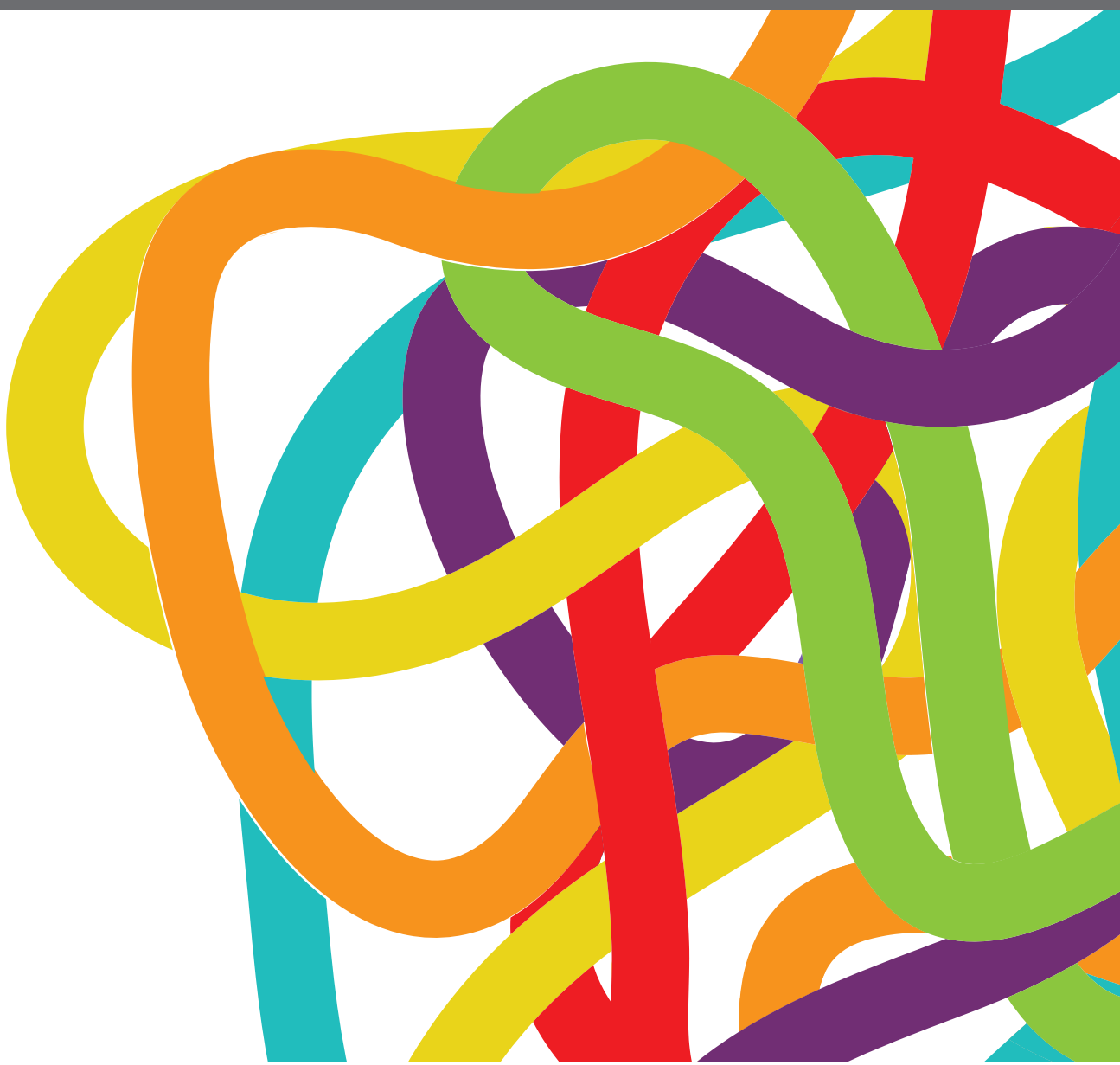
WOMEN IN GENITOURINARY ONCOLOGY: 2021

EDITED BY: Sanja Štifter and Alessia Cimadamore
PUBLISHED IN: Frontiers in Oncology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-83250-482-6
DOI 10.3389/978-2-83250-482-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

WOMEN IN GENITOURINARY ONCOLOGY: 2021

Topic Editors:
Sanja Štifter, Skejby Sygehus, Denmark
Alessia Cimadamore, Marche Polytechnic University, Italy

Citation: Štifter, S., Cimadamore, A., eds. (2022). Women in Genitourinary Oncology: 2021. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-482-6

Table of Contents

frontiers | Frontiers in Oncology

TYPE Editorial
PUBLISHED 28 September 2022
DOI 10.3389/fonc.2022.1027852

OPEN ACCESS

EDITED AND REVIEWED BY
Ronald M Bukowski,
Cleveland Clinic, United States

*CORRESPONDENCE
Sanja Štifter
stifter.sanja@gmail.com

SPECIALTY SECTION
This article was submitted to Genitourinary Oncology, a section of the journal Frontiers in Oncology

RECEIVED 25 August 2022
ACCEPTED 29 August 2022
PUBLISHED 28 September 2022

CITATION
Štifter S (2022) Editorial: Women in genitourinary oncology: 2021. *Front. Oncol.* 12:1027852. doi: 10.3389/fonc.2022.1027852

Editorial: Women in genitourinary oncology: 2021

Sanja Štifter*

Department of Pathology, Aarhus University Hospital, Aarhus, Denmark

KEYWORDS

women, genitourinary oncology, prostate carcinoma, renal cell carcinoma, penis carcinoma

Editorial on the Research Topic
Women in genitourinary oncology: 2021

Recently I had the privilege to edit a Frontiers Topic dedicated to women in genitourinary oncology research and practice. Exquisite women researchers helped us promote topics from genitourinary oncology together with their peers. The aim was to gather a collection of state-of-the-art research articles showing that women in research have a valuable position not only as a part of a team but also as leading researchers or group leaders. I can confidently say that this particular topic attracted a very interesting group of articles covering a broad spectrum of the genitourinary oncology field (Figure 1).

As it was stated in the topic introduction, at present, fewer than 30% of researchers worldwide are women. Therefore, this topic is even more relevant in strengthening efforts to overcome gender stereotypes discouraging girls and women away from science-related fields, and STEM research in particular.

Editing this research Topic was for me a very positive experience concluded with a collection of articles mostly covering prostate cancer oncology research and clinical practice. Different prostate cancer therapeutic strategies were well presented in several articles by groups of authors including Raju et al. and Maggi et al. Raju et al concluded by presenting a real-world data regional study that showed both abiraterone and enzalutamide will remain standard-of-care treatments in Australian men with metastatic castration-resistant prostate cancer (mCRPC), as the survival and disease control benefits of these agents have continued to be seen in numerous real world studies, consistent with the phase III clinical trials.

New emerging therapeutic targets with preliminary results were presented in articles by Marvaso et al. and Masson et al. It was very interesting to observe a substantially high interest in the correlation between psychosocial stress and age-influenced depression and anxiety-related behavior driving tumor inflammatory cytokines and accelerating prostate cancer growth in mice by Bellinger et al. A clinical prospective study presented by Logozzi et al., which showed that plasmatic exosome number and size distinguish prostate cancer patients from healthy individuals, gained great attention from audiences.

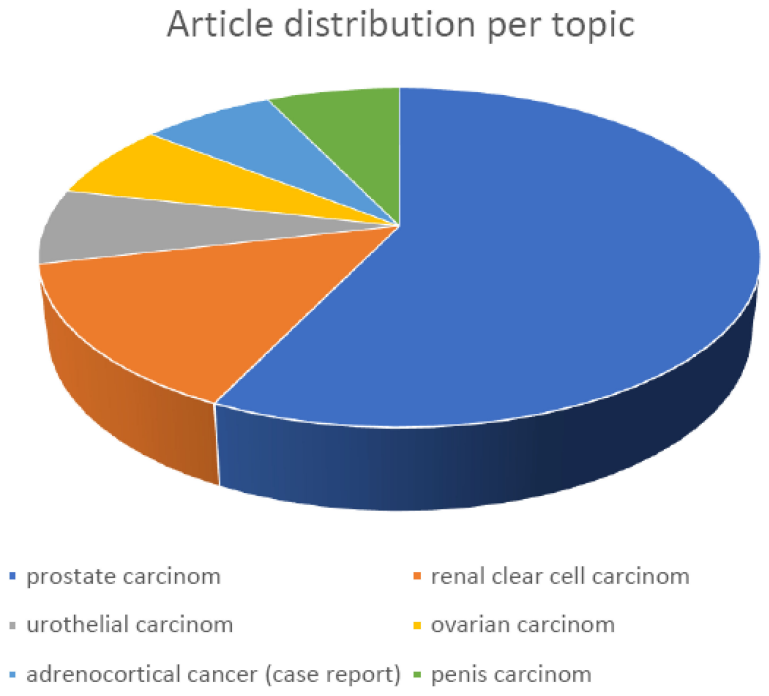


FIGURE 1
Diagram represents article distribution per topic showing the most frequent articles discussing prostate and renal cell clear cell carcinoma.

A systematic review and meta-analysis of combined therapy for renal cell carcinoma, presented by a group of authors led by Tao et al. gave an interesting perspective on balancing the risk-benefit ratio. In volume two, a mini-review on targeting strategies in the treatment of fumarate hydratase deficient renal cell carcinoma provides an authentic review of therapeutic approaches presented by a group of researchers led by Renate Pichler.

Results of a multicenter prospective study presented quality-of-life outcomes in female patients with ileal conduit or orthotopic neobladder urinary diversion by Siracusano et al.

Another interesting piece of clinical research was presented by Xiong et al., where a population-based study analyzed the prevalence and outcomes of unilateral versus bilateral oophorectomy in women with ovarian cancer.

It is not often that a Research Topic accepts a case report, but in this collection, we found one case report of interest by Tőke et al. describing complete remission of advanced adrenocortical cancer following mitotane monotherapy which, besides presenting a case report, gives a thorough literature review of predictive markers.

Finally, a very interesting original research article elucidating the patterns of treatment and outcomes in older men with penile cancer, A SEER Dataset Analysis, is part of volume two presented by Maria T. Bourlon et al.

Instead of a conclusion, I would like to point out that such platforms promoting the work of women scientists, across all fields of Oncology, are beneficial in giving more visibility to women researchers. This can be inspiring for young girls and women and, at the same time, ensures sustainable development of science too. Gender equality must be promoted as well as gender stereotypes defeated, and girls and women should be encouraged to pursue STEM careers.

I would like to dedicate this editorial to my recently departed colleague, Professor Ondrej Hess, who was an outstanding researcher, teacher, pathology expert, and nature supporter and also a strong advocate of gender equality in science and research.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

frontiers in Oncology

CASE REPORT
published: 11 May 2021
doi: 10.3389/fonc.2021.680853

Complete Remission of Advanced Adrenocortical Cancer Following Mitotane Monotherapy: A Case Report and Literature Review of Predictive Markers

Judit Tőke[1], *Zsuzsanna Jakab*[1], *Júlia Stark*[1], *Gergely Huszty*[2], *Péter Reismann*[1] *and Miklós Tóth*[1]*

[1] Department of Internal Medicine and Oncology, ENDO-ERN HCP, Semmelweis University, Budapest, Hungary,
[2] Department for Transplantation and Surgery, Semmelweis University, Budapest, Hungary

Mitotane has been used for the treatment of adrenocortical cancer (ACC) for over 50 years. Despite its widespread use both in monotherapy and in combination with chemotherapeutics, our knowledge of its mechanism of action and therapeutic efficacy is scarce. The number of patients with advanced ACC who have achieved complete remission documented by detailed clinical data is below ten. We report a case of a 64-year-old woman with a non-functional ACC. Histological examination showed vascular invasion, Ki67 of 10% and a mitotic count of 3/10 high-power field. Immunohistochemistry revealed p53 positivity. Pathological TNM grade was reported as T2N0M0, ENSAT stage 2. Nine months after the initial diagnosis, re-staging CT revealed multiple peritoneal nodules, lymph node and kidney metastases confirmed by histologic examination. Mitotane monotherapy was started with a maintenance dose between 2.0 and 2.5 grams/day. Partial remission was established at six months. Subsequently, for another 12 months, each of the three-monthly CT scans confirmed complete remission. Nineteen months after the initiation of mitotane, an unexpected sudden death occurred. A detailed autopsy work-up, performed in the full awareness of oncological history, confirmed complete remission. The authors review the molecular biomarkers and clinical features reported as predictors of response to mitotane monotherapy.

Keywords: adrenocortical cancer, complete remission, predictive markers, mitotane, monotherapy

OPEN ACCESS

Edited by:
Fabio Calabro',
San Camillo-Forlanini Hospital, Italy

Reviewed by:
Alfredo Berruti,
University of Brescia, Italy
Linda Cerbone,
San Camillo-Forlanini Hospital, Italy

***Correspondence:**
Miklós Tóth
toth.miklos@med.semmelweis-univ.hu

Specialty section:
This article was submitted to Genitourinary Oncology, a section of the journal Frontiers in Oncology

Received: *15 March 2021*
Accepted: *23 April 2021*
Published: *11 May 2021*

Citation:
Tőke J, Jakab Z, Stark J, Huszty G, Reismann P and Tóth M (2021) Complete Remission of Advanced Adrenocortical Cancer Following Mitotane Monotherapy: A Case Report and Literature Review of Predictive Markers. Front. Oncol. 11:680853. doi: 10.3389/fonc.2021.680853

INTRODUCTION

Nowadays, mitotane is the only compound registered to treat adrenocortical cancer (1). Despite its use of over 50 years, the exact mechanism of action is mostly unknown (2). Mitotane is most frequently used in combination with chemotherapeutics. For recurrent or advanced adrenocortical cancer, etoposide-doxorubicin-cisplatin therapy combined with mitotane (EDP-M) is

Abbreviations: ACC, adrenocortical carcinoma; ACTH, adrenocorticotropic hormone; CT, computed tomography; FDG-PET-CT, fluoro-deoxyglucose positron emission tomography; ENDO-ERN HCP, European Reference Network on Rare Endocrine Conditions, Health Care Provider.

recommended as the first-line treatment of choice. In second-line settings, gemcitabine plus capecitabine or streptozocin could be possible options with continued mitotane treatment (3). In patients with low tumour load or poor performance status, mitotane can be initiated as monotherapy (4). Although mitotane is not registered for adjuvant purposes, it is used with increasing frequency in the adjuvant setting (5–7).

The efficacy of EDP-M is limited in general; however, favourable response is obtained in a few cases. Terzolo et al. reported their single-centre experiences with 180 metastatic ACC patients treated with EDP-M therapy over 20 years. Four patients (2.2% of all) exhibited progression-free survival for five years. Two patients showed complete remission after EDP-M chemotherapy (8). In another single-institution series, surgery identified complete pathological response in 4 (7%) out of 58 consecutive metastatic ACC patients following EDP-M. None of them had recurred at the last follow-up (9).

Despite its widespread use in the therapy of ACC, we have limited knowledge about its efficacy as monotherapy. The most extensive study to date reported experiences with 127 patients from a German multicentre study (10). In this retrospective analysis, 26 patients (20,5%) exhibited objective response; three of them had a complete response (10). Another study from the Memorial Sloan-Kettering Cancer Center from the period between 1989 and 2015 showed that only 4 out of the 36 patients (11%) had an objective response to mitotane monotherapy; however, 3 of them showed complete response (11).

Concerning complete remission achieved by mitotane monotherapy in patients with advanced-stage ACC, there is a remarkable paucity of reported patients. El Ghorayeb et al. summarized all the nine adult patients with advanced ACC having achieved complete remission with mitotane monotherapy, published between 1974 and 2014 (12). Since this publication, we are aware of six other cases reported without further details in the two aforementioned retrospective clinical studies (10, 11). Besides reporting a new patient, we review the molecular and clinical predictors of response to mitotane monotherapy in advanced ACC patients.

CASE DESCRIPTION

A 64-year-old woman was referred to our adrenocortical cancer referral centre following left adrenalectomy. The adrenal tumour, 9.5 cm in its largest diameter, was detected incidentally during investigations for an unexplained rash (**Figure 1**). Preoperative hormonal measurements including plasma cortisol, aldosterone, ACTH, and plasma renin activity resulted in the diagnosis of a non-functional adrenal tumour. Chest and abdominal computed tomography revealed discrete peritumoral adipose tissue infiltration without pathological lymph nodes or distant metastases. The native density of the tumour on CT was +40 Hounsfield unit. Histological examination showed vascular invasion, Ki67 of 10% and a mitotic count of 3/10 high-power field. Immunohistochemistry revealed p53 positivity. Pathological TNM grade was reported as T2N0M0, ENSAT stage 2.

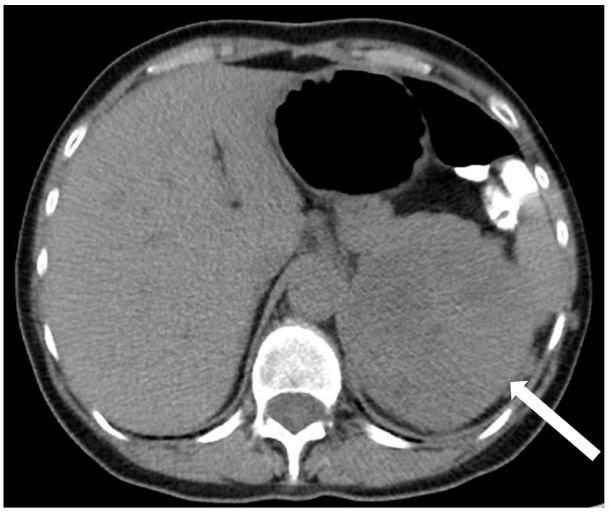


FIGURE 1 | Left adrenal tumour, 9.5 cm in its largest diameter. Unenhanced computed tomographic scan. Native density 40 Hounsfield unit.

2,5 months following adrenalectomy, the tumour bed was irradiated using a Varian Clinac iX 6 MV photon beam instrument with a total dose of 50.5 Gy. The postoperative 3- and 6-month re-staging CT did not show any tumour lesion. Nine months after the initial diagnosis, abdominal ultrasonography and re-staging CT revealed multiple peritoneal nodules raising the suspicion of peritoneal carcinomatosis, a periventricular lymph node, and a left kidney metastasis (**Figures 2** and **3**). The maximal tumoral diameter was 12 mm. Laparoscopic tissue sampling from the ascending colon and sigmoid intestine resulted in the histological diagnosis of peritoneal carcinomatosis from ACC. Therefore, twelve months following adrenalectomy, mitotane monotherapy was started according to a high-dose regimen (13).

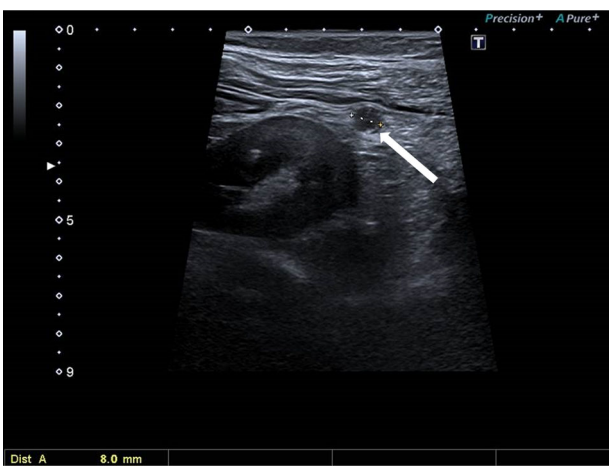


FIGURE 2 | 8 mm metastasis within the left perirenal fat. Ultrasonographic image.

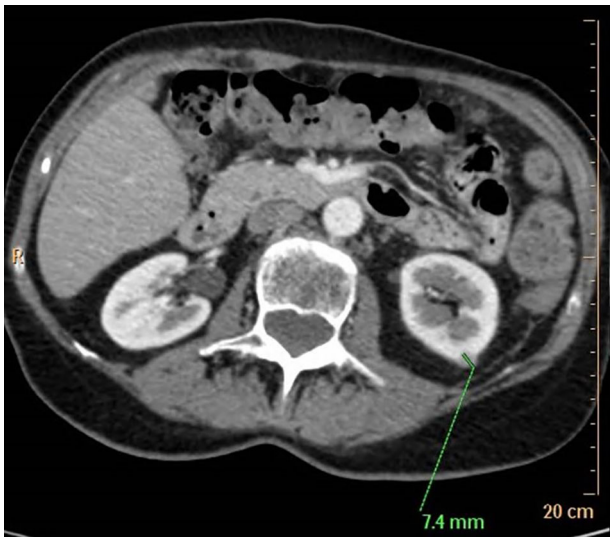


FIGURE 3 | 7.4 mm metastasis in the left renal cortex. Contrast-enhanced computed tomographic scan.

Mitotane plasma levels were monitored by the Lysosafe service (www.lysosafe.com). Therapeutic mitotane concentration was achieved within two months. Mitotane, with a maintenance dose between 2.0 and 2.5 grams/day, was well tolerated during the whole course of its administration. The patient was substituted with hydrocortisone, 30 mg/day. She was educated for signs and symptoms of adrenal failure and supplied with an emergency card and parenteral hydrocortisone KIT.

Re-staging CT performed three months following the initiation of mitotane therapy revealed stable disease, while partial remission was reported at six months. After that, each three-monthly CT confirmed complete remission.

Two and a half years following the adrenalectomy and nineteen months after the initiation of mitotane, the lone-living patient was found dead in her flat. A detailed pathological work-up, performed in the full awareness of oncological history, confirmed complete remission. Special attention was paid to the peritoneal surfaces and the liver, which was sliced into 5-mm slices. Lesions suspected of metastatic disease were not found anywhere. The autopsy could not give a definite answer regarding the cause of death. Because of the influenza epidemic at that time, a viral infection could be suspected as a cause of death. Other alternatives as possible explanations for the unexpected sudden death include adrenal insufficiency and long Q-T syndrome caused by mitotane therapy.

According to our in-house ACC registry, out of 48 patients with advanced ACC, 15 patients were initially treated with mitotane monotherapy. The patient reported here is the only one who achieved complete remission on mitotane monotherapy.

DISCUSSION

Mitotane causes selective damage to adrenocortical cells, acting primarily by the disruption of mitochondria and activating apoptosis (14). Regarding the potential molecular biomarkers of the efficacy of mitotane therapy, data are scarce. Maintaining serum mitotane levels in the target range of 14 to 20 mg/L is a strong predictor of effectiveness (15). However, we have several hints that objective response to mitotane can be achieved with lower plasma mitotane concentrations (10, 11). The human cytochrome P450 2W1 enzyme (CYP2W1) was suggested to be involved in the metabolism of mitotane. CYP2W1 immunoreactivity was associated with longer overall survival and time to progression in ACC patients treated with mitotane monotherapy (16). A recent multicentre study suggested that a specific combination of single nucleotide polymorphisms of two mitochondrial enzymes (CYP2W1 and CYP2B6) may predict therapeutic response to mitotane monotherapy (17). Next-generation sequencing proved unsuccessful in predicting response to mitotane monotherapy in two out of the three complete responders to mitotane (11). Mitotane is an inhibitor of sterol-O-acyl transferase 1 (SOAT1), which was postulated to be a key molecular target of mitotane. However, SOAT1 expression has not been correlated with clinical endpoints in a study with ACC patients on mitotane monotherapy (18). Despite numerous efforts, none of the studies established clinically useful biomarkers having the potential for predicting response to mitotane therapy. The results of these studies are summarized in **Table 1**.

Similarly to potential biochemical markers, to date, we do not have firm clinical markers predicting response to mitotane (12). Two extensive retrospective studies were published with mitotane monotherapy in their focus (10, 11), and only one paper summarized individual case reports to date (12). **Table 2** updates the main clinicopathological features of each patient reported with

TABLE 1 | Molecular markers tested so far for a response to mitotane monotherapy in patients with advanced ACC.

Presumed biomarker	No of the tested patients	Result	Reference
DNA repair enzymes RRM1 and ERCC1	92	RRM1 expression was associated with DFS and OS	Volante et al. (19)
Germline DNA	2	NGS was negative in complete responders	Reidy-Lagunes et al. (11)
CYP2W1 expression	25	CYP2W1 predicts response to mitotane	Ronchi et al. (16)
SNPs (CYP2W1*2, CYP2W1*6, CYP2B6*6 and CYP2B6 rs4803419)	182	CYP2W1*6 and CYP2B6*6 may predict individual response	Altieri et al. (17)
Sterol-O-acyl transferase 1 (SOAT1)	231	no correlation with clinical endpoints	Weigand et al. (18)

DFS, disease-free survival, ERCC1: excision repair cross-complementation group 1; NGS, next-generation sequencing; OS, overall survival; RRM1, Ribonucleotide reductase large subunit 1; SNP, single nucleotide polymorphism.

TABLE 2 | Clinicopathological features of adult patients with metastatic adrenocortical cancer reported as complete responders to mitotane monotherapy.

Reference	No of patients reported with CR to mitotane	Timing of mitotane initiation	Tumour burden at mitotane initiation	Hormonal excess
Megerle et al. (10)	3	≥360 days since initial diagnosis (3/3)	< 10 tumor lesion (2/3) ≥ 10 tumor lesion (1/3)	Cortisol (1/3)
Reidy-Lagunes et al. (11)	3	ND	Tumors in one site with low (< 3 cm) tumor volume (3/3)	Nonfunctional (3/3)
El Ghorayeb et al. (12)	9	At initial diagnosis (6/9) 2 years since initial diagnosis (1/9) ND (2/9)	ND	Nonfunctional (3/9) Cortisol (1/9) Androgens (3/9) Cortisol and androgens (2/9)
Our patient, 2021	1	1 year since initial diagnosis (1/1)	< 10 tumor lesion, low tumor burden (maximum tumor diameter 12 mm)	Non-functional (1/1)

ND, no data.

TABLE 3 | Clinical parameters suggested predicting favourable response to mitotane monotherapy in patients with advanced ACC.

	Reidy-Lagunes et al., 2017 (11)	Megerle et al., 2018 (10)
Endocrine activity	non-functional tumours probably respond better	not a predictive factor
Performance status	Patients with ECOG 0-1 probably respond better	not investigated
Tumour burden	disease limited to one site low volume disease (< 3 cm)	< 10 tumoral lesions
Timing of recurrence	not investigated	delayed (>360 days) advanced recurrence

complete response to mitotane monotherapy. In these publications, a common clinical parameter predicting response to mitotane was the tumour burden itself, expressed either as the number of sites involved or tumoral diameter or number of tumoral lesions. Late versus early recurrence of advanced disease (cut-off at 360 days) proved to be another highly significant clinical parameter (10). According to these clinical reports, hormonal activity of the primary tumour is not an important feature influencing response to mitotane as complete remission could be achieved both in functioning and in non-functioning adrenocortical cancers. The metabolic response to mitotane on FDG-PET scan was suggested to be a potential radiologic predictor of response to mitotane monotherapy (12).

Concerning the suggested clinical predictors of a favourable response to mitotane, our patient fulfilled three out of the four parameters listed in **Table 3**. She was in good condition at the tumour's recurrence; she had a non-functional tumour and had a low tumour burden with less than ten lesions at recurrence. Only one criterion was not fulfilled; namely, her tumour relapsed after 270 days.

Our case presentation has limitations. First, the cure of peritoneal carcinomatosis was initially diagnosed and followed with computed tomography scans which could not imply a complete pathological response in vivo. Nevertheless, the autopsy findings, including the inspection of the peritoneal surfaces, should be considered as strong indicators of complete remission. In addition, the follow up of this patient was short (19 months) due to the occurrence of the sudden death.

The optimal length of mitotane monotherapy following achievement of complete response is unknown. Within El Ghorayeb's compilation of patients with complete remission, disease-free survivals were between 4 and 25 years. Regarding the length of mitotane therapy following CR, we do not have any guidance. In some patients, mitotane was administered lifelong; however, long-lasting disease-free intervals were reported in patients with discontinued mitotane therapy.

