunkten in Ovarialkarzinoma von einer Internationalen Expertengruppe erstellt worden.

Zu diesem Ziel, konzentrieren sich die Autoren auf den biologischen Hintergrund von HGSOC, wie folgt:

1) Beziehung zwischen die Tumor-Mikroumgebung und das Immunsystem

2) Gewebeanalysen zur Bestimmung von neu aufgetretenen Resistenz-Mechanism

3) Integration mit omics Daten von Proben mit immunologische und andere Tumor mivroinironment Komponenten.

Demensprechend ist die Forschung die waehrend diesem internationalen Phd Programm durchgefuehrt wurde, und hier discutiert wird, zur Vorstellung von Antworten zu diesen prioritaeren Fragestellungen zum Verstaendnis vom Ovarialkarzinom gerichtet.
Die folgende Forschungslinien wurden entwickelt: Untersuchung der Modulation von der Tumor gesteuerte Neoangiogenese zur Zeit der Progession von Ovarialkarzinom und von der Rolle die von den anti-agiogenteischen Medikamenten in der Aenderung der Tumor assoziierte immunologische Hemmung spielen.
Die 3 Studien die hier vorgestellt warden* sind im Rahmen von dem "OCTIPS Consortium" einen Forschungsprojeckt der von der Europaeische Kommission 2012 finanziert wurde, durchgefuehrt worden. Die Biobank vom OPTICS Consortium ist einer der groessten in Europa und enthaelt Proben von Ovarialkarzinome zur Zeit der Diagnose sowie zur Zeit vom Rezidiv und befindert sich an der Charite' Medical University (Berlin, Germany). Zusaetzlich hat es eine Kollaboration mit dem Laboratory of Tumor Immunology and Cell therapy Unit" unter der Leitung von Frau Prof. Marianna Nuti, an der Univeritaet Sapienza (Rom, Italien) gegeben.

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* Liste ausgewählter Publikationen in der Doktorarbeit:

1) Ruscito I. et al. *Characterisation of tumor microvessel density during progression of high-grade serous ovarian cancer: clinico-pathological impact. An OCTIPS Consortium study.* British Journal of Cancer 2018;119(3):330-338.
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2) Napoletano C[§] & Ruscito I[§] et al. *Bevacizumab-Based Chemotherapy Triggers Immunological Effects in Responding Multi-Treated Recurrent Ovarian Cancer Patients by Favoring the Recruitment of Effector T Cell Subsets.* J Clin Med. 2019;8(3). pii: E380. doi: 10.3390/jcm8030380.
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INTRODUCTION

After almost 20 years of carboplatin plus paclitaxel upfront treatment for ovarian cancer [**duBois A, J Natl Cancer Inst 2003; Ozols RF, J Clin Oncol 2003**], the new millennium started with the successful introduction of targeted-therapies into the management of advanced disease [**Burger RA, New Engl J Med 2011; Perren TJ, New Engl J Med 2011**]. In 2011, the anti-angiogenetic monoclonal antibody Bevacizumab, against Vascular Endothelial Growth Factor (VEGF), was the first to be incorporated into first-line management of advanced disease (EMA, December 2011). The second decade of 2000s is witnessing a new ovarian cancer paradigm shift based on the results recently obtained in first line setting by a new categories of targeted agents: the Poly (ADP-ribose) polymerase (PARP)-Inhibitors (PARPi) [**Moore K, New Engl J Med 2018; Coleman RL, New Engl J Med 2019; Gonzalez-Martìn A, New Engl J Med 2019; Ray-Coquard I, New Engl J Med 2019**], whose major target is primarily involved in the repair of single-strand DNA breaks [**Ratnam K, Clin Cancer Res 2007**].

Despite the successful incorporation of these target therapies into the management of advanced ovarian cancer, high-grade serous ovarian carcinoma (HGSOC) still accounts for the highest mortality rate among all ovarian cancer (OC) histotypes, with almost 80% of all new deaths from OC being caused by this distinct subgroup of ovarian tumours (**Levanon K, J Clin Oncol 2008; Li J, J Hematol Oncol 2012; Cancer Genome Atlas Research Network 2011; Bowtell DD, Nat Rev Cancer 2015**).

More recently, the emerging role of immune checkpoint inhibitors (ICIs) in solid cancers is revolutionizing the scenario and the outcome of cure in oncology. The immune system, indeed, is a complex network where the several mechanisms and interactions involved are continuously discovered. Consequently, more than ever translational scientists are close to clinicians to better understand patients immune response to cancer in order to potentiate possible avenues of cure.

Up to now, clinical results of these new immunomotherapy agents in OC are still limited, but suggest that they could benefit some patients with recurrent disease. The preliminary results of two phase III trials have shown that the addition of ICIs to chemotherapy does not improve progression-free survival. For this reason, there is a primary need to look for synergistic effects between ICIs and other active drugs in OC.

In this scenario, international groups of opinion leaders have recognised the designing of new translational studies on recurrent and end-stage HGS tumour tissue samples as a key 'unmet need' in the understanding of HGSOC biology and clonal evolution, in order to maximize the effect of

available target therapies and possibly combine them with new immunotherapy drugs, with the final aim of enlarging the platform of OC patients, who could benefit from these new treatment categories (**Bowtell DD, Nat Rev Cancer 2015**). As a consequence, the research work carried out during this international PhD programme, and here discussed, aimed to add answers to these universally recognized priority issue in ovarian cancer understanding.
In particular, the leading research lines were developed and carried out aimed to explore the modulation of tumor-derived neoangiogenesis during ovarian cancer progression and the role of anti-angiogenetic agents in reverting tumor immune suppressive circuit thus triggering host's anti-tumoral immunological response.
The 3 research studies here presented were both carried out in the context of the "OCTIPS Consortium", a 7th Framework Program research Project, funded by the European Commission in 2012, which currently holds in its biobank at Charité Medical University (Berlin, Germany) one of the largest European collection of paired primary and recurrent ovarian cancers samples; and in the context of the "Laboratory of Tumor Immunology and Cell therapy Unit" directed by Prof. Marianna Nuti, at Sapienza University (Rome, Italy).

RESEARCH AIMS

After decades of paralysis in primary OC first-line chemotherapy treatment, incorporation of bevacizumab in the upfront regimen for advanced newly diagnosed disease (**Burger RA, New Engl J Med 2011**) has changed the 'standard of care paradigm' of advanced primary OC, although characterised by less survival impact than expected (**Hansen JM, Eur J Cancer 2016; Aghajanian C, J Clin Oncol 2012; Pujade-Lauraine, J Clin Oncol 2014**). Thus, understanding changes in the vasculature or identification of prognostic biomarkers of response to vasculature targeting is needed. Unfortunately, there are currently no predictive biomarkers to tailor bevacizumab treatment in OC patients. A full knowledge of molecular changes involving intratumoural vasculature from primary to recurrent HGSOC is still lacking and may provide new opportunities to: (1) tailor treatment with currently available anti-angiogenetic agents, (2) shed light on acquired resistance mechanisms, and (3) develop new targeted therapies.
In this context, the aims of the enclosed reseach studies were:

1) to identify changes occurring from primary to recurrent HGSOC in tumour tissue expression of the angiogenesis-associated biomarkers CD31, applied for detecting microvessels density (MVD) (**Abufalia O, Gynecol Oncol 1999; Lertkiatmongkol P, Curr Opin Hematol 2016; Stone P, Gynecol Oncol 2003**) and VEGF-A (**Hazelton P, Curr Oncol Rep 1999**) by analysing a large cohort of paired primary and recurrent HGSOC tissue samples. Secondary endpoints included the correlation of biomarkers expression with patients' clinico-pathological characteristics and survival data. (**Ruscito I, Br J Cancer 2018**);
2) to investigate the correlation between the clinical response to bevacizumab-based chemotherapy and the improvement of immune fitness of multi-treated ovarian cancer patients (**Napoletano C & Ruscito I, J Clin Med 2019**);
3) To give insight into new mechanism of immune-activation exerted by anti-angiogenetic agents commonly applied in oncology practice (**Zizzari IG, Cancer Immun Res 2018**). In particular this study, including only mRCC patients subjected to anti-angiogenetic TKI agents-based first line, give a proof of concept of the immune-priming effects of anti-angiogenetic drugs and open new therapeutic avenues for their integration with ICIs in solid cancers, which is what is currently hoped and waited also in ovarian cancer setting.

❖ Ruscito I et al., Br J Cancer. 2018

♦ *MATTER AND METHODOLOGY*

Samples Collection

In **Ruscito I, Br J Cancer 2018**, paired cancer tissue samples belonging to HGSOC patients were collected during primary and secondary cytoreduction. Patients were retrospectively and consecutively selected from OCTIPS (Ovarian Cancer Therapy–Innovative Models Prolong Survival, Agreement No.279113-2) Consortium database. Included patients underwent both primary (pOC) and recurrent (rOC) surgery in one of the European Gynaecologic Oncology referral Centers of the following Institutions: Charité Universitätsmedizin Berlin, Germany; Catholic University of Leuven, Belgium; Imperial College, London, UK; University of Edinburgh, UK; University Medical Center Hamburg-Eppendorf, Germany. Inclusion criteria were: availability of paired primary and recurrent cancer tissue samples from HGSOC patient together with clinical annotation. Exclusion criterion was: neoadjuvant

chemotherapy treatment, due to the need to analyse primary chemo-naïve tumours. Approval from each local ethics committee was obtained (EK207/2003, ML2524, 05/Q0406/178, EK130113, 06/S1101/16). All included samples underwent central histopathological assessment to confirm HGSOC histology and ensure tumour tissue content and quality.
Patients' clinico-pathological data, including somatic-BRCA status from 52 included patients, were retrieved from OCTIPS Consortium database.18 GCIG criteria were applied to define platinumresistance and platinum sensitivity.19 RECIST Criteria were applied during patients' follow-up to define HGSOC relapse.20 No residual tumour was defined intraoperatively by the surgeon in case no macroscopic tumour could be detected at the end of cytoreduction. In order to investigate any association between different tumour vasculature profiles and intratumoural immune infiltrate in both pOCs and rOCs, MVD and/or VEGF profiles were matched with previous OCTIPS data on tumour infiltrating lymphocytes (TILs), assessed through the immunohistochemical expression of CD3, CD4, and CD8 biomarkers, as previously reported (**Stanske M, Neoplasia 2018**). Furthermore, immunosuppressive TILs were evaluated through the expression of T-regulatory cells-specific biomarker FoxP3, using the mouse anti-human FOXP3 antibody (clone ab20034; Abcam, Cambridge, MA, USA, 1:200, 1.5 h at room temperature).

<u>Methods</u>

The immunohistochemistry methodology was applied. Tissue microarrays (TMA) were constructed for immunohistochemical staining. Each primary and recurrent tumour tissue sample was represented within the TMA by two tumour cores, each containing at least 90% of cancer cells. Sections from TMA were deparaffinised in xylol, rehydrated in graded alcohol, and boiled in pressure cooker for 5 minutes in citrate buffer (pH = 6), for CD31 staining, or in EDTA (pH = 9), for VEGF staining. Rabbit anti-human CD31 antibody (clone ab32457; Abcam, Cambridge, MA, USA) and rabbit anti-human VEGF-A antibody (clone A-20; Santa Cruz Biotechnology, Dallas, TX, USA) were diluted 1:20 and 1:250, respectively, and incubated on slides for 60 minutes at room temperature. Bound antibodies were visualised using DAKO Real Detection System and DAB + (3,3′ -diaminobenzidine; DAKO, Glostrup,Denmark) as a chromogen. Finally, the slides were co-stained with hematoxylin. CD31 stained samples were assessed in terms of MVD. MVD was determined by averaging the number of vessels from three distinct areas of tumour with highest vessels density examined at ×200 magnification (**Goodheart M, Gynecol Oncol**

2002; Crasta J, Int J Gynecol Pathol 2011; Nadkarni N, Cancer Lett 2013). Samples were further classified into 'MVDhigh' (≥16.3 vessels) or 'MVDlow' (<16.3 vessels), establishing the cut-off level of MVD count for dichotomisation at first quartile (primary samples), being the value able to maximise difference in OS hazard ratio (**Goodheart M, Gynecol Oncol 2002; Nadkarni N, Cancer Lett 2013; Bais C, J Natl Cancer Inst 2017**).
For VEGF staining evaluation, the number of stained tumour cells within the whole TMA cores (0% = 0; 1–10% = 1; 11–50% = 2; >50% = 3) was multiplied with the intensity of staining (negative = 0; weak = 1; moderate = 2; strong = 3) (**Mukherjee S, J Clin Diagn Res 2017**) resulting in a semiquantitive immunoreactivity score (IRS) ranging from 0 to 9. Samples were classified as 'VEGF(+)', for VEGF-high tumour expression (IRS = 4–9), or as 'VEGF(−)', for absent/weak focal staining (IRS = 0–3). As positive control for IHC were used human liver sections. Samples staining was assessed independently by two co-authors (IR and SDE).

Statistical Ananlysis
Statistical analysis was performed using SPSS version 22.0 (SPSS Inc,Chicago,IL,USA). Difference in biomarker expression between pOCs and rOCs was assessed through the correlation test (Spearman coefficient, 2-tailed) and 'Wilcoxon signed rank' nonparametric test for related samples. Fisher's exact test was applied to correlate MVD and/or VEGF tumour expression with patients' clinico-pathological categorical data. Patients' progression-free interval (PFI), progression-free survival (PFS), and overall survival (OS) were identified through Kaplan–Meier analysis (Log-Rank test). PFI was defined as the time interval from the last adjuvant chemotherapy to relapse, whereas progression-free survival (PFS) was established as the time interval between first recurrence diagnosis and tumour progression. Univariate and multivariate survival analyses were performed applying Cox-regression model. Multivariable models were obtained among variables reporting a p-value ≤ 0.1 in univariate analysis. p-values ≤ 0.05 were evaluated statistically significant.

♦ *RESULTS*

A total of 222 intra-patient paired primary and recurrent HGSOC tissue samples derived from 111 patients were included. To note, only 2/111 (1.8%) patients received bevacizumab in front-line chemotherapy, thus the staining of recurrent samples have not been influenced by first-line administration of anti-angiogenetic compounds.

MVD staining
MVDhigh staining was detected in 75.7% (84/111) of pOC and in 51.4% (57/111) of rOC, whereas MVDlow staining was found in 24.3% (27/111)

and in 48.6% (54/111) of pOC and rOC, respectively. MVDlow staining was twice as prevalent in relapsed tumours compared to primary disease (p = 0.0003, Fisher's exact test). Nevertheless, globally, pairwise analysis revealed no tendency towards a change in MVD to higher or lower levels in recurrent samples (p = 0.935, Wilcoxon test; Fig. 1e), as well as no significant correlation between pOCs and rOCs in MVD was reported (Spearman correlation, p = 0.920; Spearman coefficient: 0.01).

VEGF-A expression

The same percentage of VEGF(+) (20.7%, 23/111) and VEGF(−) (79.3%, 88/111) tumour samples was found between pOCs and rOCs, respectively, (p = 1, Fisher's exact test), although no significant correlation between pOCs and rOCs VEGF IRS values could be observed (p = 0.505, Spearman coefficient 0.06). Furthermore, pairwise analysis confirmed no tendency towards a change in VEGF IRS levels at tumour relapse (p = 0.121, Wilcoxon test).

MVDhigh and VEGF(+) co-expression in pOCs vs rOCs.

MVDhigh and VEGF(+) co-expression was more frequent in pOCs group (22/111, 19.8%) compared to rOCs (9/111, 8.1%) (p = 0.02, Fisher's exact test).

Relationship between MVD and/or VEGF-A expression with TILs.

Results showed that MVDhigh levels in pOCs samples were associated with higher CD3(+) (p = 0.029, Mann–Whitney test) and CD8(+) (p = 0.013) effector TILs, but not with a higher FoxP3(+) (p = 0.443) T-regulatory cells infiltrate. To note, the correlation between MVD and CD3(+)/CD8(+) TILs disappeared at tumour recurrence. No significance between pOCs or rOCs VEGF expression or MVDhigh + VEGF(+) co-staining with TILs was reported.

MVD and/or VEGF-A profiles and patients' clinico-pathological factors

To note, VEGF(+) primary HGSOCs and MVDhigh/VEGF(+) primary samples were most frequently encountered among somatic-BRCA-mutated tumours compared to somatic- BRCA wild-type cases (p = 0.019, Fisher's exact test). No further significant associations between different intratumoural residual tumour after primary debulking or first-line platinum response was identified. Decrease of VEGF expression in rOCs was observed only in BRCA-mutated patients (p = 0.053, Wilcoxon test), although this association did not reach statistical significance.

Survival

Patients, whose pOCs resulted MVDhigh, VEGF(+) or co-stained for both biomarkers, were found to have a significantly improved OS compared to patients without these intratumoural profiles at primary disease. In particular, median OS for MVDhigh and MVDlow patients was 67 and 46

months, respectively (p = 0.019), median OS for VEGF(+) and VEGF(−) patients resulted 76 vs 52 months, respectively (p = 0.036), while median OS for patients with co-stained pOCs was 76 months, compared to 52 months in women without co-expression (p = 0.021). On the contrary, no influence of pOCs or rOCs MVD and/or VEGF expression on patients' time to progression after primary (PFI) or first recurrent disease (PFS) was reported. Multivariate analysis for OS and PFI was carried out on the whole patients' population (n = 111) and also on the subgroup of patients (n = 52) with known tumour somatic-BRCA status. VEGF-A was not found to be an independent prognostic factor for OS anymore when considering also somatic-BRCA mutational status. Only somatic-BRCA mutation (HR: 0.354, CI 95%: 0.133–0.994; p = 0.038), high CD4(+) TILs (HR: 0.997, CI 95%: 0.995–1.000; p = 0.038) and first-line platinum response (HR: 0.216, CI 95%: 0.051–0.991; p = 0.037) were found to independently improve HGSOC patients' OS. When analysing the Platinum free-interval (PFI) in patients with or without BRCA somatic mutations, advanced FIGO stage (HR: 18.261, CI 95%: 1.28–260.17; p = 0.032) and low CD4(+) TILs (HR: 0.996, CI 95%: 0.993–0.998; p = 0.001) were the only independent poor prognostic factors.

❖ <u>Napoletano C & Ruscito I, J Clin Med. 2019</u>

♦ *MATTER AND METHODOLOGY*

<u>Samples Collection</u>

In **Napoletano C & Ruscito I, J Clin Med 2019**, 20 consecutive recurrent ovarian cancer patients were retrospectively selected from "Sapienza" PBMC sample collection at the "Laboratory of Tumor Immunology and Cell Therapy Unit", Department of Experimental Medicine (Sapienza University of Rome, Italy—Ethical Committee approval, protocol n° 703/2008; date of approval 07/24/2008). PBMCs belonging to all available multi-treated platinum-resistant ovarian cancer patients subjected to intraperitoneal (i.p.) bevacizumab-based chemotherapy as compassionate use were selected (Bev group; 10 patients), together with 10 patients (Ctrl group) that received non-bevacizumab-based chemotherapy. Inclusion criteria were: primary diagnosis of advanced epithelial serous ovarian cancer; having been subjected to at least three previous chemotherapy lines; diagnosis of tumor progression confirmed by CT scan; presence of malignant ascites; life expectancy of at least three months; and availability of at least three PBMCs samples per patient collected during the course of

bevacizumab-based versus non-bevacizumab-based chemotherapy. Furthermore, patients of the Bev group were matched with the Ctrl group patients for age, tumor grading, FIGO stage, type of primary treatment strategy (primary debulking surgery versus neoadjuvant chemotherapy followed by interval debulking surgery), tumor residual at first surgery, and type of recurrence at the time of blood sampling in order to minimize selection bias and avoid misinterpretations of results. Ten patients were identified as the ones that had received i.p. bevacizumab 5 mg/kg every 21 days immediately after paracentesis for treatment of malignant ascites [**Kobold S, Oncologist 2009; Bellati F, Invest New Drugs, 2010**] plus intravenous (intravenous injection, i.v.) monochemotherapy (cisplatin) [**Shamsunder S, J Obstet Gynaecol Res 2000**], while 10 other patients were identified as been treated with i.v. monochemotherapy alone (paclitaxel, topotecan, pegylated liposomal doxorubicin, cisplatin). Patients' clinicopathological data were retrieved from clinical charts. Disease progression was defined basing on the response evaluation criteria in solid tumors (RECIST) [**Eisenhauer E.A, Eur. J. Cancers 2009**]. This retrospective study received institutional review board (IRB) approval and was carried out following the rules of the Declaration of Helsinki of 1975.

Methods

- **PBMC Purification**

PBMCs were isolated from 12 to 14 mL of peripheral blood by Ficoll Hypaque gradient (1077 g/mL; Pharmacia LKB, Sweden), obtaining a yield between 10 × 106 and 12 × 106 cells for each drawing and cryopreserved until use. Samples were taken before therapy (T0) and after three (III) and six (VI) cycles of therapy.

- **Cell Phenotype**

Cell phenotype staining was performed using several directly conjugated monoclonal antibodies (MoAbs). T cells were incubated with the anti CD8-PE-Cy5.5 (RPA-T8 clone), anti-CD3-PE (UCHT1 clone), anti-CCR7-FITC (150503 clone), and anti-CD45RA-APC (HI100 clone) MoAbs, all from Becton Dickinson (Franklin Lakes, NJ, USA). Tregs were stained with the anti-CD25-PE (MA251clone), anti-CD45RA-APC (HI100 clone), anti-CD4-FITC (RPA-T4 clone), and anti-FOXP3-Alexa 647 (259D/C7 clone) MoAbs, all from Becton Dickinson. Cells were incubated with the conjugated MoAbs targeting extracellular antigens for 30 min at room temperature (RT) as indicated by the manufacturer's instruction. The staining of intracellular antigen FOXP3 was performed after the cells' permeabilization with the Human FOXP3 Buffer Set (Beckton Dickinson,

Franklin Lakes, NJ, USA). After washing, at least 1 × 104 events were evaluated using a FACSCanto flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) running FACSDiva data acquisition and analysis software (Becton Dickinson, Franklin Lakes, NJ, USA). The percentages of CD4 and CD8 T cells were calculated with respect to the entire CD3 T cell population, while the percentage of Treg was evaluated with respect to CD4 T cells.

- **Intracellular Cytokine Staining**

T cells were stimulated with the anti-CD3 (OKT3 clone, 1 μg/mL) (eBioscence, San Diego, CA, USA) and anti-CD28 (CD28.2 clone, 5 μg/mL) (BioLegend, San Diego, CA, USA) MoAbs for 16 h at 37 °C in the presence of Brefeldin (Sigma-Aldrich, St. Louis, MO, USA) (10 μg/mL). The staining of IL-10 positive cells was carried out by fixing the cells with 2% paraformaldehyde (Sigma-Aldrich, Saint Louis, MO, USA). Cells were than washed with phosphate-buffered saline (PBS) without Ca2+ and Mg2+ + 0.5% saponin (Sigma-Aldrich, Saint Louis, MO, USA) + 10% fetal bovine serum (FBS) (Sigma-Aldrich, Saint Louis, MO, USA) and incubated for 30 min with anti-IL-10-PE (JES3-19F1 clone) (BioLegend, San Diego, CA, USA) MoAb. Cells were analyzed using a FACSCanto flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) running FACSDiva data acquisition and analysis software (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical Ananlysis

Statistical analysis was performed using Graphpad Prism version 6 (Graphpad Software, Inc., San Diego, CA, USA). Descriptive statistics (average and standard deviation) were used to describe different groups of continuous data. Student's t-test was used to compare groups of continuous variables. Groups of categorical data were compared using the Fisher's exact test. Significance is indicated when $p \leq 0.05$.

♦ *RESULTS*

Patients' Characteristics and Clinical Response

Twenty patients met all inclusion criteria and were included in the study. As a result of patient matching, no differences in terms of clinicopathological variables as well the Eastern Cooperative Oncology Group (ECOG) performance status could be identified between the Bev group and the Ctrl group. At the time of blood sampling for immunological analysis, 12/20 women (60%) presented intraperitoneal tumor progression, whereas the remaining 3/20 (15%) and 5/20 (25%) patients were diagnosed with intraperitoneal plus retroperitoneal disease

worsening and widespread tumor dissemination, respectively. From a clinical point of view, and as confirmed by serial Ca125 serum levels, 50% (10/20) of patients were judged responders to chemotherapy after six cycles of treatment and were equally distributed in each group of interventions (5/10 in the Bev group and 5/10 in the Ctrl group).

