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Anti-Tumour Treatment

The ambitious role of anti angiogenesis molecules: Turning a cold tumor into a hot one

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ABSTRACT

In renal cancer emerging treatment options are becoming available and there is a strong need to combine therapies to reformulate and adjourn clinical practice. We here highlight and discuss the need to take advantage of the common immune targets to design combined strategies to increase clinical responses.

Personal view

The introduction of immune checkpoint inhibitors (ICIs) in clinical practice has revolutionized the scenario and the outcome of cure in oncology. The immune system is a complex network where the several mechanisms and interactions involved are continuously discovered. More than ever translational scientists are close to the clinicians to better understand patients immune response to cancer in order to potentiate possible avenues of cure. Several if not all standard therapies that represent today the gold standard in oncology need to be characterized from an immunological point of view for a necessary integration process that must be done to optimize protocols of cure.

From this point of view we want to discuss and share some of the critical points in the clinical management of anti angiogenic drugs, particularly Tyrosine Kinase Inhibitors (TKIs) and immunotherapy.

TKIs are important drugs that target receptors involved in the neoangiogenesis of tumors, such as the Vascular Endothelial Growth factor (VEGF) pathway, and they have contributed to changing life expectancy particularly of renal cancer patients [1,2]. In renal cancer, the activation of genes of the hypoxic response results in the downstream activation of pro-angiogenic growth factors like VEGF. Together with sunitinib, sorafenib, pazopanib, axitinib, most recently drugs targeting possible pro-angiogenic resistance mechanisms, lenvatinib and cabozantinib are also available.

The increase in treatment options including ICI has added complexity to clinical questions on how to choose first-line treatment and treatments after recurrence [2].

Tumor associated angiogenesis and immune system

Tumor progression is characterized by a number of mutational events that confer to the initially transformed cells a number of distinctive and functional features associated with invasive potential and expression of tumor associated antigens. A class of these, the neoantigens are not essential for tumor progression, but are important in generating an immune response in the patient. Indeed the presence of a relevant anti-tumor immune infiltrate is associated with an increased mutational tumor burden; moreover these tumors have been shown to respond better to ICI [3,4]. Despite immune activation, tumors are able to evade immunological control and after a phase of equilibrium that can also be prolonged in time, they finally enter in the escape phase characterized by several immunosuppressive traits and independence from any kind of immune control.

In this scenario the tumor microenvironment appears to have an important role and a number of studies are now focalizing in understanding the dynamics of these interactions during tumor progression.

The growing tumor establishes from the beginning a complex network of cross-talk between tissue resident stromal cells and immune cells, which are also resident but more frequently recruited ad hoc from the bone marrow and circulation. Tumor directed vascularization or tumor angiogenesis appears to involve several molecules, cells and signaling pathways. One of the main leaders in this crucial step, the angiogenic switch, which allows the tumor to acquire the invasive behaviour and a fully metastatic potential is the VEGF. Hypoxic cancer cells are able to secrete VEGF which engages the specific receptor on the endothelial cells that in turn proliferate, gradient guided to generate

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new blood vessel sprouts characterized by impaired vascular maturation, poor functionality and defects in endothelial architecture [5]. The immaturity of the new generated tumor associated vasculature results in excessive permeability, poor perfusion and imperfect blood flow. This has direct impact on anticancer treatment efficacy [6].

Moreover angiogenesis has an important influence on the immune system and these processes appear to be intimately linked. The vascular network with its specific components, endothelial cells, pericytes, growth factors and receptors is fundamental in the inflammatory response, in wound healing and in immune surveillance [7]. T cells, particularly antigen primed T cells, need a healthy endotelium for the trafficking to tissue districts and the cell to cell cross-talk that is established during the priming and effector phase of the immune response.

This is the basic principle of immune surveillance that relies on an efficient vascular-lymphatic circulation and endothelia that can be viewed as "critical non-hematopoietic components of the immune system" [8].