CONCLUDING REMARKS

The few reported cases of patients with advanced ACC achieving complete remissions on mitotane monotherapy are strong pieces of evidence for the therapeutic efficacy of mitotane. Molecular biomarkers predicting the success of mitotane are desperately needed.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JT and MT wrote the first draft of the manuscript. JT, ZJ, PR, and MT participated in the diagnosis and treatment of the patient, and providing follow-up. GH, PR, and JS acquired clinical data. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Paragliola RM, Corsello A, Locantore P, Papi G, Pontecorvi A, Corsello SM. Medical Approaches in Adrenocortical Carcinoma. *Biomedicines* (2020) 8 (12). doi: 10.3390/biomedicines8120551
2. Fassnacht M, Kroiss M, Allolio B. Update in Adrenocortical Carcinoma. *J Clin Endocrinol Metab* (2013) 98(12):4551–64. doi: 10.1210/jc.2013-3020
3. Fassnacht M, Assie G, Baudin E, Eisenhofer G, de la Fouchardiere C, Haak HR, et al. Adrenocortical Carcinomas and Malignant Phaeochromocytomas: ESMO-EURACAN Clinical Practice Guidelines for Diagnosis, Treatment and Follow-Up. *Ann Oncol* (2020) 31(11):1476–90. doi: 10.1016/j.annonc.2020.08.2099
4. Else T, Kim AC, Sabolch A, Raymond VM, Kandathil A, Caoili EM, et al. Adrenocortical Carcinoma. *Endocr Rev* (2014) 35(2):282–326. doi: 10.1210/er.2013-1029
5. Puglisi S, Calabrese A, Basile V, Pia A, Reimondo G, Perotti P, et al. New Perspectives for Mitotane Treatment of Adrenocortical Carcinoma. *Best Pract Res Clin Endocrinol Metab* (2020) 34(3):101415. doi: 10.1016/j.beem.2020.101415
6. Terzolo M, Angeli A, Fassnacht M, Daffara F, Tauchmanova L, Conton PA, et al. Adjuvant Mitotane Treatment for Adrenocortical Carcinoma. *N Engl J Med* (2007) 356(23):2372–80. doi: 10.1056/NEJMoa063360
7. Terzolo M, Baudin AE, Ardito A, Kroiss M, Leboulleux S, Daffara F, et al. Mitotane Levels Predict the Outcome of Patients With Adrenocortical Carcinoma Treated Adjuvantly Following Radical Resection. *Eur J Endocrinol* (2013) 169(3):263–70. doi: 10.1530/eje-13-0242
8. Terzolo M, Daffara F, Ardito A, Zaggia B, Basile V, Ferrari L, et al. Management of Adrenal Cancer: A 2013 Update. *J Endocrinol Invest* (2014) 37(3):207–17. doi: 10.1007/s40618-013-0049-2
9. Laganà M, Grisanti S, Cosentini D, Ferrari VD, Lazzari B, Ambrosini R, et al. Efficacy of the EDP-M Scheme Plus Adjunctive Surgery in the Management of Patients With Advanced Adrenocortical Carcinoma: The Brescia Experience. *Cancers (Basel)* (2020) 12(4). doi: 10.3390/cancers12040941
10. Megerle F, Herrmann W, Schloetelburg W, Ronchi CL, Pulzer A, Quinkler M, et al. Mitotane Monotherapy in Patients With Advanced Adrenocortical Carcinoma. *J Clin Endocrinol Metab* (2018) 103(4):1686–95. doi: 10.1210/jc.2017-02591
11. Reidy-Lagunes DL, Lung B, Untch BR, Raj N, Hrabovsky A, Kelly C, et al. Complete Responses to Mitotane in Metastatic Adrenocortical Carcinoma-a New Look At an Old Drug. *Oncologist* (2017) 22(9):1102–6. doi: 10.1634/theoncologist.2016-0459
12. El Ghorayeb N, Rondeau G, Latour M, Cohade C, Olney H, Lacroix A, et al. Rapid and Complete Remission of Metastatic Adrenocortical Carcinoma Persisting 10 Years After Treatment With Mitotane Monotherapy: Case Report and Review of the Literature. *Med (Baltimore)* (2016) 95(13):e3180. doi: 10.1097/md.0000000000003180
13. Fassnacht M, Dekkers OM, Else T, Baudin E, Berruti A, de Krijger R, et al. European Society of Endocrinology Clinical Practice Guidelines on the Management of Adrenocortical Carcinoma in Adults, in Collaboration With the European Network for the Study of Adrenal Tumors. *Eur J Endocrinol* (2018) 179(4):G1–g46. doi: 10.1530/eje-18-0608
14. Poli G, Guasti D, Rapizzi E, Fucci R, Canu L, Bandini A, et al. Morphofunctional Effects of Mitotane on Mitochondria in Human Adrenocortical Cancer Cells. *Endocr Relat Cancer* (2013) 20(4):537–50. doi: 10.1530/erc-13-0150
15. Hermsen IG, Fassnacht M, Terzolo M, Houterman S, den Hartigh J, Leboulleux S, et al. Plasma Concentrations of O,P'ddd, O,P'dda, and O, P'dde as Predictors of Tumor Response to Mitotane in Adrenocortical Carcinoma: Results of a Retrospective ENS@T Multicenter Study. *J Clin Endocrinol Metab* (2011) 96(6):1844–51. doi: 10.1210/jc.2010-2676
16. Ronchi CL, Sbiera S, Volante M, Steinhauer S, Scott-Wild V, Altieri B, et al. CYP2W1 is Highly Expressed in Adrenal Glands and is Positively Associated With the Response to Mitotane in Adrenocortical Carcinoma. *PloS One* (2014) 9(8):e105855. doi: 10.1371/journal.pone.0105855
17. Altieri B, Sbiera S, Herterich S, De Francia S, Della Casa S, Calabrese A, et al. Effects of Germline CYP2W1*6 and CYP2B6*6 Single Nucleotide Polymorphisms on Mitotane Treatment in Adrenocortical Carcinoma: A Multicenter ENSAT Study. *Cancers (Basel)* (2020) 12(2). doi: 10.3390/cancers12020359
18. Weigand I, Altieri B, Lacombe AMF, Basile V, Kircher S, Landwehr LS, et al. Expression of SOAT1 in Adrenocortical Carcinoma and Response to Mitotane Monotherapy: An ENSAT Multicenter Study. *J Clin Endocrinol Metab* (2020) 105(8):2642–53. doi: 10.1210/clinem/dgaa293
19. Volante M, Terzolo M, Fassnacht M, Rapa I, Germano A, Sbiera S, et al. Ribonucleotide Reductase Large Subunit (RRM1) Gene Expression May Predict Efficacy of Adjuvant Mitotane in Adrenocortical Cancer. *Clin Cancer Res* (2012) 18(12):3452–61. doi: 10.1158/1078-0432.ccr-11-2692

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

SYSTEMATIC REVIEW
published: 08 June 2021
doi: 10.3389/fonc.2021.700258

A Systematic Review and Meta-Analysis of Randomized Controlled Trials With Novel Hormonal Therapies for Non-Metastatic Castration-Resistant Prostate Cancer: An Update From Mature Overall Survival Data

Martina Maggi[1], Stefano Salciccia[1], Francesco Del Giudice[1], Gian Maria Busetto[2], Ugo G. Falagario[2], Giuseppe Carrieri[2], Matteo Ferro[3], Angelo Porreca[4], Giovanni Battista Di Pierro[1], Vittorio Fasulo[5], Viviana Frantellizzi[6], Giuseppe De Vincentis[6], Ettore De Berardinis[1] and Alessandro Sciarra[1]*

[1] Department of Maternal-Infant and Urological Sciences, Sapienza Rome University, Policlinico Umberto I, Rome, Italy, [2] Department of Urology and Renal Transplantation, University of Foggia, Policlinico Riuniti, Foggia, Italy, [3] Department of Urology, European Institute of Oncology (IEO), Milan, Italy, [4] Department of Urology, Veneto Institute of Oncology (IOV) IRCCS, Padua, Italy, [5] Department of Urology, Istituto Clinico Humanitas IRCCS-Clinical and Research Hospital, Milan, Rozzano, Italy, [6] Department Of Maternal-Infant And Urological Sciences, Sapienza Rome University, Policlinico Umberto I, Rome, Italy

OPEN ACCESS

Edited by:
Andrea Mari,
Careggi University Hospital, Italy

Reviewed by:
Antonio Andrea Grosso,
Careggi University Hospital, Italy
Luca Lambertini,
Careggi University Hospital, Italy

***Correspondence:**
Gian Maria Busetto
gianmaria.busetto@unifg.it

Specialty section:
This article was submitted to Genitourinary Oncology, a section of the journal Frontiers in Oncology

Received: 25 April 2021
Accepted: 14 May 2021
Published: 08 June 2021

Citation:
Maggi M, Salciccia S, Del Giudice F, Busetto GM, Falagario UG, Carrieri G, Ferro M, Porreca A, Di Pierro GB, Fasulo V, Frantellizzi V, De Vincentis G, De Berardinis E and Sciarra A (2021) A Systematic Review and Meta-Analysis of Randomized Controlled Trials With Novel Hormonal Therapies for Non-Metastatic Castration-Resistant Prostate Cancer: An Update From Mature Overall Survival Data. Front. Oncol. 11:700258. doi: 10.3389/fonc.2021.700258

Introduction: To get better insight into the management of non-metastatic castration-resistant prostate cancer (M0 CRPC), in this meta-analysis and review we aimed to present an updated evaluation of the efficacy and safety of novel hormonal therapies (nHT) for M0 CRPC according to final analyses with mature overall survival (OS) and safety data.

Methods: We analyzed metastasis-free survival (MFS), OS, time to prostate-specific antigen (PSA) progression, second-line therapies data, adverse events (AEs), including all AEs, serious AEs (SAEs), AEs leading to discontinuation of trial regimen, AEs leading to death, fatigue, dizziness, cardiovascular events, and fractures; moreover, we evaluated the impact of PSA doubling time (PSA-DT), Eastern Cooperative Oncology Group (ECOG) score, use of bone-targeted therapy, lymph lodes (LN) status, and prior HT on final OS data. A comparison among the placebo arms of the included trials in terms of survival and safety profiles was assessed.

Results: According to the pooled analysis with updated and mature OS data, OS was significantly improved with nHT compared to placebo (hazard ratio (HR)= 0.74, 95% confidence interval (CI)= 0.66–0.84). nHT significantly improved OS over placebo across all pre-specified subgroups. Subgroup analysis revealed a greater OS benefit in patients with PSA-DT >6 months than ≤6 months (HR= 0.69 versus HR= 0.75), ECOG 0 than 1

(HR= 0.70 versus HR= 0.80), N1 disease than N0 (HR= 0.61 versus HR= 0.78), and in those receiving bone-targeted therapy (HR= 0.65 versus HR= 0.74), and a comparable OS by number of prior HT (HR= 0.75 versus HR= 0.76, for HT= 1 and ≥2); yet, differences between pre-specified subgroups were not significant (all p> 0.05). Overall, the nHT arm was significantly associated with higher rates of AEs, when compared with the placebo arm. The long-term analysis showed a worse safety profile with nHT than the interim analysis.

Conclusions: According to final analyses, nHT have shown to improve OS over placebo in the setting of high-risk M0 CRPC. The long-term analysis showed a worse safety profile with nHT than the interim analysis, whit distinct profiles among different nHT. The lack of survival data regarding second-line therapies remains a major issue.

Keywords: prostate neoplasm, non-metastatic castration-resistant prostate cancer, hormonal therapy, overall survival, adverse events, metastasis

INTRODUCTION

Androgen deprivation therapy (ADT) is the basis of the medical treatment for advanced prostate cancer (PC), and for those men with early-stage PC who experience biochemical progression with a rising prostate-specific antigen (PSA) level after curative treatment (1–4). ADT can be achieved with either surgery (i.e. bilateral orchiectomy) or various agents (i.e. gonadotropin-releasing hormone (GnRH) agonists, GnRH antagonists and anti-androgens), and despite it is initially effective, eventually the majority of cases will experience progression to a castration-resistant PC (CRPC) (5). According to the European Association of Urology (EAU) guidelines, CRPC can be defined as castrate serum testosterone (<50 ng/dL or 1.7 nmol/L) plus either biochemical or radiological progression (6). The status with a progressive rising PSA levels, in a low testosterone environment, and in the absence of detectable metastasis on conventional imaging, is known as non-metastatic CRPC (M0 CRPC), whose prevalence has been estimated to about 10% of PC in Europe (7, 8). It has also been observed that metastasis-free survival (MFS) in this setting is 25 to 30 months, and that about one-third will develop visible bone metastases within 2 years (9).

Since metastatic CRPC (mCRPC) is fatal, with a median survival of approximately 3 years, currently prolonging as long as possible the M0 status by delaying the onset of metastasis and the need of subsequent treatments -with the related side effects- is a major treatment goal in M0 CRPC. Until recently, no approved systemic therapies existed for these cases, and observation in the context of on-going ADT was the standard of care. This scenario changed in 2018, with the sequential approvals of novel hormonal therapies (nHT) (i.e. Enzalutamide, Apalutamide, and Darolutamide), after 3 phase III randomized controlled trials (RCTs) were carried out comparing these drugs to placebo (i.e. treatment with the sole on-going ADT) in high-risk M0 CRPC cases (i.e. M0 CRPC cases with a PSA doubling time (PSA-DT) of ≤ 10 months) (10–12). Although all 3 trials met their primary endpoint (i.e. MFS), skepticism was raised regarding MFS as a clinically relevant endpoint, and whether it would reflect an improved OS. Indeed, at the primary analyses, none of the studies showed an OS benefit due to immature data (6). Updated data regarding OS were presented at the 2020 American Society of Clinical Oncology (ASCO) Annual Meeting, and then recently published, showing clearer results (13–15). Moreover, since different drugs appeared to be comparable in terms of oncological profile, and in view of the long-term treatment with these agents in asymptomatic patients, the therapeutic choice should be based on safety profile. To date, no direct comparison among these compounds has been made.

To get better insight into the management of these cases, and to further guide the future choice among these novel compounds, in this meta-analysis and review we aimed to present an updated evaluation of the efficacy and safety of nHT for M0 CRPC cases according to last publications with mature OS and safety data; moreover, we sought to assess whether there existed differences in the placebo arms of the evaluated studies in terms of oncological outcomes and safety profiles, which could have influenced comparative results among nHT trials.

METHODS

Objective

The primary aim of the present meta-analysis is to systematically analyze the current evidence on nHT for M0 CRPC cases.

In particular, in populations of M0 CRPC cases, we analyzed: MFS, OS, time to PSA progression, second-line therapies data, adverse events (AEs) (overall AEs and grade 3-4 AEs) including all AEs, serious AEs (SAEs), AEs leading to discontinuation of trial regimen, AEs leading to death, fatigue, dizziness, cardiovascular events, and fractures; moreover we evaluated the impact of PSA-DT (defined as the time required for the PSA level to double; ≤ versus >6 months), performance status (PS) (Eastern Cooperative Oncology Group (ECOG) score 0 versus 1), the use of bone-targeted therapy (yes versus no), lymph lodes (LN) status (N0 versus N1), and prior HT (1

versus ≥2) on updated OS data. A comparison among the placebo arms of the included trials in terms of survival and safety profiles was assessed.

Search Strategy

We searched in the Medline and Cochrane Library database and the American Society of Clinical Oncology (ASCO) Meeting (search terms: "prostate neoplasm" AND "castration-resistant prostate cancer" AND "non metastatic" AND "hormonal therapy" OR "apalutamide" OR "darolutamide" OR "enzalutamide"), without language restriction from the literature from January 2009 to September 2020, following The Preferred Reporting Items for Systematic Review and Meta-analyses (PRISMA) guidelines (**Figure S1**, **Supplementary Material**) (16). Original and review articles were included and critically evaluated. Additional references were identified from reference lists of these articles.

Selection of the studies and Inclusion Criteria

Entry into the analysis was restricted to data collected from original studies on RCTs including subjects with a diagnosis of M0 CRPC who subsequently underwent treatment with Apalutamide, Enzalutamide, or Darolutamide.

Two authors (MM; AS) independently screened the titles and abstracts of all articles using predefined inclusion criteria. The full-text articles were examined independently by three authors (MM; SS; VF) to determine whether or not they met the inclusion criteria. Then, two authors (VF; GB) extracted data from the selected articles. Final inclusion was determined by all investigators' evaluation discussion.

The studies selected for inclusion met the following criteria: (I) M0 CRPC cases; (II) Apalutamide, Enzalutamide, or Darolutamide as the experimental agent; (III) the comparison with placebo arm (i.e. received the sole ADT). **Table S1**, **Supplementary Material**, shows inclusion criteria following the Population, Intervention, Comparison, Outcomes and Study design (PICOS) method.

Articles were excluded if: (I) multiple reports were published on the same population, (II) data provided were insufficient for the outcomes described in the aim section, (III) animal studies, (IV) non-randomized studies.

Statistical Analysis

Risk of bias (RoB) for all included studies was evaluated using the Review Manager (RevMan) (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration) tool for the assessment of the methodological quality of trials (**Figure S2**, **Supplementary Material**).

Random effects meta-analysis of class-level effect of nHT versus placebo was performed using the inverse variance technique for meta-analysis of hazard ratios (HRs) for efficacy outcomes, and the Mantel-Haenszel method for meta-analysis of dichotomous data for AEs. To explore the pre-defined outcomes of interest, subgroup analysis was performed regarding differences in the PS (ECOG score 0 versus 1), the use of bone-targeted therapy (yes versus no), LN status (N0 *vs* N1) and PSA-DT (>6 months *vs* <6 months). To assess the variance distribution of the event rates (ERs) of survival and safety outcomes in the sole placebo arms, pooled ERs with 95% confidence intervals (CIs) were calculated.

Heterogeneity was evaluated by X^2 Q test and I^2 statistic (17). For the Q test, $p < 0.05$ indicated significant heterogeneity; for the I^2 statistics, an I^2 value >50% was considered significant. Our results are graphically displayed as forest plots, with HR with 95% CIs for the time-to-event variables, and odds ratio (ORs) or event rates (ERs) with 95% CIs for the dichotomous variables. Due to the small numbers of the included trials, no publication bias was estimated. Calculations were accomplished using RevMan version 5.4 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration) and Stata version 16.1 (Stata Corporation, College Station, TX, USA). A p value of <0.05 was regarded as statistically significant, and all tests were two-sided.

RESULTS

Search Results

The search strategy identified 2576 potentially relevant studies; after removing the duplicates, 1247 studies were screened of which 1224 were excluded based on title and abstract. For the remaining 23 studies, the full texts were obtained. The PRISMA flow diagram is presented in **Figure S1**, **Supplementary Material**. In total 3 studies fulfilled the inclusion criteria and were included in the final analysis (**Table 1**).

Design and Baseline Characteristics of the Included Studies

Three studies that met the inclusion criteria were included in this analysis (**Table 1**). A total of 4117 high-risk M0 CRPC patients were evaluated: 2694 cases were in the nHT arm (i.e. received the experimental drug plus on-going ADT), and 1423 cases were in the control arm (i.e. received the matched placebo plus on-going ADT). The enrollment of patients was performed between 2013 and 2018. Study design and inclusion criteria were similar among the studies. All the studies were international, randomized, double-blind, placebo-controlled, phase III trials. ADT was continued throughout the trial in all the studies. Based on updated data from the most recent publications on final analyses, patients were followed for a median of 29 to 52 months. As experimental drug, in 1 study was administered Apalutamide 240 mg once daily (806 cases) (11), in 1 Darolutamide 600 mg twice daily (955 cases) (12), and in 1 Enzalutamide 160 mg once daily (933 cases) (10). Patients baseline characteristics were similar among the studies, though with few subtle differences (**Table 1**). Patients in the experimental arm of SPARTAN trial showed a slightly lower median total PSA, compared with PROSPER and ARAMIS trials; patients in the experimental arm of PROSPER trial had a lower median PSA-DT, as well as a higher proportion of patients with PSA-DT ≤ 6 months and a higher percentage of patients with a better PS (ECOG= 0), when compared with PROSPER and ARAMIS trials. Median time from initial diagnosis was shorter

TABLE 1 | Patients baseline characteristics in the 3 included studies by treatment group [number of cases (%), and median (range)].

Variable	ARAMIS (12) (n= 1509)		PROSPER (10) (n= 1401)		SPARTAN (11) (n= 1207)	
Arm	DAROLUTAMIDE	PLAC	ENZALUTAMIDE	PLAC	APALUTAMIDE	PLAC
Patients, *n°*	955	554	933	468	806	401
Age (years), *median (range)*	74 (48–95)	74 (50–92)	74 (50–95)	74 (53–92)	74 (48–94)	74 (52–97)
Follow-up * (months), *median*	29.0		48.0		52.0	
Time from initial diagnosis (months), *median*	86.2	84.2	n.s.	n.s.	95.4	94.2
Total PSA level (ng/mL), *median (range)*	9.0 (0.3–858.3)	9.7 (1.5–885.2)	11.1 (0.8–1071.1)	10.2 (0.2–467.5)	7.8	8.0
Testosterone level (nmol/L), *median (range)*	0.6 (0.2–25.9)	0.6 (0.2–7.3)	n.s.	n.s.	0.8 (0.3–3.1)	0.8 (0.3–2.8)
PSA-DT, *n° (%)*						
≤6 months	667 (70)	371 (67)	715 (77)	361 (77)	576 (72)	284 (71)
>6 months	288 (30)	183 (33)	217 (23)	107 (23)	230 (29)	117 (29)
median (months)	4.4	4.7	3.8	3.6	4.4	4.5
LN status, *n° (%)*						
N0	792 (83)	396 (71)	n.s.	n.s.	673 (84)	336 (84)
N1	163 (17)	158 (29)			133 (17)	65 (16)
PS ECOG score, *n° (%)*						
0	650 (68)	391 (71)	747 (80)	382 (82)	623 (77)	311 (78)
1	305 (32)	163 (29)	185 (20)	85 (18)	183 (23)	89 (22)
Use of Bone targeted therapy, *n° (%)*						
No	924 (97)	522 (94)	828 (89)	420 (90)	724 (90)	362 (90)
Yes	31 (3)	32 (6)	105 (11)	48 (10)	82 (10)	39 (10)

PLAC, placebo; PSA, Prostate-specific antigen; PSA-DT, PSA doubling time; LN, lymph nodes; PS, performance status; ECOG, Eastern Cooperative Oncology Group; n.s., not specified.
**Follow-up is updated to final analyses of OS (13–15).*

in the ARAMIS compared with SPARTAN trial (86.2 versus 95.4 months, respectively); PROSPER trial did not report this data.

MFS and Time to PSA Progression Analyses

MFS was the primary endpoint in all included trials. The pooled analysis showed a significantly better MFS with nHT than with placebo (HR= 0.32, 95%CI= 0.25–0.41) (**Figure 1A**). Similarly, time to PSA progression was significantly improved with nHT compared to placebo (HR= 0.08, 95%CI= 0.05–0.14) (**Figure 1B**).

OS Analysis: Updated and Mature Results From Final Analyses

At the primary analyses, OS data were immature for all the trials since median OS was not reached in either treatment groups. OS data from final analyses of the included trials are summarized in **Table 2**. The median follow-up was 29 to 52 months; ARAMIS trial showed a shorter follow up time (29 months) when compared to PROSPER and SPARTAN trials (48 and 52 months, respectively). Death events occurred less frequently in ARAMIS trial (n= 148, 15%) than PROSPER and SPARTAN trials (n= 288, 31% and n= 274, 34%, respectively). According to the pooled analysis with updated and mature OS data, OS was significantly improved with nHT compared to placebo (HR= 0.74, 95%CI= 0.66–0.84) (**Figure 1C**). Moreover, nHT significantly improved OS over placebo across all pre-specified subgroups (**Figure S3**, **Supplementary Material**). Subgroup analysis revealed a greater OS benefit in patients with PSA-DT >6 months than ≤6 months (HR= 0.69 versus HR= 0.75), ECOG 0 than 1 (HR= 0.70 versus HR= 0.80), N1 disease than N0 (HR= 0.61 versus HR= 0.78), and in those receiving bone-targeted therapy (HR= 0.65 versus HR= 0.74), and a comparable OS by number of prior HT (HR= 0.75 versus HR= 0.76, for HT= 1 and ≥2); yet, differences between pre-specified subgroups were not significant (all p> 0.05) (**Figures S3 A–E**).

Stratified AEs Analysis

Figure 2 shows the comparison of AEs reported by both interim (**Figures 2A, C, E**) and final (**Figures 2B, D, F, G**) safety analyses of the included trials. Overall, the nHT arm was significantly associated with higher rates of AEs, when compared with the placebo arm. According to the pooled analysis of data from the safety final analyses of the three trials, the nHT arm was associated with a higher likelihood of experiencing grade 3–4 AEs, SAEs, AEs leading to discontinuation of trial regimen, and AEs leading to death than placebo (OR= 1.92, 95%CI= 1.30–2.85, OR= 1.748, 95%CI= 1.19–2.54, OR= 1.62, 95%CI= 0.89–2.92, and OR= 3.69, 95%CI= 0.79–17.30, respectively) (**Figures 2A–D**). Since published final analyses did not provide sufficient and consistent data to accomplish updated comparisons for all specific types of AEs, rates of specific types of AEs were assessed with data from the safety interim analyses (with the exception of fracture events, which are updated to final analyses). The likelihood of any grade and grade 3–4 fatigue was increased in the nHT arm than in the placebo arm (OR= 1.93, 95%CI= 1.23–3.04, and OR= 1.87, 95%CI= 0.37–9.37, respectively) (**Figures 3A, B**). Similarly, dizziness, cardiovascular events and fractures occurred more often with nHT than with placebo (OR= 1.63, 95%CI= 1.07–2.47, OR= 1.49, 95%CI= 1.09–2.03, and OR= 2.47, 95%CI= 1.63–3.74, respectively) (**Figures 3C–F**).

Comparison of the Placebo Arms of the Included Studies: Baseline Characteristics, Survival, and Safety Data

Patients baseline characteristics of the placebo arms included in the 3 studies are presented in **Table S2**, **Supplementary Material**.

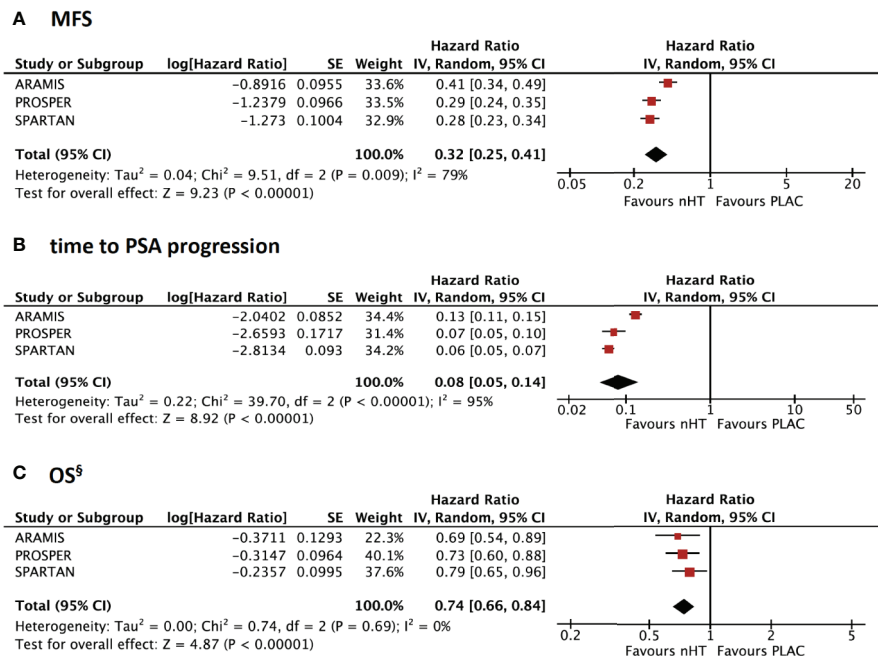


FIGURE 1 | Forest plots reporting pooled survival outcomes from the 3 included studies. **(A)** Metastasis-free survival (MFS); **(A)** time to Prostate-specific antigen (PSA) progression; **(C)** overall survival (OS). § OS data are updated to final analyses with mature OS data. [CI, confidence interval; nHT, novel hormonal therapy; PLAC, placebo].