Bevacizumab-Treated Patients Showed a Different Immunological Signature Compared with the Control Group

To understand whether bevacizumab treatment impacts the immunological status of ovarian cancer patients, the modulation of circulating CD4 and CD8 T cells was firstly analyzed in the Bev group and the Ctrl group before (T0) and after III and VI cycles of treatments. Both CD4 and CD8 T cells played a critical role in the activation of an effective antitumor immunity. CD8 lymphocytes exerted their cytotoxic activity by eliminating tumor cells, while CD4 T lymphocytes sustained and maintained a CD8 T cell response by cytokine production [**Lanzavecchia, A. Science 2000, 290, 92–97**]. A deficiency in the activation of one of these two populations induced the development of a failed immunity against the tumor. Results obtained from the cancer patients showed that therapies did not modify the percentage of CD4 and CD8 lymphocytes in both groups at different time points. CD4 T cells were significantly higher in the Bev group at T0 and III compared with the Ctrl goup, although this difference disappeared at the end of VI cycles. No difference was observed in CD8 T cells between the two groups, although the ratio CD4/CD8 remained high (>1) up to VI cycles in both groups, suggesting a predominance of CD4 T cells during therapies. CD8 and CD4 T cells were concurrently analyzed for the expression of CCR7 and CD45RA molecules, which identify four different lymphocyte subsets: effector (CCR7−CD45RA+), naïve (CCR7+CD45RA+), central memory (CCR7+CD45RA−), and effector memory (CCR7−CD45RA−) T cells. Analyzing these T cell subpopulations in the Bev and Ctrl group patients, no significant difference throughout the treatment in each patient group and between the two groups were found.

Finally, the percentage of Tregs was also examined following the expression of CD4, CD25, and FOXP3 markers. In cancers, Tregs represent one of the most important T cell populations as they are able to suppress the activation and/or expansion of antitumor CD4 and CD8 T cells through cell–cell contact or by cytokine release [10]. A high percentage of Tregs is associated with a poor prognosis in different types of solid tumors [**Shou J, BMC Cancer 2016; Zhao S, Oncotarget 2016**]. In our setting of patients, the results demonstrated that the Ctrl group showed a significant decrease in total Tregs from T0 to VI cycles (36% vs. 31%, $p = 0.03$), while

no difference was found in the Bev group throughout the therapy. Total Tregs were further analyzed according to the combined expression of CD25, FOXP3, and CD45RA markers, which identifies three important Treg subpopulations [10]: resting Treg (CD25+CD45RA+FOXP3+: rTregs), activated Tregs (CD25highCD45RA-FOXP3-: aTregs), and cytokinesecreting Tregs with no suppressive activity (CD25+CD45RA-FOXP3+: nsTregs). aTreg have been
described as terminally differentiated cells that rapidly die after exerting their suppressive activity, whereas rTreg proliferate and convert into aTreg both in vitro and in vivo [10]. The analysis revealed that bevacizumab-treated patients showed a lower percentage of aTregs and rTregs compared with the Ctrl group at T0. This difference persisted until III cycles of treatment in the rTreg subset and disappeared after VI cycles, suggesting that these patients exhibited a less suppressive immunological profile compared with the Ctrl group at the beginning and in particular after III cycles of therapies.

Bevacizumab-Treated Patients Showed a Discrete CD4 Effector T Cell Population throughout the Treatment.

Patients belonging to the Bev group and the Ctrl group were then divided in clinically responders (R) and clinically nonresponders (N-R) to therapy according to RECIST (Table S1). The modulation of CD4 and CD8 T cell was initially evaluated in R and N-R patients of both groups, followed by the analysis of the different T cell subsets (Figure 2). The results demonstrated that the CD8 T cells derived from bevacizumab-treated patients were not differently modulated in R and NR patients, while the CD4 T cells appeared to be significantly higher in the N-R group at the beginning and after VI cycles of treatment. Conversely, in the Ctrl group, the CD8 T cells seemed to be significantly higher after VI cycles in the R patients compared with the N-R ones, while no significant difference was observed in the CD4 T cell population.

Lymphocytes were also analyzed according to the expression of CCR7 and CD45RA molecules. The results demonstrated that in the Bev group and the Ctrl group, the percentage of CD4 effector T lymphocytes in R patients was higher compared with N-R patients at T0. This difference persisted until the end of the therapies for bevacizumab-treated patients, while it had already disappeared after III cycles of therapy in the control group. This suggests that Bev treatment, by favoring the normalization of the tumor vasculature [13], improves and sustains the circulation of effector T cells. The other CD4 T cell subsets and the CD8 T cell populations were not significantly modified by treatments.

Tregs Were Modulated in Bevacizumab-Treated Patients during

Therapies.

To assess whether the treatment schedule and/or the clinical response could be associated with the modulation of the Treg subsets, the percentage of circulating Tregs after III or VI treatment cycles were compared with the baseline value at T0, and the analysis was expressed as fold increase (%TregIII/%TregT0 or %TregVI/%TregT0) (Figure 4). After III cycles of treatment, the level of the entire Treg population was significantly higher in R patients compared with N-R patients in the Bev group. This increase was ascribed to the nsTreg subset being significantly higher in R compared with N-R patients. These differences between R and N-R patients disappeared after VI cycles of bevacizumab treatment. In contrast, the control group did not show any difference in the percentage of Tregs between R and N-R patients during therapies, and no difference between the Bev and Ctrl patients was observed.

Bevacizumab-Treated N-R Patients Had Higher Level of IL10+ T Cells Compared to R Patients.

Because IL10, such as TGFβ, is one of the most important cytokines released by Tregs [14] that is able to downregulate Th1 cytokine production and block NF-κB activity [15], T cell derived from patients in the Bev group were analyzed for their capacity to produce IL10 as intracellular staining. These patients exhibited a significant increase in IL10+ cells from T0 up to VI cycles of therapy. Analyzing the data as fold increase of the percentage of IL10+ cells after III and VI cycles of therapy compared with T0 (%IL10III/%IL10T0 or %IL10VI/%IL10T0) between R and N-R patients, significant high levels of IL10 were found in N-R patients after III cycles, suggesting an enhancement of the immunosuppression during the bevacizumab treatment in this group. This increase disappeared after VI cycles ($p = 0.08$) of therapy.

❖ Zizzari IG et al., Cancer Immunol Res. 2018

♦ *MATTER AND METHODOLOGY*

Samples Collection

In **Zizzari IG, Cancer Immun Res 2018**, peripheral blood mononuclear cells (PBMCs) of 6 metastatic Renal Cancer Cell (mRCC) patients (Ethical Committee Protocol, RIF.CE: 4181) were isolated from blood samples (50

mL) by Ficoll–Hypaque at different times (Ethical Committee Protocol, RIF.CE: 4181): before treatment with sunitinib or pazopanib (T0), during treatment (T1: 1 month of treatment, T2: 2 months, T3: 3 months etc.) and during progression. PBMCs isolated from healthy donors were also collected to consititute a control group.

Methods

- **Generation of DCs**

Human monocyte-derived DCs were generated from PBMCs) of healthy donors and of mRCC patients. Monocytes (CD14þ) were purified from PBMCs after Ficoll–Hypaque gradient (1,077 g/mL; Pharmacia LKB) by Human CD14-Positive Selection Kit (StemCell Technologies) and cultured (5 x 105 cells/mL) in RPMI 1640 (Hyclone) supplemented with 2 mmol/L L-glutamine, penicillin 100 U/mL, streptomycin 100 mg/mL (Sigma-Aldrich), with 5% heat-inactivated Fetal Calf Serum (FCS; Hyclone). Fifty ng/mL rhGM-CSF (R&D Systems) and 2,000 U/mL rhIL4 (R&D Systems) were added at day 0 and 2. Immature DCs (iDCs) were collected at day 5 and matured with cytokine cocktail (rhIL1b, IL6, TNFa and PGE2; all purchased by R&D Systems) for 16 hours. Sunitinib (50 ng/mL; Sigma-Aldrich) and pazopanib (19 mg/mL; Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO; Euroclone) and added to the culture during DC differentiation.

- **Immune phenotype**

DC phenotype was analyzed by flow cytometry using the following monoclonal antibodies (mAb): anti–HLAII-DR-FITC, anti–CD86-FITC, from BD Biosciences, anti–CD14-PE, anti–CCR7-FITC, anti–CD83-PE, anti–CD40-PE, anti–PD-L1-PE from BioLegend and anti–VEGR-1 from R&D Systems. MoAbs anti–IgG1-FITC and anti–IgG1-PE (BioLegend) were used as isotype controls. For immune profile evaluation, PBMCs were isolated from blood samples (50 mL) from six mRCC patients by Ficoll– Hypaque at different times: before treatment with sunitinib or pazopanib (T0), during treatment (T1: 1 month of treatment, T2: 2 months, T3: 3 months etc.) and during progression. Various T-cell subsets were analyzed:

T-cell subpopulations: anti–CD3-APC-H7/CCR7-PE/CD8 PerCp-Cy5.5/CD45RA-BB15.

Treg cells: anti–CD4-APC-H7/CD25-PE/CD45RA-BB15/FoxP3-Alexa647.

T-cell activation/proliferation: anti–CD3-APC-H7/CD8-PerCp-Cy5.5/CD137-APC/Ki67-PeCy7.

T-cell exhaustion: anti–CD3-APC-H7/CD8-PerCpCy5.5/PD1-

PE/CTLA4-APC/Tim3-BB15.
All mAbs were purchased by BD Biosciences and BioLegend. Flow cytometric analysis was performed using FACSCanto flow cytometer running FACS Diva data acquisition and analysis software (BD Biosciences).

- **Microvesicle isolation**

Microvesicles were isolated from supernatants of DCs differentiated with and without suninitib and pazopanib. Supernatants were centrifuged at 13,000 x g for 30 minutes at 4°C. Microvesicles were then stained with anti–PD-L1 (BioLegend) and acquired by FACSCanto flow cytometer and analyzed by FACS Diva software. Anti–IgG1-PE (BD Biosciences) was used as isotype control. Fluorescent Nile Red Particles (0.1–0.3 mm, Spherotech Inc.) were used as size control.

- **Western blot analysis**

Immature DCs and mature DCs (iDCs and mDCs, respectively) with and without sunitinib and pazopanib were lysed using the NP-40 solution (Biocompare) in the presence of phenylmethylsulfonyl fluoride (1 mmol/L, PMES) and protease inhibitors (1X; Sigma). Proteins obtained were quantified by Bradford assay, were resolved using 4% to 12% SDS-PAGE gel and transferred to nitrocellulose. After blocking, membranes were incubated with rabbit anti–b-catenin (Bethyl Laboratories Inc.; 1:1,000), rabbit anti-pErk42/44 (Erk1/2; Cell Signaling Technologies; 1:1,000), mouse anti–b-actin (Cell Signaling Technologies; 1:1,000) and mouse anti–NF-kB (p105/p50; Cell Signaling Technologies; 1:1,000), followed by peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (HþL; Jackson Immuno Research Laboratories; 1:20,000). Protein bands were detected with Immobilon Western (Millipore Corporation) following the manufacturer's instructions. The density of protein bands was analyzed by Image J software and was normalized in terms of average intensity of bands of each protein per average intensity of bands of b-actin.

- **Endocytosis assay**

FITC-dextran (1 mg/mL; Molecular Probes) was added to untreated iDCs and mDCs and differentiated with sunitinib and pazopanib for 2 hours at 37°C. After washing, cells were acquired by FACSCantoII flow cytometer and analyzed by FACSDiva software. DCs incubated with FITC-dextran for 2 hours at 4°C were used as negative controls.

- **Cytokine production**

Culture supernatants from iDCs and mDCs untreated or differentiated with sunitinib and pazopanib and sera from mRCC patients were collected and analyzed using the ProcartaPlex Human Inflammation Panel (20 Plex; eBioscence). Samples were measured by BioPlex Magpix

Multiplex Reader (Bio-Rad) and data analysis was performed using Bioplex Manager MP software (Bio-Rad).

- **T-cell proliferation**

T lymphocytes were purified from Ficoll–Hypaque gradient (1,077 g/mL; Pharmacia LKB) followed by CD3 immunomagnetic isolation (StemCell). T cells were then cocultured in a 96-well round-bottom microplate (Costar) with allogeneic iDC and mDCs differentiated with and without sunitinib and pazopanib (DCs: T cells, 1:5) in the presence of PHA (5 mg/mL) for 4 days at 37°C. T cells were pretreated with CarboxyFluorescein Succinimidyl Ester (1 mmol/L, CFSE; Life Technologies) and cell proliferation was monitored through progressive halving of fluorescence using FACSCantoII flow cytometer and analyzed by FACSDiva software (BD Biosciences). Results were reported as the percentage of proliferation increase of T cells cultured with DCs treated with sunitinib and pazopanib compared with T cells cultured with DCs alone. T cells were also cultured in 6-well round-bottom microplates (Costar) in the presence of sunitinib (50 ng/mL) and pazopanib (19 mg/mL) up to 24 hours. Proliferation was evaluated by FACSCantoII flow cytometer and analyzed by FACSDiva software.

- **Statistical analysis**

Descriptive statistics (average and standard deviation) were used to describe the various data. ANOVA test was used to analyze statistical differences between three groups. Student paired t test was used to compare two groups. Significance is indicated when the P value was less than 0.05.

♦ *RESULTS*

Pazopanib improves DC activation and increases expression of DC-maturation markers.

To investigate the capacity of TKIs to influence DC differentiation and maturation in vitro, the expression of CD14, HLA-DR, CD86, CD83, CCR7 and CD40 was evaluated by flow cytometry on monocyte-derived DCs of healthy donors. Untreated DCs were used as control. The exposure to concentrations of sunitinib and pazopanib found in plasma affected the phenotype of immature and mature DCs differently. Sunitinib did not affect DC differentiation and maturation, but DCs cultured in the presence of pazopanib were more activated. Pazopanib modified iDC phenotype, significantly increasing the expression of HLA-DR and CD40 molecules, compared with that of iDCs alone (HLA-DR $P < 0.01$) and iDC treated with sunitinib (HLA-DR $P < 0.05$; CD40 $P < 0.05$; Fig. 1A). The average values of mean fluorescence intensity (MFI) indicated upregulation of CD83 in DCs differentiated with pazopanib. On the other hand, CD14

expression was downregulated by pazopanib during differentiation. Similar results were obtained after DC maturation. mDCs cultured in presence of pazopanib significantly upregulated the expression of CCR7 and CD40 molecules compared with untreated mDCs (CCR7 $P < 0.05$; CD40 $P <0.05$) and sunitinib-treated mDCs (CCR7 $P < 0.05$), suggesting that pazopanib enhances activation status of both immature and mature DCs. VEGF-R1 expression, which is the target of both TKIs, was unaltered by treatment of DCs with pazopanib or sunitinib.

PD-L1 is downregulated in pazopanib-generated DCs.

The capacity of DCs to stimulate T cells depends on the balance between costimulatory and coinhibitory signals. Increased expression of costimulatory marker such as CD40 or CD83 can facilitate T-cell activation, whereas increased expression of inhibitory markers such as PD-L1 contributes to T cell-negative regulation (**Steinman RM. Apmis 2003**). To evaluate the expression of coinhibitory signals in DCs in response to TKI treatment, we analyzed the expression of the PD-L1 both on DCs and on shed microvesicles. During iDC differentiation, only pazopanib began to decrease PD-L1 expression, compared with untreated DC and DCs differentiated with sunitinib (Fig. 2A). The decrease in expression became significant ($P < 0.01$) in DCs after maturation.

Microvesicles released by the DCs also showed a decrease in PD-L1 expression: PD-L1 expression on microvesicles released by DCs treated with pazopanib was lower than that on microvesicles obtained by untreated DCs and DCs treated with sunitinib. The difference in expression of PD-L1 between microvesicles of mDCs and mDCs treated with pazopanib was statistically significant ($P < 0.05$).

Pazopanib treatment reduces immunosuppression downregulating IL10 production by DCs.

The production of cytokines such as IL10 and IL12 during DC maturation can influence the capacity of DCs to alter Th1 or Th2 immune responses (**Moser M, Nat Immunol 2000**). Several chemokines released by DCs, such as CXCL-10, promote tumor-reactive effector T-cell recruitment (**Pfirschke C, Cancer Cell 2017**). Thus, we evaluated cytokines and chemokines released by untreated DCs and DCs treated with TKIs. We observed a significant reduction ($P < 0.05$) of IL10 in iDCs and mDCs treated with pazopanib compared with untreated DCs (both iDC and mDCs) and DCs treated with sunitinib (both iDC and mDCs). The balance between IL12/IL10 and CXCL-10/IL10 favored immune activation when DCs were generated with pazopanib.

Pazopanib-treated DCs are able to increase T-cell activity.

DCs must possess specialized features to act as good antigenpresenting

cells. In addition to expression of costimulatory molecules and release of cytokines, other indicators of DC function and quality include their capacity for endocytosis and T-cell activation. The exposure to maturation stimuli induces changes including downregulation of endocytosis and increase of antigen presentation to T cells. We evaluated the endocytic capacity of DCs treated with TKIs and untreated DCs by fluorescein isothiocyanate (FITC)-dextran uptake and followed by flow cytometry. We determined the ratio of the fluorescence from positive (dextran uptake obtained after 2 hours at 37°C) and negative (dextran uptake after 2 hours at 4°C) samples. The results show that pazopanib reduced endocytosis capacity by 29% for iDCs treated with pazopanib compared with untreated DCs (1.42 ratio vs. 2, respectively), and by 40% compared with iDCs treated with sunitinib (1.42 vs. 2.33). The trend persisted after maturation: the endocytic capacity of pazopanib-treated mDCs was 42% lower than that of mDCs (0.8 ratio vs. 1.37) and 57% lower than that of sunitinib-treated mDCs (0.8 vs. 1.82). We then analyzed the capacity of DCs to stimulate the proliferation of allogeneic T cells. Lymphocytes, pretreated with CFSE, were cocultured with DCs and proliferation was evaluated after 4 days through progressive halving of fluorescence by flow cytometry. The results, plotted as percentage of fold increase (% of proliferation obtained as ratio between T cells stimulated by pazopanib-DCs/DCs or sunitinib-DCs/ DCs), showed that when DCs were differentiated in the presence of pazopanib, they acquired a greater capacity to stimulate T cells than either untreated DCs (20% fold increase for alone for 24 hours in the presence of either TKI.

<u>Pazopanib affects DC differentiation by inhibiting p-Erk/ b-catenin signaling.</u>

The Wnt-b-catenin pathway, particularly in DCs, regulates the balance between tolerance and immune response (**Swafford D, Discov Med 2015**). Loss of b-catenin impairs the ability of DCs to induce Tregs (**Hong Y, Cancer Res 2015**), instead the activation of b-catenin pathway increases the capacity of DC to release IL10 and promote immune tolerance, (**Manoharan I, J Immunol 2014; Shan MM, Science 2013**). In DCs, b-catenin signaling synergizes with other pathways, such as the Erk pathway, to induce anti-inflammatory cytokines and proliferation of Tregs. Erk1/2 signaling retards the phenotypic and functional maturation of monocyte-derived human DCs (**Puig-Kroger A, Blood 2001**). To evaluate whether changes in DC functional activity were due to signaling differences, we examined DC intracellular
pathways. Untreated DCs or DCs differentiated in the presence of sunitinib or pazopanib were lysed and probed with anti–p-Erk 1/2, anti–b-

catenin and anti–NF-kB by western blot. DCs treated with pazopanib expressed less p-Erk ½ than did DCs treated with sunitinib or untreated DCs. This downregulation associated with a significant reduction of b-catenin expression (iDCs and sunitinib-iDCs vs. pazopanib- iDCs $P < 0.05$; mDCs vs. pazopanib-mDCs $P < 0.05$). In both pazopanib-iDCs and pazopanib-mDCs, p-Erk1,2 and b-catenin antibodies detected a weaker signal than in untreated DCs and DCs treated with sunitinib, suggesting that pazopanib iDCs, 5% for mDCs) or sunitinib-treated DCs (14% for iDC, 5,1% for mDCs). No effect was observed on T cells cultured could act through these pathways. We analyzed the activation of NF-kB, which is essential for DC development and survival and regulates DC maturation. Results indicated that NF-kB activation was similar in all iDCs tested. Although the p50 signal was weaker in mDCs treated with pazopanib, the balance between all intracellular pathways favored the activation pathway.

Modulation of DCs generated from mRCC patients treated with pazopanib or sunitinib.

To confirm the pazopanib immune-priming effect in patients, we analyzed monocyte-derived DCs differentiated from mRCC patients during TKI treatment. DCs were differentiated in vitro by standard methodology without the addition of TKIs. Fig. 5 reported in published article shows the phenotype of DCs generated from PBMCs of RCC patients after one month of pazopanib or sunitinib treatment. CD14, a marker of DC immaturity, was less expressed in iDCs from patients treated with pazopanib ($P < 0.01$) than patients treated with sunitinib confirming data obtained in vitro. Moreover, DCs (both iDCs and mDCs) from pazopanibtreated patients expressed more of the activation markers HLADR and CCR7 and less PD-L1 (mDCs; $P < 0.01$) as compared with DCs generated from sunitinib-treated patients. For one patient, we evaluated the modulation of some DC markers differentiated before (T0) and during pazopanib therapy (T1: after 1 month of treatment, T2: after 2 months) and results are shown in Supplementary Fig. S3A reported in published article. As with DCs generated in vitro with pazopanib, iDCs from this mRCC patient in treatment with pazopanib showed increased expression of HLA-DR at T2 and CD40 at T1 and T2. Expression of the coinhibitory molecule PD-L1 decreased during pazopanib treatment, both in iDCs and mDCs, confirming that pazopanib boosts DC activity.

Immune profile of mRCC patients during TKI treatment.

The priming of DCs by pazopanib could impact the immune repertoire in the peripheral blood ofmRCCpatients. To verify this hypothesis, we followed and monitored longitudinally mRCC patients undergoing TKI

therapy, either pazopanib or sunitinib. Patients had different previous therapies although all of the patients belonged to the same risk group. Six patients in treatment with TKIs for mRCC underwent peripheral blood sampling at different time points and PBMCs were isolated and analyzed by flow cytometer in order to investigate the evolution of patients' immunological profile during TKI administration. Patients 1, 2, and 3 received pazopanib, whereas patients 4, 5, and 6 received sunitinib. The immunoassays were performed when possible at different time points. Two patients (patient 3 and 5) showed progressive disease and one of these died from the disease. The other four are still in treatment with TKIs. Several immunological parameters were evaluated for each patient at each time point considering the limited amount of blood sampling. In particular, we analyzed T-cell subpopulations, their activation status (CD137 expression), their proliferation ability as assessed by expression of Ki67, the immunosuppression as a percentage of Tregs, and T-cell exhaustion as assessed by expression of checkpoint inhibitors such as PD-1, CTLA-4, and Tim-3. Pro- and anti-inflammatory cytokines were evaluated in serum of patients.
We observed that pazopanib seemed to induce an increased number of CD3þCD137þ T cells (Fig. 6B). In patient 1, for example, activated (CD137þ) CD4þ T cells were 30% of the activated CD3þ T cells at time T2, compared with T0 (before pazopanib therapy, 0.3% of activated CD3þ T cells) and T1 (4.7% of CD3þ T cells). At T5, we observed a decrease of CD4þ and CD8þ CD137þ T-cell populations. In the same patient, plasma levels of IL6 dropped from T0 to T2, both IL4 and CXCL-10 increased at T1 and T2, and ICAM decreased at T2. Ki67 expression, a proliferation signal for T cells, was higher at T2 than at other time points. Patient 2 had fewer CD137þ T cells from time T0 forward. Regulatory T cells decreased during treatment and Ki67þ T cells increased. Patient 3, who was monitored during pazopanib treatment and immediately after progression, showed enhancement of CD4þCD137þ T cells at T3 (54%) compared with T0 (24.9%) and T1 (23.2%). This population decreased during progression under nivolumab treatment (T4, T5). This patient's Treg population was unchanged. PD1þ T cells increased slightly from T0 to T1, then decreased during pazopanib and nivolumab treatment. When we analyzed mRCC patients treated with sunitinib, we observed that CD137þ T cells were less evident, thus suggesting that this population could be influenced by the effect of pazopanib. In Patient 4, under sunitinib therapy, CD4þCD137þ T cells were barely detectable at all three time points. This patient presented with 29% of T cells being CD8þCD137þ T cells at T1; however, this population decreased to 14% at T2 and T3. In the same patient, the serum

concentrations of IL10 were increased at T2 compared with T1, whereas serum concentrations of IL12, IL4, and CXCL-10 decreased. In accordance with the increase of IL10, we observed an increase of Treg cells during sunitinib treatment, accompanied by upregulation of CTLA-4 expression on T cells at T3. Patient 5 showed a similar decreasing trend in CD137þ T-cell population. Coinhibitory markers, such as CTLA-4 and Tim-3, increased during sunitinib treatment. Ki67þ T cells were decreased, and Treg cells were reduced. Patient 6 presented low and stable fractions of CD137þ T cells during sunitinib treatment when there time points were analyzed, although CD3þCD137þ T cells doubled at T7. At the same time IL10 and IL4 decreased compared with T5, and IL12 slightly increased. Tregs decreased at T7 but increased at T9.