The access of immune cells into the tumor becomes a critical issue for the outcome of immunotherapeutic strategies such as adoptive cell transfer and expansion of tumor specific T cells with ICIs. Moreover also classical chemotherapeutic drugs have been shown to benefit for optimal efficacy of an intact immune system thus suggesting a synergy between cancer cell death and immune activation [9]. A normalized endothelium is preferred assuring the correct trafficking of T cells to the tumor bed since it is now well established that the presence of tumor infiltrating lymphocytes correlates with improved prognosis for most tumors [10]. The process of T cell infiltration is regulated by several variables. The type of T cell repertoire that can be described in the tumor depends on tumor released factors, on the cytokine/chemokine secreted and relies during the tumor growth, on the first encounter between the initial transformed cells and resident antigen presenting cells. Dendritic cells (DCs) as first sentinel cells are empowered to deliver the correct Th1 signalling to generate tumor specific T cells by endocytating dead neoplastic cells or cellular debris, transporting tumor antigen to the draining lymph nodes and cross-presenting antigens to T cells. Expression of an array of receptors and molecules on the surface of DCs is required to deliver the correct non tolerogenic signaling (DC maturation) [11]. Activated T cells can now expand and home to the tumor to exert the effector functions. This whole process, theoretically simple and smooth, is tightly regulated and can be perturbed by a number of factors and variables. These include all the well described immune tumor evasion mechanisms such as phenotypic changes in DCs induced for example by VEGF, PGE2, IL-10, tumor hypoxia. The T cell tumor infiltrate can also be populated by a variety of immunosuppressive cells usually attracted by the tumor and with the ability to counteract immune anti tumor activity: Regulatory T cells (Tregs), Myeloid Derived Suppressor cells (MDSCs) and Tumor associated macrophages (TAMs) [12]. So in the end the balance between immune activation and immune suppression will determine or at least influence significantly the outcome of any cancer treatment that relies or utilizes immunity networks.

The distinction between "hot" and "cold" tumors is relatively new. Some tumors have been defined as naturally "hot" such as melanoma characterized by a high mutational load in cancer cells associated with neoepitope expression and induction of tumor specific T cells [13–15]. For these tumors the possibility that ICI immunotherapy would work is relatively high since it is believed that the tumor microenvironment already has a repertoire of exhausted PD-1$^+$ T cells ready to be expanded. Responder patients are probably these, in which the immune-balance was in equilibrium and activation just needed to be unleashed to take over. A "cold" tumor has little or no T cell infiltration like prostate cancer, and therefore the efficacy of an ICI treatment can be null or very limited. Cold tumors are also those where a T cell infiltration can be observed but is localized in the periphery of the tumor suggesting a sort of limited access probably due to the tumor stroma including the vasculature network [16,17].

Tumor associated angiogenesis and anti cancer therapies

The efficacy of most of the anti cancer therapies is severely impaired by the imperfect newly generated blood vasculature. The interruption of a regulated blood flow will not allow sufficient drug delivery in the tumor particularly in all the different areas. Therefore efficacy of the treatment will be compromised. It should be emphasized that this effect is highly variable among patients and between tumors and can explain why patients respond only initially to treatment. The other important finding is that we know today that anti-angiogenic drugs can reduce dramatically cancer induced immunosuppression, simply because the targets such as VEGF and its receptors are the main actors in this process.

The initial idea that led to the generation of anti-angiogenesis drugs was to operate a pruning effect of the tumor associated blood vessels to induce tumor starvation. The dosing and schedule of antiangiogenic drugs has been revolutionized by Jain's hypothesis demonstrated in animal models that the "judicious" use of antiangiogenic agents could transiently normalize tumor vasculature, diminish hypoxia, reduce immunosuppression and improve efficacy of different therapies [18]. This hypothesis puts the basis for using anti angiogenic therapies and immunotherapies in combination more than as single agents. Clinical data confirm and sustain the validity of this thesis i.e chemotherapy and immune therapy require functional blood vessel for optimal efficacy [19]. The recent outstanding results from the combinations of ICI and chemotherapy in lung cancer strengthens furthermore this rationale and encourages the design of novel combination and /or sequential clinical trials using drugs targeting different mechanisms (tumor cell death, endothelium, immunity).