TABLE 2 | Overall Survival (OS) data from the final analyses of the three included trials.

Variable	ARAMIS (14) (n= 1509)		PROSPER (15) (n= 1401)		SPARTAN (13) (n= 1207)	
Arm	DAROLUTAMIDE	PLAC	ENZALUTAMIDE	PLAC	APALUTAMIDE	PLAC
Patients, *n°*	955	554	933	468	806	401
Death events, *n° (%)*	148 (15)	106 (19)	288 (31)	178 (38)	274 (34)	154 (38)
Median OS, *months (95%CI)*	n.s.	n.s.	67.0 (64.0-NR)	56.3 (54.4–63.0)	3.9 (61.2-NR)	59.9 (52.8-NR)
HR for OS, *(95%CI) p*	0.69 (0.53–0.88) 0.003		0.73 (0.61–0.89) 0.001		0.78 (0.64–0.96) 0.016	
Median follow-up, *months*	29.0		48.0		52.0	

PLAC, placebo; OS, overall survival; n.s., not specified; NR, not reached; HR, hazard ratio; CI, confidence interval.

A slightly higher percentage of patients in the PROSPER trial had a shorter PSA-DT of ≤ 6 months (77%), compared to ARAMIS and SPARTAN trial (67% and 71%, respectively), as well as a lower median PSA-DT value at baseline (3.6 versus 4.7 and 4.5, respectively). Regarding lymph nodes status, a higher percentage of N1 patients in the ARAMIS trial was reported, when compared to SPARTAN trial (29% versus 16%); PROSPER trial did not report this data. A worse PS was reported in the placebo arm of ARAMIS trial, with 29% of patients having an ECOG= 1, compared to 18% and 22% for PROSPER and SPARTAN trials, respectively. The use of bone targeted therapy was similar for PROSPER and SPARTAN trials (both 10% of patients), while was slightly lower for ARAMIS (6%).

With regards to survival outcomes, ERs comparing patients in the sole placebo arms showed similar results for metastasis or death events for PROSPER and SPARTAN trials (ER= 0.49, 95% CI= 0.36–0.62 and ER= 0.48, 95%CI= 0.34–0.63, respectively), while ARAMIS showed a lower rate (ER= 0.39, 95%CI= 0.25–0.53) (**Figure 4A**). Similarly, ERs for death events were comparable between PROSPER and SPARTAN trials (ER= 0.38, 95%CI= 0.23–0.53 and ER= 0.38, 95%CI= 0.22–0.55, respectively), whereas ARAMIS showed a lower rate (ER= 0.19, 95%CI= 0.00–0.39) (**Figure 4B**).

With respect to safety outcomes, there was a significant difference in ERs for fatigue reported by the trials (p=0.03) (**Figure 4C**). Fatigue was less common in the ARAMIS arm, showing the lowest ER of 0.09 (95%CI −0.21 to 0.38), when compared with the arms from PROSPER and SPARTAN trials (ER=0.14, 95%CI −0.12 to 0.39 and ER=0.21; 95% CI, −0.01 to 0.43, respectively). On the contrary, placebo arms did not significantly differ in ERs for other analyzed AEs (i.e. dizziness, cardiovascular events and fractures) (all p> 0.05) (**Figures 4D–F**).

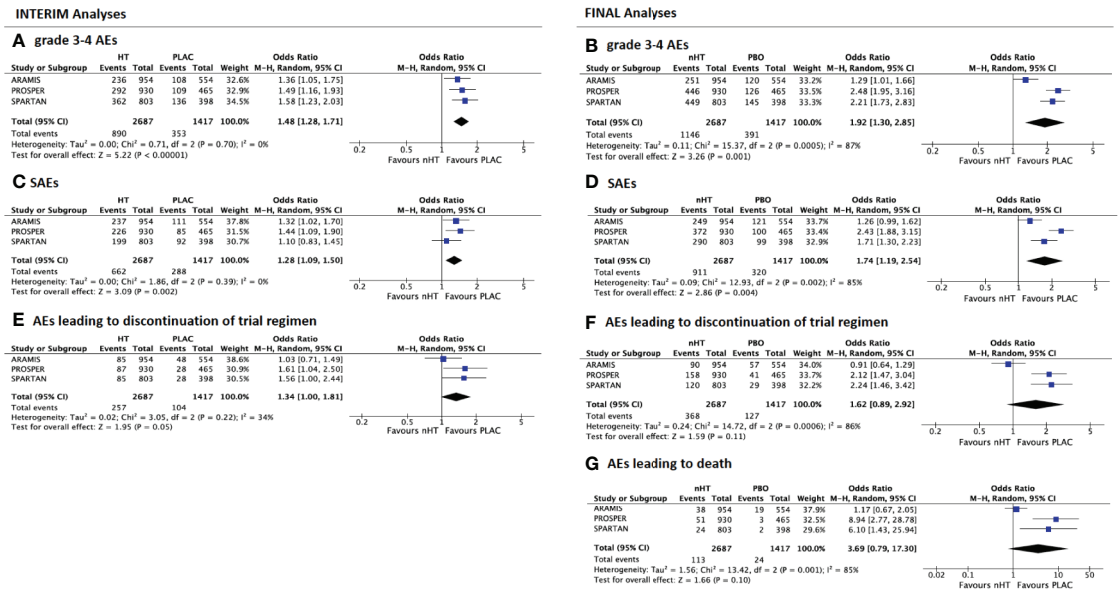


FIGURE 2 | Forest plots reporting pooled safety outcomes from both interim and final analyses of the 3 included studies. Grade 3–4 adverse events (AEs) from interim **(A)** and final analyses **(B)**; serious AEs (SAEs) from interim **(C)** and final analyses **(D)**; AEs leading to discontinuation of trial regimen from interim **(E)** and final analyses **(F)**; AEs leading to death from final analyses **(G)**. [CI, confidence interval; nHT, novel hormonal therapy; PLAC, placebo].

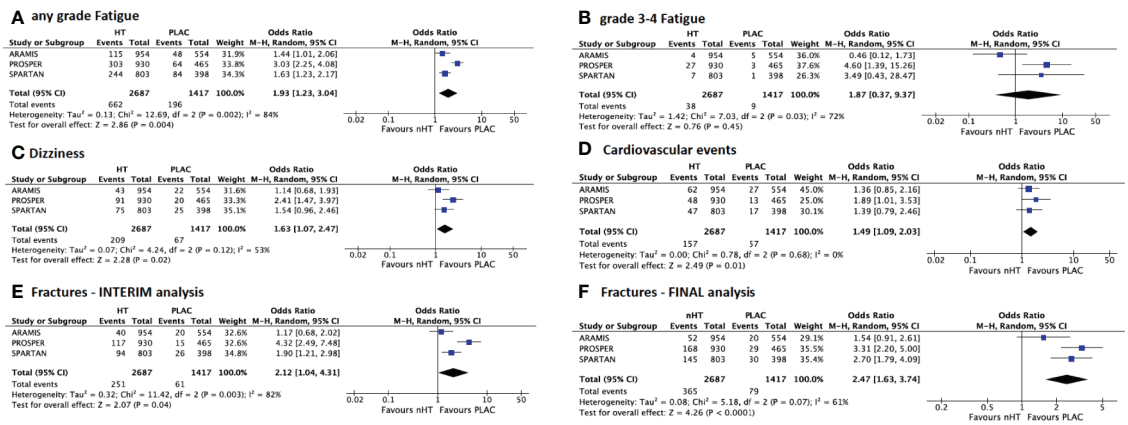


FIGURE 3 | Forest plots reporting pooled safety outcomes from interim analyses of the 3 included studies. **(A)** any grade and **(B)** grade 3–4 fatigue; **(C)** dizziness; **(D)** cardiovascular events; **(E)** fractures from interim analysis; **(F)** fractures from final analysis. [CI, confidence interval; nHT, novel hormonal therapy; PLAC, placebo].

Second-Line Therapies Analysis

Table 3 summarizes second-line therapies data updated to final analyses reported by the included trials. A higher percentage of patients in the SPARTAN trial received a second line therapy (48%), when compared to ARAMIS and PROSPER trials (15 and 33%, respectively). Chemotherapy with Docetaxel was the most common treatment used in both ARAMIS and PROSPER trials (58 and 60% of patients, respectively), whereas nHT (with either Abiraterone acetate or Enzalutamide) was the most frequent in SPARTAN trial (88% of patients); of note, only 9% of patients in the SPARTAN trial received Docetaxel as second-line treatment. SPARTAN was the sole trial reporting data on second progression-free survival (PFS) (defined as the time from randomization to investigator-assessed disease progression during the first subsequent treatment for mCRPC or death from any cause). At the final analysis, Apalutamide significantly improved second PFS over placebo (HR=0.55, 95%CI −0.46 to −0.66), with an extension of median second PFS of 14.4 months (55.6 months with Apalutamide versus 41.2 months with placebo). Data regarding second PFS were not evaluated in ARAMIS and PROSPER trials (**Table 3**).

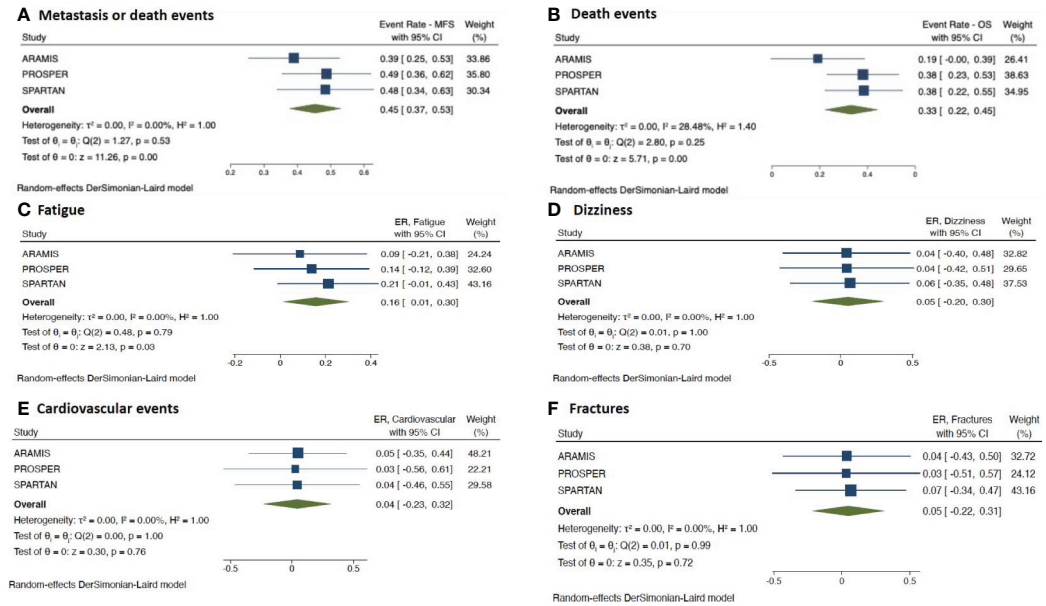


FIGURE 4 | Forest plots reporting pooled survival and safety outcomes from the sole placebo arms of the 3 included studies. **(A)** metastasis or death events; **(B)** death events; **(C)** fatigue; **(D)** dizziness; **(E)** cardiovascular events; **(F)** fractures. [ER, event rate; CI, confidence interval; nHT, novel hormonal therapy; PLAC, placebo].

TABLE 3 | Second line therapies data from the final analyses of the three included trials.

Variable	ARAMIS (DAROLUTAMIDE, n= 955)		PROSPER (ENZALUTAMIDE, n= 933)		SPARTAN (APALUTAMIDE, n= 806)	
	INTERIM analysis (12)	**FINAL analysis (14)**	**INTERIM analysis (10)**	**FINAL analysis (15)**	**INTERIM analysis (11)**	**FINAL analysis (13)**
Patients receiving subsequent therapies, *n° (%)*	100 (11)	141 (15)	138 (15)	310 (33)	165 (21)	386 (48)
Type of subsequent therapies, *n° (%)*						
- DOCETAXEL	- 49 (49)	- 82 (58)	- 37 (27)	- 185 (60) ^	- 15 (9)	- 33 (9)
- HT$	- 31 (31)	- 57 (41)	- 52 (38)	- 196 (63)	- 145 (88)	- 314 (81)
- other$	- 13 (13)	- 2 (1)	- 49 (35)	- 74 (14)	- 5 (3)	- 39 (10)
	FINAL analysis (14)		*FINAL analysis* (15)		*FINAL analysis* (13)	
Second progression events*, *n° (%)*	not evaluated		not evaluated		319 (40)	
Median second PFS, *months*	not evaluated		not evaluated		55.6	
HR for second PFS *(95%CI)*	not evaluated		not evaluated		0.55 (0.46–0.66)	
Median follow-up, *months*	29.0		48.0		52.0	

HT, hormonal therapy; n.s., not specified; PFS, progression-free survival; HR, hazard ratio; CI, confidence interval.
*$ includes: ENZALUTAMIDE or ABIRATERONE ACETATE plus PREDNISONE; $ includes other therapies such as CABAZITAXEL, BICALUTAMIDE. * defined as progression on or after the first subsequent therapy or death.*
^% are based on the number of patients who received at least one antineoplastic agent after discontinuation of the trial regimen.

DISCUSSION

The treatment scenario of high-risk M0 CRPC cases has recently and deeply changed, with the shift from the sole on-going ADT to the addition of nHT to on-going ADT. Approval was based on data from the three RCTs: ARAMIS, PROSPER, and SPARTAN, in which Darolutamide, Enzalutamide, and Apalutamide improved MFS over placebo. The pooled benefit in MFS of nHT over placebo was seen in the overall population and analyzed in subgroups analyses; MFS was improved with a greater extent in men with ECOG 0 versus 1, yet no differences were found according to PSA-DT and the use of bone-targeted therapy (18–20).

At this primary analysis, although OS data consistently favored nHT over placebo in all the mentioned trials, the results with respect to OS did not meet the criteria for significance. Recent meta-analyses showed that nHT prolonged OS in a statistically significant manner, yet at that time median OS -still not reached in all experimental arm- and the short follow-up precluded from definitive conclusions (18–20). Results

from the prespecified OS final analyses of the 3 trials were presented at the 2020 ASCO Annual Meeting, and then recently published (13–15). To the best of our knowledge, the present work is the first literature-based meta-analysis evaluating results of the final analyses with respect to OS of the three included RCTs.

After a median follow-up of 29 to 52 months, our updated pooled results demonstrated a reduction in death in 26% of patients (HR= 0.74, 95%CI = 0.66–0.84). The pooled analysis revealed the absence of heterogeneity among the studies. However, ARAMIS trial reported the lowest rate of death events in the nHT arm (15% versus 31% and 34% for PROSPER and SPARTAN, respectively), yet it had the shortest follow-up time (29 months versus 48 and 52 months for PROSPER and SPARTAN, respectively). The analysis of the variance distribution of survival data in the sole placebo arms of the trials showed a similar trend. Indeed, ERs for death events were comparable between PROSPER and SPARTAN trials (ER= 0.38 for both) and higher than that ARAMIS showed a lower rate (ER= 0.19). Analysis according to pre-specified subgroups did not show differences by PSA-DT, ECOG score, use of bone-target therapy, LN status and number of prior HT. Indeed, although results revealed a greater OS benefit in groups of patients (i.e. PSA-DT >6 months, ECOG= 0, N1 disease and the use of concomitant bone-targeted therapy), differences were not statistically significant. Therefore, further research is warranted to better define subgroups of patients who will benefit most from nHT.

In view of the long-term treatment with these agents in asymptomatic patients, safety profile covers a pivotal role in the treatment decision-making with these novel compounds.

Overall, patients receiving nHT were more likely to experience AEs, when compared with those receiving the sole on-going ADT. As expected, the long-term analysis (median follow-up of 29 to 52 months) showed a worse safety profile with nHT than the interim analysis (median follow-up of 15 to 20 months). The pooled OR for grade 3–4 AEs increased from 1.48 on interim analysis to 1.92 on final analysis. Of note, ORs in ARAMIS trial remained stable during this time frame, while increased in PROSPER and SPARTAN trials (from 1.49 to 2.48, and from 1.58 to 2.21, respectively). Similarly, the pooled OR for SAEs increased from 1.28 on interim analysis to 1.74 on final analysis; ORs in ARAMIS trial were stable, whereas increased in PROSPER and SPARTAN trials (from 1.44 to 2.43, and from 1.10 to 1.71, respectively). PROSPER trial showed the highest OR increase and value for both grade 3–4 AEs and SAEs, suggesting a higher risk of toxicity at long-term analysis; of note, among patients receiving nHT, PROSPER trial had a higher percentage of patients with a better PS (ECOG= 0) at baseline, when compared with PROSPER and ARAMIS trials. Despite the worse PS showed at baseline in patients receiving Darolutamide compared to other nHT, ARAMIS trial was associated with a more favorable long-term safety profile. Although it reported the shortest follow-up time (29 months), the safety profile appeared to be stable over the time (interim versus final analyses).

With regards to specific types of AEs, rates differed among the evaluated drugs. OR for any grade fatigue on pooled analysis was 1.93; patients in the PROSPER trial experienced more events than those in the ARAMIS and SPARTAN trials (OR 3.03 versus 1.44 and 1.63, respectively). Similarly, patients in the PROSPER trial were more likely to experience dizziness and cardiovascular events (OR 2.41 and 1.89, respectively) than ARAMIS and SPARTAN trials. Concerning fractures, at the interim analyses PROSPER trial showed a somewhat surprisingly higher risk of events (OR= 4.32), than ARAMIS and SPARTAN trials (OR= 1.17 and 1.90, respectively). At final safety analyses, although Enzalutamide was associated with the highest risk among nHT, the risk decreased (OR= 3.31), nuancing the difference among the trials (OR= 1.54 with Darolutamide and OR= 2.70 with Apalutamide).

The difference in safety profiles showed by these trials could be explained by the different structures and mechanisms of action of these novel agents. While Apalutamide and Enzalutamide are androgen receptor inhibitors, Darolutamide is an androgen receptor antagonist. Due to a distinct structure, the latter assures a low penetration of the blood-brain barrier as well as e low binding affinity for κ-aminobutyric acid type A receptors (21, 22). Indeed, data from the final analysis of the ARAMIS trial confirmed the low potential for central nervous system (CNS)-related effects expected with Darolutamide (14). This aspect might be especially important in frail patients, for whom possible CNS-related AEs should be taken into account for assessing the risk-benefit balance of treatment utilization. Although patients in the PROSPER and SPARTAN trials reported higher incidences of CNS-related AEs than those in the ARAMIS, heterogeneous duration of treatment and follow-up could have directly affected these incidences. Moreover, it should be underlined that grade 3–4 CNS-related AEs rates occurred in <1% of patients in all trials. To explore other possible explanation for this difference in toxicity, we evaluated the sole placebo arms of the trials in terms of variance distribution of the AEs rates. Placebo arms significantly differed only in ERs for fatigue, yet not for other analyzed AEs.

Given the use of these agents in asymptomatic patients, health-related quality of life (HRQoL) is a main performance measure - in addition to survival data - that should be taken into account when deciding among treatments. Indeed, HRQoL covers a main role, providing insights into the impact of treatments on patients' daily life, in terms of both physical and psychological wellbeing (23). Data from the SPARTAN trial demonstrated that HRQoL was not impaired with Apalutamide treatment, and that HRQoL deterioration was more apparent in the placebo group (24). In the PROSPER trial, Enzalutamide showed to increase the time to deterioration in HRQoL, when compared with placebo (25). According to a recent anchored matching-adjusted indirect comparison (MAIC) study, the probability of a better HRQoL with Apalutamide versus Enzalutamide was 73.1% (26). Data from the primary analysis of ARAMIS trial revealed similar QoL scores between Darolutamide and placebo groups, with scores consistently favoring Darolutamide - yet the clinically

meaningful thresholds were not reached (12). Since HRQoL is of pivotal importance for patients' care, better exploring this aspect still represent an area of unmet medical need to guide more informed treatment decisions.

About one-third of patients with M0 CRPC will develop visible bone metastases within 2 years. Currently, there are multiple available therapies for men with mCRPC (i.e. Docetaxel, Abiraterone/Prednisolone, Enzalutamide, Cabazitaxel and Radium–223), and despite the importance of sequencing systemic therapy in mCRPC has already been acknowledged, the optimal strategy of sequencing remains a major challenge. Indeed, selection of treatment for mCRPC is multifactorial and, among other factors, type of previous treatment (e.g. known cross resistance between androgen receptor targeted agents), quality of response and pace of progression on previous treatment have a main role (6, 27). Therefore - and especially after systemic therapies have been moved earlier in the treatment scenario of PC - providing data on response and progression on second-line therapies would be of particular clinical value for accurately managing PC patients over the time.

Unfortunately, only SPARTAN trial provided survival data on second-line therapies (i.e. second PFS), and currently no other data are available to help set the proper sequencing of therapeutic agents, suggesting further research in this field is required. However, results from SPARTAN trial were promising, showing that second progression or death events occurred in 15% of patients receiving Apalutamide, and that this drug extended median second PFS by 14.4 months versus placebo; the HR for second PFS with Apalutamide was reduced by 45% versus placebo (**Table 3**) (13). Owing to the lack of data from other trials, we are not able to make a comparison among different nHT.

In conclusion, to date, main limitations that may affect an optimal treatment decision-making with these novel compounds – and that should represent a field for further research, could be summarized as follow: (I) the lack of comparable HRQoL data; (II) heterogeneous follow-up period; (III) scarce survival data on second-line therapies.

Moreover, it is important to underline that in all the available trials the M0 status was assessed by conventional scans (i.e. computed tomography (CT) and bone scans). According to recent publications, it is reasonable to speculate that with more sensitive imaging modalities (e.g. PSMA PET/CT or whole-body magnetic resonance imaging (MRI)) more patients are expected to be diagnosed with early mCRPC (28), suggesting this setting is expected to evolve in the near future.

CONCLUSION

According to the available evidence, nHT have shown to improve MFS as well as - according to final analyses - OS over placebo in the setting of high-risk M0 CRPC. Owing to the importance of sequencing systemic therapy in CRPC, the lack of survival data regarding second-line therapies remains a major issue. The long-term analysis showed a worse safety profile with nHT than the interim analysis, whit distinct profiles among different nHT. Moreover, phase IV trials evaluating nHT in a real-world setting would be of particular clinical value to help guide proper treatment choices in these patients. Lastly, whether the use of novel imaging modalities will change treatment decision in this setting represents an open question for the near future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization: MM and AS. Methodology: VF and GMB, Software: UGF, Formal analysis: MM and FDG. Investigation: VF and GDV. Resources: MF and AP. Data curation: SS. Writing—original draft preparation: MM. Writing—review and editing: AS and EDB. Supervision: AS. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.700258/full#supplementary-material

REFERENCES

1. Gillessen S, Attard G, Beer TM, Beltran H, Bossi A, Bristow R, et al. Management of Patients With Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference Apccc 2017. *Eur Urol* (2018) 73(2):178–211. doi: 10.1016/j.eururo.2017.08.010
2. van den Bergh RC, van Casteren NJ, van den Broeck T, Fordyce ER, Gietzmann WK, Stewart F, et al. Role of Hormonal Treatment in Prostate Cancer Patients With Nonmetastatic Disease Recurrence After Local Curative Treatment: A Systematic Review. *Eur Urol* (2016) 69(5):802–20. doi: 10.1016/j.eururo.2015.11.023
3. Bolla M, Gonzalez D, Warde P, Dubois JB, Mirimanoff RO, Storme G, et al. Improved Survival in Patients With Locally Advanced Prostate Cancer Treated With Radiotherapy and Goserelin. *N Engl J Med* (1997) 337 (5):295–300. doi: 10.1056/NEJM199707313370502
4. Crook JM, O'Callaghan CJ, Duncan G, Dearnaley DP, Higano CS, Horwitz EM, et al. Intermittent Androgen Suppression for Rising PSA Level After Radiotherapy. *N Engl J Med* (2012) 367(10):895–903. doi: 10.1056/NEJMoa1201546
5. Anantharaman A, Small EJ. Tackling non-Metastatic Castration-Resistant Prostate Cancer: Special Considerations in Treatment. *Expert Rev Anticancer Ther* (2017) 17(7):625–33. doi: 10.1080/14737140.2017.1333903
6. Mottet N, van den Bergh RCN. *Prostate Cancer: European Association of Urology (EAU) Guidelines* (2019). Available at: https://uroweb.org/guideline/prostate-cancer.
7. Liede A, Günther O, Bennett B, Wong S. Prevalence of non-Metastatic Castration-Resistant Prostate Cancer in Europe. *European Society for Medical Oncology, Vienna Austria September 28-October 2* (2012) 2012.
8. Liede A, Arellano J, Hechmati G, Bennett B, Wong S. International Prevalence of Nonmetastatic (M0) Castration-Resistant Prostate Cancer