INTEGRATED DISCUSSION

Endothelium is recognized as a major contributor in the efficacy of the immune response and several receptors are shared between participant cells. Research efforts have been directed in understanding the immune effects of anti angiogenic drugs. The interest is becoming urgent since with the possibility to introduce the ICIs during or combined with the anti angiogenic treatments in several cancers it is mandatory to consider these drugs from the point of view of their impact on the patient's immune system. The important question is: can we use the normalization effect induced by the anti angiogenic drugs to potentiate immunotherapeutic strategies? Moreover how are the shared receptors among immune cells, i.e. VEGFR, influenced by the therapy? Can we use anti-angiogenetic drugs to turn a "cold" tumor into a "hot" one and prepare the cancer patient for a successful ICI therapy?
The best readouts are the patients. Each of them has its own immune system, which has been shaped during life starting from host genetic factors and modulated in time by history of infectious diseases, environmental and lifestyle factors, stress and microbioma repertoire [**Routy B, Science 2018**). When the patient arrives to our attention with a diagnosed cancer, we need to consider not only the nature (histotype, genomic portrait, etc.) of the malignancy but also the immunological "fitness" of the patient, particularly at the tumor level. This is a novel approach and the new immunotherapy drugs require this information. In

this context our experiments on monocyte derived DCs (**Zizzari IG, Cancer Immun Res 2018**) indicated that plasmatic concentration of pazopanib were able to improve DC differentiation and performance, by upregulating maturation markers, dowregulating co-inhibitors molecules such as PD-L1 and increasing allogenic response and Th1 cytokine production. We demonstrated that the shut down of β-catenin pathway was the mechanism involved. The activation of the β-catenin pathway has been shown to correlate with the absence of T cells from the microenvironment in metastatic melanoma and urothelial cancers. The targeting of the β-catenin pathway has therefore been suggested as an optimal strategy to reestablish lymphocyte trafficking within the tumor [**Spranger S, Nature 2015**].

In ovarian cancer setting, the clinical benefit of bevacizumab has not been completely justified from a biological point of view. In particular, its interaction with patients' immune system is still not completely elucidated, although a strong rationale about the interplay between its ligand (VEGF) and the host's immune response suppression has already been shown [**Lapeyre-Prost A, International Review of Cell and Molecular Biology 2017**]. In particular, three different mechanisms related to VEGF-mediated immunosuppression have been assessed so far: inhibition of dendritic cell maturation [**Gabrilovich DI, Nat. Med. 1996; Gabrilovich D, Blood 1998; Oyama T, J. Immunol. 1998**) reduction of T cell tumor infiltration [**Li B, Clin. Cancer. Res. 2006**) and promotion of inhibitory cells in the tumor microenvironment [**Facciabene A, Nature 2011**]. In this scenario, our study published on JCM (**Napoletano C & Ruscito I, J Clin Med 2019**) adds new evidence to the body of knowledge concerning the immune effects of bevacizumab in advanced cancer patients by showing that (1) ovarian cancer patients not treated with bevacizumab-based chemotherapy seem to have a more immunosuppressive profile with the presence of a rTreg population that persists until the end of III cycles of therapy; (2) patients that clinically respond to bevacizumab treatment show a discrete population of effector T cells at the beginning of therapy that is maintained throughout the treatment; (3) Tregs are mainly represented by non-suppressive regulatory T cells in clinically responding bevacizumab patients compared with nonresponding patients and are also stably maintained in this ratio (nsT reg > sup T reg) throughout the treatment; (4) after three cycles of treatment, nonresponding bevacizumab patients produce more immunosuppressive IL-10 cytokine compared with responding patients.

It should be pointed out that these results were obtained by comparing two groups of patients that were matched for all clinical characteristics.

Particularly important is to notice that the performance status was similar among the two groups; this variable has been significantly associated with the immunological effects and response to several therapies [24].
Our results (**Napoletano C & Ruscito I, J Clin Med 2019**) also follow and confirm the original observation by our group [6] in which a significant reduction of Tregs and an increase in the proportion and function of effector CD8 T cells were found in an end-of-life ovarian cancer patient treated with low-dose intraperitoneal bevacizumab for malignant ascites. We also showed that responding bevacizumab-treated patients reported a higher percentage of circulating CD4 effector T cells compared with nonresponding bevacizumab patients, confirming what has already been observed in metastatic colorectal cancer [**Manzoni M, Oncology 2010**]. This data has key implications in the current panorama of oncological clinical approach. Indeed, it is reasonable to suggest that the circulating effector T cells recruited and sustained by bevacizumab treatment, thanks to its ability to restore tumor microvascular normalization [**Goel S, Physiol. Rev. 2011**], could be expanded by the administration of
checkpoint inhibitor agents, thus giving a strong biological rationale for the combination of immunotherapy with bevacizumab antiangiogenetic therapy. In support of this consideration, tumor tissue derived from metastatic renal cell carcinoma (mRCC) patients treated with anti-PD-L1 atezolizumab plus bevacizumab was recently found to show an increase in intratumoral CD8 T cells as well as an increase in intratumoral MHC-I, Th1 and T-effector markers, and chemokines. Trafficking lymphocytes also increased in tumors following bevacizumab and combination treatment [**Wallin JJ, Nat. Commun. 2016**].
Another interesting results of our studies who deserve a special discussion in this context is that in **Ruscito I, Br J Cancer 2018** article, we observed that VEGFA overexpression in pOC has been most frequently found among patients with a cancer somatic mutation of BRCA1/2 genes. This finding is in line with two other previously published papers. In 2013, Danza (**Danza, K. Eur. J. Hum. Genet. 2013**) observed that BRCA-mutated breast cancer patients reported higher levels of VEGF mRNA (P = 0.04) compared with those without BRCA mutations. In 2016, another study revealed that a VEGF-dependent gene signature (VDGs) was overexpressed in OC BRCA mutation carriers (**Yin X, Sci. Rep. 2016**). An interesting hypothesis explaining the linking between BRCA1 mutation and VEGF overexpression in HGSOC has been recently proposed: in 2015 Desai A and Colleagues (**Desai A, J Gynecol Res 2015**) pointed out that wild-type BRCA1 binds to Ubc9, which induces Caveolin-1 expression, downregulates VEGF and regulates endothelial function in normal ovaries

and fallopian tubes. In HGSOC with BRCA1 dysfunction, Ubc9 is not binded and this inhibits Caveolin-1 expression causing increased VEGF levels, loss of endothelial function and accumulation of ascites. Compared to these previous studies, we also confirmed in our cohort the positive influence of BRCA mutations on OC patients' survival (**Yang D, JAMA 2011; Bolton, K. L., JAMA 2012**), as well as the significant association
between BRCA mutation and VEGF-positivity determined VEGFpositivity a good prognostic factor in our HGSOC series. This result may also reflect the highly selection of the sample analysed, which only included HGSOC patients, who can also undergo secondary cytoreductive surgery for recurrence. These patients have usually good performance status and low tumour burden, so there is a selection of patients with a better clinical outcome (**Norquist BM, Clin. Cancer Res. 2018**). Furthermore, patients have been treated in high volume centres, with high experience in surgical treatment of ovarian cancer.

In conclusion, these studies sheds a light on the strong need to routinely include immunomonitoring in ovarian cancer clinical protocols of patients during the course of antiangiogenetic therapy administrations, with the final aim being to identify early the subset of patients who can mostly benefit from its adoption. Furthermore, our studies provides a first rationale regarding the positive immunologic impact of combining bevacizumab with checkpoint inhibitors.

In light of this evidence, results of the following ongoing phase III trials exploring combination of ICIs and PARPIs and/or anti-VEGF drugs are strongly expected (Table 1, extracted from **Borella F, Diagnostics 2020**):

Table 1

Study	Setting		Enrollment	Arms	Primary Endpoints	Current Status
AGO/DUO-ENGOT Ov46; NCT03737643	Front line and maintenance	Stage III-IV OC, FTC, or PPC. UPS of IDS.	1056 patients	Carboplatin-taxol + bevacizumab + placebo followed by +bevacizumab + placebo + placebo Carboplatin-taxol + bevacizumab + durvalumab (anti PD-L1) followed by bevacizumab + durvalumab + placebo Carboplatin-taxol + bevacizumab + durvalumab followed by bevacizumab + durvalumab + olaparib	PFS in non-tBRCA mutated	Recruiting
KEYLYNK-001/ ENGOT-ov43; NCT03740165	Front line and maintenance	Stage III-IV OC, FTC, or PPC, all hystotypes excluding mucinous, germ cell, or borderline tumors. UPS of IDS.	1086 patients	Carboplatin-taxol + placebo followed by + placebo Carboplatin-taxol + pembrolizumab (anti PD-1) followed by pembrolizumab+ placebo Carboplatin-taxol + pembrolizumab followed by pembrolizumab+ olaparib	PFS and OS	Recruiting
GINECO/FIRST ENGOT Ov44; NCT03602859	Front line and maintenance	Stage III-IV OC, FTC, or PPC, all hystotypes excluding mucinous, germ cell, or borderline tumors. UPS of IDS.	912 patients	Carboplatin taxol + placebo followed by placebo Carboplatin taxol + placebo + followed by placebo+ niraparib Carbo-tax + dostarlimab (anti TSR042) followed by+ dostarlimab (anti PD1) +niraparib	PFS	Recruiting
ATHENA GOG3020/ENGOT Ov45; NCT03522246	Maintenance after front line	Stage III-IV OC, FTC, or PPC. Completed first-line platinum-based chemotherapy and surgery with a response. UPS or IDS.	1012 patients	Rucaparib + nivolumab (anti PD1) Rucaparib + placebo Nivolumab-placebo Placebo + placebo	PFS	Recruiting
GOG3015/ENGOT OV39; NCT03038100	Front line	Stage III-IV OC, FTC, or PPC with macroscopic residual disease postoperatively or neoadjuvant therapy followed by IDS.	Estimated 1300 patients	Carboplatin-taxol + bevacizumab Carboplatin-taxol + bevacizumab + atezolizumab (anti PD-L1)	PFS and PFS in PD-L1 + subpopulation; OS and OS in PD-L1 + subpopulation	Active
ENGOT-Ov41/GEICO 69-O/ANITA; NCT03598270	Recurrence Platinum sensitive	PFI > 6 months and 2 prior lines of chemotherapy. The last line of chemotherapy should have included platinum. BRCA status known	414 patients	Carboplatin combo + niraparib Carboplatin combo + niraparib + atezolizumab	PFS	Recruiting
ATALANTE/ENGOT OV29; NCT02891824	Recurrence Platinum sensitive	PFI > 6 months and 2 prior lines of chemotherapy. The last line of chemotherapy should have included platinum.	600 patients	Carboplatin combo + bevacizumab Carboplatin combo + bevacizumab + atezolizumab	PFS	Active
EORTC-1508, NCT02659384	Recurrence Platinum resistant	Platinum resistant EOC, FTC or PPC. Any number of platinum-based chemotherapy lines, but a maximum of 2 previous non-platinum containing lines. Prior treatment with bevacizumab or other targeted agents	Estimated 160 patients	Bevacizumab Bevacizumab + atezolizumab Bevacizumab + atezolizumab + aspirin	PFS at 6 months	Closed to recruitment

OC: ovarian cancer, FTC: fallopian tube cancer, PPC: primary peritoneal cancer, UPS: upfront primary surgery, IDS: interval debulking surgery, PFS: progression-free survival, OS: overall survival.

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STATUTORY DECLARATION

“I, ILARY RUSCITO, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic “HARNESSING TUMOR ANGIOGENESIS
TO EXPLORE OVARIAN CANCER IMMUNE SUPPRESSION AND ADDRESS TARGET-THERAPIES OUTCOMES”, independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I also declare this is an International Joint Dissertation (Cotutelle du thése) and for this reason this dissertation has been submitted, in identical form, also to Sapienza University of Rome, Rome, Italy.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date 10/09/2020 Signature

Declaration of your own contribution to the publications

ILARY RUSCITO contributed the following to the below listed publications:

- **Publication 1**: **Ruscito I**, Cacsire Castillo-Tong D, Vergote I, Ignat I, Stanske M, Vanderstichele A, Glajzer J, Kulbe H, Trillsch F, Mustea A, Kreuzinger C, Benedetti Panici P, Gourley C, Gabra H, Nuti M, Taube ET, Kessler M, Sehouli J, Darb-Esfahani S, Braicu EI. *Characterisation of tumor microvessel density during progression of high-grade serous ovarian cancer: clinico-pathological impact. An OCTIPS Consortium study.* British Journal of Cancer 2018;119(3):330-338.

CONTRIBUTION:
Study concepts and design: I.R. and E.I.B.. Data acquisition: I.R., H.K., F.T., A.V., M.S., I.I.. Quality control of data acquired: D.C.C-T., I.V., C.G., H.G., A.M., J.S., S.D-E.. Data analysis and interpretation: I.R., S.D-E., M.K., C.K., P.B.P, M.N., J.G.. Statistical analysis: I.R., M.S., S. D-E., E.T.T.. Manuscript writing: I.R. and E.I.B.. Manuscript editing: all co-authors.

- **Publication 2**: Napoletano C*, **Ruscito I***, Bellati F, Zizzari IG, Rahimi H, Gasparri ML, Antonilli M, Panici PB, Rughetti A, Nuti M. *Bevacizumab-Based Chemotherapy Triggers Immunological Effects in Responding Multi-Treated Recurrent Ovarian Cancer Patients by Favoring the Recruitment of Effector T Cell Subsets.* J Clin Med. 2019;8(3). pii: E380. doi: 10.3390/jcm8030380.
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Conceptualization, C.N. and F.B.; Data curation, M.L.G. and M.A.; Formal analysis, I.R.; Funding acquisition, M.N.; Investigation, I.G.Z. and H.R.; Methodology, C.N. and I.R.; Project administration, C.N. and I.R.; Resources, F.B., M.G., M.A. and P.B.P.; Supervision, M.N.; Validation, A.R.; Visualization, C.N. and I.R.; Writing – original draft, C.N. and I.R.; Writing – review & editing, C.N., I.R., A.R. and M.N.

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Signature of doctoral candidate

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BJC British Journal of Cancer

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ARTICLE
Molecular Diagnostics

Characterisation of tumour microvessel density during progression of high-grade serous ovarian cancer: clinico-pathological impact (an OCTIPS Consortium study).

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BACKGROUND: High-grade serous ovarian cancer (HGSOC) intratumoural vasculature evolution remains unknown. The study investigated changes in tumour microvessel density (MVD) in a large cohort of paired primary and recurrent HGSOC tissue samples and its impact on patients' clinico-pathological outcome.
METHODS: A total of 222 primary (pOC) and recurrent (rOC) intra-patient paired HGSOC were assessed for immunohistochemical expression of angiogenesis-associated biomarkers (CD31, to evaluate MVD, and VEGF-A). Expression profiles were compared between pOCs and rOCs and correlated with patients' data.
RESULTS: High intratumoural MVD and VEGF-A expression were observed in 75.7% (84/111) and 20.7% (23/111) pOCs, respectively. MVD^{high} and $VEGF^{(+)}$ samples were detected in 51.4% (57/111) and 20.7% (23/111) rOCs, respectively. $MVD^{high}/VEGF^{(+)}$ co-expression was found in 19.8% (22/111) and 8.1% (9/111) of pOCs and rOCs, respectively ($p = 0.02$). Pairwise analysis showed no significant change in MVD ($p = 0.935$) and VEGF-A ($p = 0.121$) levels from pOCs to rOCs. MVD^{high} pOCs were associated with higher $CD3^{(+)}$ ($p = 0.029$) and $CD8^{(+)}$ ($p = 0.013$) intratumoural effector TILs, while $VEGF^{(+)}$ samples were most frequently encountered among BRCA-mutated tumours ($p = 0.019$). Multivariate analysis showed VEGF and MVD were not independent prognostic factors for OS.
CONCLUSIONS: HGSOC intratumoural vasculature did not undergo significant changes during disease progression. High concentration of $CD31^{(+)}$ vessels seems to promote recruitment of effector TILs. The study also provides preliminary evidence of the correlation between VEGF-positivity and BRCA status.

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INTRODUCTION

High-grade serous ovarian carcinoma (HGSOC) still accounts for the highest mortality rate among all ovarian cancer (OC) histotypes, with almost 80% of all new deaths from OC being caused by this distinct subgroup of ovarian tumours.[1–4] International groups of opinion leaders have recognised the designing of new translational studies on recurrent and end-stage HGS tumour tissue samples as a key 'unmet need' in the understanding of HGSOC biology and clonal evolution.[4]

In this scenario, analysis of the evolution process affecting intratumoural vasculature during HGSOC progression is a pivotal issue to be still elucidated.

After decades of paralysis in primary OC first-line chemotherapy treatment, indeed, incorporation of bevacizumab in the upfront regimen for advanced newly diagnosed disease[5] has changed the 'standard of care paradigm' of advanced primary OC, although characterised by less survival impact than expected.[6–8] Thus, understanding changes in the vasculature or identification of

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prognostic biomarkers of response to vasculature targeting is needed. Unfortunately, there are currently no predictive biomarkers to tailor bevacizumab treatment in OC patients.

A full knowledge of molecular changes involving intratumoural vasculature from primary to recurrent HGSOC is still lacking and may provide new opportunities to: (1) tailor treatment with currently available anti-angiogenetic agents, (2) shed light on acquired resistance mechanisms, and (3) develop new targeted therapies.

The aim of this study was to identify changes occurring from primary to recurrent HGSOC in tumour tissue expression of the angiogenesis-associated biomarkers CD31, applied for detecting microvessels density (MVD),[9–11] and VEGF-A,[12] by analysing a large cohort of paired primary and recurrent HGSOC tissue samples. Secondary endpoints included the correlation of biomarkers expression with patients' clinico-pathological characteristics and survival data.

MATERIALS AND METHODS

Sample Collection

Paired cancer tissue samples belonging to HGSOC patients were collected during primary and secondary cytoreduction. Patients were treated with primary debulking surgery followed by platinum-based chemotherapy between 1985 and 2013, and were retrospectively and consecutively selected from OCTIPS (Ovarian Cancer Therapy–Innovative Models Prolong Survival, Agreement No.279113-2) Consortium database. Included patients underwent both primary (pOC) and recurrent (rOC) surgery in one of the European Gynaecologic Oncology referral Centers of the following Institutions: Charité Universitätsmedizin Berlin, Germany; Catholic University of Leuven, Belgium; Imperial College, London, UK; University of Edinburgh, UK; University Medical Center Hamburg-Eppendorf, Germany.

Inclusion criteria were: availability of paired primary and recurrent cancer tissue samples from HGSOC patient together with clinical annotation. Exclusion criterion was: neoadjuvant chemotherapy treatment, due to the need to analyse primary chemo-naïve tumours. Approval from each local ethics committee was obtained (EK207/2003, ML2524, 05/Q0406/178, EK130113, 06/S1101/16). All included samples underwent central histopathological assessment to confirm HGSOC histology and ensure tumour tissue content and quality.

Immunohistochemistry

Tissue microarrays (TMA) were constructed for immunohistochemical staining. Each primary and recurrent tumour tissue sample was represented within the TMA by two tumour cores, each containing at least 90% of cancer cells.

Sections from TMA were deparaffinised in xylol, rehydrated in graded alcohol, and boiled in pressure cooker for 5 minutes in citrate buffer (pH = 6), for CD31 staining, or in EDTA (pH = 9), for VEGF staining. Rabbit anti-human CD31 antibody (clone ab32457; Abcam, Cambridge, MA, USA) and rabbit anti-human VEGF-A antibody (clone A-20; Santa Cruz Biotechnology, Dallas, TX, USA) were diluted 1:20 and 1:250, respectively, and incubated on slides for 60 minutes at room temperature. Bound antibodies were visualised using DAKO Real Detection System and DAB + (3,3′-diaminobenzidine; DAKO, Glostrup,Denmark) as a chromogen. Finally, the slides were co-stained with hematoxylin.

CD31 stained samples were assessed in terms of MVD. MVD was determined by averaging the number of vessels from three distinct areas of tumour with highest vessels density examined at ×200 magnification.[13–15]

Samples were further classified into 'MVD^{high}' (≥16.3 vessels) or 'MVD^{low}' (<16.3 vessels), establishing the cut-off level of MVD count for dichotomisation at first quartile (primary samples), being the value able to maximise difference in OS hazard ratio[13,15,16] (Table S1).

For VEGF staining evaluation, the number of stained tumour cells within the whole TMA cores (0% = 0; 1–10% = 1; 11–50% = 2; >50% = 3) was multiplied with the intensity of staining (negative = 0; weak = 1; moderate = 2; strong = 3),[17] resulting in a semiquantitive immunoreactivity score (IRS) ranging from 0 to 9. Samples were classified as 'VEGF$^{(+)}$', for VEGF-high tumour expression (IRS = 4–9), or as 'VEGF$^{(-)}$', for absent/weak focal staining (IRS = 0–3).

As positive control for IHC were used human liver sections. Samples staining was assessed independently by two co-authors (IR and SDE).

Table 1. Patient characteristics

Patients *n*	111
Age	
Median (range)	56 y (33y-77y)
FIGO Stage (%)	
I	2 (1.8%)
II	5 (4.5%)
III	93 (83.8%)
IV	11 (9.9%)
Residual tumour after PDS:	
No Residual Tumour	89 (80.2%)
Any Residual Tumour	22 (19.8%)
Type of first-line CHT	
With bevacizumab	2 (1.8%)
Without bevacizumab	109 (98.2%)
Type of second-line CHT	
With bevacizumab	8 (7.2%)
Without bevacizumab	103 (92.8%)
Platinum response after primary treatment	
Platinum sensitive	90 (81.1%)
Platinum resistant	18 (16.2%)
Unknown	3 (2.7%)
Platinum response after treatment for disease relapse	
Platinum sensitive	59 (53.2%)
Platinum resistant	12 (10.8%)
Missing	40 (36%)
Somatic-BRCA status	
BRCA wt	31 (27.9%)
BRCA 1/2 mutation	21 (18.9%)
Unknown	59 (53.2%)
Maximum follow-up time	214 months
Median OS	63 months

CHT Chemotherapy, *OS* Overall survival, *PDS* Primary debulking surgery, *wt* wild type

Patients' clinico-pathological data

Patients' clinico-pathological data, including somatic-BRCA status from 52 included patients, were retrieved from OCTIPS Consortium database.[18] GCIG criteria were applied to define platinum-resistance and platinum-sensitivity.[19] RECIST Criteria were applied during patients' follow-up to define HGSOC relapse.[20] No residual tumour was defined intraoperatively by the surgeon in case no macroscopic tumour could be detected at the end of cytoreduction.