In the last few years we have also learned that chemotherapy can influence immunity and is able to target and normalize cancer endothelium, when given in a metronomic fashion [8]. The real innovative and relevant application of metronomic chemotherapy is the combination with immunotherapy by directly affecting the immunosuppressive tumor microenvironment. Cyclophosphamide, with its well known effect in depleting the Treg population, is one of the most used drugs in a variety of cancer vaccine protocols as a tool to make space for the newly activated specific T effector cells, which would be liberated by the immunosuppressive milieu [20].

Targeting angiogenesis and the immune system: Learning from patients' immune system

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 of course 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 [21]. 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

Table 1
Main ongoing trials in renal cancer with combination strategies: immunotherapy, TKI and anti-angiogenic targeted therapy

Trial	Phase	NCT#	Status
A Study of Atezolizumab Combination With Bevacizumab Versus Sunitinib in Participants With Untreated Advanced Renal Cell Carcinoma [IMmotion151]	III	02420821	Active, not recruiting
Atezolizumab in Combination With Entinostat and Bevacizumab in Patients With Advanced Renal Cell Carcinoma	I/II	03024437	Recruiting
Study of Atezolizumab + Bevacizumab in Patients With Advanced Non-Clear Cell Renal Cell Carcinoma	II	02724878	Recruiting
A Study of the Safety and Efficacy of Atezolizumab Administered in Combination With Bevacizumab and/or Other Treatments in Participants With Solid Tumors	I	02715531	Recruiting
A Study of Atezolizumab (an Engineered Anti-Programmed Death-Ligand 1 [PD-L1] Antibody) as Monotherapy or in Combination WithBevacizumab (Avastin®) Compared to Sunitinib (Sutent®) in Participants With Untreated Advanced Renal Cell Carcinoma	II	01984242	Active, not recruiting
Study to Evaluate Safety, Pharmacokinetics and Therapeutic Activity of RO6874281 as a Combination Therapy in Participants WithUnresectable Advanced and/or Metastatic Renal Cell Carcinoma (RCC)	I	03063762	Recruiting
Nivolumab vs Nivolumab + Bevacizumab vs Nivolumab + Ipilimumab in Metastatic Renal Cell Carcinoma (mRCC)	I	02210117	Active, not recruiting
Nivolumab (BMS-936558; MDX-1106) in Combination With Sunitinib, Pazopanib, or Ipilimumab in Subjects With Metastatic Renal Cell Carcinoma (RCC) (CheckMate 016)	I	01472081	Active, not recruiting
A BIOmarker Driven Trial With Nivolumab and Ipilimumab or VEGFR tKi in Naïve Metastatic Kidney Cancer	II	02960906	Recruiting
Study in Which Therapy is Either Switched to Nivolumab After 3 Months of Treatment or Therapy is Continued With a Tyrosine Kinase Inhibitor in Patients With MetastaticRenal Cell Carcinoma (RCC) and Disease Control	II	02959554	Recruiting
A Proof of Principle Study of Pembrolizumab With SBRT in TKI mRCC Patients	II	02599779	Recruiting
Safety and Efficacy Study of Pazopanib and MK 3475 in Advanced Renal Cell Carcinoma (RCC; KEYNOTE-018)	I/II	02014636	Active, not recruiting
Study to Evaluate the Efficacy and Safety of Pembrolizumab (MK-3475) in Combination With Axitinib Versus Sunitinib Monotherapy in Participants With Renal Cell Carcinoma (MK-3475-426/KEYNOTE-426)	III	02853331	Active, not recruiting
A Study Of Avelumab In Combination With Axitinib In Advanced Renal Cell Cancer (JAVELIN Renal 100)	I	02493751	Active, not recruiting
A Study of Avelumab With Axitinib Versus Sunitinib In Advanced Renal Cell Cancer (JAVELIN Renal 101)	III	02684006	Active, not recruiting
Neoadjuvant AXITINIB and AVELUMAB for Patients With Localized Clear-cell RCC	II	03341845	Recruiting
Lenvatinib/Everolimus or Lenvatinib/Pembrolizumab Versus Sunitinib Alone as Treatment of Advanced Renal Cell Carcinoma (CLEAR)	III	02811861	Recruiting
CPI-006 Alone and in Combination With CPI-444 and With Pembrolizumab for Patients With Advanced Cancers	I	03454451	Recruiting
Pembrolizumab Combined With Itacitinib (INCB039110) and/or Pembrolizumab Combined With INCB050465 in Advanced Solid Tumors	I	02646748	Recuiting
Pembrolizumab (Anti-PD-1) and AMG386 (Angiopoietin-2 (Ang-2) in Patients With Advanced Solid Tumor	I	03239145	Recruiting
Phase 1b Trial of Lenvatinib Plus Pembrolizumab in Subjects With Selected Solid Tumors	I	03006887	Active, not recruiting
Arginase Inhibitor INCB001158 as a Single Agent and in Combination With Immune Checkpoint Therapy in Patients With Advanced/Metastatic Solid Tumors	I/II	02903914	Recruiting