(CRPC). *J Clin Oncol* (2013) 31:e16052–e16052. doi: 10.1200/jco.2013.31.15_suppl.e16052
9. Smith MR, Kabbinavar F, Saad F, Hussain A, Gittelman MC, Bilhartz DL, et al. Natural History of Rising Serum Prostate-Specific Antigen in Men With Castrate Nonmetastatic Prostate Cancer. *J Clin Oncol* (2005) 23(13):2918–25. doi: 10.1200/JCO.2005.01.529
10. Hussain M, Fizazi K, Saad F, Rathenborg P, Shore N, Ferreira U, et al. Enzalutamide in Men With Nonmetastatic, Castration-Resistant Prostate Cancer. *N Engl J Med* (2018) 378(26):2465–74. doi: 10.1056/NEJMoa1800536
11. Smith MR, Saad F, Chowdhury S, Oudard S, Hadaschik BA, Graff JN, et al. Apalutamide Treatment and Metastasis-free Survival in Prostate Cancer. *N Engl J Med* (2018) 378(15):1408–18. doi: 10.1056/NEJMoa1715546
12. Fizazi K, Shore N, Tammela TL, Ulys A, Vjaters E, Polyakov S, et al. Darolutamide in Nonmetastatic, Castration-Resistant Prostate Cancer. *N Engl J Med* (2019) 380(13):1235–46. doi: 10.1056/NEJMoa1815671
13. Smith MR, Saad F, Chowdhury S, Oudard S, Hadaschik BA, Graff JN, et al. Apalutamide and Overall Survival in Prostate Cancer. *Eur Urol* (2021) 79 (1):150–8. doi: 10.1016/j.eururo.2020.08.011
14. Fizazi K, Shore N, Tammela TL, Ulys A, Vjaters E, Polyakov S, et al. Nonmetastatic, Castration-Resistant Prostate Cancer and Survival With Darolutamide. *N Engl J Med* (2020) 383(11):1040–9. doi: 10.1056/NEJMoa2001342
15. Sternberg CN, Fizazi K, Saad F, Shore ND, De Giorgi U, Penson DF, et al. Enzalutamide and Survival in Nonmetastatic, Castration-Resistant Prostate Cancer. *N Engl J Med* (2020) 382(23):2197–206. doi: 10.1056/NEJMoa2003892
16. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, et al. The PRISMA Statement for Reporting Systematic Reviews and Meta-Analyses of Studies That Evaluate Health Care Interventions: Explanation and Elaboration. *J Clin Epidemiol* (2009) 62(10):e1–34. doi: 10.1016/j.jclinepi.2009.06.006
17. Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring Inconsistency in Meta-Analyses. *BMJ* (2003) 327(7414):557–60. doi: 10.1136/bmj.327.7414.557
18. Roviello G, Gatta Michelet MR, D'Angelo A, Nobili S, Mini E. Role of Novel Hormonal Therapies in the Management of non-Metastatic Castration-Resistant Prostate Cancer: A Literature-Based Meta-Analysis of Randomized Trials. *Clin Transl Oncol* (2020) 22(7):1033–9. doi: 10.1007/s12094-019-02228-2
19. Hird AE, Magee DE, Bhindi B, Ye XY, Chandrasekar T, Goldberg H, et al. A Systematic Review and Network Meta-Analysis of Novel Androgen Receptor Inhibitors in Non-metastatic Castration-Resistant Prostate Cancer. *Clin Genitourin Cancer* (2020) 18(5):343–50. doi: 10.1016/j.clgc.2020.02.005
20. Kumar J, Jazayeri SB, Gautam S, Norez D, Alam MU, Tanneru K, et al. Comparative Efficacy of Apalutamide Darolutamide and Enzalutamide for Treatment of Non-Metastatic Castrate-Resistant Prostate Cancer: A Systematic Review and Network Meta-Analysis. *Urol Oncol* (2020) 38 (11):826–34. doi: 10.1016/j.urolonc.2020.03.022
21. Zurth C, Sandmann S, Trummel D, Seidel D, Gieschen H. Blood-Brain Barrier Penetration of [14C]Darolutamide Compared With [14C]Enzalutamide in Rats Using Whole Body Autoradiography. *J Clin Oncol* (2018) 36:Suppl 6S: 345. abstract. doi: 10.1200/JCO.2018.36.6_suppl.345
22. Moilanen AM, Riikonen R, Oksala R, Ravanti L, Aho E, Wohlfahrt G, et al. Discovery of ODM-201, a New-Generation Androgen Receptor Inhibitor Targeting Resistance Mechanisms to Androgen Signaling-Directed Prostate Cancer Therapies. *Sci Rep* (2015) 5:12007. doi: 10.1038/srep12007
23. Maggi M, Gentilucci A, Salciccia S, Gatto A, Gentile V, Colarieti A, et al. Psychological Impact of Different Primary Treatments for Prostate Cancer: A Critical Analysis. *Andrologia* (2019) 51(1):e13157. doi: 10.1111/and.13157
24. Saad F, Cella D, Basch E, Hadaschik BA, Mainwaring PN, Oudard S, et al. Effect of Apalutamide on Health-Related Quality of Life in Patients With non-Metastatic Castration-Resistant Prostate Cancer: An Analysis of the SPARTAN Randomised, Placebo-Controlled, Phase 3 Trial. *Lancet Oncol* (2018) 19(10):1404–16. doi: 10.1016/S1470-2045(18)30456-X
25. Tombal B, Saad F, Penson D, Hussain M, Sternberg CN, Morlock R, et al. Patient-Reported Outcomes Following Enzalutamide or Placebo in Men With non-Metastatic, Castration-Resistant Prostate Cancer (PROSPER): A Multicentre, Randomised, Double-Blind, Phase 3 Trial. *Lancet Oncol* (2019) 20(4):556–69. doi: 10.1016/S1470-2045(18)30898-2
26. Chowdhury S, Oudard S, Uemura H, Joniau S, Pilon D, Lefebvre P, et al. Matching-Adjusted Indirect Comparison of Health-Related Quality of Life and Adverse Events of Apalutamide Versus Enzalutamide in Non-Metastatic Castration-Resistant Prostate Cancer. *Adv Ther* (2020) 37(1):512–26. doi: 10.1007/s12325-019-01157-4
27. Sciarra A, Gentilucci A, Silvestri I, Salciccia S, Cattarino S, Scarpa S, et al. Androgen Receptor Variant 7 (AR-V7) in Sequencing Therapeutic Agents for Castratrion Resistant Prostate Cancer: A Critical Review. *Med (Baltimore)* (2019) 98(19):e15608. doi: 10.1097/MD.0000000000015608
28. Fendler WP, Weber M, Iravani A, Hofman MS, Calais J, Czernin J, et al. Prostate-Specific Membrane Antigen Ligand Positron Emission Tomography in Men With Nonmetastatic Castration-Resistant Prostate Cancer. *Clin Cancer Res* (2019) 25(24):7448–54. doi: 10.1158/1078-0432.CCR-19-1050

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 18 June 2021
doi: 10.3389/fonc.2021.656146

Real-World Data on Outcomes in Metastatic Castrate-Resistant Prostate Cancer Patients Treated With Abiraterone or Enzalutamide: A Regional Experience

Rachel Raju[1], *Arvind Sahu*[1,2]*, *Myron Klevansky*[1,2] *and Javier Torres*[1,2]

[1] Department of Oncology, Goulburn Valley Health, Shepparton, VIC, Australia, [2] Department of Rural Health, Faculty of Medicine, Dentistry and Health Sciences, University of Melbourne, Shepparton, VIC, Australia

OPEN ACCESS

Edited by:
Young E. Whang, University of North Carolina at Chapel Hill, United States

Reviewed by:
Daniel Crona, UNC Eshelman School of Pharmacy, United States
Benjamin Maughan, University of Utah, United States

***Correspondence:**
Arvind Sahu
Arvind.Sahu@gvhealth.org.au

Specialty section:
This article was submitted to Genitourinary Oncology, a section of the journal Frontiers in Oncology

Received: *20 January 2021*
Accepted: *19 April 2021*
Published: *18 June 2021*

Citation:
Raju R, Sahu A, Klevansky M and Torres J (2021) Real-World Data on Outcomes in Metastatic Castrate-Resistant Prostate Cancer Patients Treated With Abiraterone or Enzalutamide: A Regional Experience. Front. Oncol. 11:656146. doi: 10.3389/fonc.2021.656146

Background: Both abiraterone and enzalutamide have shown to improve overall survival (OS), progression-free survival (PFS) and prostate-specific antigen (PSA) response in patients with metastatic castration-resistant prostate cancer (mCRPC) regardless of previous treatment with chemotherapy (COU-AA301[1], COU-AA302[2], AFFIRM[3] and PREVAIL[4]). The data regarding the impact of these treatments in the real world setting is scarce. This study assessed the real world survival and disease outcomes in mCRPC patients in a regional health service in Victoria with the use of abiraterone and enzalutamide.

Methods: This retrospective clinical audit included 75 patients with diagnosis of mCRPC treated with either abiraterone or enzalutamide between January 1, 2014, and December 31, 2019, at Goulburn Valley Health. Patients were stratified according to the drug received, Eastern Cooperative Oncology Group (ECOG) performance status, Gleason score, burden of disease at diagnosis, presence of visceral metastases and use of previous chemotherapy. The primary end point was PSA response (defined as a reduction in the PSA level from baseline by 50% or more). The secondary outcomes were PSA PFS, radiographic PFS, and OS.

Results: Thirty-seven patients received enzalutamide, and the other 38 received abiraterone. Only 20% of patients in either group had visceral metastases. 32% of patients receiving enzalutamide had a high burden of disease, compared to 53% receiving abiraterone. 38% of patients in the enzalutamide group and 53% in the abiraterone group had received prior chemotherapy. PSA response rates were higher in the enzalutamide group than abiraterone group (70.3% vs 37.8%). Both PSA and radiographic PFS were longer in the enzalutamide group than abiraterone group; 7 months vs 5 months for both end points. OS was also found to be longer in patients receiving enzalutamide; 30 months compared to only 13 months in patients receiving abiraterone.

Conclusion: Both abiraterone and enzalutamide have shown to result in significant PSA response rates, as well as PFS and OS benefit in mCRPC patients in the real world setting. The difference in responses and survival benefit are probably impacted by the unbalanced burden of disease.

Keywords: real world, regional, metastatic prostate cancer, abiraterone, enzalutamide

INTRODUCTION

Both abiraterone and enzalutamide are current standard of care treatments for patients with metastatic castrate-resistant prostate cancer (mCRPC), and are widely used in clinical practice. These agents have shown to improve overall survival (OS), prostate-specific antigen (PSA) response, and radiographic and PSA progression-free survival (PFS) in mCRPC patients regardless of prior chemotherapy use, as reflected in large phase III clinical trials; COU-AA301 (1), COU-AA302 (2), AFFIRM (3), and PREVAIL (4). In the COU trials, abiraterone with prednisolone compared to placebo and prednisolone resulted in a PFS and OS benefit in mCRPC patients who had prior docetaxel chemotherapy, but only a PFS benefit was demonstrated in chemotherapy naïve mCRPC patients (1, 2). AFFIRM and PREVAIL demonstrated a PFS and OS benefit of enzalutamide over placebo in mCRPC patients, with or without prior use of docetaxel (3, 4). Quality of life improvement has also been shown in these studies with abiraterone and enzalutamide, with reduction in time to first skeletal related event and improved pain management in this group of patients. This quality of life data is especially important in patients with metastatic prostate cancer, as bony metastases can be extensive and symptomatic, and can result in acute neurological sequelae such as cord compression and cauda equina syndrome.

The decision of choosing one agent over the other is individualised, as to date there are no prospective studies evaluating the sequencing of abiraterone and enzalutamide. A randomised phase II sequencing trial involving 202 chemotherapy naïve patients with mCRPC assigned to abiraterone plus prednisolone or enzalutamide with crossover allowed, demonstrated no significant difference between first-line abiraterone and first-line enzalutamide in terms of time to PSA progression [median 11·2 vs 10·2 months, Hazard ratio (HR) = 0·95, 95% CI 0·66–1·36, p = 0·78]. The abiraterone-first arm had longer time from start of first-line therapy to second PSA progression (median 28·4 months vs 14·2 months, HR = 0·65, 95% CI 0·36–1·17, p = 0·15) and higher second PSA responses. However, there was no statistically significant difference in OS between the two arms (5). A meta-analysis aimed at comparing the efficacies between abiraterone and enzalutamide in mCRPC patients, using pooled results of 19 studies, found that treatment with first-line enzalutamide was associated with an increase in median OS of 5.9 months (HR 0.81, p<0.001) and an increase in median PFS of 8.3 months (HR = 0.47, p<0.001) compared to abiraterone in the pre-docetaxel mCRPC setting. In the post-docetaxel setting, enzalutamide was shown to have a small but statistically significant (especially after adjusting for baseline Gleason score) advantage over abiraterone with respect to PFS (6).

Prospective trial validation comparing efficacies of one androgen receptor blocker to the other, however, is lacking. The PFS and OS outcomes of abiraterone and enzalutamide in clinical practice especially in regional health centres in Australia have not been studied. Variability in drug tolerability due to differences in ECOG performance status and comorbidities, as well as compliance in the real world population can affect outcomes. This retrospective real world study assessed the PSA response, PFS and OS outcomes in mCRPC patients on enzalutamide and abiraterone in a regional health service in Victoria (Australia).

METHODS

Participants and Data Definitions

Patients with the diagnosis of mCRPC treated with either abiraterone or enzalutamide between the period January 1, 2014, and December 31, 2019, at Goulburn Valley Health were included in this retrospective audit. Any prior treatment including chemotherapy was allowed. Individual patient electronic records were reviewed and data recorded on to an Excel spreadsheet. The demographic data and baseline patient and tumour characteristics were collected from the electronic medical record system. Patient characteristics including age and ECOG performance status, as well as tumour characteristics including Gleason score, burden of disease at diagnosis (high volume defined as presence of visceral metastases and/or four or more bony metastases with one or more beyond vertebral body and pelvis), presence of visceral metastasis, prior systemic therapies were recorded from patient hospital files and hospital electronic medical records. Radiological and biochemical response to treatment, as well as tolerability was recorded.

The primary outcome was PSA response rates. The secondary outcomes were PSA PFS, radiographic PFS, and OS. PSA response was defined as a reduction in the PSA level from baseline by 50% or more (3). PFS was defined as time from treatment initiation with abiraterone or enzalutamide to disease progression, measured either biochemically *via* PSA readings alone or in combination with radiological staging utilising CT and whole body bone scans. The definition of biochemical disease progression was based on the Prostate Cancer Clinical Trials Working Group (PCWG-3) criteria (7). The definition of radiological progression was based on the WHO criteria in tumour response (8). OS was defined as time from treatment

initiation with abiraterone or enzalutamide to time of death of any cause.

Statistical Analysis

Survival was assessed in using the Kaplan-Meier method, and tested by means of a two-sided log-rank test. A Cox proportional hazards model was used to perform multivariable analysis of various factors affecting OS, including study intervention. All analyses were performed using SPSS Statistics for Windows software, version 26.0 (SPSS, Chicago, IL).

RESULTS

Patient and Disease Characteristics

Information was collected on 86 patients in total, but 11 patients were ultimately excluded due to various reasons (**Figure 1**). A total of 75 patients were divided into two groups based on whether they received abiraterone or enzalutamide, and stratified according to ECOG performance, Gleason score, burden of disease, presence of visceral metastases and use of previous systemic therapy including chemotherapy and other androgen blockade therapies (**Table 1**). Median age was 80 years old (61–94 years old), with most patients having an ECOG performance status of 1 (39%) or 2 (36%). About half of the patients in either group had a Gleason score of at least 8 or above. 55% of patients on abiraterone and 64% on enzalutamide were previously treated with other anti-androgen blockers. Median follow up duration was 37 months.

TABLE 1 | Baseline patient and disease characteristics in the abiraterone and enzalutamide groups.

Characteristic	Abiraterone (N = 38)	Enzalutamide (N = 37)
Age, year		
* **Median**	80	80
* **Range**	61–94	61–94
ECOG status- no. (%)		
* **0**	1 (2)	1 (3)
* **1**	12 (32)	17 (46)
* **2**	17 (45)	10 (27)
* **3**	8 (21)	9 (24)
Gleason score (%)	6 (16)	9 (24)
* **6–7**	19 (50)	21 (57)
* **8–10**	13 (34)	7 (18)
* **Unknown**		
Burden of disease* at diagnosis- no. (%)		
* **High**	20 (53)	12 (32)
* **Low**	18 (47)	25 (68)
Visceral metastases- no. (%)	8 (21)	7 (19)
Prior systemic treatment- no. (%)		
* **Docetaxel**	20 (53)	14 (38)
* **Abiraterone**	–	2 (5)
* **Enzalutamide**	9 (24)	–
* **Other antiandrogens**	21 (55)	24 (64)

**Defined as high volume defined as presence of visceral metastases and/or four or more bony metastases with one or more beyond vertebral body and pelvis.*

Disease and Survival Outcomes

PSA response occurred in 54% of the entire study population (41 out of 75 patients). A higher proportion of patients in the enzalutamide group had a PSA response; 26 out of 37 patients (70.3%) compared to only 15 out of 38 patients (39.5%) in the abiraterone group. The PSA PFS was 6 months (95% CI, 4.5–7.5) in the entire cohort. Patients on enzalutamide experienced a PSA PFS of 7 months (95% CI, 4.7–9.3), compared to 5 months (95% CI, 3.3–6.7; p=0.022) for patients on abiraterone. Radiographic PFS in the enzalutamide group was 7 months (95% CI, 3.6–10.4) compared to 5 months in the abiraterone group (95% CI, 2.0–8.0; p=0.036) (**Figures 2A, B**).

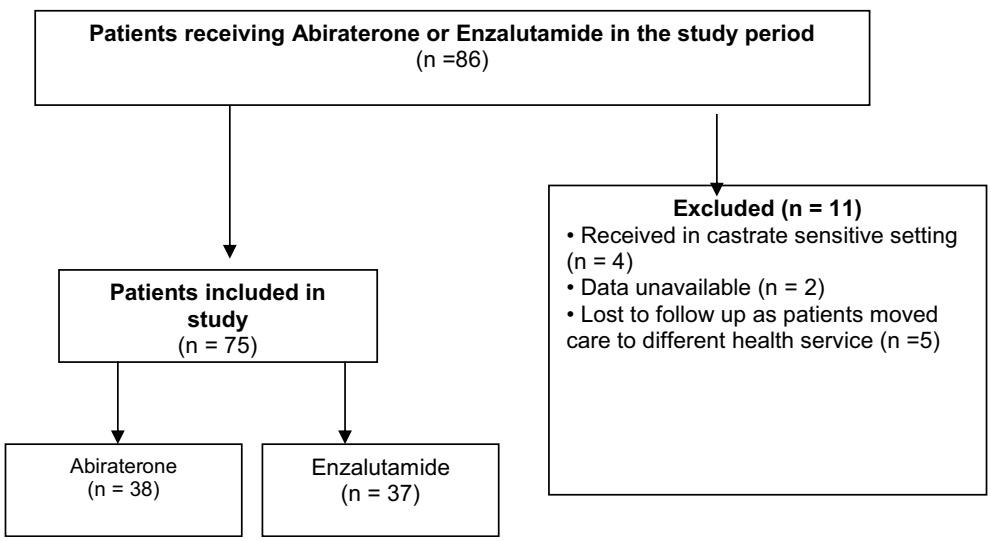


FIGURE 1 | CONSORT diagram.

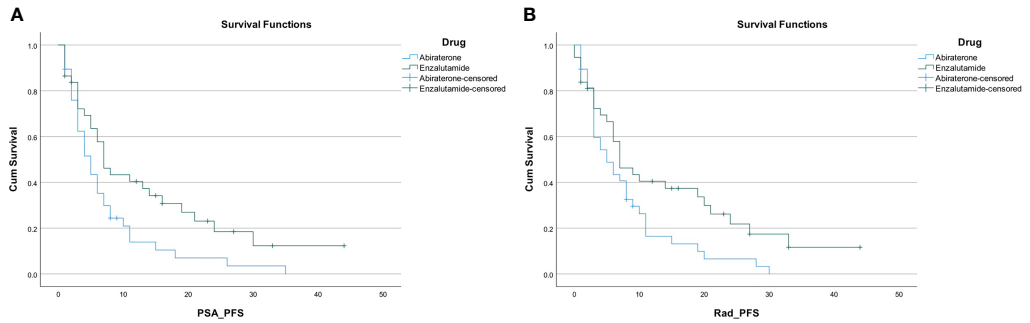


FIGURE 2 | **(A)** PSA progression free survival. **(B)** Radiographic progression free survival.

PSA PFS was found to be 7 months in the subgroup with low burden of disease, and 4 months in the subgroup with high burden of disease. PSA PFS was 7 months in the chemotherapy naïve subgroup, and 4 months in the chemotherapy experienced subgroup. Radiographic PFS was found to be 8 months in those with low burden of disease, and 6 months in those with high burden of disease. Radiographic PFS was 7 months in the chemotherapy naïve group, and 6 months in those who have had prior chemotherapy.

Overall survival was 24 months (95% CI, 15.5–32.5) in the entire cohort. Overall survival was found to be longer in patients receiving enzalutamide compared to abiraterone regardless of previous chemotherapy use; 30 months (95% CI, 23.3–36.7) versus 15 months (95% CI, 9.7–20.3; p=0.002) in those who were chemotherapy naïve; and 29 months (95% CI 21.3–36.7) versus 7 months (95% CI, 0–18.5; p=0.002) in those with prior chemotherapy use (**Figures 3A, B**).

On univariate analysis, enzalutamide use (HR 0.405; p value 0.002), dose reduction (HR 1.68; p value 0.05), ECOG performance status <2 (HR 0.71; p value 0.03) and high disease burden (HR 1.56; p value 0.05) had significant association with OS. Only ECOG < 2 (HR 0.66; p value 0.03) showed significant independent effects on survival on multivariate analysis. None of the other factors (age, presence or absence of visceral metastasis, Gleason's score or prior chemotherapy were associated with impact on OS on both univariate and multivariate analysis.

Tolerability

No patients were started on upfront dose reductions of either abiraterone or enzalutamide. Dose reductions subsequently occurred in 24% of the entire study population (18 out of 75 patients). A higher proportion of patients receiving enzalutamide required a dose reduction; 13 patients (35%) compared to 5 patients (13%) receiving abiraterone. Dose interruptions or delays occurred in 21% of the entire cohort (16 out of 75 patients); this appeared to be similar; 8 patients (21%) in each of the enzalutamide and abiraterone groups. The most common reasons for dose reductions or delays for patients on enzalutamide were fatigue (8 patients); 2 patients experienced drowsiness and 1 patient's enzalutamide was ceased after a haemorrhagic stroke. As for abiraterone, liver function test derangement (2 patients), drowsiness (2 patients), fatigue (1 patient) and an unrelated acute medical illness requiring hospital admission (1 patient) were reasons for dose reductions or delays. Reasons for dose reductions or delays in the other patients were not clear from the medical records.

DISCUSSION

The positive survival outcomes of abiraterone and enzalutamide have long been proven in the mCRPC population in large phase III clinical trials [COU-AA301 (1), COU-AA302 (2), AFFIRM

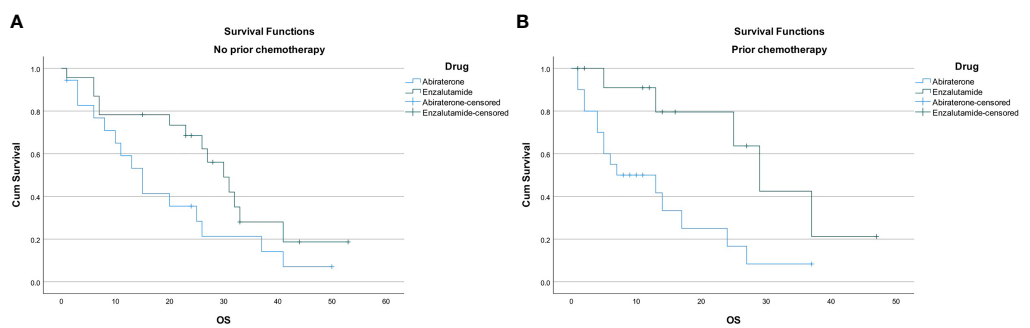


FIGURE 3 | **(A)** Overall survival in chemotherapy naive patients. **(B)** Overall survival in patients with prior chemotherapy use.

(3) and PREVAIL (4)]. Our study found that despite the variability in both patient and disease factors in the regional Australia real world setting, abiraterone and enzalutamide remain effective treatment options in our clinical practice, and provide a significant survival benefit and disease control in this group of patients with mCRPC.

To date, there is no evidence suggesting one drug is superior to the other in terms of survival outcomes. The four landmark clinical trials in this space (AFFIRM, PREVAIL, COU-AA301 and COU-AA302) demonstrated that the median OS in chemotherapy naïve mCRPC patients was close to 3 years for both enzalutamide and abiraterone; 32.4 months and 34.7 months respectively. PSA response rates were observed to be higher with enzalutamide; 54% versus 38% with abiraterone in the post docetaxel setting. Similarly, in a real world retrospective study conducted in the United Kingdom (9), a greater PSA50 (defined as the percentage of patients who had a PSA decline of at least 50% from baseline) was seen in the enzalutamide group compared to abiraterone group (58% versus 31% $p<0.0005$), but there was no significant median OS difference between the groups (enzalutamide 13.8 months versus abiraterone 12.5 months $p=0.065$). Responses on enzalutamide were further supported by a retrospective cohort study (10), that showed a PSA50 of 55% in 931 men with mCRPC on enzalutamide therapy. A meta-analysis (11) demonstrated superiority of enzalutamide over abiraterone in terms of radiographic PFS, time until PSA progression, and PSA response rate in both the pre- and post-docetaxel settings, but again OS did not differ significantly between the two drugs.

In our study, the survival outcomes from enzalutamide appear to match the results from the phase III trials more closely than abiraterone, which is likely due to the unbalanced disease burden and ECOG performance status between the two groups. Specifically, the OS with enzalutamide was about 30 months with or without prior chemotherapy, which is similar to results from AFFIRM and PREVAIL. PSA responses for enzalutamide seen in our study appear to be similar to other retrospective trials mentioned above (9, 10). Patients receiving abiraterone however appeared to have a much poorer survival outcome; only 15 months and 7 months for no chemotherapy and prior chemotherapy respectively. Interestingly, similar to our study, real world studies on abiraterone in mCRPC have showed poorer outcomes than in the COU trials. A Singaporean retrospective audit (12) looking at abiraterone in the real world mCRPC population of 200 patients demonstrated a median OS of 20 months for men who were chemotherapy naïve and only 11.3 months for men who have had prior chemotherapy. The variability in patient population in terms of ECOG performance status and comorbidities in the real world are important factors to consider given the majority of patients with prostate cancer are elderly often with multiple medical problems. These chronic medical issues particularly active cardiovascular comorbidities such as ischemic heart disease, congestive heart failure and strokes can affect the type of anti-cancer therapy these patients with mCRPC receive. Abiraterone is associated with more frequent cardiac events, myocardial infarction, arrythmia and heart failure (13), hence it is usually contraindicated in patients with cardiac comorbidities in particular congestive heart failure. In the phase III trials, the median age was 70 years old and majority of participants (up to 90%) were ECOG 0–1. In contrast, our real world study included patients with a median age of 80 years old, with majority being ECOG 1–2 with 20% of patients being ECOG 3. Compliance rates due to side effect profile and psychosocial factors can also be variable in the real world population, and can ultimately affect survival outcomes (14). As a result, the real world patient population at times are under represented in large phase III clinical trials where disease factors (such as Gleason score, burden of disease, and presence of visceral metastases) are the key differentiating mechanisms affecting outcomes.

One of the limitations to this study is that it was a small single-centre retrospective study. The unbalanced patient and disease characteristics between the two groups likely contributed to the differences in outcomes. There was a higher proportion of patients taking abiraterone who were classified as having high burden of disease, and more patients on abiraterone had prior chemotherapy (docetaxel) use compared to those on enzalutamide. This would suggest that the group taking abiraterone likely had a more aggressive biology of their metastatic prostate cancer, requiring more lines of treatment prior to abiraterone. Majority of patients on abiraterone were ECOG 2 compared to those on enzalutamide who were ECOG 1 and thus more medically fit. The greater proportion of ECOG 1 and 2 patients noted in our audit compared to the COU trials would be consistent with our practice of commencing these less medically fit patients on androgen receptor targeted agents rather than chemotherapy. In the real word setting, mCRPC patients with advanced age and poorer performance status are sequenced to a different anti-androgen rather than chemotherapy on disease progression due to concerns about tolerance. This can have impact on survival outcomes. A subset analysis in Chan et al. of chemotherapy naive patients with an ECOG 2–4 showed a poorer OS and PFS (12). Similarly, Boegemann et al. (15), also showed that poorer ECOG was associated with shorter time to treatment failure.

There has been a few sequencing studies of abiraterone and enzalutamide in the mCRPC space. Khalaf et al. (5) suggest that enzalutamide may be used effectively after abiraterone (rather than vice versa), based on the improved second PSA response and time to second PSA progression. Another single arm, multicentre study (16) included 214 mCRPC patients who commenced on enzatalumide 24 weeks or more after progressing on abiraterone and prednisolone, with or without prior chemotherapy. This study showed a median radiographic PFS of 8.1 months (95% CI: 6.1–8.3) and a median time-to-PSA progression of 5.7 months (95% CI: 5.6–5.8), however the median OS had not been reached. The anti-tumour activity of enzalutamide after abiraterone was further confirmed by Azad et al. (17), demonstrating a median time to PSA progression of 4.63 months (95% CI: 3.11–6.15) and 6.64 months (95% CI: 2.82–10.46), and a median OS of 10.58 months (7.16–14.00) and 8.64 months (6.57–11.71) for both chemotherapy experienced

and chemotherapy naïve patients respectively. However, the CARD randomised control trial showed a significantly longer median PFS and OS in mCRPC patients given cabazitaxel compared to those who had abiraterone after enzalutamide or vice versa (18), concluding that in this group of patients if fit enough, further chemotherapy is still the preferred option. In our study, only 9 (24%) of patients in the abiraterone group received previous enzalutamide and 2 (5%) of patients in the enzalutamide group received previous abiraterone; hence making the numbers too small to draw any conclusions.