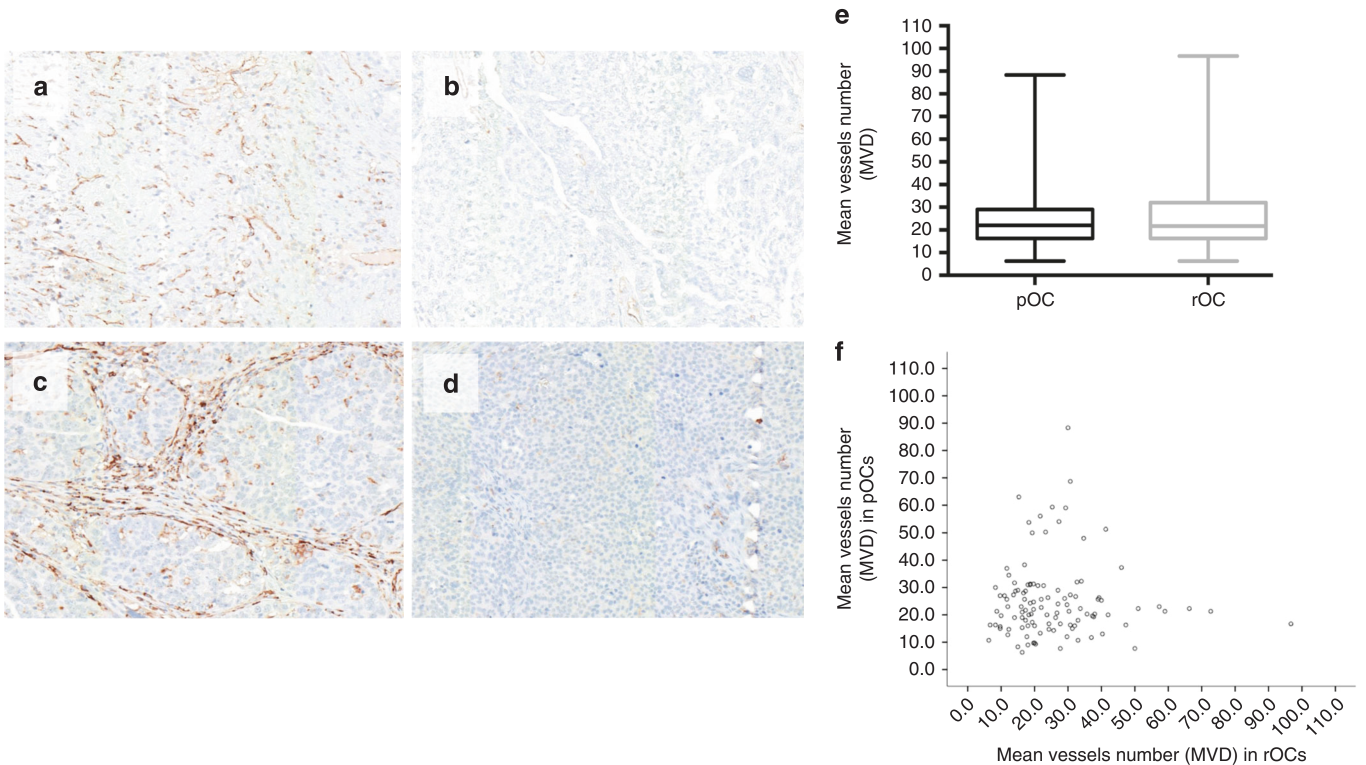

Fig. 1 CD31 immunohistochemistry staining for intratumoural MVD assessment: MVD^{high} (**a**) and MVD^{low} (**b**) pOC samples; MVD^{high} (**c**) and MVD^{low} (**d**) rOC samples. ×400 magnification; MVD count among primary and recurrent tumours (box plot (**e**) and scatter plot (**f**))

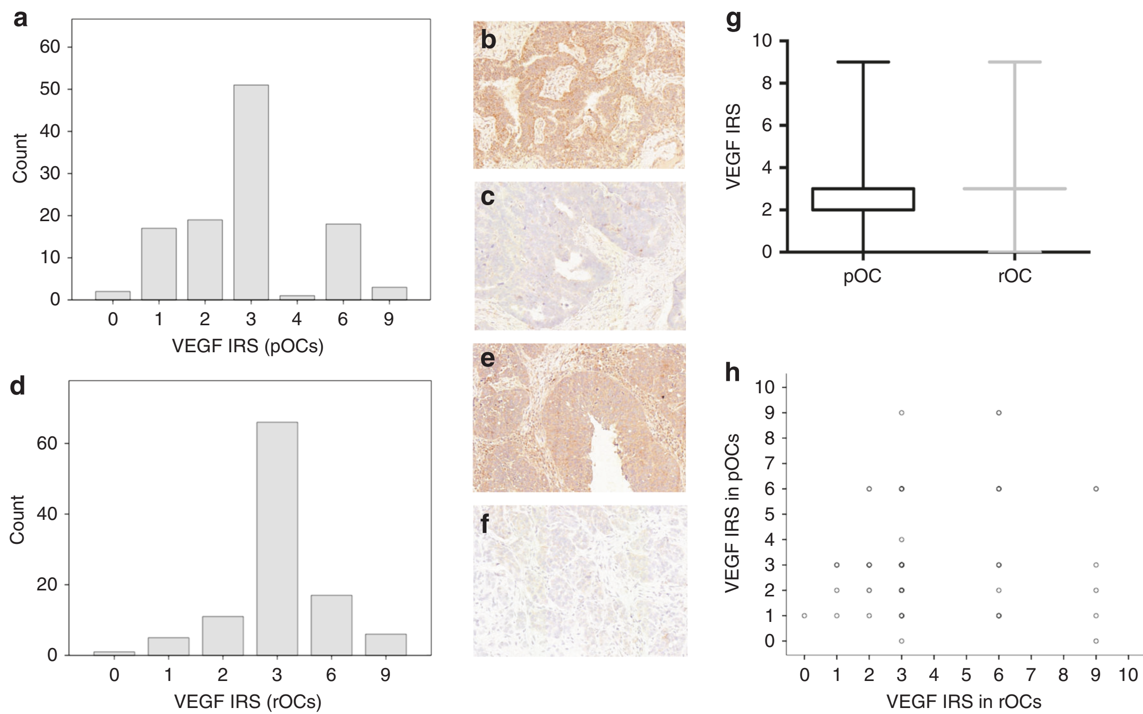

Fig. 2 VEGF-A immunohistochemistry staining. VEGF-A IRS distribution in primary (**a**) and recurrent (**d**) tumour samples. pOCs, VEGF$^{(+)}$ (**b**) and VEGF$^{(-)}$ (**c**); rOCs, VEGF$^{(+)}$ (**e**) and VEGF$^{(-)}$ (**f**); VEGF-A IRS among primary and recurrent tumours (box plot (**g**) and scatter plot (**h**))

In order to investigate any association between different tumour vasculature profiles and intratumoural immune infiltrate in both pOCs and rOCs, MVD and/or VEGF profiles were matched with previous OCTIPS data on tumour infiltrating lymphocytes (TILs), assessed through the immunohistochemical expression of CD3, CD4, and CD8 biomarkers, as previously reported.[21] Furthermore, immunosuppressive TILs were evaluated through the expression of T-regulatory cells-specific biomarker FoxP3,

using the mouse anti-human FOXP3 antibody (clone ab20034; Abcam, Cambridge, MA, USA, 1:200, 1.5 h at room temperature). The count of stained FoxP3-positive TILs was then performed automatically with the *VM Scope Quantifier*, as previously reported.[21]

Statistical Analysis
Statistical analysis was performed using SPSS version 22.0 (SPSS Inc,Chicago,IL,USA). Difference in biomarker expression between pOCs and rOCs was assessed through the correlation test (Spearman coefficient, 2-tailed) and 'Wilcoxon signed rank' non-parametric test for related samples. Fisher's exact test was applied to correlate MVD and/or VEGF tumour expression with patients' clinico-pathological categorical data. Patients' progression-free interval (PFI), progression-free survival (PFS), and overall survival (OS) were identified through Kaplan–Meier analysis (Log-Rank test). PFI was defined as the time interval from the last adjuvant chemotherapy to relapse, whereas progression-free survival (PFS) was established as the time interval between first recurrence diagnosis and tumour progression. Univariate and multivariate survival analyses were performed applying Cox-regression model. Multivariable models were obtained among variables reporting a p-value ≤ 0.1 in univariate analysis. p-values ≤ 0.05 were evaluated statistically significant.

RESULTS

A total of 222 intra-patient paired primary and recurrent HGSOC tissue samples derived from 111 patients were included. Patients' characteristics are listed in Table 1. To note, only 2/111 (1.8%) patients received bevacizumab in front-line chemotherapy, thus the staining of recurrent samples have not been influenced by first-line administration of anti-angiogenetic compounds.

MVD staining
MVD^{high} staining was detected in 75.7% (84/111) of pOC and in 51.4% (57/111) of rOC, whereas MVD^{low} staining was found in 24.3% (27/111) and in 48.6% (54/111) of pOC and rOC, respectively. MVD^{low} staining was twice as prevalent in relapsed tumours compared to primary disease ($p = 0.0003$, Fisher's exact test, Fig. 1a–d). Nevertheless, globally, pairwise analysis revealed no tendency towards a change in MVD to higher or lower levels in recurrent samples ($p = 0.935$, Wilcoxon test; Fig. 1e), as well as no significant correlation between pOCs and rOCs in MVD was reported (Spearman correlation, $p = 0.920$; Spearman coefficient: 0.01).

VEGF-A expression
VEGF IRS distribution in both pOCs and rOCs is shown in Fig. 2a, d. The same percentage of $VEGF^{(+)}$ (20.7%, 23/111) and $VEGF^{(-)}$ (79.3%, 88/111) tumour samples was found between pOCs and rOCs, respectively, ($p = 1$, Fisher's exact test, Fig. 2b, c, e, f), although no significant correlation between pOCs and rOCs VEGF IRS values could be observed ($p = 0.505$, Spearman coefficient 0.06). Furthermore, pairwise analysis confirmed no tendency towards a change in VEGF IRS levels at tumour relapse ($p = 0.121$, Wilcoxon test; Fig. 2g).

MVD^{high} and $VEGF^{(+)}$ co-expression in pOCs vs rOCs.
MVD^{high} and $VEGF^{(+)}$ co-expression was more frequent in pOCs group (22/111, 19.8%) compared to rOCs (9/111, 8.1%) ($p = 0.02$, Fisher's exact test, Fig. S1).

Relationship between MVD and/or VEGF-A expression with TILs.
Results showed that MVD^{high} levels in pOCs samples were associated with higher $CD3^{(+)}$ ($p = 0.029$, Mann–Whitney test) and $CD8^{(+)}$ ($p = 0.013$) effector TILs, but not with a higher $FoxP3^{(+)}$ ($p = 0.443$) T-regulatory cells infiltrate. To note, the correlation between MVD and $CD3^{(+)}/CD8^{(+)}$ TILs disappeared at tumour recurrence. No significance between pOCs or rOCs VEGF expression or $MVD^{high} + VEGF^{(+)}$ co-staining with TILs was reported (Fig. S2, Table S2).

MVD and/or VEGF-A profiles and patients' clinico-pathological factors
Analysis on the correlation between MVD and/or VEGF expression in pOCs with patients' clinico-pathological characteristics is shown in Table 2. In particular, $VEGF^{(+)}$ primary HGSOCs and $MVD^{high}/VEGF^{(+)}$ primary samples were most frequently encountered among somatic-BRCA-mutated tumours compared to somatic-BRCA wild-type cases ($p = 0.019$, Fisher's exact test). No further significant associations between different intratumoural

Table 2. Association of MVD and/or VEGF expression with patients' clinico-pathological characteristics (pOCs)

Clinico-pathological factors	Total *N*	MVD (pOC)			VEGF (pOC)			MVD high + VEGF pos co-expression (pOC)		
		High	Low	*P*	High	Low	*P*	Yes	No	*P*
Patients' Age										
<56 y	53	39	14	0.663	13	40	0.360	13	40	0.246
≥56 y	58	45	13		10	48		9	49	
FIGO Stage										
I/II	7	4	3	0.358	2	5	0.633	2	5	0.624
III/IV	104	80	24		21	83		20	84	
Residual tumour after first cytoreductive surgery										
No residual	89	67	22	1	18	71	0.775	17	72	0.767
Any residual	22	17	5		5	17		5	17	
Platinum-sensitivity status after primary treatment										
Platinum sensitive	90	71	19	0.133	18	72	0.530	17	73	0.521
Platinum resistant	18	11	7		5	13		5	13	
Somatic-BRCA status										
BRCA-WT	31	26	5	0.105	3	28	0.019	3	28	0.019
mBRCA1/2	21	13	8		8	13		8	13	

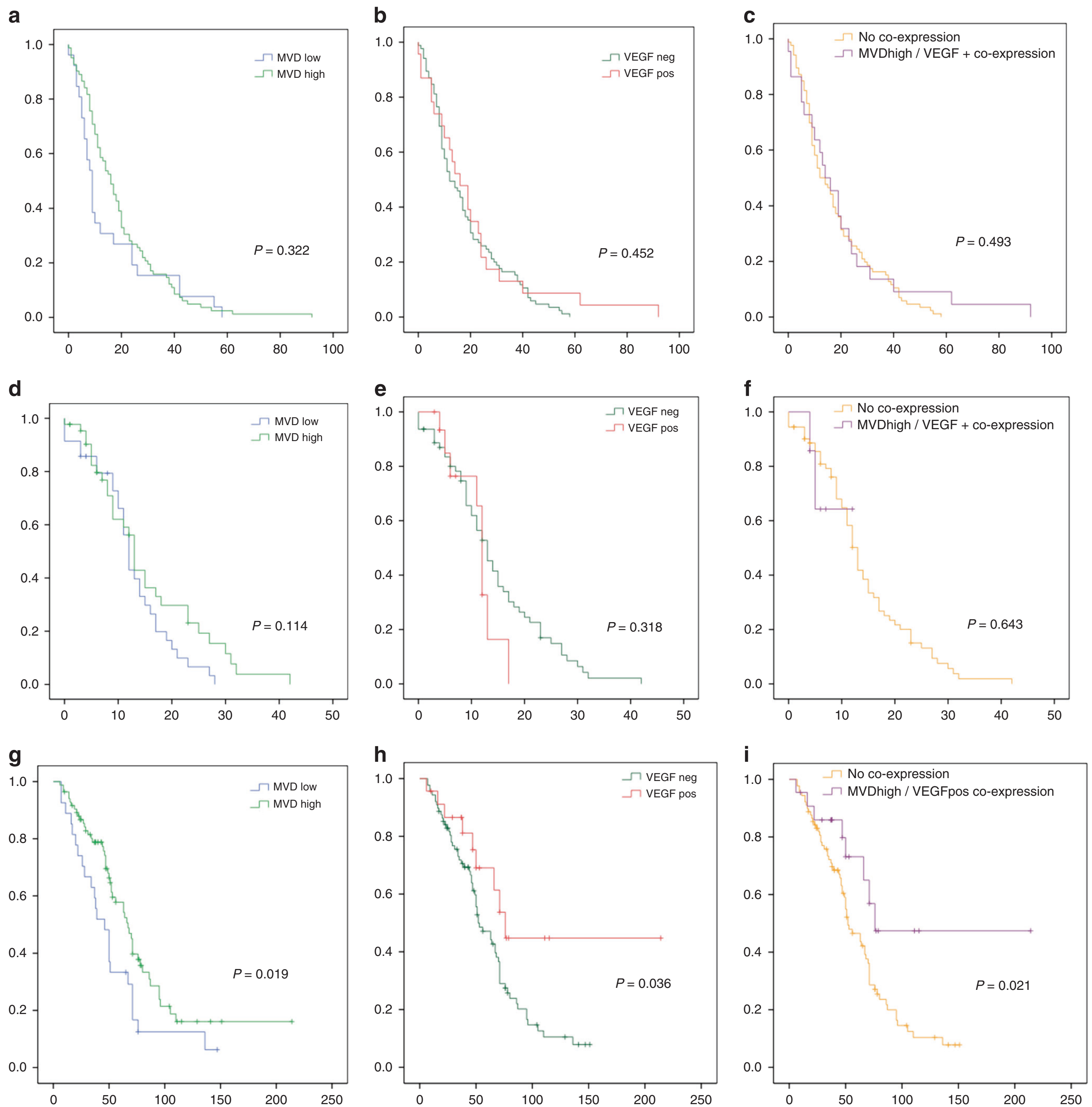

Fig. 3 MVD and/or VEGF status and progression-free survival after primary (PFI (**a**), (**b**), (**c**)) and recurrent (PFS, (**d**), (**e**), (**f**)) disease. **g**–**i** MVD and/or VEGF status at primary disease and overall survival. '*x*-axis': months, '*y*-axis': survival probability

vasculature profiles and patients' age at diagnosis, FIGO stage, residual tumour after primary debulking or first-line platinum response was identified.

Decrease of VEGF expression in rOCs was observed only in BRCA-mutated patients ($p = 0.053$, Wilcoxon test), although this association did not reach statistical significance (Fig. S3).

Survival

Patients, whose pOCs resulted MVD^{high}, $VEGF^{(+)}$ or co-stained for both biomarkers, were found to have a significantly improved OS compared to patients without these intratumoural profiles at primary disease (Fig. 3g–i). In particular, median OS for MVD^{high} and MVD^{low} patients was 67 and 46 months, respectively ($p = 0.019$), median OS for $VEGF^{(+)}$ and $VEGF^{(-)}$ patients resulted 76 vs 52 months, respectively ($p = 0.036$), while median OS for patients with co-stained pOCs was 76 months, compared to 52 months in women without co-expression ($p = 0.021$).

On the contrary, no influence of pOCs or rOCs MVD and/or VEGF expression on patients' time to progression after primary (PFI) or first recurrent disease (PFS) was reported (Fig. 3a–f).

Multivariate analysis for OS and PFI was carried out on the whole patients' population ($n = 111$) and also on the subgroup of patients ($n = 52$) with known tumour somatic-BRCA status. Table 3a, b shows that VEGF-A was not found to be an

Table 3. Multivariate analysis for OS

	HR (95% CI)	*P*
a: Whole population ($n = 111$)		
Overall survival		
Age (≥56 y vs <56 y)	1.155 (0.683–1.953)	0.590
FIGO stage (III/IV vs I/II)	2.507 (0.621–10.127)	0.197
Residual tumour (any residual vs no residual)	1.610 (0.875–2.962)	0.126
MVD (high vs low)	0.818 (0.417–1.604)	0.558
VEGF (positive vs negative)	0.420 (0.178–0.991)	**0.048**
FoxP3 mean number	0.963 (0.778–1.191)	0.727
CD3 mean number	1.000 (0.998–1.002)	0.786
CD4 mean number	1.000 (0.999–1.001)	0.925
CD8 mean number	1.000 (0.998–1.002)	0.846
Platinum response (Plat. Sens. vs Plat. Resist)	0.229 (0.104–0.506)	**< 0.001**
b: Only somatic-BRCA-tested population ($n = 52$)		
Overall survival		
Age (≥56 y vs <56 y)	1.017 (0.410–2.524)	0.971
FIGO stage (III/IV vs I/II)	1.506 (0.091–24.829)	0.775
Residual tumour (any residual vs no residual)	1.417 (0.259–7.755)	0.687
MVD (high vs low)	0.747 (0.243–2.291)	0.609
VEGF (positive vs negative)	0.440 (0.127–1.526)	0.196
FoxP3 mean number	0.683 (0.439–1.061)	0.090
CD3 mean number	0.998 (0.994–1.001)	0.132
CD4 mean number	0.997 (0.995–1.000)	**0.038**
CD8 mean number	0.998 (0.994–0.997)	0.438
Somatic-BRCA status (BRCA–mut vs BRCA wt)	0.354 (0.133–0.994)	**0.038**
Platinum response (Plat. Sens. vs Plat. Resist)	0.216 (0.051–0.991)	**0.037**
c: Whole population ($n = 111$)		
Progression-free interval		
Age (≥56 y vs <56 y)	1.067 (0.692–1.644)	0.770
FIGO stage (III/IV vs I/II)	2.447 (0.892–6.711)	0.082
Residual tumour (any residual vs no residual)	1.009 (0.568–1.794)	0.974
MVD (high vs low)	1.445 (0.832–2.511)	0.191
VEGF (positive vs negative)	0.945 (0.541–1.652)	0.843
FoxP3 mean number	0.984 (0.832–1.162)	0.845
CD3 mean number	1.000 (0.999–1.001)	0.835
CD4 mean number	1.000 (0.999–1.001)	0.698
CD8 mean number	1.000 (0.998–1.002)	0.845
d: Only somatic-BRCA-tested population ($n = 52$)		
Progression-free interval		
Age (≥ 56 y vs < 56 y)	1.121 (0.542–2.318)	0.759
FIGO stage (III/IV vs I/II)	18.261 (1.282–260.172)	**0.032**
Residual tumour (any residual vs no residual)	1.391 (0.280–6.918)	0.687
MVD (high vs low)	0.884 (0.375–2.081)	0.777
VEGF (positive vs negative)	0.916 (0.400–2.095)	0.834
FoxP3 mean number	0.868 (0.659–1.145)	0.317
CD3 mean number	0.998 (0.995–1.001)	0.159
CD4 mean number	0.996 (0.993–0.998)	**0.001**
CD8 mean number	0.999 (0.995–1.003)	0.719
Somatic-BRCA status (BRCA-mut vs BRCA wt)	0.982 (0.462–2.087)	0.962

Multivariate analysis for OS carried out on (a) the whole patients' population ($n = 111$), (b) only somatic-BRCA-tested population ($n = 52$) and multivariate analysis for PFI carried out on (c) the whole patients' population ($n = 111$), (d) only somatic-BRCA-tested population ($n = 52$). Bold values indicate significant p values (<0.05)

independent prognostic factor for OS anymore when considering also somatic-BRCA mutational status. Only somatic-BRCA mutation (HR: 0.354, CI 95%: 0.133–0.994; $p = 0.038$), high CD4$^{(+)}$ TILs (HR: 0.997, CI 95%: 0.995–1.000; $p = 0.038$) and first-line platinum response (HR: 0.216, CI 95%: 0.051–0.991; $p = 0.037$) were found to independently improve HGSOC patients' OS.

When analysing the PFI in patients with or without BRCA somatic mutations, advanced FIGO stage (HR: 18.261, CI 95%: 1.28–260.17; $p = 0.032$) and low CD4$^{(+)}$ TILs (HR: 0.996, CI 95%: 0.993–0.998; $p = 0.001$) were the only independent poor prognostic factors (Table 3c, d).

DISCUSSION

In the last decade, 'omics' sciences provided fundamental insight into the understanding of HGSOC biology,[3] showing as one distinct malignancy with its own characteristic phenotype, aetiology and progression profile.[22] Although known for its aggressive behaviour, HGSOC has a higher change to show durable response after first-line chemotherapy, compared to other OC histologies,[23] as well as its common platinum-sensitivity allows it to access a more varied panel of experimental second-line combinations.[24] Unfortunately, progression from HGSOC is often rapid and chemo-resistance develops.[4]

In this context, understanding the biological changes occurring to HGSOC during disease progression is an essential issue through which new identified biomolecular signatures, marking the HGSOC clinical evolution, could help developing new tailored treatment strategies.

In this study, OCTIPS Consortium aimed to identify modifications involving HGSOC intratumoural vasculature from primary to recurrent disease, by assessing the evolution of cancer MVD and VEGF-A expression. Results showed that: (1) MVD and/or VEGF levels did not undergo significant changes from pOC to rOC (being in line with already available clinical findings, as bevacizumab is showing mild improvement in PFS, in both primary and relapsed situation);[5,7,8] (2) High MVD levels in pOC seems to sustain the intratumoural recruitment of effector TILs and were associated with better OS in HGSOC patients; (3) VEGF$^{(+)}$ HGSOCs were most frequently encountered among somatic-BRCA-mutated tumours and VEGF-positivity correlates with better OS in this HGSOC cohort; (4) MVD and VEGF were not independent prognostic factor for OS when taking into account the BRCA mutational status and TILs profile.

The definition of 'intratumoural microvessel density' has been coined in the middle of 90's to objectivise the entity of blood supply available within the tumour mass to sustain cancer growth.[25] Intratumoural vessels are usually characterised by impaired vascular maturation, poor functionality and defects in endothelial architecture. Immaturity of the new generated tumour-associated vasculature results in excessive permeability, poor perfusion and imperfect blood flow.[26]

During the last 20 years, different studies recognised 'high' MVD a poor prognostic factor for cancer patients,[27–29] including women affected by OC.[30] Different biomarkers have been adopted to assess MVD in OC, including Von Willebrand Factor, CD105, CD34 and CD31, being CD34 the most used MVD detector and the biomarker associated with the poorest HR for OS (HR: 1.67, CI 95%: 1.36–2.35) compared to other MVD detectors (HR: 1.32, CI 95%: 0.82–1.82).[30]

CD31, also known as 'platelet endothelial cell adhesion molecule-1' (PECAM-1) is a transmembrane glycoprotein expressed on endothelial cells, platelets, neutrophils and T-cells. It is a key factor to maintain the integrity of endothelial cells permeability barrier and to promote the controlled activation of T-cells and their survival,[11,31,32] thus being expression of a normalised endothelium able to sustain the correct trafficking of T-cells into the tumour. In line with CD31 biological role, we observed that MVD^{high} levels in pOCs samples correlated with higher CD3$^{(+)}$ and CD8$^{(+)}$ TILs, but not with a higher FoxP3$^{(+)}$ T-lymphocytes infiltrate, thus suggesting that a high concentration of intratumoural CD31$^{(+)}$ vessels might be able to promote the intratumoural recruitment of effector T-cell populations, thus ultimately improving patients' survival.[33] Recently, Bais et al.[16] identified CD31-dependent MVD as a predictive biomarker for bevacizumab response in first-line treated OC patients. This finding might be consequence of intratumoural endothelial maturity, represented by high CD31-dependent MVD levels, able to ensure a normalised blood flow, which is pivotal for intratumoural drug delivery and efficacy.[26]

Vascular Endothelial Growth Factor (VEGF) is a key angiogenetic cytokine that regulates cell mitosis and endothelial cells permeability.[34] Overexpression of VEGF has been found to correlate with cancer relapse and decreased survival in patients affected by different solid tumours, including OC.[35] Despite previous studies, absence of significant changes in MVD and VEGF profile following disease progression of this unique cohort, indicates that these markers are not major drivers of molecular cancer evolution in vivo, but rather remain supportive factors.