approach and the new immunotherapy drugs require this information.

In our laboratory we have performed longitudinal studies on pheripheral blood of cancer patients undergoing anti-angiogenetic and/or chemo-immuno therapies. Recently, we reported results on the immunological effect of the TKI pazopanib, a kinase inhibitor targeting mainly the VEGF-R molecule [22].

Preclinical experiments on monocyte derived DCs 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 [23].

A strong DC immunopriming treatment could release from the tolerogenic state DCs in the tumor microenvironment and impact the functionality of effector T cells, presumably tumor specific T cells. We investigate changes occurring in the immune T cell repertoire of mRCC during TKI treatment with the intention to capture changes occurring in the pheripheral blood as a consequence of the treatment. Among the different biomarkers tested, we were able to detect a $CD137^+$ T cell subset in mRCC arising during pazopanib treatment. The impact of these findings needs to be confirmed and validated in an appropriately designed study, which is currently ongoing. However, we can speculate that differences in the T cell $CD137^+$ repertoire in cancer patients could be linked to the rescued potency of DC due to pazopanib treatment and the appearance of exhausted T cell recirculating in the pheripheral blood [24].

If this observation is confirmed in larger studies we could propose to follow with a specific therapy targeting CD137 to expand the anti tumor T cell population that was mobilized by the TKI treatment.

In our study we did not see similar effect on DC with sunitinib, the other TKI similarly used in mRCC with identical clinical efficacy. Two patients treated with Sunitinib however showed upregulation of CTLA4 on pheripheral T cells after one and two months of treatment.

Immunosuppression appears to be downregulated in mRCC patients treated with sunitinib or axitinib, whose Treg and MSDC cell populations are affected. Sorafenib has the opposite effect by reducing antigen-specific T-cell induction *in vitro* [25–30].

An Identical clinical outcome using the two TKI is then justified although results from CheckMate 025 which was not powered to test the difference suggest that Pazopanib immunoactivating treatment in first line in mRCC allows the following ICI treatment to have improved overall survival [31].

The other consideration that can be made is the timing of the immunological effect. We found that immunological changes could be detected quite early in the pheripheral blood of the patients. This suggests that the impact on the immunological repertoire is an early event and this could simplify the immune monitoring analysis since we would be able to have quite quickly a possible marker of overall response. In other words, the rapid changes in the immunological setting of a patient indergoing ICI therapy could anticipate the direction and outcome of the immuno treatment.

Immune monitoring technology is continuously improving, adding novel potential biomarkers that need to be validated in larger studies. It is however quite clear that it will be extremely difficult to rely on single "universal" biomarker and that longitudinal studies and immunomonitoring of each patient can be of valuable help specially in the early discovery of novel immune indicators [32]. The longitudinal evaluation of the immune-tumor status of the patient appears to be particularly significant since tumor undergoes progression and immuno-editing as well as immunosuppression. Therefore a static picture of the tumor and its microenvironment is totally unrepresentative.