In terms of tolerability, the REAAcT prospective, real-world study showed grade 3 and 4 adverse events appeared to be similar for both abiraterone and enzalutamide, although fatigue was more commonly reported by patients on enzalutamide compared to those on abiraterone (26% vs 8%). In this study, there was found to be a statistically significant worsening of fatigue for patients on enzalutamide using the FACIT (Functional Assessment of Chronic Illness Therapy Fatigue subscale)-Fatigue score, but not using the other two patient reported outcome instruments. Dose reductions were more common in enzalutamide (16% vs 6%) but dose adjustments and interruptions were similar (19). Similarly, a retrospective cohort study from the British Columbia Cancer Agency looking at abiraterone and enzalutamide in elderly patients with mCRPC showed more patients treated with enzalutamide needed dose reductions due to fatigue (20). We observed in our study that dose reductions were also more frequent for patients on enzalutamide than those on abiraterone, but dose interruptions and delays appeared to be similar in both groups. Similar to previous real world studies, we observed that fatigue was the main reason for dose reductions or delays in the enzalutamide group.

Recently, an electronic CRPC Australian database (ePAD), which is a multi-site, national prospective cohort study, has been commenced to analyse treatment patterns and outcomes from real-world patients with CRPC. Data is being collected regarding baseline patient characteristics, details at diagnosis, pathological characteristics, local treatment and use of androgen deprivation therapy, diagnosis of castration-resistance, prescription of and effectiveness of each systemic therapy and survival (21). This will aim to provide further guidance to Australian medical oncologists when it comes to decision making around systemic treatment selection and rationale for change of treatments in our CRPC patients.

CONCLUSION

Both abiraterone and enzalutamide will remain standard of care treatments in Australian men with mCRPC, as the survival and disease control benefits of these agents have continued to be seen in numerous real world studies, consistent with the phase III clinical trials. Although some retrospective studies demonstrate the superior efficacy of enzalutamide over abiraterone, to date very limited prospective trials with head-to-head comparison between these agents exist to adequately support these results. ECOG performance status and to a lesser extent age, which are key variability factors in the real world population do have an impact on survival outcomes. We await data from the Australian ePAD registry to further provide us with real world patient outcomes to support and improve our clinical practice in the mCRPC space.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

RR, first author, involved in performing all data collection and preparation of manuscript and poster (eposter) presentations for various conferences AS, second and corresponding author, involved in performing data analysis and review of manuscript and provision of feedback and comments to first author. MK, subsequent author, involved in review of manuscript and provision of feedback. JT, subsequent author, involved in review of manuscript and provision of feedback. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.656146/full#supplementary-material

REFERENCES

1. De Bono J, Logothetis C, Molina A, Fizazi K, North S, Chu L, et al. Abiraterone and Increased Survival in Metastatic Prostate Cancer. *N Engl J Med* (2011) 364:1995–2005. doi: 10.1056/NEJMoa1014618
2. Ryan C, Smith M, Fizazi K, Saad F, Mulders P, Sternberg C, et al. Abiraterone in Metastatic Prostate Cancer Patients Without Previous Chemotherapy. *N Engl J Med* (2013) 368:138–48. doi: 10.1056/NEJMoa1209096
3. Beer T, Armstrong A, Rathkopf D, Loriot Y, Sternberg C, Higano C, et al. Enzalutamide in Metastatic Prostate Cancer Patients Before Chemotherapy. *N Engl J Med* (2014) 371:424–33. doi: 10.1056/NEJMoa1405095
4. Scher H, Fizazi K, Saad F, Taplin M, Sternberg C, Miller K, et al. Increased Survival With Enzalutamide in Prostate Cancer After Chemotherapy. *N Engl J Med* (2012) 367:1187–97. doi: 10.1056/NEJMoa1207506
5. Khalaf DJ, Annala M, Taavitsainen S, Finch DL, Oja C, Vergidis J, et al. Optimal Sequencing of Enzalutamide and Abiraterone Acetate Plus Prednisone in Metastatic Castration-Resistant Prostate Cancer: A Multicentre, Randomised, Open-Label, Phase 2, Crossover Trial. *Lancet Oncol* (2019) 20(12):1730–9. doi: 10.1016/S1470-2045(19)30688-6
6. Fang M, Nakazawa M, Antonarakis E, Li C. Efficacy of Abiraterone and Enzalutamide in Pre- and Postdocetaxel Castration-Resistant Prostate Cancer: A Trial-Level Meta-Analysis. *Prostate Cancer* (2017) 3:1–8. doi: 10.1155/2017/8560827

7. Scher H, Morris M, Stadler W, Higano C, Fizazi K, Antonarakis E, et al. Trial Design and Objectives for Castration-Resistant Prostate Cancer: Updated Recommendations From the Prostate Cancer Clinical Trials Working Group 3. *J Clin Oncol* (2016) 34:1402–18. doi: 10.1200/JCO.2015.64.2702
8. Miller AB, Hoogstraten B, Staquet M, Winkler A. Reporting Results of Cancer Treatment. *Cancer* (1981) 47:207–14. doi: 10.1002/1097-0142(19810101)47:1
9. Rowe M, Walter S, Hidayat A, Donkers H, Browne A, Norris T, et al. Real World Patterns of PSA Response and Survival With Abiraterone and Enzalutamide in Metastatic Castrate Resistant Prostate Cancer. *Sci Repository* (2020) 3(7):2–6. doi: 10.31487/j.COR.2020.07.11
10. Freedland S, Schultz N, Coutinho A, Fuldeore R, Hedlund N, Feng Q, et al. Real-World Analysis of Prostate-Specific Antigen (PSA) Outcomes Among Patients With Metastatic Castration-Resistant Prostate Cancer (mCRPC) Treated With Enzalutamide (ENZA). *J Clin Oncol* (2019) 38(suppl 6):33. doi: 10.1200/JCO.2020.38.6_suppl.33
11. Chopra A, Haaland B, Lopes G. Comparative Effective Analysis Between Enzalutamide and Abiraterone in the Treatment of Metastatic Castration-Resistant Prostate Cancer. *J Clin Oncol* (2013) 31(6_suppl):217–7. doi: 10.1200/jco.2013.31.6_suppl.217
12. Chan J, Yap S, Fong Y, Lim H, Toh C, Ng Q, et al. Real-World Outcome With Abiraterone Acetate Plus Prednisone in Asian Men With Metastatic Castrate-Resistant Prostate Cancer: The Singapore Experience. *Asia Pac J Clin Oncol* (2020) 16:75–9. doi: 10.1111/ajco.13241
13. Cone E, Reese S, Marchese M, Nabi J, Kilbridge K, Trinh QD, et al. Association of Abiraterone and Higher Odds of Cardiac Complications Compared to Enzalutamide. *J Clin Oncol* (2020) 38(6_suppl):70–0. doi: 10.1200/JCO.2020.38.6_suppl.70
14. Behl A, Ellis L, Pilon D, Xiao Y, Lefebvre P. Medication Adherence, Treatment Patterns and Dose Reduction in Patients With Metastatic Castrate- Resistant Prostate Cancer Receiving Abiraterone Acetate or Enzalutamide. *Am Health Drug Benefits* (2017) 10(6):296–303.
15. Boegemann M, Khaksar S, Bera G, Birtle A, Dopchie C, Dourthe L, et al. Abiraterone Acetate Plus Prednisone for the Management of Metastatic Castration-Resistant Prostate Cancer (mCRPC) Without Prior Use of Chemotherapy: Report From a Large, International, Real-World Retrospective Cohort Study. *BMC Cancer* (2019) 19(1):60. doi: 10.1186/s12885-019-5280-6
16. De Bono J, Chowdhury S, Feyerabend S, Eliott T, Grande E, Melhem-Bertrandt A, et al. Antitumour Activity and Safety of Enzalutamide in Patients With Metastatic Castration-Resistant Prostate Cancer Previously Treated With Abiraterone Acetate Plus Prednisone for ≥24 Weeks in Europe. *Eur Urol* (2018) 74(1):37–45. doi: 10.1016/j.eururo.2017.07.035
17. Azad A, Eigl BJ, Murray N, Kollmannsberger CK, Chi KN. Efficacy of Enzalutamide (Enza) Following Abiraterone Acetate (Abi) in Chemotherapy-Naïve Metastatic Castration-Resistant Prostate Cancer (mCRPC) Patients (Pts). *Prostate Cancer Prostatic Dis* (2015) 67(1):23–9. doi: 10.1038/pcan.2014.53
18. De Wit R, de Bono J, Sternberg C, Fizazi K, Tombal B, Wülfing C, et al. Cabazitaxel Versus Abiraterone or Enzalutamide in Metastatic Prostate Cancer. *N Engl J Med* (2019) 381:2506–18. doi: 10.1056/NEJMoa1911206
19. Shore N, Saltzstein D, Sieber P, Mehlhaff B, Gervasi L, Phillips J, et al. Real-World Study of Enzalutamide and Abiraterone Acetate (With Prednisone) Tolerability (ReaacT): Results. *J Clin Oncol* (2018) 36(suppl 6):296–6. doi: 10.1200/JCO.2018.36.6_suppl.296
20. Khalaf D, Zou K, Struss WJ, Eigl BJ, Kollmannsberger CK, Finch D, et al. Efficacy and Tolerability of First-Line Abiraterone + Prednisone (ABI) Versus Enzalutamide (ENZ) for Metastatic Castration-Resistant Prostate Cancer (mCRPC) in Men ≥ 80 Years: A Retrospective Cohort Study. *J Clin Oncol* (2018) 36(15_suppl):5051–1. doi: 10.1200/JCO.2018.36.15_suppl.5051
21. *Prostate Cancer Registry (Electronic CRPC Australian Database, ePAD) 2016-2019.*

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 16 September 2021
doi: 10.3389/fonc.2021.703848

Psychosocial Stress and Age Influence Depression and Anxiety-Related Behavior, Drive Tumor Inflammatory Cytokines and Accelerate Prostate Cancer Growth in Mice

Denise L. Bellinger[1]*, *Melissa S. Dulcich*[2], *Christine Molinaro*[1], *Peter Gifford*[1], *Dianne Lorton*[3], *Daila S. Gridley*[4] *and Richard E. Hartman*[2]

[1] Department of Pathology & Human Anatomy, School of Medicine, Loma Linda University, Loma Linda, CA, United States, [2] Department of Psychology, School of Behavioral Health, Loma Linda University, Loma Linda, CA, United States, [3] Department of Psychology, Kent State University and the Kent Summa Initiative for Clinical and Translational Research, Summa Health System, Akron, OH, United States, [4] Departments of Radiation Medicine and Biochemistry and Microbiology, School of Medicine, Loma Linda University, Loma Linda, CA, United States

OPEN ACCESS

Edited by:
Benyi Li,
University of Kansas Medical Center, United States

Reviewed by:
Yu H. Sun,
University of Rochester, United States
Qiuyang Zhang,
Tulane University, United States

***Correspondence:**
Denise L. Bellinger
dbellinger@llu.edu

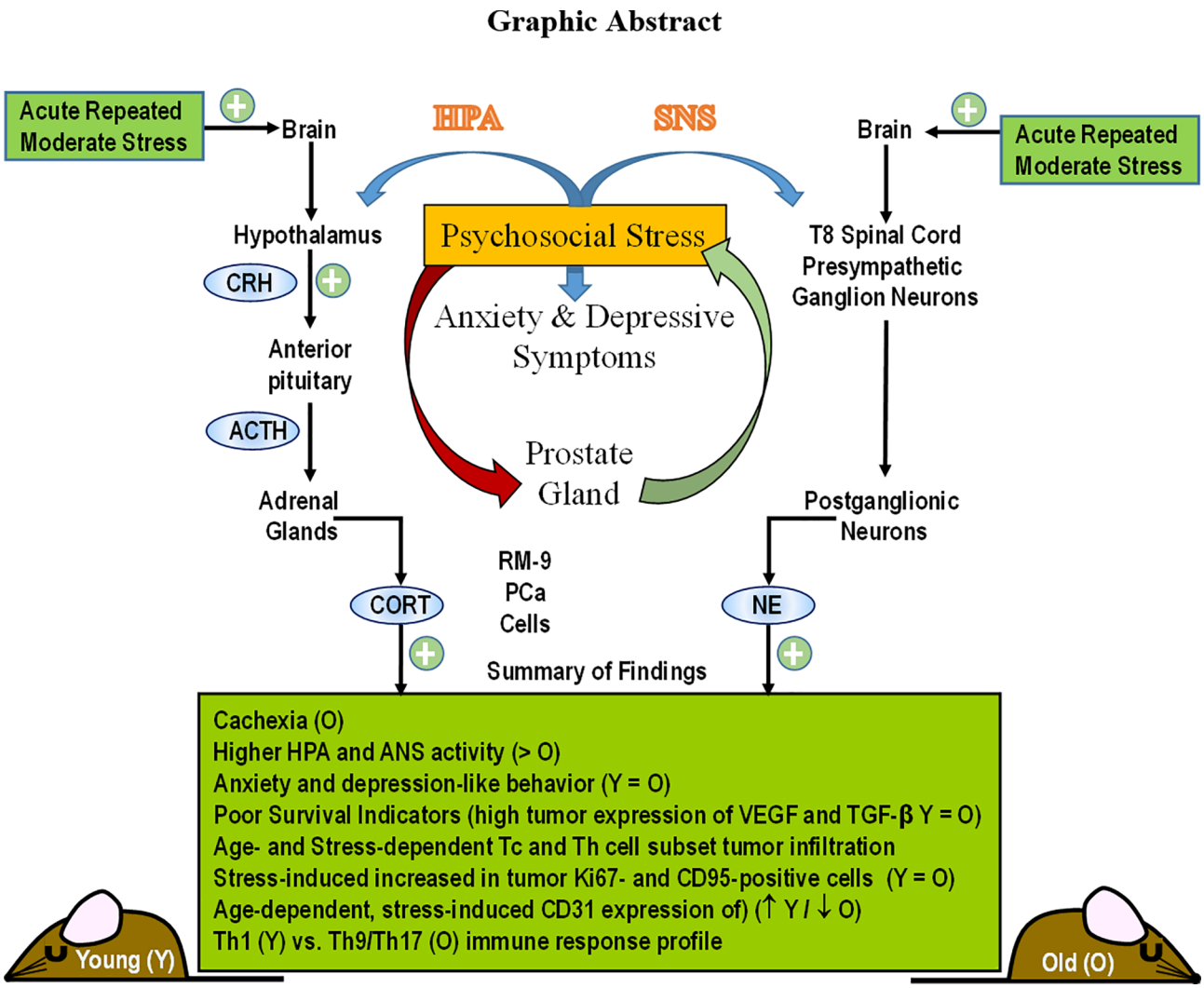
Specialty section:
This article was submitted to Genitourinary Oncology, a section of the journal Frontiers in Oncology

Received: 30 April 2021
Accepted: 09 July 2021
Published: 16 September 2021

Citation:
Bellinger DL, Dulcich MS, Molinaro C, Gifford P, Lorton D, Gridley DS and Hartman RE (2021) Psychosocial Stress and Age Influence Depression and Anxiety-Related Behavior, Drive Tumor Inflammatory Cytokines and Accelerate Prostate Cancer Growth in Mice. Front. Oncol. 11:703848. doi: 10.3389/fonc.2021.703848

Prostate cancer (PCa) prevalence is higher in older men and poorer coping with psychosocial stressors effect prognosis. Yet, interactions between age, stress and PCa progression are underexplored. Therefore, we characterized the effects of age and isolation combined with restraint (2 h/day) for 14 days post-tumor inoculation on behavior, tumor growth and host defense in the immunocompetent, orthotopic RM-9 murine PCa model. All mice were tumor inoculated. Isolation/restraint increased sympathetic and hypothalamic-pituitary-adrenal cortical activation, based on elevated serum 3-methoxy-4-hydroxyphenylglycol/norepinephrine ratios and corticosterone levels, respectively. Elevated zero maze testing revealed age-related differences in naïve C57Bl/6 mice, and increased anxiety-like behavior in tumor-bearing mice. In open field testing, old stressed mice were less active throughout the 30-min test than young non-stressed and stressed, and old non-stressed mice, suggesting greater anxiety in old stressed mice. Old (18 month) mice demonstrated more depression-like behavior than young mice with tail suspension testing, without effects of isolation/restraint stress. Old mice developed larger tumors, despite similar tumor expression of tumor vascular endothelial growth factor or transforming growth factor-beta1 across age. Tumor chemokine/cytokine expression, commonly prognostic for poorer outcomes, were uniquely age- and stress-dependent, underscoring the need for PCa research in old animals. Macrophages predominated in RM-9 tumors. Macrophages, and $CD4^+$ and $CD4^+FoxP3^+$ T-cell tumor infiltration were greater in young mice than in old mice. Stress increased macrophage infiltration in old mice. Conversely, stress reduced intratumoral $CD4^+$ and $CD4^+FoxP3^+$ T-cell numbers in young mice. $CD8^+$ T-cell infiltration was similar across treatment groups. Our findings support that age- and psychological stress interacts to affect PCa outcomes by interfering with neural-immune mechanisms and affecting behavioral responses.

Keywords: psychosocial stress, aging, tumor immunity, IL-9/IL-17 balance, anxiety/depression-related behavior



GRAPHICAL ABSTRACT |

INTRODUCTION

Prostate cancer (PCa) is the most prevalent cancer, and third most common cause of cancer-related death in men (1). Psychological stress and depression, which can alter the expression of cancer-linked genes in the prostate, is prevalent in patients with PCa (2). PCa incidence is directly linked to patient age, and perceived stress can increase with increasing age (3). Studies investigating the effects of age- and psychosocial stress-related changes on the microenvironment of PCa is limited, despite PCa being the most prevalent cancer in men. In this population there is a five-year relative survival rate for all stages of PCa. The 5-year survival rate for men with metastatic PCa is only 30% (4). Identifying mechanisms in which psychosocial stress affects the pathophysiology and disease outcomes of PCa in animal models may lead to improved patient care.

Anxiety and depression are major challenges for PCa survivors, particularly in the first 5 to 10 years post-cancer diagnosis (5–8). However, men rarely seek mental health care (5), despite that depression negatively impacts survival of men with metastatic PCa (6). Acute repetitive or chronic stress, anxiety and depression are relatively high in men with PCa (8), and may be predictive of cancer progression and/or mortality (9). Murine models of stress have significantly advanced our knowledge of mechanisms responsible for stress-induced changes in inflammation and immunity in other types of cancer, but research in this area for PCa is comparatively limited (2, 6–10).

Mood disorders in cancer survivors are proposed to evolve from combinations of tumor pathophysiology, cancer interventions, and stress (10). The impact of each of these is difficult to dissect out in the clinical setting. Animal research can control for confounding variables difficult to control for in clinical settings and may be used to disentangle mechanistic

interactions between neural, immune and endocrine processes. Among these factors are chronic inflammation and anti-tumor immunity that are reported to correlate with fatigue and persistent negative affect (11). Mood, comorbidities and inflammation exist before cancer diagnosis and treatments (12, 13).

Hypothalamic-pituitary-adrenal (HPA), vagal nerve, and sympathetic nervous system (SNS) activity and their response to cancer- and/or treatment-related challenges are altered in cancer patients (14–19). Age-related changes in autonomic innervation of the prostate gland and changes in nerve activity also can influence PCa development and progression (15, 19). Autonomic and HPA pathways exert potent anti-inflammatory actions and influence behavior (20, 21). Mechanistic interactions between these factors remain unresolved.

Rodent models are the mainstay research tools to systematically identify the etiology of behaviors comorbid with cancer. Psychosocial stressors promote prostate carcinogenesis in mice that is sympathetically-mediated *via* regulation of anti-apoptotic signal pathways (22). For example, restraint stress altered the expression of cancer-related genes in the prostate (2). Herrara-Corvarrubias et al. (23) reported that an immune stressor during puberty promotes precancerous lesions in adult rats. Decker et al. (24) found that the SNS reactivated quiescent PCa cancer cells and promoted their metastases to the bone marrow. This research supports the idea that psychosocial interactions can significantly influence prostate physiology and PCa progression, consistent with breast cancer research that supports stress as an important moderator of tumor progression (25–27).

Preclinical research has targeted neural-immune-mediated mechanisms in tumor biology. Altered neural functions that manifest as depressive and anxiety-like behaviors are present in many rodent models of solid tumors (28–30). Given the rising and aging population of cancer survivors, it is important to understand the behavioral consequences of a cancer diagnosis and progression that contributes to disease pathophysiology.

One hallmark of the aging prostate is tissue remodeling and greater inflammatory cell infiltration that contribute to the age-related pathology observed in the prostate (31–33). Anti-tumor immunity can markedly differ in prostate tumor models using young or old mice. During aging, both molecular and structural changes develop to disrupt matrix components, and promote a proinflammatory microenvironment. These changes include stromal proliferation, robust T cell and macrophage infiltration and up-regulated proinflammatory cytokines and growth factors that are contributory to benign hyperplasia, prostatitis, and PCa (34). Remodeling of the extracellular matrix in the aged prostate microenvironment is also linked with greater PCa growth and invasion. Compared with young mice, prostate tumor cells orthotopically inoculated into the prostate, grow at an accelerated rate in old mice (31–35). Taken together, these findings demonstrate an aged prostatic microenvironment whereby resident immune cells, particularly macrophages and their polarization, adopt a protumorigenic phenotype that collaborates with the extracellular matrix to advance PCa in aging mice, and by extension aging men. Understanding the regulation of key mediators of PCa progression in the tumor microenvironment of the aged prostate is necessary to improve treatment of elderly men with PCa.

The aim of this study was to use an age-appropriate, syngeneic, immunocompetent, orthotopic animal model of PCa to evaluate the effects of age and chronic psychological stress that induces anxiety- and depressive-like behaviors on cancer progression and anti-tumor immunity. Using the RM9 prostate cancer cell line, a murine prostate reconstitution (MPR3) model was established young and aging male C57BL/6 mice for this purpose. In this paper, we report significant effects of both age and chronic stress on (1) depression-like behaviors, (2) stress pathway activation, (3) PCa progression based on local measures of proliferation, cell death, vascularization, and immune cell infiltration into RM-9 tumors cell. Collectively, our findings indicate that both age and psychosocial factors can interact to affect anti-tumor immunity and PCa outcome.

MATERIALS AND METHODS

Animals

Two- and 18-month-old male C57BL/6 mice (young and old, respectively) were purchased from the NIA colony (Charles Rivers Laboratories, Wilmington, MA). Upon arrival, mice were housed 4-5 per cage in the vivarium at Loma Linda University; Mice shipped in the same containers were housed together to minimize fighting, and were acclimated to vivarium conditions for 1 week (temperature, 22 ± 1°C; humidity, ~50%; 12-h light/dark cycling, environmental enrichment, and food and water provided *ad libitum*). Mice were then acclimated to handling for one week to minimize distress during the study, and then inoculated orthotopically with syngeneic PCa cells (**Figure 1**). Mice were observed for general health throughout surgery and post-surgery recovery for tumor inoculation. Feeding, drinking, and grooming behaviors were monitored and recorded. Animal procedures were approved by the Institutional Animal Care and Use Committee, in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

RM-9 Cells

The mouse prostate reconstitution or MPR^3 model system using RM-9 prostate tumor cells was chosen to closely mimic complex, morphological, immunological, and molecular changes that underlie PCa (36–38). RM-9 tumor cells have similar mutations or aberrant activities of ras, myc, and p53 as in human PCa cells (36–38), and a low MHC class I profile as in many human PCa cell lines (36–38). The RM-9 PCa cell line is derived from a ras$^+$ myc transformed/wild-type p53 primary prostate tumor induced in the Zipras/myc-9-infected C57BL/6 murine prostate reconstitution (MPR^3) model (36–38). RM-9 cells were generously provided by Dr. Timothy C. Thompson at the Baylor College of Medicine in Houston, TX.

RM-9 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose and L-glutamine (GIBCO,

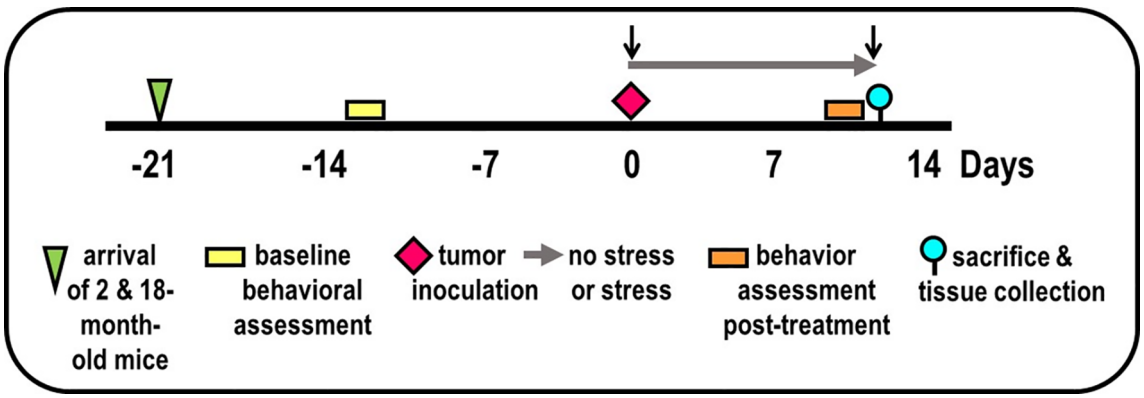


FIGURE 1 | Experimental Design. Experimental timeline of events is illustrated, including mice arrival (triangle), accommodation to vivarium conditions, pre- and post-treatment behavioral testing (yellow/orange bars, respectively), tumor inoculation (diamond), collection of blood by retroorbital bleeding (↓), and tissue collection for endpoint assessments (circle).

Grand Island NY) and supplemented with 10% fetal bovine serum (Omega Scientific Inc., Tarzana, CA), 10 mM HEPES buffer (Hyclone Laboratories, Inc., Logan, UT), 50 international units (IU)/ml penicillin and 50 μg/ml streptomycin (Mediatech Inc., Manassas, VA) at 37°C in a humidified atmosphere containing 5% CO_2. RM-9 cells were passaged by trypsinization with 0.05% trypsin/0.53 mM EDTA in HBSS without sodium bicarbonate, calcium or magnesium (Mediatech Inc., Manassas, VA). RM-9 cells were counted, viability assessed using the trypan blue exclusion method (Sigma-Aldrich, St. Louis, MO) and then resuspended in medium. RM-9 cells were frozen at passage 14, thawed out and cultured prior to each experiment.

Tumor Induction

RM-9 cells were trypsinized, washed 1X with 10 ml DMEM, resuspended in medium and counted. The viability was 90-94%. One ml of cells, adjusted to a concentration of 5 x 10^5 cells/ml, were centrifuged at 200g for 8 min at 4°C. Supernatants were discarded, and the cells resuspended in 1 ml sterile saline.

After vivarium accommodation and baseline behavioral evaluation (**Figure 1**), mice were anesthetized with sodium pentobarbital. A low transverse abdominal incision was made, and the dorsolateral prostate was exposed. A 10-μl suspension of 5,000 RM-9 cells was injected into the dorsolateral prostate to induce an *in situ* primary prostate adenocarcinoma in immune-competent mice (37). The incision was closed with wound clips. The viability of the RM-9 cells used for inoculation was 83-89% post-tumor inoculation. To control for surgery effects, (i.e., tissue repair, anesthesia), additional mice (n=8) that received no treatment or that were inoculated with the vehicle minus RM-9 cells were included in the study to control for surgical effects on behaviors assessed in this study.

Affective-Like Behavior

Two standardized tests of anxiety-like behavior (open field test (OFT)) and elevated zero maze (EZM)) and one test of depressive-like behavior (tail suspension test (TST)) (39) were administered in that order over 3 consecutive days between 900-1100 h Pacific Standard Time. This affective-like behavior battery was administered D14 before tumor inoculation and prior to the study endpoint (see **Figure 1**). Two researchers, who were blinded to the treatment groups, scored all behavior videos. Prior to restraint-stressing, mice were tested for baseline stress activity in the EZM. Post restraint-stressing, anxiety and learned helplessness were tested with the EZM, OFT, and the TST.