One of the most intriguing outcomes of our study is that VEGF-A overexpression in pOC has been most frequently found among patients with a cancer somatic mutation of BRCA1/2 genes. This finding is in line with two other previously published papers. In 2013, Danza[36] observed that BRCA-mutated breast cancer patients reported higher levels of VEGF mRNA ($P = 0.04$) compared with those without BRCA mutations. In 2016, another study revealed that a VEGF-dependent gene signature (VDGs) was overexpressed in OC BRCA mutation carriers.[37] An interesting hypothesis explaining the linking between BRCA1 mutation and VEGF overexpression in HGSOC has been recently proposed: in 2015 Desai A and Colleagues[38] pointed out that wild-type BRCA1 binds to Ubc9, which induces Caveolin-1 expression, downregulates VEGF and regulates endothelial function in normal ovaries and fallopian tubes. In HGSOC with BRCA1 dysfunction, Ubc9 is not binded and this inhibits Caveolin-1 expression causing increased VEGF levels, loss of endothelial function and accumulation of ascites. Compared to these previous studies, we also confirmed in our cohort the positive influence of BRCA mutations on OC patients' survival,[39,40] as well as the significant association between BRCA mutation and VEGF-positivity determined VEGF-positivity a good prognostic factor in our HGSOC series. This result may also reflect the highly selection of the sample analysed, which only included HGSOC patients, who can also undergo secondary cytoreductive surgery for recurrence. These patients have usually good performance status and low tumour burden, so there is a selection of patients with a better clinical outcome.[41] Furthermore, patients have been treated in high volume centres, with high experience in surgical treatment of ovarian cancer. Most Centers have been also approved and allowed to participate in the LION (ClinicalTrials.gov Identifier: NCT00712218), DESKTOP III (ClinicalTrials.gov Identifier: NCT01166737) and TRUST (ClinicalTrials.gov Identifier: NCT02828618) studies, based on the high quality of the tumour debulking.

Nevertheless, further studies aiming to assess the association between BRCA mutation and VEGF overexpression would provide new instrument to personalise treatment with anti-angiogenetic agents among BRCA-mutated and BRCA wild-type OC patients.[42] In this scenario, the randomised phase III clinical trial ENGOT-ov25/PAOLA-1 (ClinicalTrials.gov Identifier: NCT02477644), which combines in advanced OC patients bevacizumab-based first-line treatment with or without the PARP-Inhibitor olaparib, could be able to add evidence concerning functional impact of VEGF expression in tumours with impaired homologous DNA repair mechanism.

To our knowledge, this is the first study analysing the changes occurring in intratumoural vasculature during disease progression in the largest cohort of paired primary and recurrent HGSOC samples. It firstly demonstrated that the vascular architecture within the tumour mass, in absence of anti-angiogenic agents administration, is maintained relatively stable during the natural course of the disease. Furthermore, the subanalysis on patients with known somatic-BRCA status increases the value of findings by taking into account the impact of BRCA status on patients' survival[39,40] and provides preliminary evidence of the correlation between VEGF-positivity and BRCA mutation.

The main limitation of the study is its retrospective nature. One of the strengths of this analysis is the large sample size of paired primary and recurrent tumour tissue samples belonging to the same cancer subtype ($n = 222$), the high quality of specimens and the systematisation of multicentric patients' clinico-pathological data. Furthermore, inclusion of patients not subjected to the bevacizumab-based first-line chemotherapy, increase the reliability of the results in comparing intratumoural vasculature profiles from primary to recurrent disease.

Future study on a larger population with known BRCA status, who has been subjected to bevacizumab-based first-line chemotherapy, is warranted to clarify the role of MVD and VEGF in predicting bevacizumab response in both BRCA-wt and BRCA-mutated HGSOC patients.

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AUTHOR CONTRIBUTIONS

Study concepts and design: I.R. and E.I.B.. Data acquisition: I.R., H.K., F.T., A.V., M.S., I.I.. Quality control of data acquired: D.C.C-T., I.V., C.G., H.G., A.M., J.S., S.D-E.. Data analysis and interpretation: I.R., S.D-E., M.K., C.K., P.B.P, M.N., J.G.. Statistical analysis: I.R., M.S., S. D-E., E.T.T.. Manuscript writing: I.R. and E.I.B.. Manuscript editing: all co-authors.

ADDITIONAL INFORMATION

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Competing interests: The authors declare that they have no conflict of interest.

Availability of data and materials: Data supporting the results reported are stored in the OCTIPS Consortium database. The documentation of clinical and patient's data was managed with "AlcedisTRIAL the web based documentation system" of Alcedis GmbH, Winchesterstr. 3, 35394 Giessen, Germany.

Consent for publication: Included patients had previously signed written informed consent regarding the anonymous publication of their clinico-pathological data for translational research purposes.

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Ethics approval and consent to participate: Included patients were previously treated in one of the European Gynaecologic Oncology referral Centers of the following Institutions: Charité Universitätsmedizin Berlin, Germany; Catholic University of Leuven, Belgium; Imperial College, London, UK; University of Edinburgh, UK; University Medical Center Hamburg-Eppendorf, Germany. Patients had previously signed written informed consent regarding tumour tissue sampling and the collection of their clinico-pathological data for translational research purposes. Approval from each local ethics committee was obtained (EK207/2003, ML2524, 05/Q0406/178, EK130113, 06/S1101/16). The study was performed in accordance with the Declaration of Helsinki.

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Bevacizumab-Based Chemotherapy Triggers Immunological Effects in Responding Multi-Treated Recurrent Ovarian Cancer Patients by Favoring the Recruitment of Effector T Cell Subsets

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Abstract: Increasing evidence strongly suggests that bevacizumab compound impacts the immunological signature of cancer patients and normalizes tumor vasculature. This study aims to investigate the correlation between the clinical response to bevacizumab-based chemotherapy and the improvement of immune fitness of multi-treated ovarian cancer patients. Peripheral blood mononuclear cells (PBMCs) of 20 consecutive recurrent ovarian cancer patients retrospectively selected to have received bevacizumab or non-bevacizumab-based chemotherapy (Bev group and Ctrl group, respectively) were analyzed. CD4, CD8, and regulatory T cell (Treg) subsets were monitored at the beginning (T0) and after three and six cycles of treatment, together with IL10 production. A lower activated and resting Treg subset was found in the Bev group compared with the Ctrl group until the third therapy cycle, suggesting a reduced immunosuppressive signature. Indeed, clinically responding patients in the Bev group showed a high percentage of non-suppressive Treg and a significant lower IL10 production compared with non-responding patients in the Bev group after three cycles. Furthermore, clinically responding patients showed a discrete population of effector T cell at T0 independent of the therapeutic regimen. This subset was maintained throughout the therapy in only the Bev group. This study evidences that bevacizumab could affect the clinical response of cancer patients, reducing the percentage of Treg and sustaining the circulation of the effector T cells. Results also provide a first rationale regarding the positive immunologic synergism of combining bevacizumab with immunotherapy in multi-treated ovarian cancer patients.

Keywords: bevacizumab; ovarian cancer; effector T cells; target therapy; immunotherapy; chemotherapy

1. Introduction

Ovarian cancer still accounts for the highest mortality rate among all gynecological malignancies, with 295,414 estimated new cases and 184,799 estimated new deaths in 2018 worldwide [1].

During the last 10 years, bevacizumab, a monoclonal antibody that binds to vascular endothelial growth factor (VEGF), has revolutionized the treatment approach in ovarian cancer, obtaining US Food and Drug Administration (FDA)/ European Medicines Agency (EMA) approval in all advanced disease settings (multi-treated/compassionate, platinum-resistant/platinum-sensitive recurrent, and primary International Federation of Gynecology and Obstetrics (FIGO) stage IIIB-IV ovarian cancer) (www.fda.gov; www.ema.europa.eu).

The biological mechanism underpinning the clinical efficacy of bevacizumab addition in ovarian cancer setting is still a matter of intense investigation. Increasing evidence supports the hypothesis that this biological compound modulates patients' immune system, reducing immunosuppression and activating acquired immunity [2]. Several authors have shown the immune effects of bevacizumab in multiple cancer settings, such as decrease in patients' regulatory T cells (Tregs) [3,4] and expansion of B and T cells [2].

Multi-treated recurrent ovarian cancer patients constitute an extremely fragile disease setting. Their immune system is weakened by multiple lines/types of treatment strategies, and the succession of therapeutic choices for them is currently discussed without a common consensus among oncologists.

In this study, we show that the clinical response to bevacizumab-based chemotherapy in this poor-prognostic disease correlates with improvement of patients' immune fitness, thus providing new evidence that the benefit of such treatment can be ascribed also to its fine immune modulation.

2. Experimental Section

2.1. Patient Selection

This retrospective study received institutional review board (IRB) approval and was carried out following the rules of the Declaration of Helsinki of 1975. Patients included in this study were treated at the Gynecologic Oncology Unit of the Department of Gynecology, Obstetrics and Urology (Sapienza University of Rome, Italy) between 2008 and 2012. Since 2007, all gynecological cancer patients admitted in this department have been regularly subjected to donation of peripheral blood samples every three cycles of chemotherapy treatment for research purposes with their written informed consent. Patients' peripheral blood mononuclear cells (PBMCs) were regularly collected and stored in liquid nitrogen at the Laboratory of Tumor Immunology and Cell Therapy Unit, Department of Experimental Medicine (Sapienza University of Rome, Italy—Ethical Committee approval, protocol n° 703/2008; date of approval 07/24/2008).

For this study, 20 consecutive recurrent ovarian cancer patients were retrospectively selected from "Sapienza" PBMC sample collection. All available multi-treated platinum-resistant ovarian cancer patients subjected to intraperitoneal (i.p.) bevacizumab-based chemotherapy as compassionate use were selected (Bev group; 10 patients), together with 10 patients (Ctrl group) that received non-bevacizumab-based chemotherapy.

Inclusion criteria were as follows: primary diagnosis of advanced epithelial serous ovarian cancer; having been subjected to at least three previous chemotherapy lines; diagnosis of tumor progression confirmed by CT scan; presence of malignant ascites; life expectancy of at least three months; and availability of at least three PBMC samples per patient collected during the course of bevacizumab-based versus non-bevacizumab-based chemotherapy. Furthermore, patients of the Bev group were matched with the Ctrl group patients for age, tumor grading, FIGO stage, type of primary

treatment strategy (primary debulking surgery versus neoadjuvant chemotherapy followed by interval debulking surgery), tumor residual at first surgery, and type of recurrence at the time of blood sampling in order to minimize selection bias and avoid misinterpretations of results.

Ten patients were identified as the ones that had received i.p. bevacizumab 5 mg/kg every 21 days immediately after paracentesis for treatment of malignant ascites [5,6] plus intravenous (intravenous injection, i.v.) monochemotherapy (cisplatin) [7], while 10 other patients were identified as been treated with i.v. monochemotherapy alone (paclitaxel, topotecan, pegylated liposomal doxorubicin, cisplatin). Patients' clinicopathological data were retrieved from clinical charts. Disease progression was defined basing on the response evaluation criteria in solid tumors (RECIST) [8].

2.2. PBMC Purification

PBMCs were isolated from 12 to 14 mL of peripheral blood by Ficoll–Hypaque gradient (1077 g/mL; Pharmacia LKB, Sweden), obtaining a yield between 10×10^6 and 12×10^6 cells for each drawing and cryopreserved until use. Samples were taken before therapy (T0) and after three (III) and six (VI) cycles of therapy.

2.3. Cell Phenotype

Cell phenotype staining was performed using several directly conjugated monoclonal antibodies (MoAbs). T cells were incubated with the anti-CD8-PE-Cy5.5 (RPA-T8 clone), anti-CD3-PE (UCHT1 clone), anti-CCR7-FITC (150503 clone), and anti-CD45RA-APC (HI100 clone) MoAbs, all from Becton Dickinson (Franklin Lakes, NJ, USA). Tregs were stained with the anti-CD25-PE (M-A251clone), anti-CD45RA-APC (HI100 clone), anti-CD4-FITC (RPA-T4 clone), and anti-FOXP3-Alexa 647 (259D/C7 clone) MoAbs, all from Becton Dickinson. Cells were incubated with the conjugated MoAbs targeting extracellular antigens for 30 min at room temperature (RT) as indicated by the manufacturer's instruction. The staining of intracellular antigen FOXP3 was performed after the cells' permeabilization with the Human FOXP3 Buffer Set (Beckton Dickinson, Franklin Lakes, NJ, USA). After washing, at least 1×10^4 events were evaluated using a FACSCanto flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) running FACSDiva data acquisition and analysis software (Becton Dickinson, Franklin Lakes, NJ, USA). The percentages of CD4 and CD8 T cells were calculated with respect to the entire CD3 T cell population, while the percentage of Treg was evaluated with respect to CD4 T cells.

2.4. Intracellular Cytokine Staining

T cells were stimulated with the anti-CD3 (OKT3 clone, 1 μg/mL) (eBioscence, San Diego, CA, USA) and anti-CD28 (CD28.2 clone, 5 μg/mL) (BioLegend, San Diego, CA, USA) MoAbs for 16 h at 37 °C in the presence of Brefeldin (Sigma-Aldrich, St. Louis, MO, USA) (10 μg/mL). The staining of IL-10 positive cells was carried out by fixing the cells with 2% paraformaldehyde (Sigma-Aldrich, Saint Louis, MO, USA). Cells were than washed with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} + 0.5% saponin (Sigma-Aldrich, Saint Louis, MO, USA) + 10% fetal bovine serum (FBS) (Sigma-Aldrich, Saint Louis, MO, USA) and incubated for 30 min with anti-IL-10-PE (JES3-19F1 clone) (BioLegend, San Diego, CA, USA) MoAb. Cells were analyzed using a FACSCanto flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) running FACSDiva data acquisition and analysis software (Becton Dickinson, Franklin Lakes, NJ, USA).

2.5. Statistical Analysis

Statistical analysis was performed using Graphpad Prism version 6 (Graphpad Software, Inc., San Diego, CA, USA).

Descriptive statistics (average and standard deviation) were used to describe different groups of continuous data. Student's *t*-test was used to compare groups of continuous variables. Groups of categorical data were compared using the Fisher's exact test. Significance is indicated when $p \leq 0.05$.

3. Results

3.1. Patients' Characteristics and Clinical Response

Twenty patients met all inclusion criteria and were included in the study. Patients' characteristics are listed in Table 1. As a result of patient matching, no differences in terms of clinicopathological variables as well the Eastern Cooperative Oncology Group (ECOG) performance status could be identified between the Bev group and the Ctrl group. At the time of blood sampling for immunological analysis, 12/20 women (60%) presented intraperitoneal tumor progression, whereas the remaining 3/20 (15%) and 5/20 (25%) patients were diagnosed with intraperitoneal plus retroperitoneal disease worsening and widespread tumor dissemination, respectively.

From a clinical point of view, and as confirmed by serial Ca125 serum levels (Table S1), 50% (10/20) of patients were judged responders to chemotherapy after six cycles of treatment and were equally distributed in each group of interventions (5/10 in the Bev group and 5/10 in the Ctrl group).

Table 1. Patients' characteristics.

	Bevacizumab-Treated Patients	Control Group	*p*-Value
Patient n°	10	10	
Age (median, range)	54 years (42y–67y)	48.5 years (45y–71y)	0.845
ECOG Performance Status			
1	1/10 (10%)	2/10 (20%)	
2	7/10 (70%)	5/10 (50%)	
3	2/10 (20%)	3/10 (30%)	
Tumor Grading at primary diagnosis			
I	0	0	0.628
II	4/10 (40%)	2/10 (20%)	
III	6/10 (60%)	8/10 (80%)	
FIGO stage at primary diagnosis			
IIIC	8/10 (80%)	7/10 (70%)	1
IV	2/10 (20%)	3/10 (30%)	
PDS	5/10 (50%)	6/10 (60%)	1
NACT	5/10 (50%)	4/10 (40%)	
RT at first surgery (cm)			
=0	9/10 (90%)	8/10 (80%)	1
>0	1/10 (10%)	2/10 (20%)	
Type of recurrence at the time of blood sampling			
Intraperitoneal only	7/10 (70%)	5/10 (50%)	0.061
intraperitoneal + retroperitoneal	1/10 (10%)	2/10 (20%)	
widespread	2/10 (20%)	3/10 (30%)	

NACT: Neoadjuvant chemotherapy; PDS: Primary Debulking Surgery; RT: Residual Tumor.

3.2. Bevacizumab-Treated Patients Showed a Different Immunological Signature Compared with the Control Group

To understand whether bevacizumab treatment impacts the immunological status of ovarian cancer patients, the modulation of circulating CD4 and CD8 T cells was firstly analyzed in the Bev group and the Ctrl group before (T0) and after III and VI cycles of treatments (Figure 1A). Both CD4 and CD8 T cells played a critical role in the activation of an effective antitumor immunity. CD8 lymphocytes exerted their cytotoxic activity by eliminating tumor cells, while CD4 T lymphocytes sustained and maintained a CD8 T cell response by cytokine production [9]. A deficiency in the activation of one of these two populations induced the development of a failed immunity against the tumor. Results obtained from the cancer patients showed that therapies did not modify the percentage of CD4 and CD8 lymphocytes in both groups at different time points. CD4 T cells were significantly higher in the Bev group at T0 and III compared with the Ctrl goup, although this difference disappeared at the end of VI cycles. No difference was observed in CD8 T cells between

the two groups, although the ratio CD4/CD8 remained high (>1) up to VI cycles in both groups, suggesting a predominance of CD4 T cells during therapies.

CD8 and CD4 T cells were concurrently analyzed for the expression of CCR7 and CD45RA molecules, which identify four different lymphocyte subsets: effector ($CCR7^-CD45RA^+$), naïve ($CCR7^+CD45RA^+$), central memory ($CCR7^+CD45RA^-$), and effector memory ($CCR7^-CD45RA^-$) T cells. Analyzing these T cell subpopulations in the Bev and Ctrl group patients, no significant difference throughout the treatment in each patient group and between the two groups were found (data not shown).

Finally, the percentage of Tregs was also examined following the expression of CD4, CD25, and FOXP3 markers (Figure 1B). In cancers, Tregs represent one of the most important T cell populations as they are able to suppress the activation and/or expansion of antitumor CD4 and CD8 T cells through cell–cell contact or by cytokine release [10]. A high percentage of Tregs is associated with a poor prognosis in different types of solid tumors [11,12]. In our setting of patients, the results demonstrated that the Ctrl group showed a significant decrease in total Tregs from T0 to VI cycles (36% vs. 31%, $p = 0.03$), while no difference was found in the Bev group throughout the therapy. Total Tregs were further analyzed according to the combined expression of CD25, FOXP3, and CD45RA markers, which identifies three important Treg subpopulations [10]: resting Treg ($CD25^+CD45RA^+FOXP3^+$: rTregs), activated Tregs ($CD25^{high}CD45RA^-FOXP3^-$: aTregs), and cytokine-secreting Tregs with no suppressive activity ($CD25^+CD45RA^-FOXP3^+$: nsTregs). aTreg have been described as terminally differentiated cells that rapidly die after exerting their suppressive activity, whereas rTreg proliferate and convert into aTreg both in vitro and in vivo [10]. The analysis revealed that bevacizumab-treated patients showed a lower percentage of aTregs and rTregs compared with the Ctrl group at T0. This difference persisted until III cycles of treatment in the rTreg subset and disappeared after VI cycles, suggesting that these patients exhibited a less suppressive immunological profile compared with the Ctrl group at the beginning and in particular after III cycles of therapies.

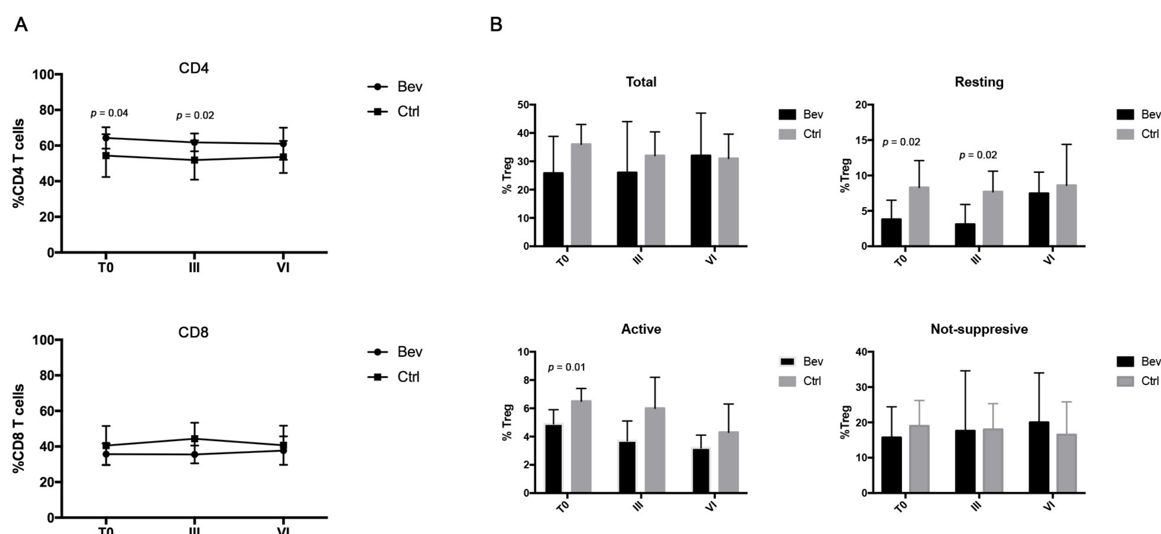

Figure 1. Evaluation of CD4 and CD8 T cell in the bevacizumab (Bev) group and the control (Ctrl) group by cytofluorimetry. (**A**) Analysis of the percentage of CD4 and CD8 T cells derived from patients belonging to the Bev group and the Ctrl group before (T0) and after III and VI cycles of therapies. CD8 T cells were identified by gating the $CD3^+CD8^+$ cells, while the CD4 T cells were identified as $CD3^+CD8^-$. (**B**) Histograms represent the percentage of the different regulatory T cell subset (total, active, resting, and nonsuppressive) calculated on $CD4^+CD25^+$cells. Bev group and Ctrl group are represented with black and grey histograms, respectively.

3.3. Bevacizumab-Treated Patients Showed a Discrete CD4 Effector T Cell Population throughout the Treatment

Patients belonging to the Bev group and the Ctrl group were then divided in clinically responders (R) and clinically nonresponders (N-R) to therapy according to RECIST (Table S1). The modulation of CD4 and CD8 T cell was initially evaluated in R and N-R patients of both groups, followed by the analysis of the different T cell subsets (Figure 2). The results demonstrated that the CD8 T cells derived from bevacizumab-treated patients were not differently modulated in R and N-R patients, while the CD4 T cells appeared to be significantly higher in the N-R group at the beginning and after VI cycles of treatment. Conversely, in the Ctrl group, the CD8 T cells seemed to be significantly higher after VI cycles in the R patients compared with the N-R ones, while no significant difference was observed in the CD4 T cell population.

Lymphocytes were also analyzed according to the expression of CCR7 and CD45RA molecules (Figure 3). The results demonstrated that in the Bev group and the Ctrl group, the percentage of CD4 effector T lymphocytes in R patients was higher compared with N-R patients at T0. This difference persisted until the end of the therapies for bevacizumab-treated patients, while it had already disappeared after III cycles of therapy in the control group. This suggests that Bev treatment, by favoring the normalization of the tumor vasculature [13], improves and sustains the circulation of effector T cells.

The other CD4 T cell subsets and the CD8 T cell populations were not significantly modified by treatments (data not shown).