Immunotherapy as "first choice": Is it always the best choice? The paradigmatic example of mRCC

Several studies are now being conducted to assess efficacy of combined immuno and target therapies. These will probably result in novel insights that could help to answer several clinical issues (Table 1).

Recently first line treatment in untreated intermediate and poor-risk renal-cell carcinoma patients with Ipilimumab and Nivolumab has shown significantly higher overall survival and objective responses as compared to treatment with the TKI inhibitor Sunitinib [33]. The question here is: can clinical parameters drive the choice of immunotherapy? Or should we consider more the immunological characteristics of tumors and patients?

Interestingly, a better response with ICI treatment was not observed in the favorable-risk patients. This is most probably to be ascribed as discussed in the editorial accompanying the paper by Motzer to the amount of mutational load that could be increased in high risk patients [4].

Furthermore, the results of the IMmotion 151 phase II trial demonstrated an increased PFS in the combination arm Bevacizumab plus Atezolizumab as compared with the monotherapy both with Atezolizumab and Sunitinib [34].

In particular, the PFS of the Bevacizumab plus Atezolizumab arm is 11.2 months (HR was 0.83) the same PFS of the combination arm of Ipilimumab plus Nivolumab (11.6 months and HR 0.82) In spite of the limitation of a comparison between a phase II and phase III trials these data could lead to considering the combination therapy as the best strategy for this category of patients, also with regards to the safety profile.

The real today challenge of oncology is to determine on the one hand what will be the best association (combo-immuno vs immuno plus target therapy) and on the other hand what is the right patient for the right combination. We must take into consideration that the efficacy of immunotherapy depends strictly on the immunological history of tumor/microenvironment interaction and on the immune system of the patients.

Furthermore, these "two sides of the same coin" are extremely dynamic so we should consider not only the right patient but also the right moment for a right therapy.

The only way to understand and define personalized immunotherapy is to explore the role of the tumor microenvironment, the lymphocyte trafficking as well as the different cells involved in immune priming. This needs to be done in pilot studies focalizing on the understanding of the biological mechanisms involved in the response.

New points of view: The challenge of oncologists

As has happened in the past as new emerging treatment options become available the most challenging task in medicine is to combine the new therapies with the standard ones and create, design and adjourn new clinical practice.

The intuition and research contribution on ICI of Jim Allison and his group has changed the life of several patients and has given researchers, immunologists and oncologists important drugs that have the capability to unleash tumor specific T cells [35]. The question is now how to increase the number of responder patients, understand the immunity underlying the non-responder patients, introduce the immunotherapy drug at the right moment in the cancer therapy protocols, and adjust doses and combinations to limit toxicities. The other complication is the number of immunotherapeutic drugs coming onto the market that will need to be validated, compared to other similar drugs, inserted in different protocols and registered for specific tumors. New clinical trials often are launched without really looking at possible combined immunological toxicities or without solid biological knowledge apparently just for the sake of combination, i.e. more is better than one [36]. In fact, in the plethora of recently finished or ongoing clinical trials very few have given significant insights that will change clinical practice.

In renal cancer it is now quite clear that this tumor is very heterogeneous and the heterogeneity has become evident using ICI treatment in the first clinical trials. For this reason, studies on the impact of the different drugs on the immune cells and immune monitoring of patients during treatment can give relevant insights in which setting we can achieve the best response using the right combination [10].

The best example of the critical issues raised by the several immunotherapy targeted therapies waiting for registration, and in the process of proving efficacy in the clinic, is the possibility to have negative results from phase II/III clinical trials, This is primarily because these drugs were combined using an obsolete rationale that was used for chemotherapy and were not optimally designed with an immunologic rationale. The worst implication is that important drugs will never go to the clinic because we simply did not test them in the right setting (i.e. with patients immunologically prepared to receive the drug) [37].