Anxiety-Related Behavior

The EZM consists of a 10-cm wide circular plastic track (100-cm outer diameter and elevated off the floor) with 35-cm tall walls enclosing 2 opposing quadrants. The room lights were dimmed, and halogen lights directly illuminated the open spaces of the maze. Animals were initially placed in the center of one of the open quadrants and their activity was monitored for 5 min. Time spent within the enclosed quadrants was calculated.

General Activity Levels/Movement Patterns

The OFT was used to assess anxiety-like behaviors and locomotion. Each animal was placed in a 49x36-cm^2 opaque open-topped plastic bin for 30 min. The movements of each animal were recorded by an overhead camera and analyzed by a computerized tracking system (Noldus Ethovision, Leesburg, VA). A loose layer of bedding was added, and the arena was cleaned with 10% bleach between mice. The distance the animal moved, percent time spent moving, time spent in the perimeter and center of the test area were measured.

Learned Helplessness/Depression

The standardized TST assesses depression-like behaviors and learned helplessness (40). The animal is placed in an inescapable, uncomfortable situation, and immobility (lack of struggling) is measured. Mice were suspended for 6 min by the tail with adhesive tape attached approximately 1 cm from the tip of the tail. The other end of the tape was wrapped around a hook embedded in the center of the ceiling of a wooden box measuring 19L x 21W x 40H cm. When suspended, the animal's nose was

approximately 20 cm from the floor. The box was partitioned and enclosed on all sides except one (for viewing). Room lighting and sound were kept to a minimum. A partition visually isolated each mouse.

While the animal struggled to escape its position, two researchers blinded to treatment group individually rated the mouse on immobility and agitation. The percent time immobile was calculated for the final 4 min. Immobility was defined as a complete lack of voluntary movement by the mouse. An animal was also rated immobile if it was curled up, appearing to rest while holding its front paws to its back paws, but was not struggling or moving.

Experimental Design

Two replicate experiments were performed, each with 25 mice per age per experiment (100 mice total), i.e., the maximum number per purchase by NIA. Mice were assigned to groups based on (1) obtaining equivalent mean and variance for baseline EZM performance (→) between treatment groups (to reduce the possibility of pre-existing differences in affective behaviors), and (2) maintaining existing housing for group-housed mice. Maintaining housing conditions for group-housed mice alleviated the effects that housing rearrangement would have on aggressive behavior and fighting between cage mates. Baseline behavioral testing (▭) using the EZM was performed 1-week after arrival (▽) of mice to the vivarium (**Figure 1**). On D0, mice were anesthetized, blood was collected by retro-orbital bleeding (↓), then tumor cells were orthotopically implanted into the prostate (◆) in all mice. After surgery, mice in the non-stressed group were returned to their home cage (i.e., grouped), whereas mice in the stressed group were place individually in a novel cage (→). Mice were weighed every 2 days. Young and old tumor-bearing mice were group-housed (non-stressed) and isolated/restraint-stressed (stressed). On D1, individually housed mice were restrained by placing the animal in well-ventilated, capped PVC tubes (2.54 cm in diameter for young and 3.175 cm in for old) for 2 h/day for 13 days. For each day of restraint stress, the restraint was randomized both in time of day and order of mice. Before restraint stress on D13, EZM testing (▭) was performed on all mice. On D14 (▭), all mice were evaluated with the OFT, followed by the TST. At the time of sacrifice (D15), mice were weighed and then euthanized by an overdose of Nembutal (50 mg/kg, i.p.), bled retro-orbitally within 5 min after injection, and targeted tissues collected dissected (⚲).

Tissue Collection and Tumor-Related Assessments

After cardiac puncture, blood was collected (800-1100 h PST) in heparin-coated syringes. Serum glucocorticoid and catecholamines were quantified using an enzyme-linked immunosorbent assay (ELISA) or high-performance liquid chromatography with coulometric detection (HPLC-CD). Spleens and tumors were dissected, weighed and frozen on dry ice. The brain and visceral organs (lung, liver, adrenal and pituitary glands) were grossly examined for age-related tumors or overt pathology. No age-related pathologies were observed in 18 month-old C57Bl/6 mice, and as previously reported for young mice (36–38), RM-9 tumors were non-metastatic in old mice.

Body, spleen, and tumor weights, circulating stress hormones, relevant organ weights, and tumor cytokine expression were end-point measures. The *n*'s were 10 mice per non-stressed or 15 mice per stressed treatment groups per replicate study. Primary tumor and organs with metastatic potential – the pelvic and retroperitoneal lymph nodes that drain the tumor site, femur bone marrow and lungs, were dissected and weighed. Tissues were fixed in 10% buffered formalin, paraffin embedded, cut on a rotary microtome at 5 μm, and stained with hematoxylin and eosin (H&E) for light microscopy. A piece of the primary tumor was immunohistochemically-stained for specific immune cell subsets including T-helper, T-regulatory, T-cytotoxic cells, and F480, M1 and M2 macrophages.

Serum Stress Hormones

Corticosterone

To determine treatment group differences in corticosterone, blood from retro-orbital sinus bleeding was centrifuged at 2,000*g* for 10 min at 4°C, and serum was aliquoted and stored at -80°C. After thawing and diluting samples 1:25 with assay diluent, duplicate serum samples were assayed for corticosterone using an AssayMax ELISA kit following manufacturer's instructions (AssayPro, St. Charles, MO; minimal detection level: 40 pg/ml; intra-assay and inter-assay coefficients of variation: 5.0 and 7.0%, respectively). Samples with a coefficient variance greater than 15% were repeated. Absorbance was read on a microplate reader at wavelengths of 450 and 570 nm immediately after adding the stopping solution. A wavelength correction was made by subtracting readings at 570 nm from those at 450 nm to correct for optical imperfections. Corticosterone concentrations were determined from standard curves generated from serially diluted standards run in duplicate on each plate.

Catecholamines

Serum catecholamine concentrations were determined after alumina extraction by HPLC-CD using a CouleChem HPLC System (ESA, Chelmsford, MA). The peak heights and area under the curves were analyzed using EZChrom Elite Software (Scientific Software Inc., Pleasanton, CA). Known standards for norepinephrine, dopamine, epinephrine, and the norepinephrine catabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG) were used to determine sample levels and were corrected for recovery using 3, 4-dihydroxybenzylamine as the internal standard. The ratio of norepinephrine-to-MHPG concentration served as an index of norepinephrine turnover and SNS activity.

Tumor Growth Factor, Chemokine and Cytokine Expression

We evaluated tumor expression of growth factors, chemokines and cytokines known to be prognostic for poorer prostate cancer outcomes, as well as screening for novel immune markers that may potentially influence tumor growth. Frozen prostate tissue samples were homogenized using a PowerGen 125 tissue homogenizer (Fisher Scientific, Pittsburg, PA). Ten μl of 10 mM

Tris lysis buffer (pH 7.5) containing protease inhibitors (One Complete Mini tablet; Roche Mannheim, Germany) per 10 ml of buffer per mg tissue were added to each tissue sample. Samples were homogenized on ice. Homogenates were centrifuged in 1.5-ml Eppendorf tubes (4°C, 12,000*g* for 10 min). The supernatants were aliquoted into prelabeled 0.5-ml Eppendorf tubes and frozen at -80°C.

For TGF-β1, a 25-µl aliquot of prostate tissue homogenate of each sample was diluted with 75 µl of the Tris Lysis Buffer. Ten µl of 0.1 M HCl was added, per the manufacturer's recommendation (R&D Systems, Minneapolis, MN). The samples were briefly vortexed, and after 10 min, neutralized with 13 µl of a 1.2 M NaOH/0.5M HEPES solution for a final dilution factor of 4.92. The samples were assayed in 96-well plates using Quantikine TGF-β1 ELISA kits (R&D Systems). The optical density of each well was read within 30 min of adding the stop solution using a microplate reader set at 450 and 540 nm. Samples were run in duplicate. Samples with a coefficient variance greater than 15% were repeated. The average TGF-β1 concentrations from the duplicate sample readings were determined from the values of standards present in each 96-well plate. The lower limit of detection for TGF-β1 was 4.61 pg/ml (R&D Systems).

For tumor cytokine expression, multiplexed immunoassay kits were employed. Prostate tumors were homogenized in protein extraction buffer [phosphate-buffered saline (PBS)], 0.05% Triton-X, Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA) using acid-washed 1.4-mm zirconium beads and a benchtop BeadBug™ tissue homogenizer (Benchmark Scientific, Sayreville, NJ). Homogenates were sonicated for 1 min in a sonication bath (Branson M1800, Branson Ultrasonics, Danbury, CT) and centrifuged (10,000*g*, 20 *min*, 4°C). Multiplexed magnetic bead-based immunoassay kits (Catalog# MCYTMAG-70K-P X 32, Millipore Sigma, Burlington MA) were run to evaluate tumor cytokine expression, according to the manufacturer's instructions. Analytes assessed were granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial factor (VEGF), chemokine C-X-C motif ligand 10 (CXCL10) or interferon gamma-induced protein-10 (CXCL10/IP-10), keratinocyte-derived chemokine (CXCL1/KC), leukemia inhibitory factor (LIF), lipopolysaccharide-induced CXC chemokine (CXCL5/LIX), chemokine C-C motif ligand 2 or monocyte chemoattractant protein 1 (CCL2/MCP-1), macrophage colony-stimulating factor (M-CSF), monokine induced by γ-interferon (CXCL9/MIG), macrophage inhibitory protein-1α (CCL3/MIP-1α), macrophage inhibitory protein-1α (CCL4/MIP-1β), macrophage inhibitory protein-2 (CXCL2/MIP-2), regulated upon activation, normal T cell expressed and presumably secreted (CCL5/RANTES), tumor necrosis factor (TNF-α), interferon-γ (IFN-γ), and interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, and IL-17.

Quantitative Immunostaining of Tumor Progression Markers and Infiltrating Leukocytes

Tumor Progression and Leukocytes Markers

Tumor cell growth, apoptosis, and vascularization were evaluated with quantitative immunofluorescence staining for Ki-67, CD95 (apoptosis antigen 1), and endothelial cell-specific vascular marker, CD31, respectively, as prognostic/predictive markers for PCa progression (41–43). F4/80CD8a, and CD4 with or without FoxP3 antibodies were used to evaluate immune cell tumor infiltration (see **Table 1** for detailed antibody information and dilutions).

Tissue Preparation and Immunostaining

Orthotopic prostate tumors were isolated, dissected, and weighed. A portion of the tumor was placed in a 1.5-ml microfuge tube; the remainder was flash frozen in liquid nitrogen and stored in a -80°C freezer. Frozen tissue was mounted in embedding medium and sectioned at 6 µm using a cryostat (Leica CM 1900, Leica Microsystems Inc., Buffalo Grove, IL) set at -20°C. Tissue sections were taken starting at mid-tumor so that the cross-sections were closely matched between samples and representative of intratumoral tissue. Cut sections were thaw-mounted onto charged slides (Surgipath Medical Industries, Richmond, IL), and stored at -20°C.

For immunohistochemical staining, slide-mounted tissues were rinsed briefly in cold 0.15M PBS, (pH 7.2-7.4) to remove embedding medium, fixed for 10 min in acetone at -20 °C, and then rinsed in PBS (3x2 min). The slides were placed in Coplin jars containing 10% normal goat serum in PBS (30 min) to block nonspecific binding, and were rinsed in PBS (3x2 min). Slides were removed, wiped dry around the tissue, and each section was circumscribed with generic nail polish using a 3-ml syringe with a 26-gauge needle and allowed to dry. The primary antibody (or antibodies, if double-labeled) was (were) diluted following the manufacturer's recommendation in antibody diluent (1% Triton-X™ and 5% bovine serum albumin in PBS) (see antibody information in **Table 1**). The antibody was applied to the tissue, and slides were incubated in a humidified chamber (2 h),

TABLE 1 | Primary antibodies for immunohistochemical staining in orthotopic RM-9 tumors.

1° Antibody	Clone	Isotype	Immunogen	Host	Dilution	Supplier
Ki67	PA5-19462	Rabbit IgG	Human residues 1200-1300	rat	1:100	Invitrogen
CD95 (Fas)	SolA15	Rat IgG2a, κ	Recombinant protein epitope	rabbit	1:200	Millipore/Sigma
CD31 (PeCAM-1)	—	Goat IgG	Mouse myeloma cell line	goat	1:100	R&D Systems
F4/80	(clone CI:A3-1)	IgG2b	Mouse F4/80 antigen	rat	1:100	AdD Serotec
CD4	(L3T4; Rat (LOU,) clone H129.19)	IgG2a, κ	A.TH mouse CTL clone A15.17	rat	1:50	BD Bioscience
CD8a	(Lyt-2; Rat LOU, clone 53-6.7)	IgG2a, κ	Mouse thymus/spleen cells	rat	1:50	BD Bioscience
FoxP3	(clone FJK-16s)	IgG	Amino acid sequence 75-125	rabbit	1:50	Invitrogen

then rinsed in PBS (5x2 min). Next, a fluorescently-tagged secondary antibody (goat anti-rat Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 555, Life Technologies, Carlsbad, CA) was diluted 1:500 with the antibody diluents and applied to the tissue. The slides were incubated in a humidified chamber (2h), and then rinsed in PBS (5x2 min), and coverslipped with Prolong™ Gold Antifade containing DAPI (4', 6-diamidino-2-phenylindole) Mountant (Life Technologies, Carlsbad, CA). Slides were placed in the dark at room temperature overnight to dry, then stored at -20°C.

Imaging of stained tissue was carried out blinded to treatment group using an Olympus BH-2 microscope equipped with a digital camera (Optronics, Goleta, CA). Quantitative analyses were performed on images captured within the tumors, specifically avoiding peritumoral regions. All images were captured using 200X total magnification. An average of 5-6 non-overlapping fields (0.162 mm^2) was randomly sampled in RM-9 tumors. Fields used for analyses were selected using the DAPI filter to avoid bias toward immune cell markers of interest. Immunohistochemically-stained cells in each field were enumerated using Image-Pro Plus™ Version 3.1 software (Media Cybernetics, Bethesda, MD). Cell counts from each field sampled per tumor were averaged for each subject (mean total number of positive cells per sample field), and group means ± SEM were calculated (i.e., mean of a mean) with an *n* of 6-13 mice per group.

Statistical Analysis

For hormone and cytokine analyses, two-way ANOVA and Tukey's post-hoc tests were used to determine statistical significance between groups. Behavioral data were analyzed with SPSS 17.0 using a mixed design ANOVA with two between-group variables (Stressed/Non-stressed and Old/Young) and one repeated measures variable (test day or pre-post treatment). Huynh-Feldt degrees of freedom controlled for assumptions of compound symmetry and sphericity due to repeated measures with more than two levels (40). Additionally, comparisons between group means were performed using Student t-tests or one-way ANOVA to evaluate differences in baseline data or age-related differences, where appropriate. For significant one-way ANOVA, Bonferroni's posthoc testing was performed to determine significant between-group differences. Pearson's product-moment coefficients were used to relate various variables. All data were expressed as means ± standard error. An alpha level of 0.05 was considered statistically significant. The level of statistical significance is indicated as follows: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

RESULTS

Stressed Tumor-Bearing Mice Were Cachexic and Had Greater Spleen Mass, Without Affecting Tumor Mass

Old mice weighed significantly more than young mice (**Figure 2A**; $p<0.001$). Over the 15-day post-surgery period, non-stressed mice maintained their original body weights to a greater extent than stressed mice (**Figures 2A, B**). Young stressed mice weighed significantly less than young non-stressed mice on D4-12 (**Figure 2A**; $p<0.0001$). The amount of weight loss from pretreatment weights was ~5%, with an age-related difference in young and old stressed mice, and stress-related difference in old mice (**Figure 2C**; $p<0.0001$), indicating greater stress-induced cachexia in old than young mice.

Psychological stress can cause corticosterone-mediated apoptosis of spleen cells, reducing spleen weight (44). However, mean spleen weights in young and old non-stressed mice were comparable to age-matched control mice (data not shown). Moreover, spleen weight was greater in stressed than the age-matched non-stressed mice (**Figure 2D**; *, $p<0.05$). Tumors were larger in old stressed mice than young stressed mice, (**Figure 2E**; **, $p<0.01$).

Stress Increased Anxiety in Tumor-Bearing Mice Regardless of Age

Baseline EZM testing revealed that young mice spent significantly more time in the dark than old mice, suggesting that young mice act more anxious than old mice based on their avoidance of the open spaces and preference for the closed dark spaces (**Figure 3A**; $p<0.0001$). All groups showed increased ($p<0.05$) anxiety-like behavior in the EZM 13 days after tumor cell inoculation (**Figure 3A**). In young non-stressed mice, serum corticosterone levels positively correlated with time spent in the dark ($r=0.55$, $p=0.035$). However, in young stressed mice, tumor progression was significantly related to: a) the amount of time animals spent in the dark during D2 of the EZM ($r=0.508$, $p=0.013$); b) the amount of learned helplessness displayed during the TST ($r=-0.454$, $p=0.034$); and c) between the amount of activity in the open field ($r=-0.423$, $p=0.045$). Tumor progression was also correlated with time spent in the dark on D2 of EZM in old non-stressed mice ($r=0.525$, $p=0.037$).

Regardless of age, non-stressed and stressed mice spent significantly more time in the dark post- than pre-tumor inoculation (baseline; **Figure 3B**). Repeated restraint and isolation stress did not alter the amount of time young or old mice spent in the dark compared with young or old non-stressed groups. When data for young and old mice were collapsed across baseline and across tumor inoculation groups, all mice spent significantly more time in the dark ($p<0.0001$) after developing tumors than before tumor inoculation (**Figure 3B**).

Tumor weight correlated with the percent time spent in the dark for all groups except old stressed mice (young non-stressed: $r=-0.55$, $p<0.035$; young stressed: $r=0.51$, $p<0.013$; old non-stressed: $r=0.53$, $p<0.037$). In addition, there was a significant positive relationship between corticosterone levels post-treatment, and how much learned helplessness an animal demonstrated during the TST ($r=0.512$, $p=0.018$). Older non-stressed mice displayed greater activity levels in both behavioral tests.

Greater Activity in the Open Field Supports Higher Anxiety in Old Stressed Mice

Old non-stressed mice were significantly less active throughout the OFT than old stressed mice or young non-stressed and stressed mice (**Figure 3C**; $p<0.05$). No significant differences were found for age or groups regarding the time spent in the perimeter or center of the open field (data not shown). Tumor

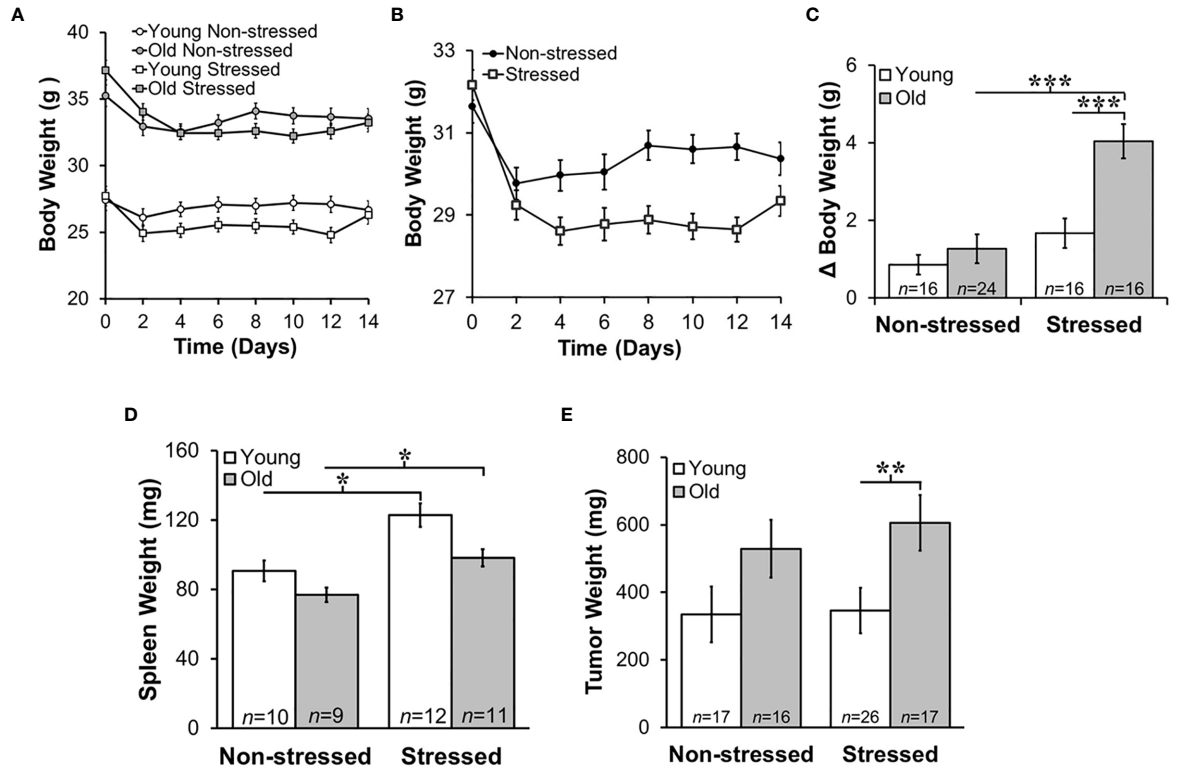


FIGURE 2 | Stress Most Affected Body and Tumor Weights in Old Mice and Increased Young and Old Spleen Weight. Mean body weights ± SEM in g (n=16-25 per group) did not differ across time post-RM9 tumor inoculation between age-matched groups **(A)** or after collapsing data across age **(B)**. **(C)** However, there was a greater mean change (Δ) in body weight (p<0.001) from baseline to study endpoint in old stressed mice compared with old non-stressed or young stressed mice. **(D)** Mean spleen weights (mg) were stress-dependently increased in young and old mice (p<0.05). **(E)** Tumor weights (mg) were greater (p<0.01) in old than in young stressed mice. *p < 0.05, **p < 0.01, ***p < 0.01.

weight in each treatment group was negatively correlated with the amount of activity in the open field (e.g., larger tumors were associated with less activity; young non-stressed: r=-0.45, p<0.045; young stressed: r=-0.42, p<0.045; old non-stressed: r=-0.595, p<0.12; old stressed: r=-0.49, p<0.044).

Old Mice Exhibited More Depressed-Like Behavior

Old mice demonstrated more depression-like learned helplessness behavior than young mice during the TST p<0.02). Stress status did not influence depression-like behavior in either age group (**Figure 3D**). In young stressed mice, tumor weight (r=0.454, p<0.034) and serum corticosterone (r=0.51, p<0.02) was associated with depression-like behavior.

HPA Activity Increased Post-Treatment When Data Is Collapsed for Age

Prior to tumor inoculation, old mice had lower baseline corticosterone levels than young mice (**Figures 4A, B**; p<0.02). A one-way ANOVA to assess treatment differences in 18-month-old mice revealed that repeated restraint and isolation stress significantly increased corticosterone levels (**Figure 4A**; p<0.04). In young stressed and non-stressed mice, corticosterone levels were not significantly different compared with baseline levels prior to tumor inoculation. Although not significant, there was a trend for old mice to display increased corticosterone levels compared with young mice post-tumor inoculation. When corticosterone levels were collapsed for stressed and non-stressed mice across age and compared to baseline values, there was a positive interaction between pre-post tumor inoculation and treatment group (p<0.02). Stressed mice had increased corticosterone levels compared with non-stressed mice (**Figure 4B**; p<0.05). However, old, but not young, stressed mice (**Figure 4C**) had significantly increased serum corticosterone levels compared with old non-stressed mice post-treatment (p<0.04). RM-9 tumor weight was correlated with circulating corticosterone levels in young and old stressed and old non-stressed mice (young stressed: r=0.73, p<0.005; old non-stressed: r=0.65, p<0.001; old stressed: r=0.43, p<0.01).

Sympathetic Nerve Activity in Prostate Tumors Increased in Old Stressed Mice

Mean prostate norepinephrine and epinephrine concentrations (**Figures 4D, E**, respectively) remained stable in young and old

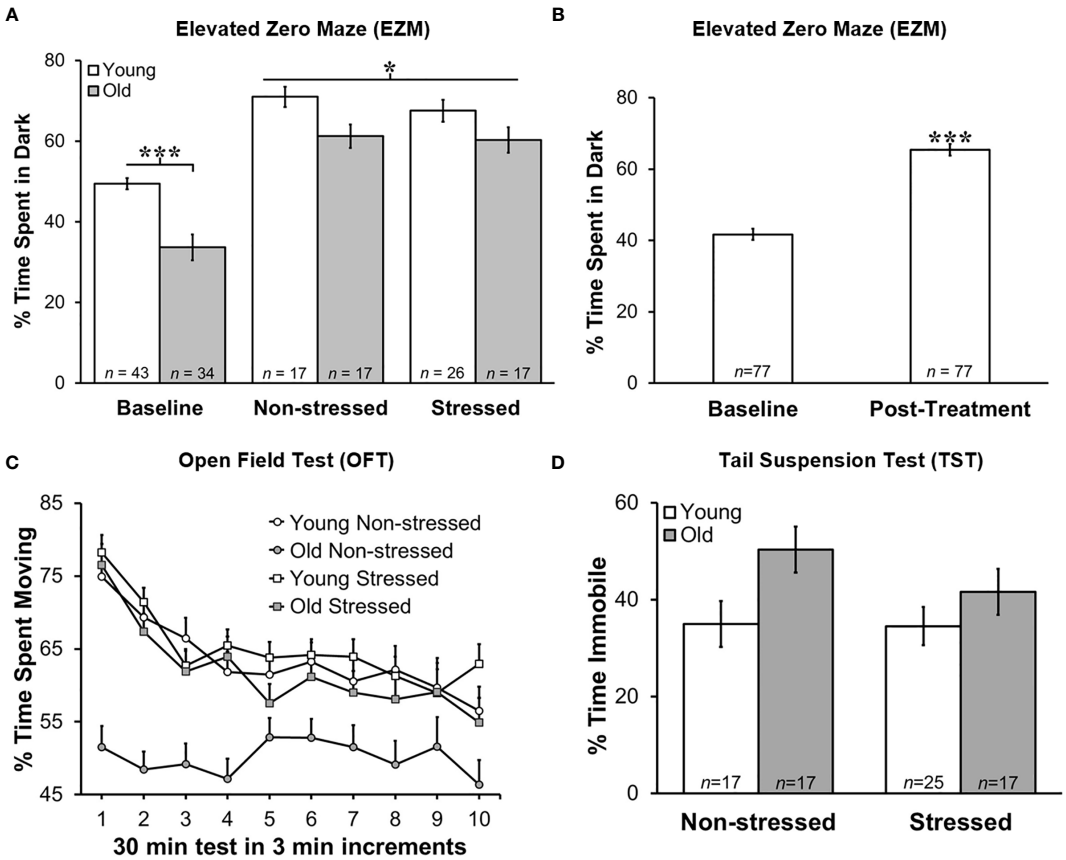


FIGURE 3 | Greater Anxiety-like Behavior in Young Mice in Zero Maze, and Post-treatment than at Baseline, but No Difference in Time Immobile on Tail Suspension Test. Behavioral effects of restraint stress and isolation in young and old tumor-bearing C57BL/6 mice. **(A)** Young mice spent significantly more time in the dark post-tumor inoculation compared with old mice ($p<0.0001$). Stress did not significantly alter the time spent in the dark for either young or old mice. Young mice spent significantly more time in the dark compared with old mice overall ($p<0.0001$). **(B)** When groups were collapsed, all mice spent significantly more time in the dark post-treatment compared with baseline ($p<0.0001$). Data are expressed as the mean % of time spent in the dark ± S.E.M. **(C)** Old non-stressed mice were significantly less active throughout the test compared with old stressed mice ($p<0.05$) and with young mice regardless of being stressed or non-stressed ($p<0.05$). Data are expressed as the mean % spent moving ± S.E.M. for each treatment group for each 3 min increment over the 30 min time period for the OFT (n=17-26 mice per group). **(D)** There were no group differences in percent time immobile on TST. $^{*}p < 0.05$, $^{***}p < 0.001$.

non-stressed and stressed mice. However, in old mice, the mean prostate concentration of the norepinephrine precursor, dopamine, was lower in stressed than in non-stressed mice (**Figure 4F**; $p<0.01$). In contrast, stress in old mice more than doubled the prostate levels of the norepinephrine catabolite, MHPG, compared with age-matched non-stressed mice (**Figure 4G**; $p<0.01$). Likewise, in old mice, MHPG/norepinephrine ratio, an indicator of norepinephrine turnover, was greater (**Figure 4H**; $p<0.01$) in stressed than non-stressed mice; this ratio was also greater in old than young stressed mice (**Figure 4H**; $p<0.05$).