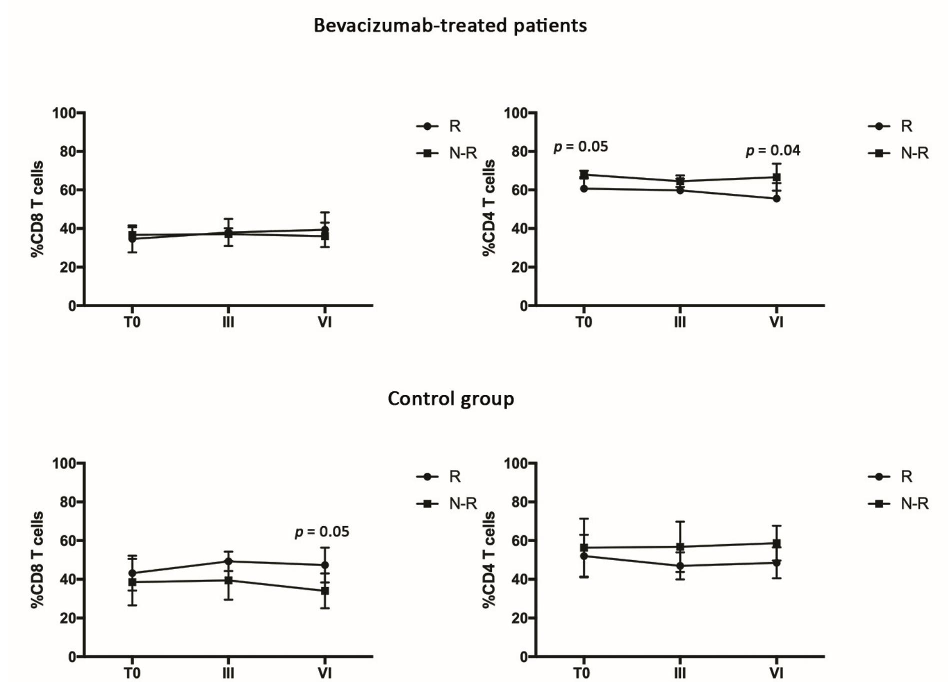

Figure 2. Evaluation of CD4 and CD8 T cells in responding (R) and nonresponding (N-R) patients of the bevacizumab-treated group and the control group by cytofluorimetry. CD8 T cells were identified by gating the $CD3^+CD8^+$ cells, while the CD4 T cells were identified as $CD3^+CD8^-$.

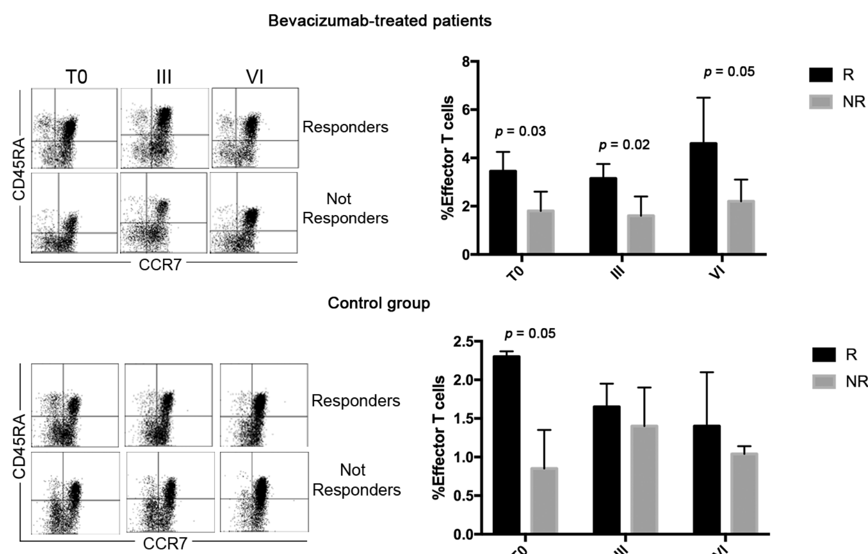

Figure 3. Analysis of CD4 and CD8 T cell subsets were carried out using the anti-CD3, anti-CD8, anti-CCR7, and anti-CD45RA MoAbs. CD8 T cells were identified by gating the $CD3^+CD8^+$ cells, while the CD4 T cells were identified as $CD3^+CD8^-$. Dot plots show the expression of CD45RA and CCR7 molecules that identify different T cell subsets (T effector: $CD45RA^+CCR7^-$; T central memory: $CD45RA^-CCR7^+$, T naive: $CD45RA^+CCR7^+$, and T effector memory: $CD45RA^-CCR7^-$) at T0 and after III and VI cycles of therapy. Histograms represent the median values of the percentage of effector T cells ($CD45RA^+CCR7^-$) of 10 patients (five patients of R and N-R of both Bev group and Ctrl group) ± standard deviation. Black and grey columns correspond to responding and nonresponding patients, respectively.

3.4. *Tregs Were Modulated in Bevacizumab-Treated Patients during Therapies*

To assess whether the treatment schedule and/or the clinical response could be associated with the modulation of the Treg subsets, the percentage of circulating Tregs after III or VI treatment cycles were compared with the baseline value at T0, and the analysis was expressed as fold increase (%TregIII/%TregT0 or %TregVI/%TregT0) (Figure 4). After III cycles of treatment, the level of the entire Treg population was significantly higher in R patients compared with N-R patients in the Bev group. This increase was ascribed to the nsTreg subset being significantly higher in R compared with N-R patients. These differences between R and N-R patients disappeared after VI cycles of bevacizumab treatment. In contrast, the control group did not show any difference in the percentage of Tregs between R and N-R patients during therapies, and no difference between the Bev and Ctrl patients was observed.

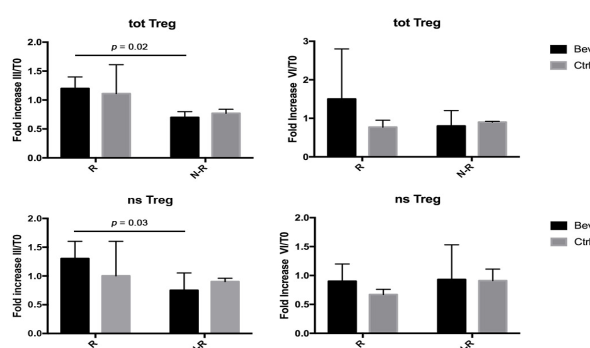

Figure 4. Total and non-suppressive Treg (totTreg and nsTreg, respectively) evaluated as fold increase after III or VI cycles of therapy compared with T0 (%TregIII or %TregVI/%TregT0). Black and grey columns correspond to Bev group and Ctrl group, respectively.

3.4. Bevacizumab-Treated N-R Patients Had Higher Level of IL10⁺ T Cells Compared to R Patients

Because IL10, such as TGFβ, is one of the most important cytokines released by Tregs [14] that is able to downregulate Th1 cytokine production and block NF-κB activity [15], T cell derived from patients in the Bev group were analyzed for their capacity to produce IL10 as intracellular staining (Figure 5). These patients exhibited a significant increase in $IL10^+$ cells from T0 up to VI cycles of therapy. Analyzing the data as fold increase of the percentage of $IL10^+$ cells after III and VI cycles of therapy compared with T0 ($\%IL10_{III}/\%IL10_{T0}$ or $\%IL10_{VI}/\%IL10_{T0}$) between R and N-R patients, significant high levels of IL10 were found in N-R patients after III cycles, suggesting an enhancement of the immunosuppression during the bevacizumab treatment in this group. This increase disappeared after VI cycles ($p = 0.08$) of therapy.

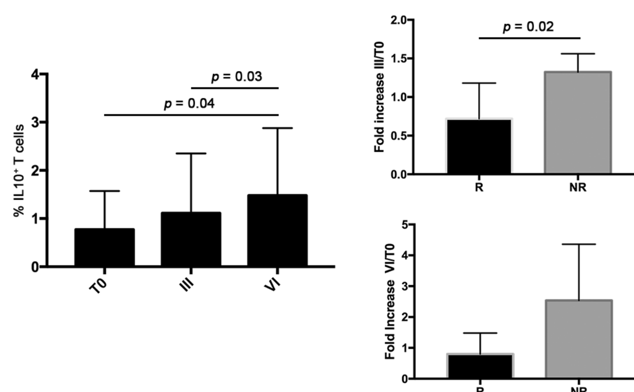

Figure 5. Evaluation of $IL10^+$cells at T0 and after III and VI cycles of treatment in bevacizumab patients and fold increase of the percentage of $IL10^+$cells after III and VI cycles of therapy compared with T0 (%IL10 III or %IL10 VI/%IL10 T0) in R and N-R patients belonging to the Bev group.

4. Discussion

Multi-treated progressive ovarian cancer still remains the most challenging disease setting for gynecologic oncologists, and so far, no global consensus has been met about how/how long patients should be continued to be treated [16]. Indeed, all these patients progressively develop pharmacoresistance for the majority of conventional chemotherapy drugs, and the only option left (if they are not eligible for phase I clinical trials) is to retreat them with previously adopted compounds [17]. Among biological agents that have obtained FDA/EMA approval in compassionate ovarian cancer setting, bevacizumab has been the first to enter into clinical practice after showing its ability to improve patients' quality of life and also reduce paracentesis frequency for women suffering malignant ascites [5].

The effects of bevacizumab on patients' immune system are still not completely elucidated, although a strong rationale about the interplay between its ligand (VEGF) and the host's immune response suppression has already been shown [18]. In particular, three different mechanisms related to VEGF-mediated immunosuppression have been assessed so far: inhibition of dendritic cell maturation [19–21], reduction of T cell tumor infiltration [22], and promotion of inhibitory cells in the tumor microenvironment [23].

In this scenario, the present study adds new evidence to the body of knowledge concerning the immune effects of bevacizumab in advanced cancer patients by showing that (1) ovarian cancer patients not treated with bevacizumab-based chemotherapy seem to have a more immunosuppressive profile with the presence of a rTreg population that persists until the end of III cycles of therapy; (2) patients that clinically respond to bevacizumab treatment show a discrete population of effector T cells at the beginning of therapy that is maintained throughout the treatment; (3) Tregs are mainly represented by non-suppressive regulatory T cells in clinically responding bevacizumab patients compared with nonresponding patients and are also stably maintained in this

ratio (nsT reg > sup T reg) throughout the treatment; (4) after three cycles of treatment, nonresponding bevacizumab patients produce more immunosuppressive IL-10 cytokine compared with responding patients.

It should be pointed out that these results were obtained by comparing two groups of patients that were matched for all clinical characteristics. Particularly important is to notice that the performance status was similar among the two groups; this variable has been significantly associated with the immunological effects and response to several therapies [24]

Other authors have observed an impact of bevacizumab-based regimens on the immunosuppressive status of cancer patients in different cancer settings. In particular, it was recently reported that, for glioblastoma patients subjected to radiation plus temozolomide (TMZ) and bevacizumab, the absolute number of peripheral Tregs significantly decreased following treatment [25]. Furthermore, the addition of bevacizumab to standard radiation and TMZ appeared to decrease the number of circulating Tregs compared with radiation plus TMZ alone. On the contrary, they also noticed a significant decrease in the absolute number of cytotoxic CD8 (CD107a$^+$), effector memory CD8, and naïve CD4 T cells in the group of bevacizumab-treated patients.

Our results follow and confirm the original observation by our group [6] in which a significant reduction of Tregs and an increase in the proportion and function of effector CD8 T cells were found in an end-of-life ovarian cancer patient treated with low-dose intraperitoneal bevacizumab for malignant ascites.

We also showed that responding bevacizumab-treated patients reported a higher percentage of circulating CD4 effector T cells compared with nonresponding bevacizumab patients, confirming what has already been observed in metastatic colorectal cancer [2]. This data has key implications in the current panorama of oncological clinical approach. Indeed, it is reasonable to suggest that the circulating effector T cells recruited and sustained by bevacizumab treatment, thanks to its ability to restore tumor microvascular normalization [13], could be expanded by the administration of checkpoint inhibitor agents, thus giving a strong biological rationale for the combination of immunotherapy with bevacizumab antiangiogenetic therapy. In support of this consideration, tumor tissue derived from metastatic renal cell carcinoma (mRCC) patients treated with anti-PD-L1 atezolizumab plus bevacizumab was recently found to show an increase in intratumoral CD8 T cells as well as an increase in intratumoral MHC-I, Th1 and T-effector markers, and chemokines. Trafficking lymphocytes also increased in tumors following bevacizumab and combination treatment [26].

We finally observed that bevacizumab responding patients showed significant lower circulating immunosuppressive IL-10 cytokine levels compared with non-responding patients, thus confirming the effect of bevacizumab in reducing patients' immune suppression. A similar finding was recently obtained in breast cancer neoadjuvant setting [27]. Patients treated with bevacizumab-included neoadjuvant chemotherapy showed a global decrease in circulating cytokines levels, such as VEGF-A, IL-12, IP-10, and IL-10. In addition, the decrease in IL-10 serum levels was confirmed to be even greater in response to bevacizumab treatment in metastatic colorectal cancer setting [28].

To our knowledge, this is the first study analyzing the immunological effects of bevacizumab-based treatment in women with advanced ovarian cancer in relation to their clinical response.

However, several significant limitations are present in this study. The first is the restricted number of patients that were retrospectively and not randomized selected. Moreover, there were no data regarding the immune fitness of the patients at diagnosis before the beginning of all therapies. Finally, although no differences in clinicopathological variables were identified, the two groups analyzed showed several differences in their immunological signature at T0. These differences could be ascribed to the several chemotherapy treatments (e.g., taxol, bemcitabine, pegilated liposomal, doxorubicin etc.) that differently impact the immunological system [29], together with the capacity of the immunological signature of each patient to differently respond to the same environmental factor, such as chemotherapy and surgery. In this setting, a discrete population of effector CD4 T cell is present in any case in both populations of patients independently from previous treatment. This cell subset is the one that appears to be affected by the bevacizumab regimen, and this could impact clinical outcome.

Another important point is the occurrence of leukopenia in multi-treated patients, which represents an important prognostic factor in patients with advance malignances [30]. In our setting, patients were affected by mild/moderate leukopenia that was non-clinically significant.

The strengths of this study can be summarized as follows: (1) the population involved was homogeneous for histology and clinical stage; (2) the patients belonging to the two groups (bevacizumab-treated and control) were matched for age, tumor grading, FIGO stage, type of primary treatment strategy (primary debulking surgery versus neoadjuvant chemotherapy followed by interval debulking surgery), tumor residual at first surgery, and type of recurrence at the time of blood sampling, thus minimizing selection bias; (3) blood sampling was carried out for all patients at the same times of treatment, i.e., at T0 and after three and six cycles of therapy.

5. Conclusions

In conclusion, this study sheds a light on the strong need to routinely include immunomonitoring in oncological clinical protocols of patients at follow-up during the course of antiangiogenetic therapy administrations, with the final aim being to identify early the subset of patients who can mostly benefit from its adoption. Furthermore, the study provides a first rationale regarding the positive immunologic impact of combining bevacizumab with checkpoint inhibitors. Confirmatory studies carried out on larger cancer patient populations are warranted.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Ca125 levels of patients belonging to Bev group and Ctrl group.

Author Contributions: Conceptualization, C.N. and F.B.; Data curation, M.L.G. and M.A.; Formal analysis, I.R.; Funding acquisition, M.N.; Investigation, I.G.Z. and H.R.; Methodology, C.N. and I.R.; Project administration, C.N. and I.R.; Resources, F.B., M.G., M.A. and P.B.P.; Supervision, M.N.; Validation, A.R.; Visualization, C.N. and I.R.; Writing – original draft, C.N. and I.R.; Writing – review & editing, C.N., I.R., A.R. and M.N.

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Conflicts of Interest: All authors declare no conflict of interest.

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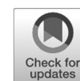
Research Article

Cancer Immunology Research

TK Inhibitor Pazopanib Primes DCs by Downregulation of the β-Catenin Pathway



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Abstract

Tyrosine kinase inhibitors (TKIs) target angiogenesis by affecting, for example, the VEGF receptors in tumors and have improved outcomes for patients with metastatic renal cell carcinoma (mRCC). Immune checkpoint inhibitors (ICIs) have also been proposed for treatment of mRCC with encouraging results. A better understanding of the activity of immune cells in mRCC, the immunomodulatory effects of TKIs, and the characteristics defining patients most likely to benefit from various therapies will help optimize immunotherapeutic approaches. In this study, we investigated the influence of the TKI pazopanib on dendritic cell (DC) performance and immune priming. Pazopanib improved DC differentiation and performance by promoting upregulation of the maturation markers HLA-DR, CD40, and CCR7; decreasing IL10 production and endocytosis; and increasing T-cell proliferation. PD-L1 expression was also downregulated. Our results demonstrate that pazopanib inhibits the Erk/β-catenin pathway, suggesting this pathway might be involved in increased DC activation. Similar results were confirmed in DCs differentiated from mRCC patients during pazopanib treatment. In treated patients pazopanib appeared to enhance a circulating $CD4^+$ T-cell population that expresses CD137 (4-1BB). These results suggest that a potentially exploitable immunomodulatory effect induced by pazopanib could improve responses of patients with mRCC in customized protocols combining TKIs with ICI immunotherapy. *Cancer Immunol Res; 6(6); 711–22. ©2018 AACR.*

Introduction

Tumor progression requires angiogenesis. Proangiogenic factors, such as VEGF, that are induced by hypoxia or oncoproteins can alter the equilibrium between pro- and antiangiogenic factors, resulting in the generation of new blood vessels, mostly with altered endothelium structure. Proangiogenic factors are involved in the generation of immunosuppression in tumors. Tumor neoangiogenesis is associated with immature and tolerogenic dendritic cells (DCs) and increased number of myeloid-derived suppressor cells (MDSCs), the activation of regulatory T cells (Tregs), and recruitment of tumor-associated macrophages (TAMs) in the tumor bed (1).

Various antiangiogenic and multitargeted compounds, including bevacizumab, sunitinib, pazopanib, sorafenib, axitinib, lenvatinib, and cabozantinib, have entered the clinic for use against tumors that depend on angiogenesis (2–4). For metastatic renal cell carcinoma (mRCC), VEGFR-directed tyrosine kinase inhibitors (TKIs) have demonstrated clinical benefits including improvements in progression-free survival and overall survival (5). These compounds, which target VEGF and its receptors, are likely to affect the immune repertoire of cells and molecules that interact with the growing tumor. Immunosuppression appears to be downregulated in mRCC patients treated with sunitinib or axitinib, whose Treg and MDSC cell populations are affected. Sorafenib has the opposite effect by reducing antigen-specific T-cell induction *in vitro* (6–11). The different selectivities and affinities of the various drugs are thought to account for the diverse effects on myelopoiesis and immune cells (12).

As immunotherapy using immune checkpoint inhibitors is moving to clinical application for mRCC, we must understand the immune consequences of TKI therapy. In the CheckMate 025 randomized phase III trial, Escudier and colleagues observed in mRCC patients improved overall survival and favorable hazard ratio for the anti–PD-1 nivolumab group that had previously received first-line treatment with pazopanib (HR, 0.60; 95% CI, 0.42–0.84); such results suggest an immune effect on the tumor microenvironment (13).

TKIs seem to be more effective in mRCC, suggesting that the requirement for angiogenesis increases as the disease progresses. In order to achieve maximum response from anti–PD-1 immunotherapy, the patient must be prepared to receive an immunotherapeutic regime that will expand activated and specific T cells. We have addressed this issue by studying DC performance at concentration of sunitinib and pazopanib found in plasma. DCs are antigen-presenting cells that prime antigen-naive

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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T cells and perform cross-priming, thus presenting antigens both in HLA class I and II to activate immune responses. Optimal DCs express costimulatory molecules such as CD40, CD83, CD86, HLA-DR, and to a lesser extent CD14 and PD-L1. In order to migrate to the lymph node for cross talk with effector cells, DCs must express the CCR7 marker. These parameters relate to T-cell priming and activation. Failure to sustain these hallmarks will lead to tolerogenic DCs that will dampen antitumor immunity (14, 15).

We report here results that identify the TKI pazopanib as an immune stimulator, which exerts its effects by influencing DC differentiation and maturation. This activation is mediated by targeting and downregulating p-Erk and β-catenin pathway. The impact of this immune activation mediated by DCs was investigated in mRCC patients undergoing TKI treatment. Our results might influence the design of first- and second-line therapies for mRCC.

Materials and Methods

Generation of DCs

Human monocyte-derived DCs were generated from peripheral blood mononuclear cells (PBMCs) of healthy donors (Ethical Committee Protocol, RIF.CE: 4212) and of mRCC patients (Ethical Committee Protocol, RIF.CE: 4181). Monocytes ($CD14^+$) were purified from PBMCs after Ficoll–Hypaque gradient (1,077 g/mL; Pharmacia LKB) by Human CD14-Positive Selection Kit (StemCell Technologies) and cultured (5×10^5 cells/mL) in RPMI 1640 (Hyclone) supplemented with 2 mmol/L L-glutamine, penicillin 100 U/mL, streptomycin 100 μg/mL (Sigma-Aldrich), with 5% heat-inactivated Fetal Calf Serum (FCS; Hyclone). Fifty ng/mL rhGM-CSF (R&D Systems) and 2,000 U/mL rhIL4 (R&D Systems) were added at day 0 and 2. Immature DCs (iDCs) were collected at day 5 and matured with cytokine cocktail (rhIL1β, IL6, TNFα and PGE_2; all purchased by R&D Systems) for 16 hours. Sunitinib (50 ng/mL; Sigma-Aldrich) and pazopanib (19 μg/mL; Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO; Euroclone) and added to the culture during DC differentiation.

Immune phenotype

DC phenotype was analyzed by flow cytometry using the following monoclonal antibodies (mAb): anti–HLAII-DR-FITC, anti–CD86-FITC, from BD Biosciences, anti–CD14-PE, anti–CCR7-FITC, anti–CD83-PE, anti–CD40-PE, anti–PD-L1-PE from BioLegend and anti–VEGR-1 from R&D Systems. MoAbs anti–IgG1-FITC and anti–IgG1-PE (BioLegend) were used as isotype controls.

For immune profile evaluation, PBMCs were isolated from blood samples (50 mL) from six mRCC patients by Ficoll–Hypaque at different times (Ethical Committee Protocol, RIF.CE: 4181): before treatment with sunitinib or pazopanib (T0), during treatment (T1: 1 month of treatment, T2: 2 months, T3: 3 months etc.) and during progression. Various T-cell subsets were analyzed:

- T-cell subpopulations: anti–CD3-APC-H7/CCR7-PE/CD8 PerCp-Cy5.5/CD45RA-BB15.
- Treg cells: anti–CD4-APC-H7/CD25-PE/CD45RA-BB15/FoxP3-Alexa647.
- T-cell activation/proliferation: anti–CD3-APC-H7/CD8-PerCp-Cy5.5/CD137-APC/Ki67-PeCy7.
- T-cell exhaustion: anti–CD3-APC-H7/CD8-PerCpCy5.5/PD1-PE/CTLA4-APC/Tim3-BB15.

All mAbs were purchased by BD Biosciences and BioLegend. Flow cytometric analysis was performed using FACSCanto flow cytometer running FACS Diva data acquisition and analysis software (BD Biosciences). Catalog numbers and clones for every antibody used are listed in Supplementary Table S1.

Microvesicle isolation

Microvesicles were isolated from supernatants of DCs differentiated with and without suninitib and pazopanib. Supernatants were centrifuged at 13,000 × *g* for 30 minutes at 4°C. Microvesicles were then stained with anti–PD-L1 (BioLegend) and acquired by FACSCanto flow cytometer and analyzed by FACS Diva software. Anti–IgG1-PE (BD Biosciences) was used as isotype control. Fluorescent Nile Red Particles (0.1–0.3 μm, Spherotech Inc.) were used as size control.

Western blot analysis

Immature DCs and mature DCs (iDCs and mDCs, respectively) with and without sunitinib and pazopanib were lysed using the NP-40 solution (Biocompare) in the presence of phenylmethylsulfonyl fluoride (1 mmol/L, PMES) and protease inhibitors (1X; Sigma). Proteins obtained were quantified by Bradford assay, were resolved using 4% to 12% SDS-PAGE gel and transferred to nitrocellulose. After blocking, membranes were incubated with rabbit anti–β-catenin (Bethyl Laboratories Inc.; 1:1,000), rabbit anti-pErk42/44 (Erk1/2; Cell Signaling Technologies; 1:1,000), mouse anti–β-actin (Cell Signaling Technologies; 1:1,000) and mouse anti–NF-κB (p105/p50; Cell Signaling Technologies; 1:1,000), followed by peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (H+L; Jackson Immuno Research Laboratories; 1:20,000). Protein bands were detected with Immobilon Western (Millipore Corporation) following the manufacturer's instructions. The density of protein bands was analyzed by Image J software and was normalized in terms of average intensity of bands of each protein per average intensity of bands of β-actin.