We all agree that we need biomarkers, particularly the predictive one's. Biomarkers of response, of toxicity, of resistance. The literature is overloaded with reports suggesting possible biomarkers. Research in this field must be enforced and algorithms designed. Most probably we will have to work with several biomarkers that can design the immune fitness of the patient rather than a single biomarker. PD-L1 is one of the most discussed biomarkers which was initially proposed and encouraged by pharma for the registrative studies but has failed to show its reliability. PD-L1 should be regarded as a sign of the degree of immunoevasion present in the tumor/immune microenvironment and probably it would be more appropriate to define it as a prognostic biomarker. The story of the PD-L1 biomarker clearly shows what immunologists know very well: the immune system is easily modulated, is characterized by redundancy and by the presence of pleiotropic activity on different cells.

The only way we can successfully proceed in this complicated scenario is to perform studies in strict collaboration with academic institutions, in close interaction with the laboratory, where there are strong scientific interests in defining mechanisms and with minimum conflicts of interests.

We need to carry out immune monitoring guided trials, at least for small sets of homogeneous patients, where both the immunological and the tumor responses as well as toxicities, have to be taken into account for clinical decision making. Methods and technology to assess the immune fitness of cancer patients need to be implemented and validated following the patients' immune responses during the different treatments (Fig. 1) [38–41]. This could allow for a more defined and personalized approach including from an immunological point of view given the possibility of understanding the mechanisms of efficacy or failure of a given treatment.

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Authors contributions

MN wrote the first draft of the manuscript based on data interpretation and discussion with other authors taking opportunity from recent results from our laboratory and recently published in Cancer Immunology Research. All authors contributed equally to literature

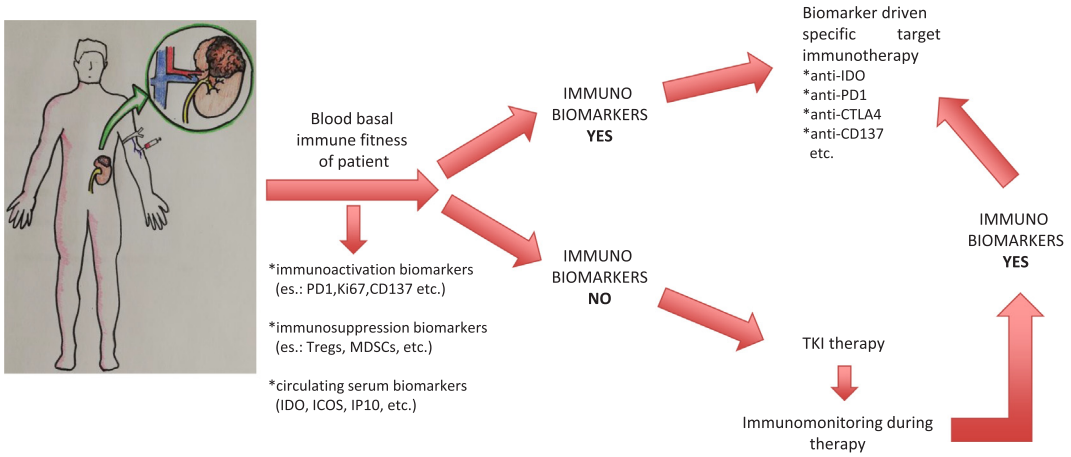

Fig. 1. Model for biomarker/immunomonitoring driven pilot clinical trials: is this the future?

search, revision of drafts and approval of final manuscript.

Conflict of interest

Dr. Marchetti reports personal fees from ROCHE, SANOFI, IPSEN, BMS, NOVARTIS, PFIZER, MSD, AMGEN, INCYTE, ASTRAZENECA, BOERINGHER outside the submitted work.

Dr. Nuti reports personal fees from Novartis, outside the submitted work; Dr. Botticelli reports personal fees from Roche and Ipsen outside the submitted work.

Dr. Rughetti and Dr. Zizzari have nothing to disclose.

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