Proliferation, Apoptosis and Endothelial Cell Markers Support Positive Effects of Stress RM-9 Tumor Progression

Quantitative immunofluorescence staining in RM9 tumors for proliferation (Ki-67), apoptosis antigen 1 (CD95), and microvessel density (CD31) markers (**Figures 5A–C**, respectively), support a tumor-promoting effect of restraint stress in young and old mice. Stress dramatically increased the percentage of Ki-67^{+} cells in RM-9 tumors in both young and old mice ($p<0.001$; **Figure 5A**). In contrast, stress reduced expression of CD95 in RM-9 tumors from young and old stressed mice ($p<0.001$; **Figure 5B**), consistent with tumor cell shedding of this ligand to escape immune-mediated apoptotic cell death. The mean number of CD95^{+} cells per field was similar in young and old non-stressed mice, about ~50-60 per field (**Figure 5B**). Expression of CD31 was lower ($p<0.001$; **Figure 5C**) in prostate tumors from stressed than non-stressed young mice, but CD31 expression was conversely higher ($p<0.001$) in prostate tumors from stressed than in non-stressed old mice.

Stress Differentially Altered Immune Cell Infiltration into Tumors in Young and Old Mice

Myeloid and T cells are crucial components of the immune response to cancer, as they play major roles in PCa initiation and

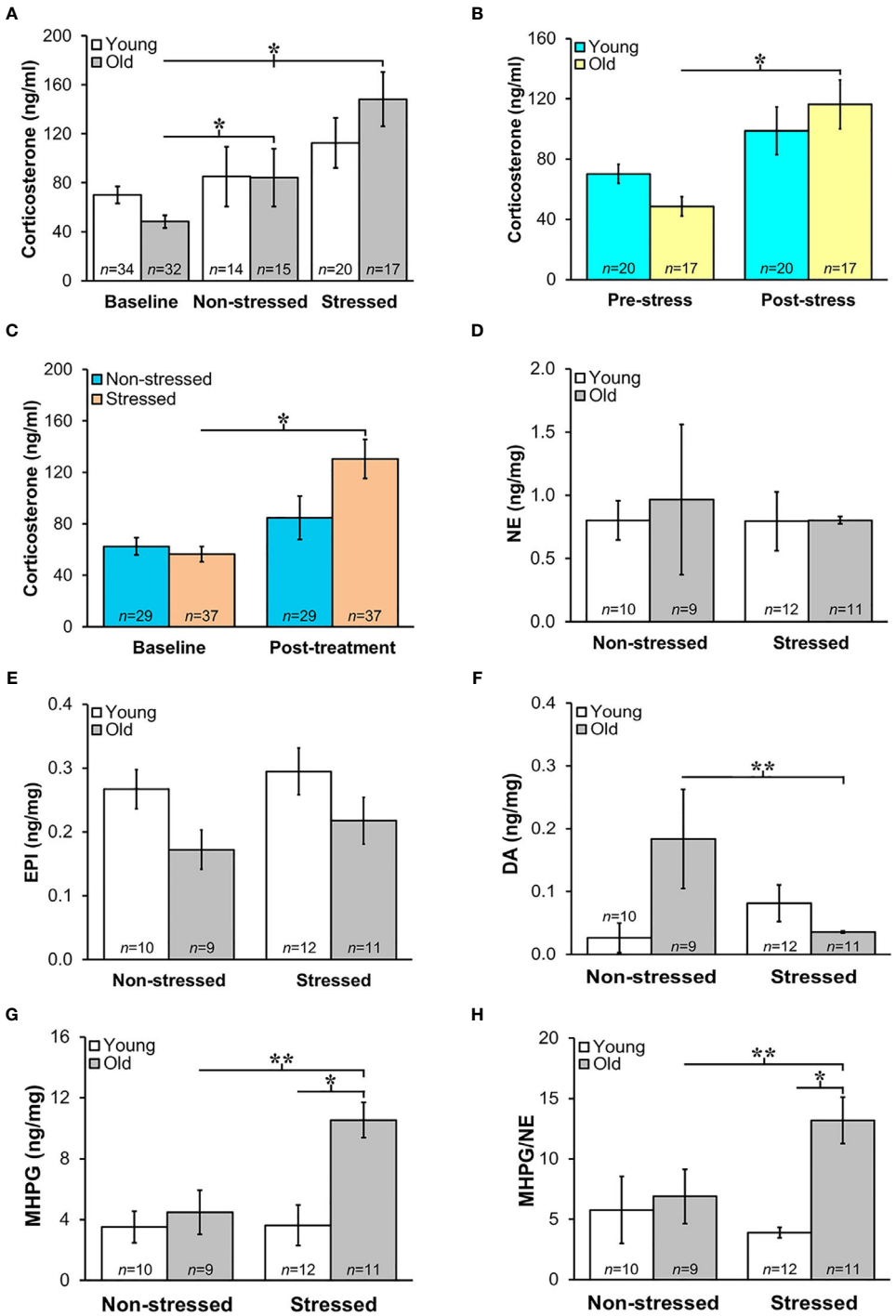


FIGURE 4 | **(A)** Increased HPA and SNS Activation in Stressed Old Mice. Mean circulating corticosterone levels (ng/ml ± S.E.M) from young and old non-stressed and stressed mice at baseline and D15 post-tumor inoculation. Old stressed mice had a significant increase in corticosterone levels post-treatment than at baseline. **(B)** After data are collapsed across age, Stressed mice had significantly higher corticosterone levels than Non-stressed mice (**$p < 0.02$). Data are expressed as corticosterone in ng/ml ± S.E.M. *Daily restraint stress began1 day after tumor inoculation. **(C)** Corticosterone expressed in ng/ml was higher (*$p < 0.05$) post-treatment compared with baseline levels in stressed compared with non-stressed mice. **(D, E)** Tumor expression of NE and EPI (ng/mg) were similar in all treatment groups. **(F, G)** However, in old mice, prostate dopamine, a precursor in NE synthesis, was lower (**$p < 0.01$) in stressed than non-stressed mice, and prostate MHPG, a metabolite of NE degradation, was elevated in old stressed mice compared with old non-stressed or young stressed mice (**$p < 0.01$ or *$p < 0.05$, respectively). **(H)** Likewise, MHPG/NE ratios, used to estimate NE turnover, was also higher in prostate tumors from old stressed mice than from old non-stressed or young stressed mice (**$p < 0.01$ or *$p < 0.05$, respectively). *$p < 0.05$, **$p < 0.01$.

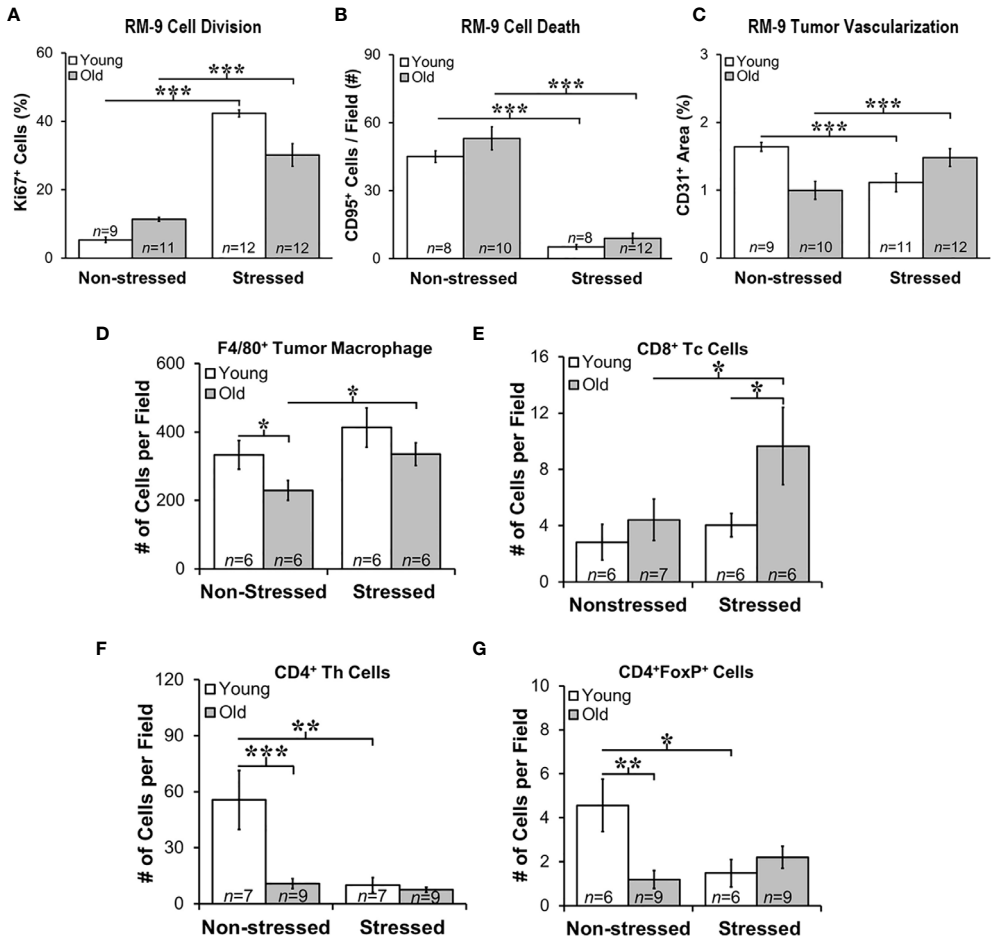


FIGURE 5 | Stress Promoted Tumor Proliferation and Apoptosis, but Age-dependent Infiltration of Immune Cell Subsets and Vascularization Support Age-dependent Anti-tumor Defense Mechanisms. Quantitation of immunostaining for proliferation **(A)**, apoptosis **(B)**, angiogenesis **(C)**, F4/80$^+$ macrophages, and CD8$^+$, CD4$^+$, and CD4$^+$FoxP$^+$ cells **(D–G**, respectively) in young (open bar) and old (gray bar) non-stressed and stressed mice D14 after orthotopic prostate tumor inoculation. **(A)** Stress increased prostate tumor expression of Ki67 in young and old mice compared with non-stress age-matched mice. **(B)** Conversely, CD95 expression was lower in RM-9 tumors from stressed than in non-stressed young and old mice. **(C)** CD31 immunoreactivity was higher in stressed than non-stressed old mice, but in young mice expression was greater in non-stressed than the stressed group. **(D)** F4/80$^+$ tumor macrophages were fewer (*$p < 0.05$) in old than young non-stressed mice, but were higher in stressed than non-stressed old mice. **(E)** CD8$^+$ Tc cell numbers were similar in non-stressed mice, but were higher in old than young in stressed mice. Moreover, more ($p<0.05$) CD8$^+$ cells were present in old than young stressed mice. **(F)** CD4$^+$, including those expressing FoxP3$^+$ **(G)**, were lower in old than young non-stress mice, and in young stressed than non-stressed mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

progression (42). Tumor-associated macrophages (TAMs) are well represented in PCa, their presence in murine models promotes tumor progression, and their presence generally strongly correlates with poor prognosis. T cell infiltration in tumors is essential for the immunologic response to tumor tissue. Therefore, we evaluated immune cell infiltrates in RM-9 tumors. F4/80$^+$ macrophages, and CD4, CD4/FoxP3$^+$ and CD8$^+$ T cells revealed tumor infiltration of immune cells into RM-9 tumors as shown in **Figures 5D–G).**

Most of the leukocyte infiltrates into the tumors were macrophages (**Figures 5D**). There were main effects of age (p=0.041) and stress (p=0.036), but no significant interaction between age and treatment for intratumoral F4/80$^+$ macrophage number (**Figure 5D**). There were fewer F4/80$^+$ macrophages in RM-9 tumors from old compared with young non-stressed groups (p<0.05). In contrast, tumors from old stressed mice had more (p<0.05) F4/80$^+$ macrophages than in non-stressed controls (**Figures 5D**) (p<0.02). There were main effects of age (p<0.03) and stress (p<0.05) for tumor CD8$^+$ Tc cells, which were sparse and widely dispersed (data not shown). In old mice, stress increased (p=0.05) tumor CD8$^+$ T cell numbers, and they were more abundant (p=0.05) in old than young stressed mice (**Figure 5E**). CD8+ Tc cells in RM9 tumors were more numerous (p<0.05) in old than young stressed mice, and in stressed mice were greater (p<0.05) in old than young mice (**Figure 5E**). In contrast, tumor CD4+ Th cells were most abundant in young non-stressed mice (**Figure 5F**), but were significantly reduced (p<0.01) by stress. Tumor CD4+ Th cells

were lower ($p<0.001$) in old than young non-stressed mice (**Figure 5F**).

$CD4^+FoxP3^+$ T cells were sparse in RM-9 tumors (**Figure 5G**). Two-way ANOVA revealed a significant interaction between age and stress status ($p=0.02$) in tumor $CD4^+FoxP3^+$ cells number (**Figure 5F**). In young mice, stress reduced tumor $CD4^+FoxP3^+$ cell infiltration ($p<0.03$). Fewer $CD4^+FoxP3^+$ cells infiltrated tumors from old than from young non-stressed mice. Unlike young mice, there was no effect of stress on $CD4^+FoxP3^+$ cell tumor infiltration in old mice (**Figure 5G**).

Growth Factors and Chemokine Expression in Prostate Tumors Were Age- and/or Stress-Dependent

Tumor growth and progression are influenced in a complex way by a multitude of growth factors and cytokines in the prostate microenvironment. Here, we evaluated tumor expression of growth factors and cytokines prognostic for PCa progression and/or poorer survival. Additionally, we assessed how stress and age influences expression of cytokine/chemokines that may be influenced by age and/or stress and hence serves as novel markers for age- and/or stress-related tumor progression. VEGF was used to assess angiogenesis (45), whereas TGF-β and IL-6 can serve as tumor promoters (46) that induce VEGF to regulate prostate growth (45, 47), and promote Th2 cell-mediated humoral immunity (48). Stress hormones can regulate both growth factors in PCa models (49). In our orthotopic PCa model, VEGF and TGF-β were highly expressed in prostate tumors, with similar levels regardless of age and treatment group (**Figures 6A, B**, respectively). Tumor VEGF and TGF-β concentrations were higher in all mice than non-treated control mice (indicated for VEGF and TGF-β by the gray horizontal bar; **Figures 6A, B**), but were comparable across age and treatment group. (The gray horizontal bars in **Figures 6A, B** represent the mean cytokine levels ± SD; $N=10$ untreated young and old C57Bl/6 mice).

GM-CSF is a potent cytokine with anti-tumor activity that works by inducing expression of TNF-α and IL-1 (50). In contrast, G-CSF is a poor prognosticator in human PCa as it promotes PCa development *via* neurogenic influence on autonomic nerves (51). G-CSF modulates the growth/sprouting and survival of sympathetic nerves, which promotes PCa growth and dissemination in metastatic models. RM-9 prostate tumors expressed both G-CSF and GM-CSF (**Figures 6C, D**, respectively); however, tumor expression of poor survival marker, G-CSF was ten-fold higher than GM-CSF. Restraint stress increased (*, $p<0.05$) tumor G-CSF in young mice (**Figure 6C**), but stress increased (*, $p<0.05$) tumor GM-CSF in old mice (**Figure 6D**), respectively.

Several chemokines that can promote tumor growth, angiogenesis and metastases were expressed in RM-9 prostate tumors (**Figures 6E–I**) (52, 53). From highest to lowest concentration they were MIP-1α > MCP-1 > MIP-2 > MIP-1α = or > RANTES. Effects of stress were found for CCL3/MIP-1α (**Figure 6E**) and CCL4/MIP-1β (**Figure 6F**). Among the greatest expressed chemokines of the five evaluated, were CCL3/MIP-1α and CCL2/MCP-1. CCL3/MIP-1α was expressed at high levels in non-stressed young and old and stressed old mice. Interestingly, CCL3/MIP-1α, a cytokine marker of poor prognosis (53), was reduced by about half in young stressed mice compared with both young non-stressed (**, $p<0.01$) and stressed old mice ($p<0.01$). Surprisingly, stress failed to reduce this chemokine in old mice. Expression of CCL4/MIP-1β was about ten-fold lower than CCL3/MIP-1α. Also in contrast to CCL3/MIP-1α, significant differences were observed only in tumors from old stressed mice, which had higher tumor CCL4/MIP-1β levels than tumors in young non-stressed mice ($p<0.05$). Although tumor CCL4/MIP-1β expression in old stressed mice were greater than in young stressed mice, this finding did not reach statistical significance.

Largely tumor derived, CCL2/MCP-1 exerts autocrine-mediated promotion of tumor growth and invasion (53, 54), and paracrine-mediated recruitment of myeloid-derived suppressor cells (MDSCs) that enhance tumor cell survival (55, 56). CCL2/MCP-1 is expressed in prostatic stromal cells, where it stimulates prostatic epithelial cells growth. In this study, age-related differences were uncovered for CCL2/MCP-1 and CXCL2/MIP-2. Tumor levels of CCL2/MCP-1 were lower in old than young stressed mice. Tumor-derived CCL2/MCP-1 produced in tumors in this study ranged from ~10 to 20 pg/ml). CCL2/MCP-1 was ~2-fold lower in old than young stressed mice (**Figure 6G**: **, $p<0.01$). No differences in this chemokine were observed between non-stressed and stressed mice.

CXCL2/MIP-2 ranged from ~ 0.75 to 2.5 pg/mg tumor tissue. In both stressed and non-stressed, old mice expressed higher levels of CXCL2/MIP-2 than young mice (**Figure 6H**: *, $p<0.05$), supporting an age-related increase of this chemokine. Secreted by endothelial cells, CCL5/RANTES induces autophagy in PCa cell lines (57). Low levels of CCL5/RANTES were produced by RM-9 tumors across all treatment groups. Both age and stress effects were demonstrated for tumor expression of CCL5/RANTES (**Figure 6I**: *, $p<0.05$). Tumors from old non-stressed mice had higher levels of CCL5/RANTES (*, $p<0.05$) than young non-stressed mice. In stressed mice, CCL5/RANTES reduced (*, $p<0.05$) in old compared with young mice. No difference in CCL5/RANTES levels was observed between non-stressed and stressed young mice or between young and old stressed mice. CXCL10/IP10, a negative regulator of tumor growth and promoter of $CD8^+$ T cell tumor infiltration in human prostate LNCaP cells (56), was expressed at comparable levels (~6 pg/mg) in all treatment groups (data not shown), despite low tumor levels of its chief inducer, IFN-γ (fg/mg; see **Figure 7B**). There were no effects of age or stress on CXCL10/IP-10 (data not shown).

Stress Enhanced Tumor IL-6, IL-9, 12p40 and IL-17, and Suppressed IL-12p70 in Old Mice

In the aged microenvironment, the transition of the prostate to a hyperplastic state is characterized by inflammatory infiltrates with distinct immune cytokine-secreting signatures that support immune cell polarization toward unfavorable disease outcomes (58). To assess age and stress effects on the tumor cytokine

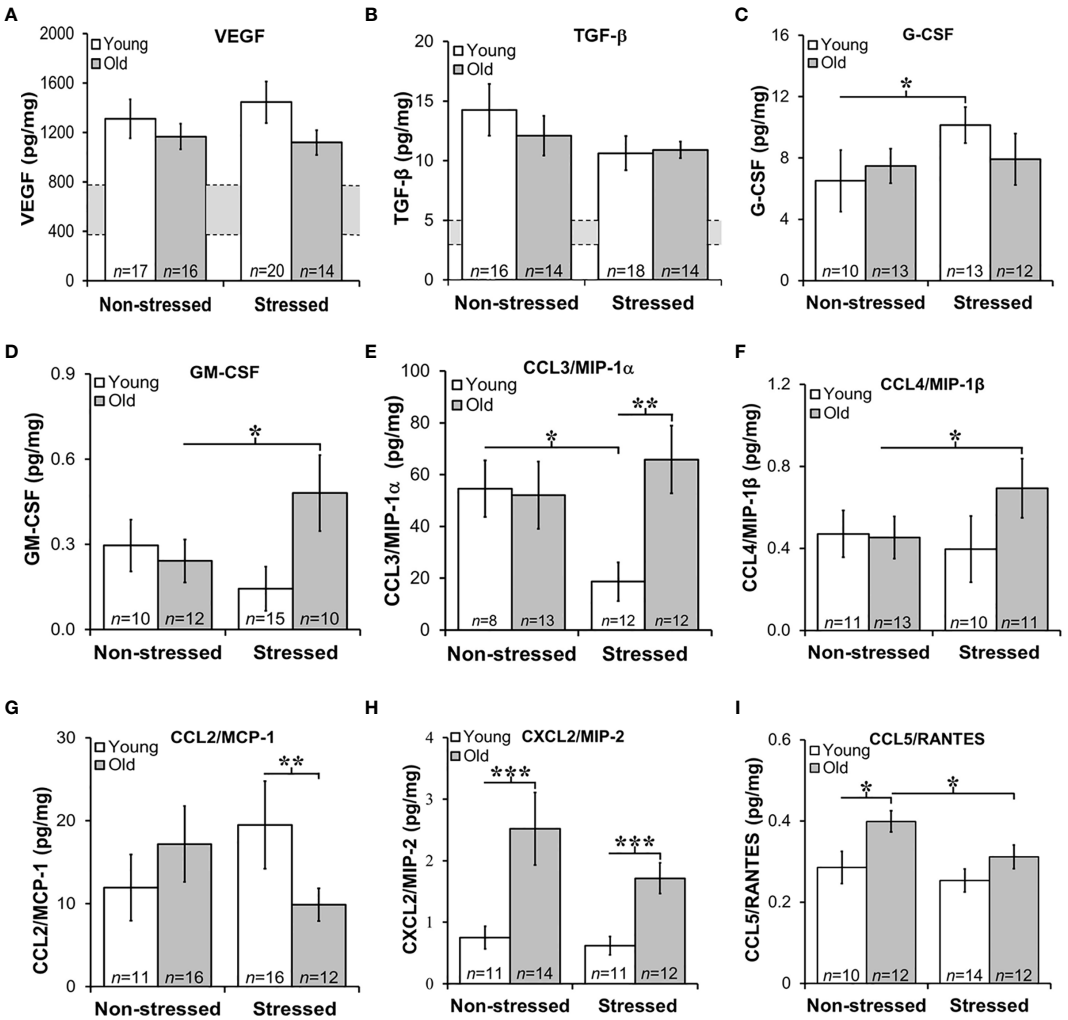


FIGURE 6 | Growth Factors and Chemokines Expression in Prostate Tumors. **(A, B)** VEGF and TGF-β were highly expressed in RM-9 tumors, regardless of age or treatment group. The gray bar indicates the range of VEGF **(A)** and TGF-β **(B)** expression in the murine prostate gland in the absence of RM9 tumors (based on collapsed data from an n of 8 per age group of normal prostate samples; no age-related differences were identified in these control samples). Mean tumor expression of VEGF and TGF-β expression were higher than in basal prostate levels, but no age- or stress-related differences were uncovered. **(C)** In young mice, RM-9 tumor G-CSF levels were higher (*$p < 0.05$) in stressed than non-stressed mice. **(D)** In contrast, tumor GM-CSF in old mice was greater (*$p < 0.05$) in stressed than non-stressed mice. **(E)** CCL3/MIP-1α was one of the most highly expressed chemokine that was quantified, and one or two chemokines where effects of both age and stress were identified. In young mice, stress reduced (*$p < 0.05$) tumor CCL3/MIP-1α compared with levels in non-stress mice. In tumors from stress mice, CCL3/MIP-1α was higher (**$p < 0.01$) in old than young mice. **(F)** An increase (*$p < 0.05$) in tumor CCL4/MIP-1β levels was observed in old compared with young stressed mice. **(G)** In the stressed groups, CCL2/MCP-1 was lower (**$p < 0.01$) in old than young mice. **(H)** Only effects of age were observed for CXCL2/MIP-2 such that levels were higher in old than young mice regardless of stressed group (***$p < 0.001$). **(I)** Tumor CCL5/RANTES concentrations were reduced in old compared with young stressed mice, but in non-stressed mice, levels were higher in old than in young mice.

milieu, multiplex antibody-based affinity protein cytokine arrays were used. The cytokine milieu was consistent with immunohistochemical observations of increased macrophages and T cell infiltration.