Endocytosis assay

FITC-dextran (1 mg/mL; Molecular Probes) was added to untreated iDCs and mDCs and differentiated with sunitinib and pazopanib for 2 hours at 37°C. After washing, cells were acquired by FACSCantoII flow cytometer and analyzed by FACSDiva software. DCs incubated with FITC-dextran for 2 hours at 4°C were used as negative controls.

Cytokine production

Culture supernatants from iDCs and mDCs untreated or differentiated with sunitinib and pazopanib and sera from mRCC patients were collected and analyzed using the ProcartaPlex Human Inflammation Panel (20 Plex; eBioscence). Samples were measured by BioPlex Magpix Multiplex Reader (Bio-Rad) and data analysis was performed using Bioplex Manager MP software (Bio-Rad).

T-cell proliferation

T lymphocytes were purified from Ficoll–Hypaque gradient (1,077 g/mL; Pharmacia LKB) followed by CD3 immunomagnetic isolation (StemCell). T cells were then cocultured in a 96-well round-bottom microplate (Costar) with allogeneic iDC and mDCs differentiated with and without sunitinib and pazopanib (DCs: T cells, 1:5) in the presence of PHA (5 μg/mL) for 4 days at 37°C. T cells were pretreated with CarboxyFluorescein

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Succinimidyl Ester (1 μmol/L, CFSE; Life Technologies) and cell proliferation was monitored through progressive halving of fluorescence using FACSCantoII flow cytometer and analyzed by FACSDiva software (BD Biosciences). Results were reported as the percentage of proliferation increase of T cells cultured with DCs treated with sunitinib and pazopanib compared with T cells cultured with DCs alone.

T cells were also cultured in 6-well round-bottom microplates (Costar) in the presence of sunitinib (50 ng/mL) and pazopanib (19 μg/mL) up to 24 hours. Proliferation was evaluated by FACSCantoII flow cytometer and analyzed by FACSDiva software.

Statistical analysis

Descriptive statistics (average and standard deviation) were used to describe the various data. ANOVA test was used to analyze statistical differences between three groups. Student paired t test was used to compare two groups. Significance is indicated when the P value was less than 0.05.

Results

Pazopanib improves DC activation and increases expression of DC-maturation markers

To investigate the capacity of TKIs to influence DC differentiation and maturation *in vitro*, the expression of CD14, HLA-DR, CD86, CD83, CCR7 and CD40 was evaluated by flow cytometry on monocyte-derived DCs of healthy donors. Untreated DCs were used as control. The exposure to concentrations of sunitinib and pazopanib found in plasma affected the phenotype of immature and mature DCs differently (Fig. 1A and B). Sunitinib did not affect DC differentiation and maturation, but DCs cultured in the presence of pazopanib were more activated. Pazopanib modified iDC phenotype, significantly increasing the expression of HLA-DR and CD40 molecules, compared with that of iDCs alone (HLA-DR $P < 0.01$) and iDC treated with sunitinib (HLA-DR $P < 0.05$; CD40 $P < 0.05$; Fig. 1A). The average values of mean fluorescence intensity (MFI) indicated upregulation of CD83 in DCs differentiated with pazopanib. On the other hand, CD14 expression was downregulated by pazopanib during differentiation. Similar results were obtained after DC maturation (Fig. 1B). mDCs cultured in presence of pazopanib significantly upregulated the expression of CCR7 and CD40 molecules compared with untreated mDCs (CCR7 $P < 0.05$; CD40 $P < 0.05$) and sunitinib-treated mDCs (CCR7 $P < 0.05$), suggesting that pazopanib enhances activation status of both immature and mature DCs.

VEGF-R1 expression, which is the target of both TKIs, was unaltered by treatment of DCs with pazopanib or sunitinib (Supplementary Fig. S1A)

PD-L1 is downregulated in pazopanib-generated DCs

The capacity of DCs to stimulate T cells depends on the balance between costimulatory and coinhibitory signals. Increased expression of costimulatory marker such as CD40 or CD83 can facilitate T-cell activation, whereas increased expression of inhibitory markers such as PD-L1 contributes to T cell-negative regulation (16). To evaluate the expression of coinhibitory signals in DCs in response to TKI treatment, we analyzed the expression of the PD-L1 both on DCs and on shed microvesicles (Fig. 2). During iDC differentiation, only pazopanib began to decrease PD-L1 expression, compared with untreated DC and DCs differentiated with sunitinib (Fig. 2A). The decrease in expression became significant ($P < 0.01$) in DCs after maturation (Fig. 2B).

Microvesicles released by the DCs also showed a decrease in PD-L1 expression: PD-L1 expression on microvesicles released by DCs treated with pazopanib was lower than that on microvesicles obtained by untreated DCs and DCs treated with sunitinib (Fig. 2C). The difference in expression of PD-L1 between microvesicles of mDCs and mDCs treated with pazopanib was statistically significant ($P < 0.05$).

Pazopanib treatment reduces immunosuppression downregulating IL10 production by DCs

The production of cytokines such as IL10 and IL12 during DC maturation can influence the capacity of DCs to alter Th1 or Th2 immune responses (17). Several chemokines released by DCs, such as CXCL-10, promote tumor-reactive effector T-cell recruitment (18). Thus, we evaluated cytokines and chemokines released by untreated DCs and DCs treated with TKIs (Fig. 3A). We observed a significant reduction ($P < 0.05$) of IL10 in iDCs and mDCs treated with pazopanib compared with untreated DCs (both iDC and mDCs) and DCs treated with sunitinib (both iDC and mDCs). The balance between IL12/IL10 and CXCL-10/IL10 favored immune activation when DCs were generated with pazopanib (Supplementary Fig. S1B).

Pazopanib-treated DCs are able to increase T-cell activity

DCs must possess specialized features to act as good antigen-presenting cells. In addition to expression of costimulatory molecules and release of cytokines, other indicators of DC function and quality include their capacity for endocytosis and T-cell activation. The exposure to maturation stimuli induces changes including downregulation of endocytosis and increase of antigen presentation to T cells. We evaluated the endocytic capacity of DCs treated with TKIs and untreated DCs by fluorescein isothiocyanate (FITC)-dextran uptake and followed by flow cytometry (Fig. 3B). We determined the ratio of the fluorescence from positive (dextran uptake obtained after 2 hours at 37°C) and negative (dextran uptake after 2 hours at 4°C) samples. The results show that pazopanib reduced endocytosis capacity by 29% for iDCs treated with pazopanib compared with untreated DCs (1.42 ratio vs. 2, respectively), and by 40% compared with iDCs treated with sunitinib (1.42 vs. 2.33). The trend persisted after maturation: the endocytic capacity of pazopanib-treated mDCs was 42% lower than that of mDCs (0.8 ratio vs. 1.37) and 57% lower than that of sunitinib-treated mDCs (0.8 vs. 1.82).

We then analyzed the capacity of DCs to stimulate the proliferation of allogeneic T cells (Fig. 3C). Lymphocytes, pretreated with CFSE, were cocultured with DCs and proliferation was evaluated after 4 days through progressive halving of fluorescence by flow cytometry. The results, plotted as percentage of fold increase (% of proliferation obtained as ratio between T cells stimulated by pazopanib-DCs/DCs or sunitinib-DCs/DCs), showed that when DCs were differentiated in the presence of pazopanib, they acquired a greater capacity to stimulate T cells than either untreated DCs (20% fold increase for iDCs, 5% for mDCs) or sunitinib-treated DCs (14% for iDC, 5,1% for mDCs). No effect was observed on T cells cultured

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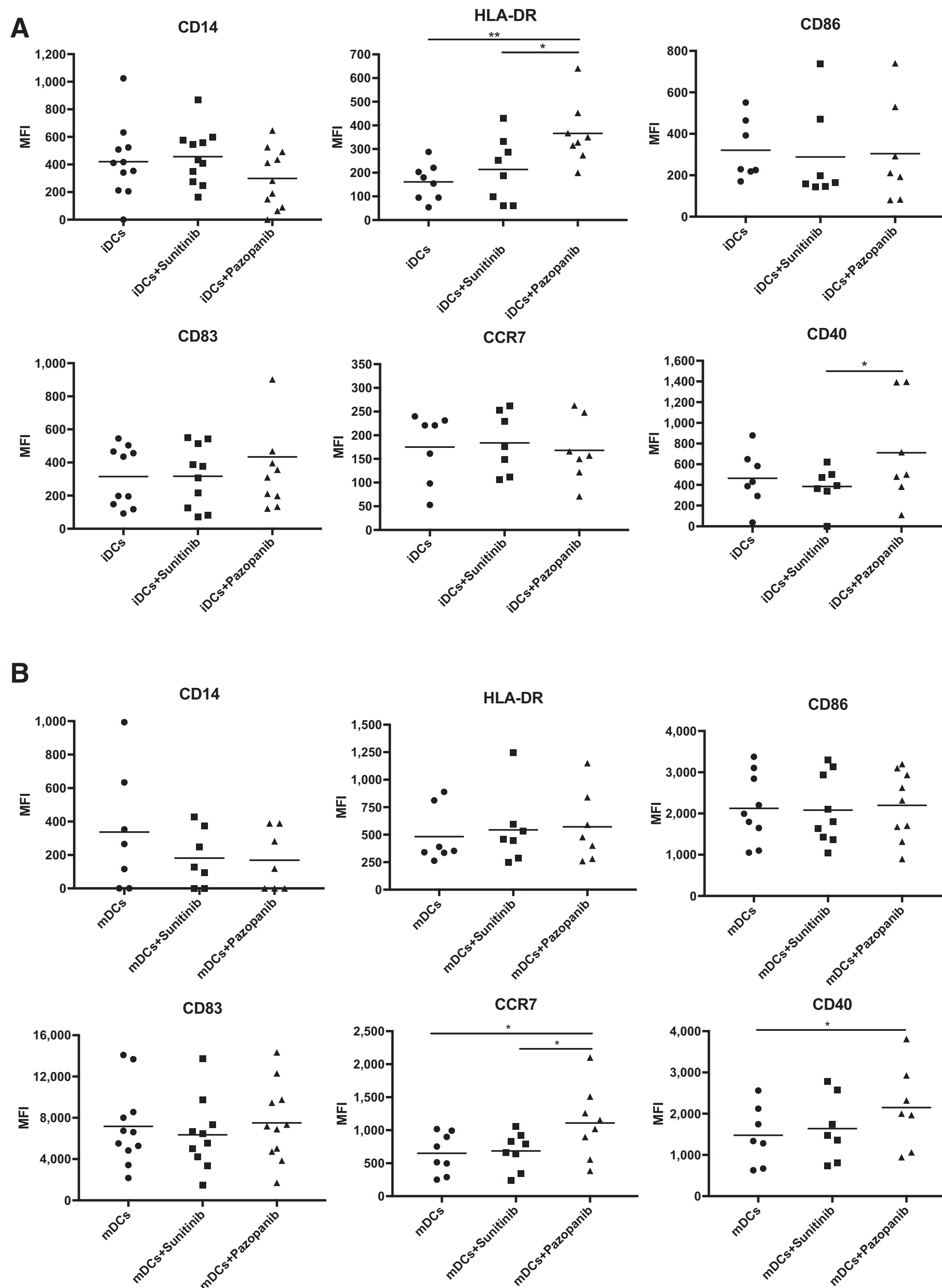

Figure 1.

Pazopanib modulates DC phenotype. The MFI values of DC phenotypic markers from healthy donors are shown. The cells were differentiated from monocytes in presence of pazopanib (20 μg/mL) and at day 5 iDCs were collected and matured with cytokine cocktail (rhIL1β, IL6, TNFα, and PGE_2). Untreated DCs and DCs differentiated with sunitinib (50 ng/mL) were used as controls. The concentration of pazopanib and sunitinib used for the culture corresponds to serum levels achieved in TKI-treated patients. **A,** The phenotype of iDCs; **B,** The phenotype of mDCs. The bars correspond to the average MFI values among donors. Statistical significance was determined by ANOVA test when comparing three groups and by Student paired *t* test for two groups. *, $P < 0.05$; **, $P < 0.01$, Student *t* test.

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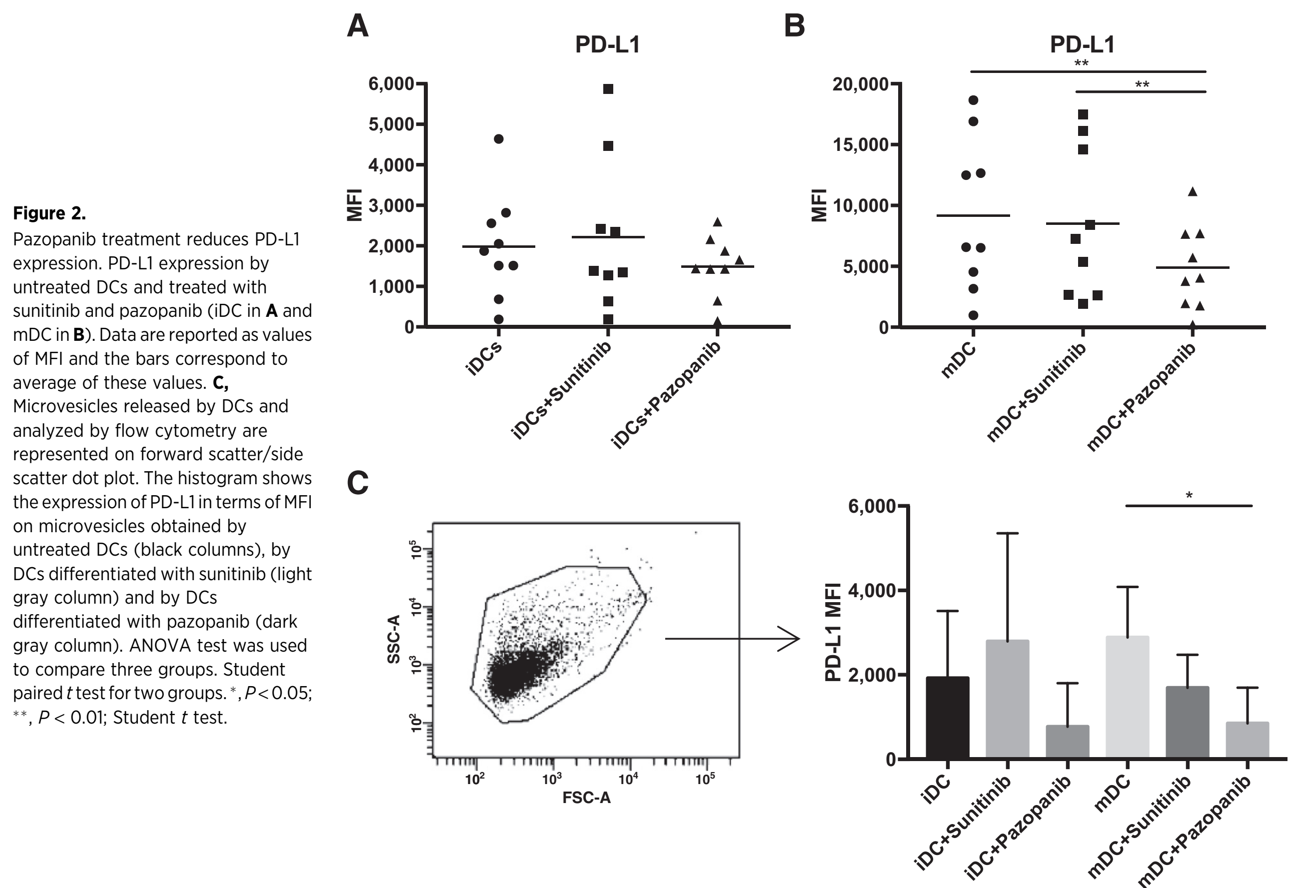

Figure 2.
Pazopanib treatment reduces PD-L1 expression. PD-L1 expression by untreated DCs and treated with sunitinib and pazopanib (iDC in **A** and mDC in **B**). Data are reported as values of MFI and the bars correspond to average of these values. **C,** Microvesicles released by DCs and analyzed by flow cytometry are represented on forward scatter/side scatter dot plot. The histogram shows the expression of PD-L1 in terms of MFI on microvesicles obtained by untreated DCs (black columns), by DCs differentiated with sunitinib (light gray column) and by DCs differentiated with pazopanib (dark gray column). ANOVA test was used to compare three groups. Student paired *t* test for two groups. *, $P < 0.05$; **, $P < 0.01$; Student *t* test.

alone for 24 hours in the presence of either TKI (Supplementary Fig. S2).

Pazopanib affects DC differentiation by inhibiting p-Erk/β-catenin signaling

The Wnt-β-catenin pathway, particularly in DCs, regulates the balance between tolerance and immune response (19). Loss of β-catenin impairs the ability of DCs to induce Tregs (20), instead the activation of β-catenin pathway increases the capacity of DC to release IL10 and promote immune tolerance, (21, 22). In DCs, β-catenin signaling synergizes with other pathways, such as the Erk pathway, to induce anti-inflammatory cytokines and proliferation of Tregs. Erk1/2 signaling retards the phenotypic and functional maturation of monocyte-derived human DCs (23).

To evaluate whether changes in DC functional activity were due to signaling differences, we examined DC intracellular pathways. Untreated DCs or DCs differentiated in the presence of sunitinib or pazopanib were lysed and probed with anti–p-Erk 1/2, anti–β-catenin and anti–NF-κB by western blot (Fig. 4). DCs treated with pazopanib expressed less p-Erk 1/2 than did DCs treated with sunitinib or untreated DCs. This downregulation associated with a significant reduction of β-catenin expression (iDCs and sunitinib-iDCs vs. pazopanib-iDCs $P < 0.05$; mDCs vs. pazopanib-mDCs $P < 0.05$). In both pazopanib-iDCs and pazopanib-mDCs, p-Erk1,2 and β-catenin antibodies detected a weaker signal than in untreated DCs and DCs treated with sunitinib, suggesting that pazopanib could act through these pathways. We analyzed the activation of NF-κB, which is essential for DC development and survival and regulates DC maturation. Results indicated that NF-κB activation was similar in all iDCs tested. Although the p50 signal was weaker in mDCs treated with pazopanib, the balance between all intracellular pathways favored the activation pathway.

Modulation of DCs generated from mRCC patients treated with pazopanib or sunitinib

To confirm the pazopanib immune-priming effect in patients, we analyzed monocyte-derived DCs differentiated from mRCC patients during TKI treatment. DCs were differentiated *in vitro* by standard methodology without the addition of TKIs. Fig. 5 shows the phenotype of DCs generated from PBMCs of RCC patients after one month of pazopanib or sunitinib treatment. CD14, a marker of DC immaturity, was less expressed in iDCs from patients treated with pazopanib ($P < 0.01$) than patients treated with sunitinib confirming data obtained *in vitro*. Moreover, DCs (both iDCs and mDCs) from pazopanib-treated patients expressed more of the activation markers HLA-DR and CCR7 and less PD-L1 (mDCs; $P < 0.01$) as compared with DCs generated from sunitinib-treated patients.

For one patient, we evaluated the modulation of some DC markers differentiated before (T0) and during pazopanib therapy (T1: after 1 month of treatment, T2: after 2 months) and results are shown in Supplementary Fig. S3A. As with DCs generated *in vitro* with pazopanib, iDCs from this mRCC patient in treatment with pazopanib showed increased

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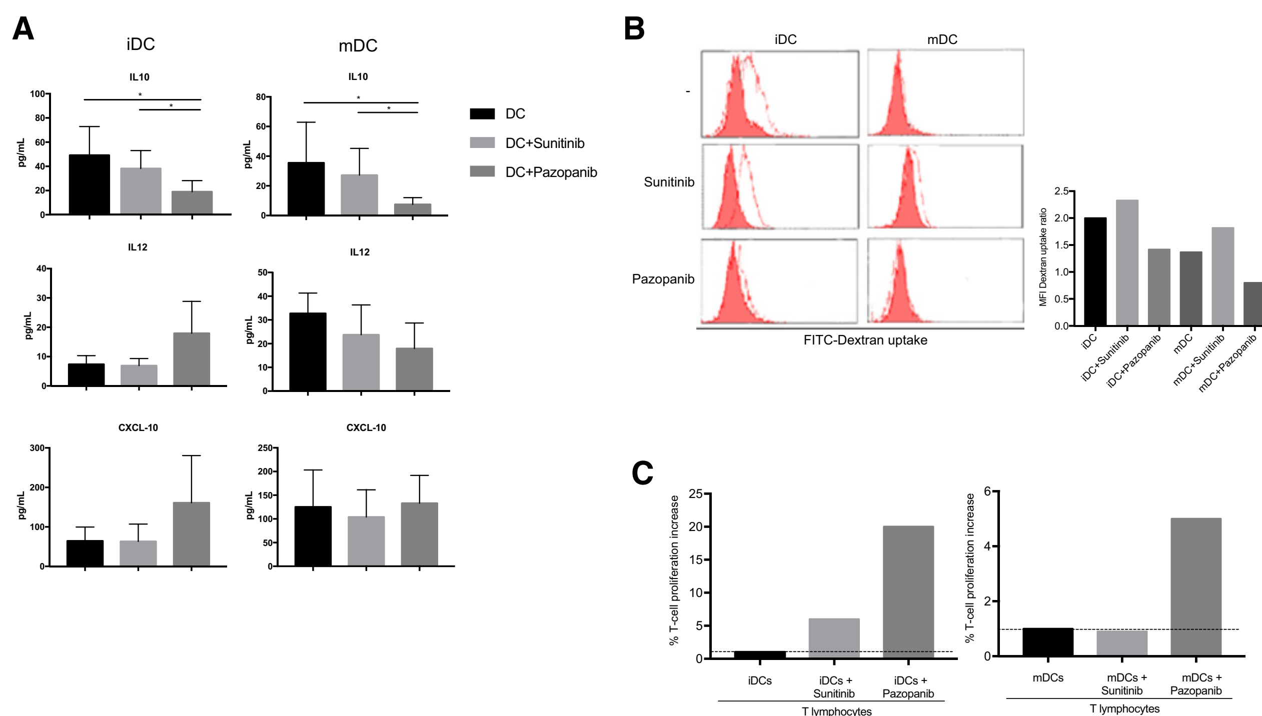

Figure 3.
DCs activated by pazopanib modulate cytokine and chemokine release, reduce endocytic capacity and increase T-cell proliferation. **A,** The amount of IL10, IL12, and CXCL-10 was evaluated in differently generated DC supernatant by Luminex multiplex beads analysis. The results correspond to the mean obtained from 10 independent experiments ± SD. **B,** The endocytic capacity of iDCs and mDCs differently generated was evaluated as FITC-dextran uptake (1 mg/mL/10^6 cells). Open histograms represent the dextran uptake obtained after 2 hours at 37°C (positive), whereas filled histograms show the dextran endocytosis after 2 hours at 4°C (negative control). Results are representative of one donor out of four. The values reported on histogram indicate the ratio between MFI of positive and negative control of four independent experiments for each DC condition. **C,** The capacity of DCs to prime allogenic T-cell response *in vitro* was tested evaluating T-cell proliferation ability through progressive halving of CFSE fluorescence using FACSCanto flow cytometry. Results are reported as a percentage of increase of T-cell proliferation capacity of three independent experiments of DCs treated with pazopanib compared with sunitinib-differentiated DCs and untreated DCs. ANOVA test was used to compare three groups. Student paired *t* test for two groups. *, $P < 0.05$.

expression of HLA-DR at T2 and CD40 at T1 and T2. Expression of the coinhibitory molecule PD-L1 decreased during pazopanib treatment, both in iDCs and mDCs, confirming that pazopanib boosts DC activity.

Immune profile of mRCC patients during TKI treatment

The priming of DCs by pazopanib could impact the immune repertoire in the peripheral blood of mRCC patients. To verify this hypothesis, we followed and monitored longitudinally mRCC patients undergoing TKI therapy, either pazopanib or sunitinib. Patients had different previous therapies although all of the patients belonged to the same risk group. Six patients in treatment with TKIs for mRCC underwent peripheral blood sampling at different time points and PBMCs were isolated and analyzed by flow cytometer in order to investigate the evolution of patients' immunological profile during TKI administration. The clinical profile and the schedule of blood sampling is showed in Fig. 6A. Patients 1, 2, and 3 received pazopanib, whereas patients 4, 5, and 6 received sunitinib. The immunoassays were performed when possible at different time points, as shown in the figure. Two patients (patient 3 and 5) showed progressive disease and one of these died from the disease. The other four are still in treatment with TKIs.

Several immunological parameters were evaluated for each patient at each time point considering the limited amount of blood sampling (Fig. 6). In particular, we analyzed T-cell subpopulations, their activation status (CD137 expression), their proliferation ability as assessed by expression of Ki67, the immunosuppression as a percentage of Tregs, and T-cell exhaustion as assessed by expression of checkpoint inhibitors such as PD-1, CTLA-4, and Tim-3. Pro- and anti-inflammatory cytokines were evaluated in serum of patients. Only the assays for which we had sufficient patient samples are shown in Fig. 6.

We observed that pazopanib seemed to induce an increased number of $CD3^+CD137^+$ T cells (Fig. 6B). In patient 1, for example, activated ($CD137^+$) $CD4^+$ T cells were 30% of the activated $CD3^+$ T cells at time T2, compared with T0 (before pazopanib therapy, 0.3% of activated $CD3^+$ T cells) and T1 (4.7% of $CD3^+$ T cells). At T5, we observed a decrease of $CD4^+$ and $CD8^+$ $CD137^+$ T-cell populations. In the same patient, plasma levels of IL6 dropped from T0 to T2, both IL4 and CXCL-10 increased at T1 and T2, and ICAM decreased at T2. Ki67 expression, a proliferation signal for T cells, was higher at T2 than at other time points. Patient 2 had fewer $CD137^+$ T cells from time T0 forward. Regulatory T cells decreased during treatment and

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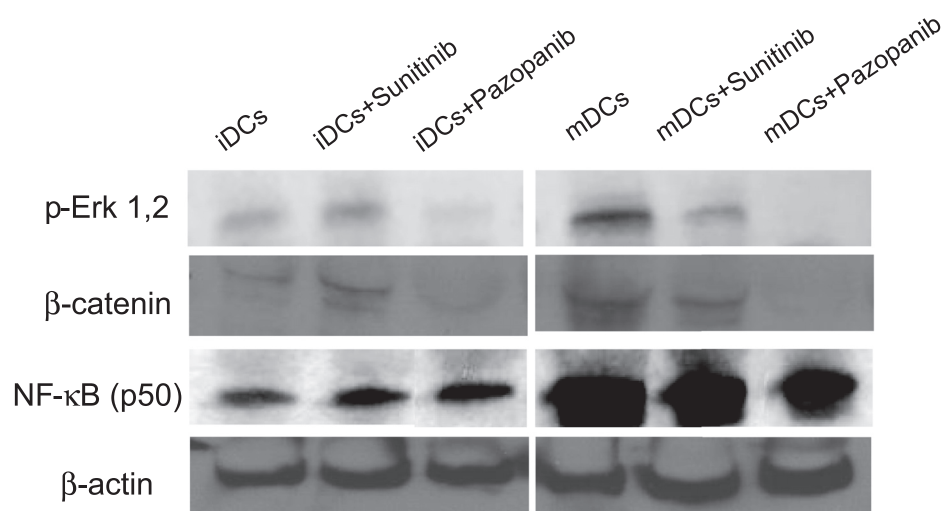

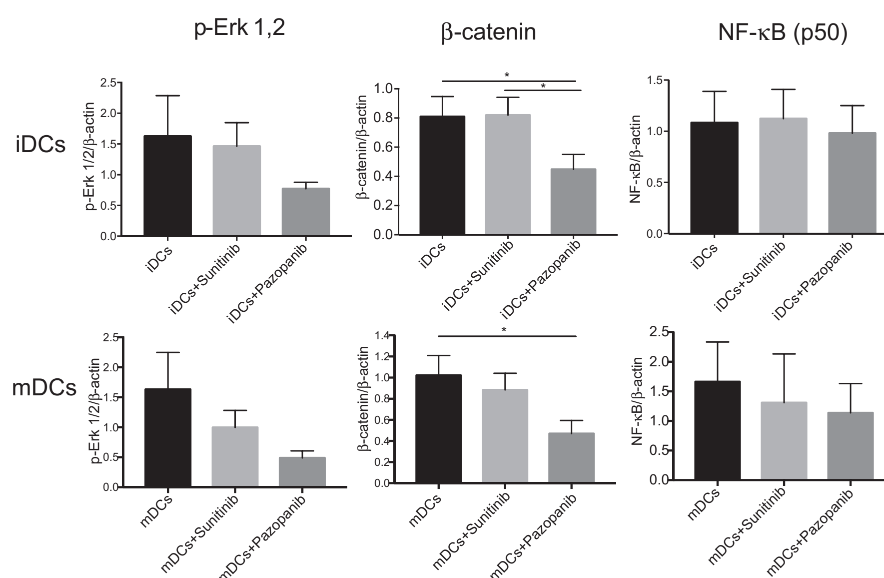

Figure 4.
Pazopanib modulates DC signaling turning off the p-Erk/β-catenin pathway. Western blotting of iDCs (left) and mDCs (right) untreated and generated in presence of sunitinib and pazopanib. Samples were analyzed for p-Erk 1/2 (42–44 kDa), β-catenin and for the presence of NF-κB. β-Actin was used as a loading control. Proteins were resolved in 4% to 12% SDS-PAGE gel. Densitometric evaluation of the signals for β-catenin, p-Erk1/2 and p50 were normalized to the levels of β-actin (under the blot). ANOVA test was used to compare three groups. Student paired *t* test for two groups. *, $P < 0.05$.

$Ki67^+$ T cells increased. Patient 3, who was monitored during pazopanib treatment and immediately after progression, showed enhancement of $CD4^+CD137^+$ T cells at T3 (54%) compared with T0 (24.9%) and T1 (23.2%). This population decreased during progression under nivolumab treatment (T4, T5). This patient's Treg population was unchanged. $PD1^+$ T cells increased slightly from T0 to T1, then decreased during pazopanib and nivolumab treatment.

When we analyzed mRCC patients treated with sunitinib, we observed that $CD137^+$ T cells were less evident, thus suggesting that this population could be influenced by the effect of pazopanib. In Patient 4, under sunitinib therapy, $CD4^+CD137^+$ T cells were barely detectable at all three time points. This patient presented with 29% of T cells being $CD8^+CD137^+$ T cells at T1; however, this population decreased to 14% at T2 and T3. In the same patient, the serum concentrations of IL10 were increased at T2 compared with T1, whereas serum concentrations of IL12, IL4, and CXCL-10 decreased. In accordance with the increase of IL10, we observed an increase of Treg cells during sunitinib treatment, accompanied by upregulation of CTLA-4 expression on T cells at T3. Patient 5 showed a similar decreasing trend in $CD137^+$ T-cell population. Coinhibitory markers, such as CTLA-4 and Tim-3, increased during sunitinib treatment. $Ki67^+$ T cells were decreased, and Treg cells were reduced. Patient 6 presented low and stable fractions of $CD137^+$ T cells during sunitinib treatment when there time points were analyzed, although $CD3^+CD137^+$ T cells doubled at T7. At the same time IL10 and IL4 decreased compared with T5, and IL12 slightly increased. Tregs decreased at T7 but increased at T9.

Discussion

RCC is the tumor that has most benefited from clinical use of TKIs. The clear cell histotype drives VEGF overproduction via inactivation of the tumor-suppressor Hippel–Lindau (VHL) gene

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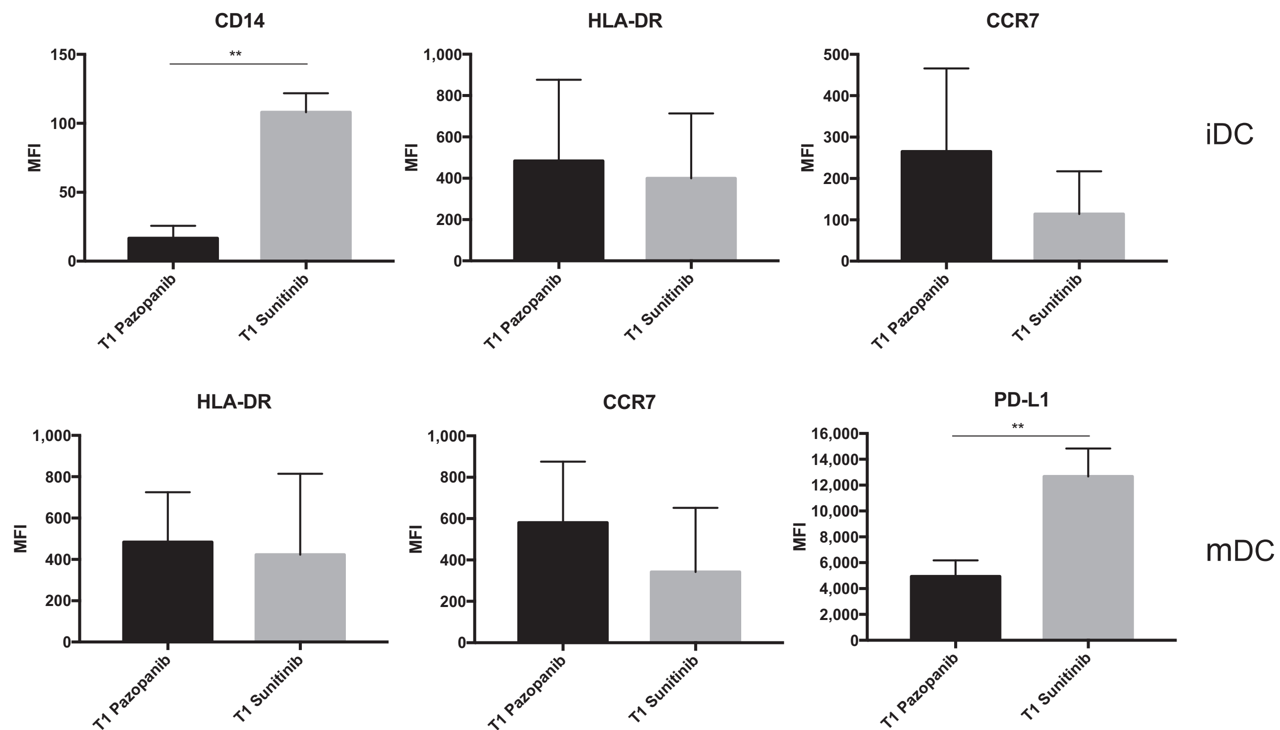

Figure 5.
DC phenotype obtained from TKI-treated patients. Comparison of iDCs and mDCs at T1 of pazopanib (black column) and sunitinib (gray column) treatment. Data are reported as values of MFI ± SD. Statistical significance was determined by Student paired *t* test. **, $P < 0.01$.

(24, 25). Immune-targeting molecules have added additional possible treatment choices for RCC. The increase in treatment options has added complexity to clinical questions on how to choose first-line treatment and treatments after recurrence (26). Although VEGF- and mTOR-targeted therapies have improved clinical outcome in metastatic RCC, durable responses remain rare despite efforts to design sequential or combined treatment modalities.

RCC is been considered an immunogenic tumor and therapy with cytokines such as IL2 and IFNα has been used although the responses have been mild and associated with toxicity. Long-term responses and complete remissions have been occasionally observed in mRCC patients treated with immunotherapy (27, 28). The induction of an immune response such as the one elicited by the multi-peptide IMA901 vaccine was associated with a clinical benefit. Over the next decade, immunotherapy trials in kidney cancer will focus on learning how to combine PD-1/PD-L1 inhibitors with immune-modifying agents such as those targeting the VEGF pathway (29, 30).

In this scenario, and with the possibility to introduce the immune checkpoint inhibitors (ICI) in RCC patient treatment, we must consider the impact of TKIs on the immune system of the patient. TKIs currently approved for first-line treatment of mRCC inhibit tumor cell growth and angiogenesis but also show immune-regulatory effects. How these immune effects impact subsequent immune-targeted therapies and affect treatment outcome needs to be understood.

Several molecules and immune cell types are involved in the interaction between the growing tumor and the immune system. The balance between immunosuppression and immune activation dictates to some extent the prognosis and the response to treatment, particularly for immune-based treatments. Several biomarkers and or immune-molecular portraits have been studied in the attempt to identify responder patients, such as those who most likely will benefit from anti–PD-L1 treatment (31). One of the mechanism of tumor silencing of immunity involves DCs, the main orchestrators of the immune system (32, 33). DCs in the tumor microenvironment may be tolerized and therefore unable to present tumor antigens or unable to present antigens in the appropriate setting within a costimulatory background. Several molecules released by the tumor can induce this freezing effect (34, 35). VEGF is one of them and this mechanism has been well characterized in RCC (36). The immediate consequence of induction of tolerogenic DCs will be the lack of T-cell infiltration, particularly tumor-specific T cells that represent the target for ICI immunotherapy.

Results reported in this article show that the TKI pazopanib could potentiate and differentiate human DCs. Immature and mature DCs showed increased expression of HLA-DR, CCR7, and CD40. PD-L1 decreased in the DCs after maturation, as compared with untreated mature DCs or DCs differentiated in the presence of sunitinib. PD-L1 was expressed in microvesicles released by DCs confirming the role of cell particles in delivering remote signaling (37). The phenotypic changes observed in the pazopanib-treated DCs were accompanied with biological behavior consistent with an immune priming effect. IL10 production was reduced, whereas IL12 and CXCL10 production increased. The process of endocytosis was reduced as well, and

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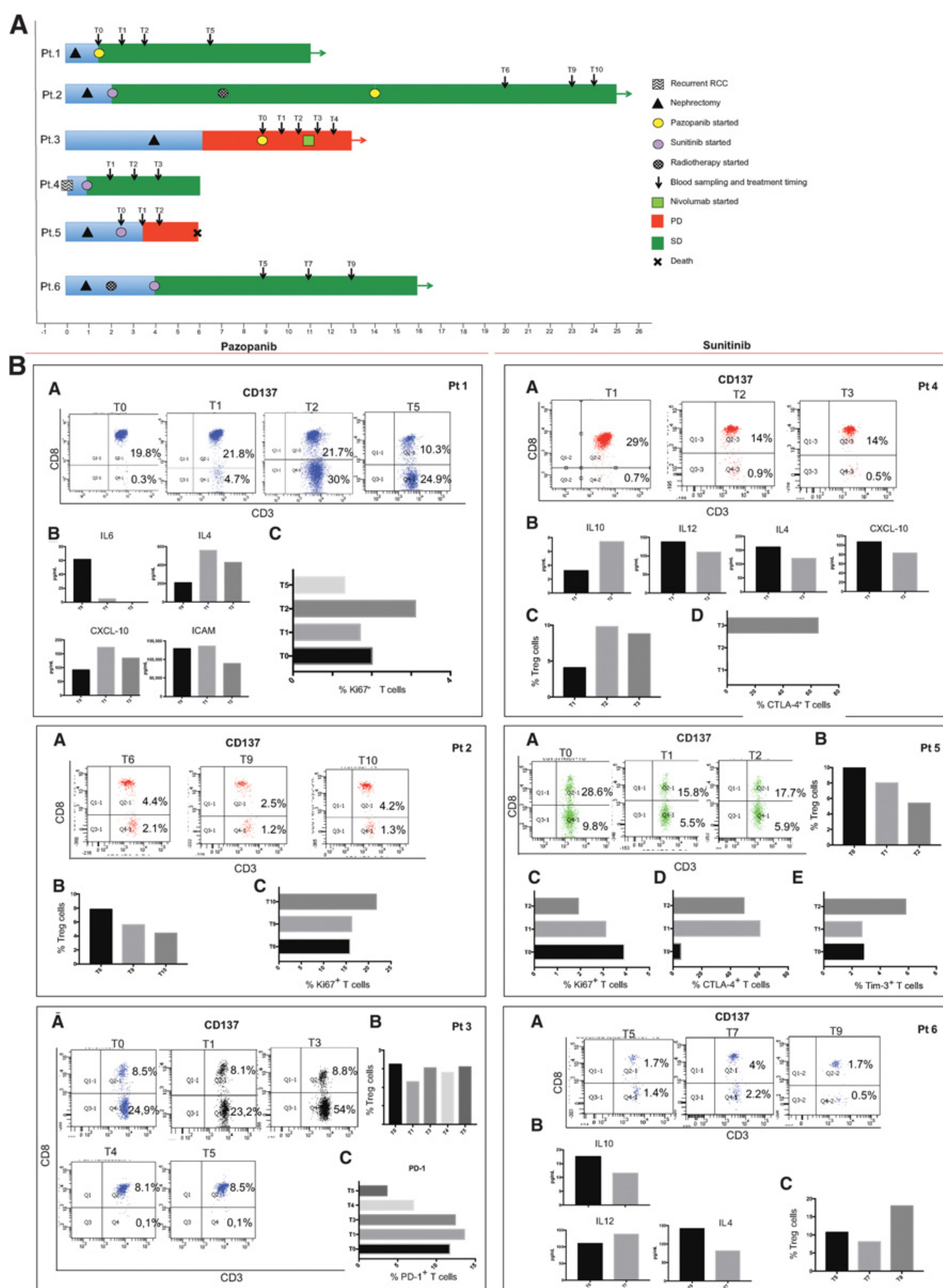

Figure 6.

Immunological profile of six mRCC patients during TKI treatment. **A,** Swimmer Plot showing the clinical history and the blood sampling of six mRCC patients: Patients 1, 2, and 3 treated with pazopanib, patients 4, 5, and 6 with sunitinib. **B,** Immunological analysis performed on PBMCs and sera collected from blood samples for each patient. Sera were used to evaluate cytokines and chemokines released during TKIs treatment and analyzed by Luminex multiplex beads. Immune cell subpopulations were monitored by flow cytometer and analyzed to FACSDiva Software. To analyze $CD137^+$ T cells, lymphocytes were first gated on FSC-A and SSC-A, then the $CD3^+$ T-cell subpopulation was selected on lymphocytes. These $CD137^+CD3^+$ T cells were then selected and analyzed for CD4 and CD8 (gating scheme in Supplementary Fig. S3B).

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amplified MLRs indicated an overall acquisition of immunogenicity by DCs.

DCs are the critical cells in immune activation as they are responsible for antigen processing/presentation, cross-priming and trafficking to the lymph nodes (38, 39). The pazopanib *in vitro* and *in vivo* "treated" DCs, both from donors and from patients, have all these hallmarks.

Sunitinib, another TKI used in mRCC treatment, did not have the effects on DC differentiation as have been described by others (11). Sunitinib and pazopanib have a similar pattern of receptor recognition but display different affinities for VEGF-R1, which could explain the diversity of immune modulation effect (12). In particular, sunitinib affects the immunosuppressive repertoire, Treg and MSDC, in RCC patients. The two drugs appear, however, to be similar in terms of clinical outcome when used in first-line therapy in mRCC (40, 41). Immunologically this could be explained by arguing that one (pazopanib) is more active in the rescue of DC from a tolerogenic state, inducing activation signals, whereas the other (sunitinib) is more efficient in making space for the antitumor effector cells by eliminating immunosuppression in the microenvironment.

The consequences of receptor inhibition were studied in the pazopanib-treated DC, as we investigated the signal transduction involved. We found that Erk signaling was shut down, leading to downregulation of β-catenin. Wnt/β-catenin signaling plays a role in cell differentiation, proliferation, survival, and immune cell function. In tumors, the Wnt/β-catenin pathway is activated in DCs leading to immune tolerance and immune evasion. This results in suppression of effector T cells and recruitment of Treg cells. Increased Wnt ligands released in the tumor microenvironment activate β-catenin signaling in DCs, resulting in production of IL10 and induction of tumor tolerance (22). In melanoma, intrinsic activation of the Wnt/β-catenin pathway correlates with absence of T cells in the microenvironment (42). The same pathway is involved in bladder cancers, generating the non-T cell–inflamed tumors that represent most cases in which tumors are unresponsive to ICI therapy (43). A DC subtype is enriched in RCC that expresses CD14, high TNFα, and low CXCL-10, which is responsible for preventing a T-cell infiltrate from mediating antitumor functions (35). Indeed, the presence of circulating, intratumoral, and peritumoral $CD14^+$ cells was a prognostic factor for decreased survival in a cohort of 375 RCC patients (44).

Pazopanib can therefore have an antagonistic effect in the tumor, releasing DCs from the tolerogenic/immature/$CD14^+$ state and tentatively restoring antitumor immune activation. The goal of such therapy is to turn cold tumors into hot tumors, which would be more responsive to ICI therapy. To understand if pazopanib treatment could indeed exert this effect *in vivo*, we utilized two different approaches. First, we selected $CD14^+$ cells from peripheral blood of mRCC patients during treatment with pazopanib or sunitinib. The immune-priming effect of pazopanib in DCs was confirmed. DCs derived from mRCC patients in treatment with sunitinib expressed high amounts of $CD14^+$ marker and PD-L1. This may suggest no effect by sunitinib on the DCs that remain in a more immature state as seen in other cancer patients (44).

The second approach was to longitudinally follow and monitor mRCC patients undergoing TKI therapy. This was not a homogeneous patient population because patients had different previous therapies, although all of them belong to the same risk group. Nevertheless, we wanted to assess if we could detect immunological changes in the peripheral blood that could be associated with pazopanib treatment. Various conclusions are supported although results are preliminary. First, patients 1, 2, and 3 who received pazopanib showed a $CD137^+$ T-cell population. Patient 1 presented an ideal setting to study the effect of pazopanib *in vivo* because this patient had no previous possibly confounding therapy. Patient 2 had a recent radiotherapy to control bone pain, which might have influenced the immune system (45). Patient 3 is the only patient we have monitored during anti–PD-1 nivolumab treatment. This patient had a drop of $CD137^+$ $CD4^+$ T-cell population at time T4 and T5 just after treatment with ICIs for progression. PD-1 expression also decreased. This could be explained by recruitment of exhausted T cells to the tumor or by difficulty in detecting PD-1 cells due to the covering of the circulating nivolumab antibody.

CD137 (4-1BB, a member of the TNF-receptor family) is considered a biomarker of tumor-reactive cells. The signaling with its ligand or an agonistic antibody promotes expansion of T cells, sustains survival, and enhances cytolytic function. This marker has been used to select tumor-specific T cells and can be upregulated in an antigen-dependent fashion (46, 47). Agonist antibodies recognizing the CD137 receptor are part of the vast repertoire of immune-modulatory antibodies being prepared for the clinic. The Treg population was unchanged or diminished in all mRCC patients. Immune monitoring on patient 4, the only patient with recurrent mRCC we tested, showed the worst-case scenario during sunitinib treatment, characterized by no $CD137^+$ T cells, high IL10, high percentages of Treg, low IL12, and upregulation of CTLA-4 at 3 months of therapy.

The other consideration is the upregulation of checkpoint inhibition markers on T cells as a sign of T-cell exhaustion. This evaluation is relevant for clinical decisions because ICI are proposed as second-line therapies for mRCC after TKIs. We saw upregulation of CTLA-4 early in 2 of 3 sunitinib-treated patients. In patient 5, we observed upregulation of both CTLA-4 and Tim-3. This patient progressed rapidly and died from the disease.

Pazopanib can have an immunological effect as early as the second month of treatment. Our experience with ICIs showed that immune modifications can happen sooner than their impact on the tumor becomes evident (48). Our data are part of an ever-evolving clinical arena. CheckMate 214, a phase III, randomized, open-label study evaluating the combination of nivolumab (anti–PD-1) plus ipilimumab (anti–CTLA-4) versus sunitinib in patients with previously untreated advanced or mRCC, showed improved OS, ORR, and PFS in the nivolumab plus ipilimumab group in intermediate/poor risk patients, with the greatest improvements in those with PD-L1 expression on $\geq$ 1% of biopsied tumor cells. These data suggest that ICIs could be used as front-line treatment in mRCC even though only the knowledge of the biological and immunological features of the tumor and patient could indicate the best individual sequence/combination of treatment.

In this context, we are recruiting mRCC patients with no previous therapies and collecting tumor specimens to confirm the immunological observations that we observed from this preliminary study. Longitudinal study methods combined with an individualized approach based on the immunological fitness of the patient hold the greatest likelihood of clinical success. Confounding influences that might affect the immune status of the patient, such as other therapies, could limit our understanding

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of the activity and efficacy of the immune-modulating agents we wish to study.

In conclusion, the immune-priming effects of pazopanib open therapeutic avenues for this TKI in the mRCC and probably for other cancers. Variations of treatment sequences, schedules, doses, and combinations with other TKIs or immunotherapy compounds or vaccines should be tested, taking into account the immunological effects of TKIs and the updated insights into oncology and the interactive tumor microenvironment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Cancer Immunology Research

TK Inhibitor Pazopanib Primes DCs by Downregulation of the β -Catenin Pathway

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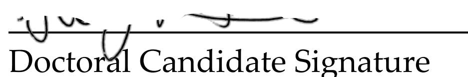
CONFLICT OF INTEREST DISCLOSURE

Dr. Ilary Ruscito has no conflict of interest to declare.



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