Chronic low-level production of TNF-α in the tumor microenvironment is a hallmark feature of PCa (59), and is consistent with low TNF-α expression in RM-9 tumors and no effects of age or stress (data not shown). Elevated IL-1β expression has been implicated in human prostate pathology, particularly linked to human prostatic proliferative inflammatory atrophy (60). In RM-9 prostate tumors, IL-1β expression was low in RM-9 tumors in young and old mice (**Figure 7A**). Still, IL-1β was significantly reduced in old compared with young non-stress and stressed mice (**Figure 7A**). IL-1β-induced IFN-γ from activated immune cells sensitizes PCa cells toward Fas-mediated cell death (61), but is also reported to induce immune escape by neuroendocrine-like differentiation in human prostate basal-epithelial cells (62). IFN-γ can upregulate MHC class I expression and induce Th1 cell polarization, driving anti-tumor-specific immune responses

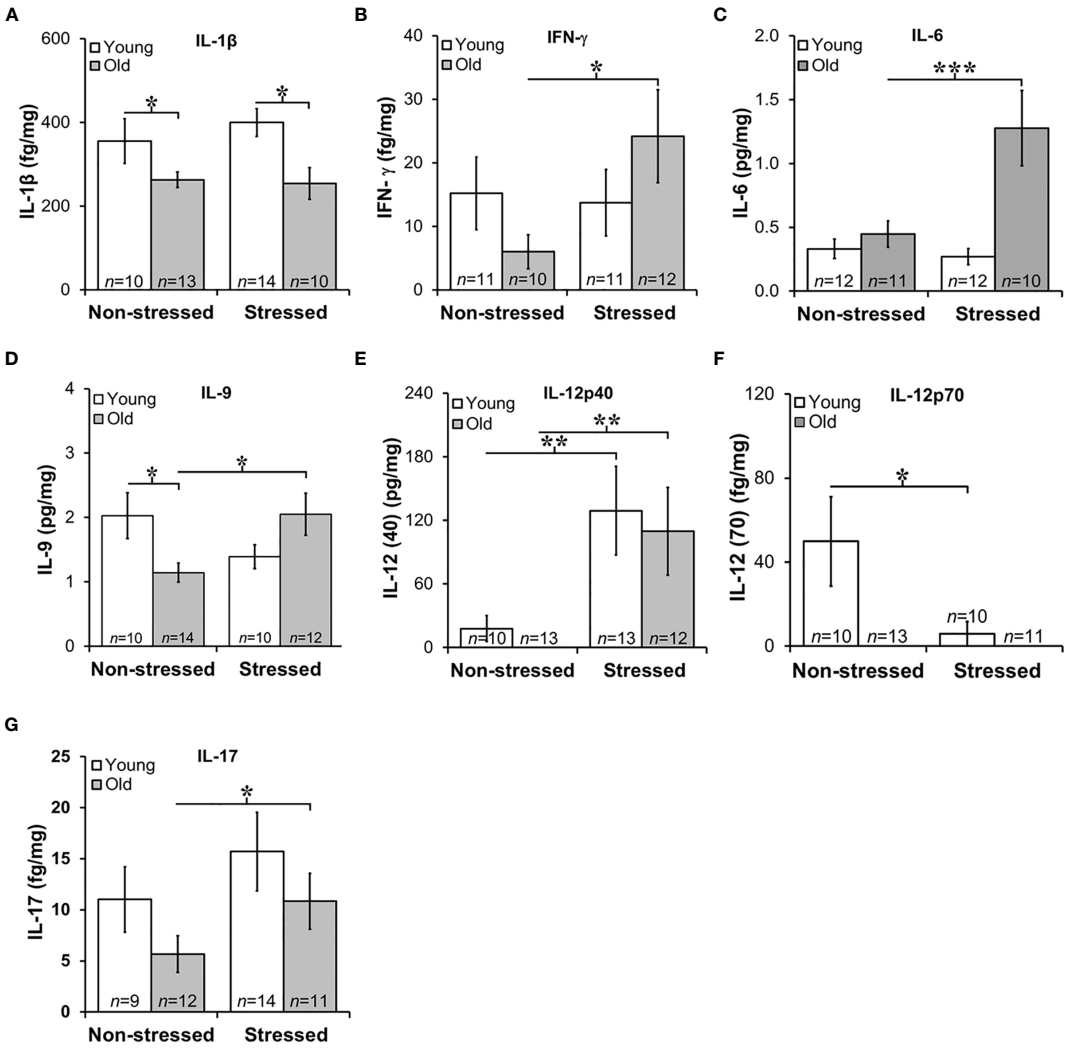


FIGURE 7 | Expression of Interleukins in Prostate Tumors. **(A)** IL-1β expression was lower ($p<0.05$) in old than in young tumors, regardless of the stress-related group. **(B, C)** There was an effect of stress on IFN-γ and IL-6 tumor expression in old mice such that stress mice had elevated tumor expression than non-stressed mice (***$p < 0.001$). **(D)** Tumors from old stressed mice expressed higher levels of IL-9 than young stressed mice, but in non-stressed mice, tumor IL-9 levels were lower in old than young mice. **(E)** Tumor levels of IL-12p40 were low in non-stressed mice, regardless of age. In both young and old mice stress significantly increased (**$p < 0.01$) tumor IL-12(p40) expression compared with non-stress age-matched mice **(F)** Overall, IL-12p70 expression in prostate tumors was lower than IL-12p40 shown in **(E)**, with low levels in old mice regardless of stress group. Still, in young and old mice, psychosocial stress reduced (**$p < 0.01$) tumor IL-12p70 expression. **(G)** Prostate tumor IL-17 levels were low in all treatment groups, but a stress-related increase (*$p < 0.05$) in tumor levels of IL-17 was observed in old compared with young mice.

mediated in part *via* IL-12 (63). Consistent with the low IL-1β production within the tumors, IFN-γ was suppressed in RM-9 tumors. However, IFN-γ was significantly elevated in the prostate tumors of stressed old mice (**Figure 7B**; *, $p<0.05$).

Several interleukins expressed in RM-9 tumors were affected by stress. IL-6 is associated with an aggressive PCa phenotype by inducing VEGF, promoting tumor cell proliferation, suppressing apoptosis, and driving Th cell differentiation of regulatory Th cells (64). IL-9 has both anti- and pro-tumor actions that are not well understood (65), and IL-9 has complicated anti-tumor and pro-tumor properties mediated by innate and adaptive immunity (66), including in PCa (67). In RM-9 tumors from stressed groups, IL-6, and IL-9 expression was greater in old than in young mice (**Figures 7C, D**, respectively; **Figure 7C**: *, $p<0.05$; **Figure 7D**: *, $p<0.05$). Additionally, IL-9 levels were significantly less in old non-stressed mice than young non-stressed mice, suggesting an age- and stress-related difference in production of this cytokine.

Primarily produced by activated myeloid cells, IL-10 and IL-12 play immunoregulatory roles in host defense and immune homeostasis, typically with opposing immune suppressive and enhancing actions, respectively. IL-10 induces anti-tumor actions by inhibiting angiogenesis and cell proliferation of PCa cells (68). PCa expression of IL-10 (mean levels ~0.06 pg/mg)

was not affected by age or stress, (data not shown). Two forms of IL-12 were expressed in RM-9 tumors, prostate tumor-promoting IL-12p40 and tumor growth suppressing IL-12p70 (63) (**Figures 7E, F**, respectively). RM-9 tumor expression of the tumor promoting IL-12p40 was greater in stressed young and old mice than age-matched non-stressed mice (**Figure 7E**: *, $p<0.05$). The tumor suppressor, IL-12p70 was below the level of detection in old mice regardless of whether these mice were stressed or not stressed. IL-12p70 was detectible in tumors from young mice. IL-12p70 was lower in RM-9 tumors from stressed young mice, and non-stressed young mice (**Figure 7F**: *, $p<0.05$).

In several murine models, IL-17 is reported to promote PCa progression (69). In this study, IL-17 expression in RM9 tumor was low (fg/mg range) regardless of age or stress, but stress significantly increased tumor expression of IL-17 in old, but not young mice (**Figure 7G**: *, $p<0.05$). No differences were observed in IL-17 between non-stressed and stressed young mice or non-stressed old and stressed young mice. IL-1α, IL-2, IL-4, IL-5, and IL-7 were expressed in prostate tumors in the fg/mg range, but there were no effects of age or stress on these cytokines (data not shown).

DISCUSSION

The primary goals of this research were to establish a novel age-relevant rodent model of PCa and to identify how psychological stress interacts with the age-dependent tumor microenvironment to influence cancer progression. To achieve these goals, we repurposed a non-metastatic, orthotopic, syngeneic mouse model of PCa previously used to study the efficacy of cytokine therapies on tumor growth. To our knowledge, this is the first study to evaluate interactions between age and mild repeated acute psychosocial stress in PCa. Stress age-dependently influenced the immune response in RM-9 tumors. The intratumoral immune response differed through its suppressive effect on the Th1-type cytokine production from $CD4^+$ T cells; stress-increased tumor IL-12p40; this effect was consistent across age. This study is the first to report the significant effects of age and psychosocial stress on PCa progression in an orthotopic mouse model. PCa is largely a disease of older men, and age often plays a role in treatment choice. In fact, the most common high-risk factor for PCa and lower overall survival is age. Our findings provide support for age-related pathophysiologic differences in RM-9 tumors. Moreover, they underscore the use of age-appropriate animal models to better understand the clinical effects of stress and their consequences for the efficacy of anti-cancer drugs than young adult rodent cancer models.

In this murine prostate model, stressed mice weighed less than non-stressed mice, supporting stress-promoting cachexia, as defined as >5% weight loss; this effect was greater in old than in young mice (~10% vs. ~7%, respectively). This finding is consistent with reports in other rodent models of PCa (70, 71) and breast cancer (27), which in the latter was reversed by G-CSF inhibition (72). The stress-induced splenomegaly in the present report is consistent with tumor detection and systemic age-dependent tumor-host interaction that can influence host defense against tumor development.

We found that restraint- and isolation-induced stress increased the activity of the SNS (based on mean MHPG/NE ratios) and HPA axis to a greater degree in old than young mice, negatively affected behavior, and promoted tumor progression in an age-dependent manner. Although RM-9 prostate tumor growth, as assessed by tumor weight, was not significant, more refined assessments of tumor status (cell proliferation/death) and cytokine expression supports that isolation/restraint stress increased RM-9 cell proliferation and reduced apoptosis regardless of age. Moreover, our findings indicate opposing age-dependent effects of moderate repeated psychosocial stress on angiogenesis, based on stress-mediated suppression and enhancement of angiogenesis in young and old mice, respectively. Overall, our findings of an exaggerated increase in SNS activity in old stressed mice are consistent with Hassan et al. (22, 73), who demonstrated that stress accelerated PCa development in another murine PCa model that was mediated *via* increased activity of the SNS.

Our findings also indicate that chronologic age moderated the effects of combined isolation and restraint stress on the infiltration of leukocyte subsets important for tumor immunity. Surprisingly, all mice displayed increased anxiety-like behaviors from baseline measures. One interpretation of this finding is that surgical stress from tumor inoculation increased anxiety-like behavior, and that there was no additive effect of isolation combined with restraint on this measure. However, we cannot exclude the possibility that the mice simply learned that there was nothing to be gained in exploration of the open and brightly-lit quadrants. However, we cannot discount that tumor presence (possibly *via* cytokines entering the circulation) induced or influenced a stress response. Cytokines released during the immune response can alter both physiology and behavior [reviewed in (74)], and mouse behavior can also change in the presence of tumor development (75). Previous studies with mice have shown that the presence of subcutaneous RM-9 tumors can significantly alter the profile of secreted cytokines (76).

In the present experiment, larger tumors were correlated with increased anxiety-like behaviors. Older men with PCa can experience both cancer-related physical and psychological stress (77). Moreover, stress reduction techniques can minimize the negative effects of both physical and cognitive reactions to stress (78). Thus, the link between tumor presence and stress is likely to be clinically important. Notably, despite increased post-inoculation anxiety-like behavior in all groups, stressed mice exhibited more corticosterone and anxiety-like behaviors than non-stressed mice, indicating that the isolation and restraint successfully produced non-tumor-related changes possibly detrimental to cancer recovery.

There were also important age-related differences in response to stress. Old mice had lower baseline levels of circulating corticosterone than young mice. Although corticosterone levels did not differ between young and old non-stressed mice with PCa. Given the lower baseline corticosterone levels, old mice had a greater increase in corticosterone levels than young mice.

Similarly, old stressed mice with PCa had a greater change in corticosterone levels than the young stressed mice with PCa. These findings support that although old mice started at a lower level of circulating corticosterone, they had a greater stress response compared with young mice.

Old stressed mice were significantly more active in a novel environment than old non-stressed mice. In fact, old stressed mice were as active as young mice, in which stress did not change activity levels. This suggests that in old mice stress prevented the normal decline in activity, but did not affect overall activity levels in young mice. These findings confirm previous research that old mice can display increased locomotion and exploratory behavior when exposed to a stressor (79). In addition, old mice demonstrated greater depression-like behaviors in an inescapable learned helplessness situation. Old mice gave up struggling on the TST much sooner than young mice. Anxiety and depressive behaviors are often co-morbid; indeed, one predictor for cancer-related depression risk is the presence of anxiety disorders (80). Studying the dynamic interplay of these behaviors, especially in the context of age, can increase our understanding of how individuals from different age groups respond to negative news, such as a cancer diagnosis and the subsequent effects this response might have on the patient's prognosis. The dynamic interplay between the mind and body is well known, and psychological distress has been found to be a predictor in cancer mortality (81). In fact, patients with high levels of psychological distress, including depression and anxiety, have a 27% increase in cancer mortality (82).

Our data infers that age also appears to be a factor in tumor size. RM-9 tumors grew faster in old mice than in young mice. A study by Reed and colleagues (83) showed that subcutaneously injected prostate TRAMP-C2 tumor cells grow at a similar rate in 20-month-old as in 4-month-old syngeneic mice. The lack of age-related difference in tumor growth between these models may be due to differences in subcutaneous compared with orthotopic placement of tumor cells. Age-related differences in tumor growth rates appear to be tumor type-specific. Whereas the growth of some tumors is slower in old animals (84, 85), certain tumors, e.g. some sarcomas, melanoma and liver cancer, grow more rapidly in older animals. However, no age-related differences were observed in the growth of melanoma and AKR lymphoma cells (84–86). Age-related differences have been linked with age-associated decline in anti-tumor immunity, different immunogenic properties and the tumor microenvironment of different types of tumors. Factors shown to be contributory to age-related differences in tumor progression include the local microenvironment, angiogenesis, apoptosis, inflammation, and host anti-tumor immunity (84–86). Greater expression of several chemokines may contribute to the age-related increase in tumor growth in old compared with young stressed mice. A summary of age- and stress-related changes in prostate tumor cytokines is illustrated in **Figure 8**.

Our finding of higher concentrations of CCL3/MIP-1α, CCL4/MIP-1β and CXCL2/MIP-2 in old versus young stressed mice are consistent with greater tumor growth in old stressed mice and poorer prognosis (87). Similarly, IL-1α and IL-9 expression were influenced by age. These findings suggest age-specific tumor immunity response profiles that can be differentially influenced by psychosocial stressors. Th1- and Th9/17-driven immune responses were observed in young and old mice, respectively. Several studies have reported that secretion of these chemokines from tumor-infiltrating macrophages promote prostate tumorigenesis *via* their effects on VEGF-mediated tumor

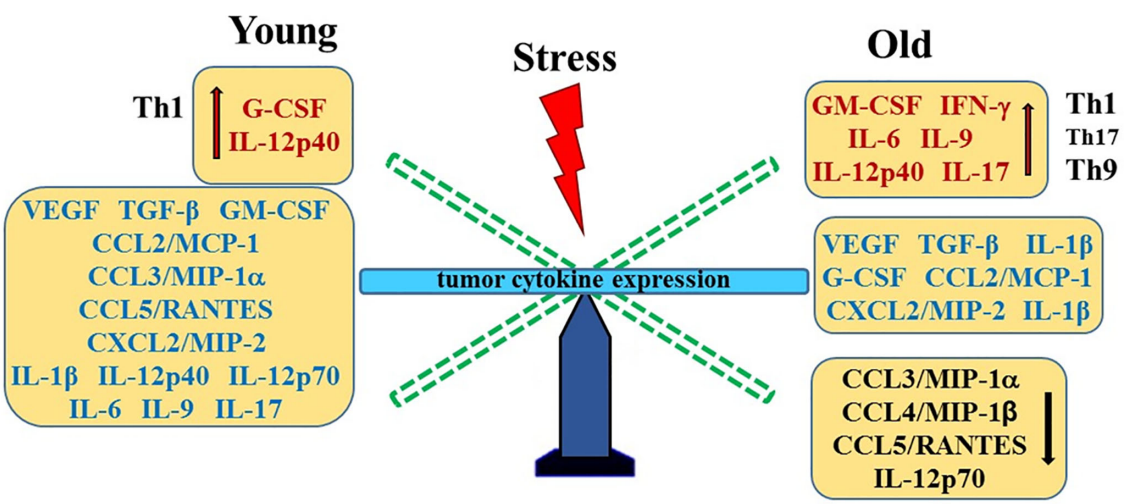


FIGURE 8 | Stress- and Age-Mediated Changes in Prostate Cytokine Expression. A summary of the changes in tumor chemokine/cytokine expression in young (left) and old (right) stressed mice is represented as a "teeter-totter' with an increase or decrease in expression represented as an elevated or depressed bars, respectively. CCL, C-C Motif Chemokine Ligand; CXCL, (C-X-C motif) ligand 1; G-CSF, Granulocyte Colony-stimulating Factor; GM-CSF, Granulocyte/Monocyte Colony-stimulating Factor; IFN, interferon; IL, interleukin; MCP, Monocyte Chemoattractant Protein-1; MIP, Macrophage Inflammatory Protein; RANTES, Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted; TGF, Transforming Growth Factor; Th, T-helper; VEGF, Vascular Endothelial Growth Factor (VEGF).

vascularization (52, 88–90). In the present study, tumor VEGF and TGF-α1 expression were higher than in non-tumor-bearing mice, but were not affected by age or stress. In contrast, age-dependent effects of stress were demonstrated for G- and GM-CSF, which may differentially affect tumor progression in young and old mice. Age- and/or stress-related influences on tumors have implications for cancer treatment. For example, aging differences in tumor vascularization with certain types of cancer (91) could affect therapeutics delivered *via* the circulation, and research supports that psychosocial stressors can affect cancer outcomes (80–82).

Antitumor host defense may provide an explanation, at least in part, for age- and/or stress-related differences, as it is well established that inflammation, innate and adaptive immunity regulates tumor progression (92–95). The numbers of tumor infiltrating F4/80$^+$ macrophages and CD8$^+$ T cells reported in this study are consistent with those reported by Nasu and colleagues (94, 95). F4/80$^+$ macrophages were the major leukocyte infiltrate into the RM-9 tumor. The number of intratumoral macrophages was lower by about 30% in old versus young non-stressed mice, but increased by ~30% in old, stressed mice. No significant correlation between RM-9 tumor weight and tumor macrophage numbers was observed; however, increased intratumoral macrophage density was associated with poor prognosis (96).

Consistent with a role for adaptive immunity in regulating RM-9 tumor growth, altered tumor CD4$^+$ T cell number and/or phenotype can affect the regulation of CD8$^+$ T cell priming (97, 98). IL-2-mediated CD8$^+$ T cell activation, clonal expansion, and maintenance of memory effector function was required for eliciting effective and specific immunity against tumors (98, 99). Still, the role of psychosocial stress and interaction with age and the cellular immune response against prostate cancer progression remains unclear. Like CD8$^+$ T cells, CD4$^+$FoxP3$^+$ Treg cell infiltration was low in all treatment groups, but both age and stress effects were revealed, as well as relationships with circulating corticosterone and CD4$^+$ T cell and macrophages tumor infiltration.

Few clinical studies have investigated links between psychosocial stressors on cytokine profiles in patients with PCa (100–102). Our data highlight the importance of using old animals to study PCa, as well as looking specifically at age-related differences in behaviors and physical response to the tumor. Human subjects participating in clinical studies are generally older men, since PCa is an age-related disease. Understanding age-related anxiety-like and depression-like behaviors and the subsequent effects of patient's attitudes on cancer can lead to better therapeutic interventions for these older men. PCa studies using animal models tend to use young mice as subjects, which our data suggest may lead to invalid conclusions. Our data further supports that identifying age-related differences will enable researchers to discern valid parameters when using younger mice.

Future studies should include control animals to determine how the tumor affects behavior and possibly masks some of the response to other stressors. One of the limitations of this research is the lack of non-tumor groups with or without exposure to stress, which prevents assessment of surgery-related stress. Additionally, refined methods that increase the time interval between tumor inoculation and cancer-induced mortality are needed to optimize this model for studying how physiological reactions to stress vary with age and their consequences for behavior and tumor immunity.

To summarize, in the present study, all mice displayed increased anxiety-like symptoms above baseline measures. Age and stress affected general activity levels, and old mice demonstrated greater depression-like behaviors. Critically, age correlated with tumor size, as tumor growth was greater in old than young mice, although there were no age-related differences in tumor TGF-β1 or VEGF levels. Furthermore, although our model did not demonstrate that stress affected tumor size *per se*, the model was successful in showing that: (a) behavior was affected by stress; (b) stress hormone levels increased due to isolation and restraint-stressing, and (c) both age and stress differentially affected intratumoral leukocyte infiltration in a complex way possibly attributed to unique cytokine profiles. Differences in the infiltration of immune cell subsets in young and old mice strongly suggest age-dependent mechanisms in tumor immunity. Collectively, these findings highlight the importance of using age-relevant orthotopic models in understanding mind-body interactions on PCa outcome in the development of effective immune-based treatments in PCa that are prevalent in the elderly population.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, DLB, upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by Loma Linda University Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

DB: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, writing original draft, review and editing. MD: data curation, formal analysis, investigation, methodology, manuscript writing reviewing and editing. CM: data curation, formal analysis, funding acquisition, investigation, investigation, methodology, validation, manuscript review and editing. PG: data curation, formal analysis, investigation, methodology, validation, manuscript review and editing. DL: formal analysis, investigation, manuscript review and editing. DG: conceptualization, investigation, methodologies, resources, manuscript review and editing. RH: conceptualization data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, manuscript writing original draft, review

and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the National Cancer Institute at the National Institutes of Health [R21CA116698].

ACKNOWLEDGMENTS

We graciously thank Dr Timothy Thompson, Professor and Director of the Prostate Cancer Basic Science Research in the Department of Genitourinary Medical Oncology and Division of Cancer Medicine for providing the RM9 cell line. We also thank Dr. Jeff Cao, Melba Andres, and Linda Healy for their technical assistance.

REFERENCES

1. Miller KD, Nogueira L, Mariotto AB, Rowland JH, Yabroff KR, Alfano CM, et al. Cancer Treatment and Survivorship Statistics, 2019. *CA Cancer J Clin* (2019) 69(5):363–85. doi: 10.3322/caac.21565
2. Flores IE, Sierra-Fonseca JA, Davalos O, Saenz LA, Castellanos MM, Zavala JK, et al. Stress Alters the Expression of Cancer-Related Genes in the Prostate. *BMC Cancer* (2017) 17(1):621. doi: 10.1186/s12885-017-3635-4
3. Osmanovic-Thunstrom A, Mossello E, Åkerstedt T, Fratiglioni L, Wang HX. Do Levels of Perceived Stress Increase With Increasing Age After Age 65? A Population-Based Study. *Age Ageing* (2015) 44:828–34. doi: 10.1093/ageing/afv078
4. Available at: https://www.cancer.net/cancer-types/prostate-cancer/statistics.
5. Erim DO, Bensen JT, Mohler JL, Fontham ETH, Song L, Farnan L, et al. Prevalence and Predictors of Probable Depression in PCa Survivors. *Cancer* (2019) 125(19):3418–27. doi: 10.1002/cncr.32338
6. Lin PH, Liu JM, Hsu RJ, Chuang HC, Chang SW, Pang ST, et al. Depression Negatively Impacts Survival of Patients With Metastatic PCa. *Int J Environ Res Public Health* (2018) 15(10):pii: E2148. doi: 10.3390/ijerph15102148
7. Wang YH, Li JQ, Shi JF, Que JY, Liu JJ, Lappin JM, et al. Depression and Anxiety in Relation to Cancer Incidence and Mortality: A Systematic Review and Meta-Analysis of Cohort Studies. *Mol Psychiatry* (2020) 25(7):1487–99. doi: 10.1038/s41380-019-0595-x
8. Watts S, Leydon G, Birch B, Prescott P, Lai L, Eardley S, et al. Depression and Anxiety in PCa: A Systematic Review and Meta-Analysis of Prevalence Rates. *BMJ Open* (2014) 4(3):e003901. doi: 10.1136/bmjopen-2013-003901
9. Batty GD, Russ TC, Stamatakis E, Kivimäki M. Psychological Distress in Relation to Site Specific Cancer Mortality: Pooling of Unpublished Data From 16 Prospective Cohort Studies. *BMJ* (2017) 356:j108. doi: 10.1136/bmj.j108
10. Sharpley CF, Bitsika V, Christie DRH, Bradford R, Steigler A , Denham JW. Total Depression and Subtypes in Prostate Cancer Survivors 10 Years After Treatment. *Eur J Cancer Care (Engl)* (2017) 26(6):10.1111/ecc.12630. doi: 10.1111/ecc.12630
11. Lacourt TE, Vichaya EG, Escalante C, Manzullo EF, Gunn B, Hess KR, et al. An Effort Expenditure Perspective on Cancer-Related Fatigue. *Psychoneuroendocrinology* (2018) 96:109–17. doi: 10.1016/j.psyneuen.2018.06.009
12. Lutgendorf SK, Weinrib AZ, Penedo F, Russell D, DeGeest K, Costanzo ES, et al. Interleukin-6, Cortisol, and Depressive Symptoms in Ovarian Cancer Patients. *J Clin Oncol* (2008) 26(29):4820–7. doi: 10.1200/JCO.2007.14.1978
13. Wefel JS, Kesler SR, Noll KR, Schagen SB. Clinical Characteristics, Pathophysiology, and Management of Noncentral Nervous System Cancer-Related Cognitive Impairment in Adults. *CA Cancer J Clin* (2015) 65(2):123–38. doi: 10.3322/caac.21258
14. Reeves FA, Battye S, Roth H, Peters JS, Hovens C, Costello AJ, et al. Prostatic Nerve Subtypes Independently Predict Biochemical Recurrence in Prostate Cancer. *J Clin Neurosci* (2019) 63:213–9. doi: 10.1016/j.jocn.2019.01.052
15. Gidron Y, De Couck M, De Greve J. If You Have an Active Vagus Nerve, Cancer Stage may No Longer be Important. *J Biol Regul Homeost Agents* (2014) 28(2):195–201.
16. Ali A, Pisipati S, Tewari A. Words of Wisdom: Re: Autonomic Nerve Development Contributes to Prostate Cancer Progression. *Eur Urol* (2014) 65(3):665–6. doi: 10.1016/j.eururo.2013.11.021
17. Wang AW, Hoyt MA. Benefit Finding and Diurnal Cortisol After Prostate Cancer: The Mediating Role of Positive Affect. *Psychooncology* (2018) 27(4):1200–5. doi: 10.1002/pon.4647
18. Hoyt MA, Bower JE, Irwin MR, Weierich MR, Stanton AL. Sleep Quality and Depressive Symptoms After Prostate Cancer: The Mechanistic Role of Cortisol. *Behav Neurosci* (2016) 130(3):351–6. doi: 10.1037/bne0000107
19. White CW, Xie JH, Ventura S. Age-Related Changes in the Innervation of the Prostate Gland: Implications for Prostate Cancer Initiation and Progression. *Organogenesis* (2013) 9(3):206–15. doi: 10.4161/org.24843
20. Miller AH, Ancoli-Israel S, Bower JE, Capuron L, Irwin MR. Neuroendocrine-Immune Mechanisms of Behavioral Comorbidities in Patients With Cancer. *J Clin Oncol* (2008) 26(6):971–82. doi: 10.1200/JCO.2007.10.7805
21. Bower JE, Ganz PA, Aziz N. Altered Cortisol Response to Psychologic Stress in Breast Cancer Survivors With Persistent Fatigue. *Psychosom Med* (2005) 67(2):277–80. doi: 10.1097/01.psy.0000155666.55034.c6
22. Hassan S, Karpova Y, Baiz D, Yancey D, Pullikuth A, Flores A, et al. Behavioral Stress Accelerates Prostate Cancer Development in Mice. *J Clin Invest* (2013) 123(2):874–86. doi: 10.1172/JCI63324
23. Herrera-Covarrubias D, Coria-Avila G, Hernandez ME, Ismail N. Stress During Puberty Facilitates Precancerous Prostate Lesions in Adult Rats. *Exp Oncol* (2017) 39(4):269–75. doi: 10.31768/2312-8852.2017.39(4):269-275
24. Decker AM, Jung Y, Cackowski FC, Yumoto K, Wang J, Taichman RS. Sympathetic Signaling Reactivates Quiescent Disseminated Prostate Cancer Cells in the Bone Marrow. *Mol Cancer Res* (2017) 15(12):1644–55. doi: 10.1158/1541-7786.MCR-17-0132
25. Pyter LM, Suarez-Kelly LP, Carson WE3rd, Kaur J, Bellisario J, Bever SR. Novel Rodent Model of Breast Cancer Survival With Persistent Anxiety-Like Behavior and Inflammation. *Behav Brain Res* (2017) 330:108–17. doi: 10.1016/j.bbr.2017.05.011
26. Sumis A, Cook KL, Andrade FO, Hu R, Kidney E, Zhang X, et al. Social Isolation Induces Autophagy in the Mouse Mammary Gland: Link to Increased Mammary Cancer Risk. *Endocr Relat Cancer* (2016) 23(10):839–56. doi: 10.1530/ERC-16-0359
27. Madden KS, Szpunar MJ, Brown EB. Early Impact of Social Isolation and Breast Tumor Progression in Mice. *Brain Behav Immun* (2013) 30 Suppl: S135–41. doi: 10.1016/j.bbi.2012.05.003
28. Schrepf A, Lutgendorf SK, Pyter LM. Pre-Treatment Effects of Peripheral Tumors on Brain and Behavior: Neuroinflammatory Mechanisms in Humans and Rodents. *Brain Behav Immun* (2015) 49:1–17. doi: 10.1016/j.bbi.2015.04.010
29. Seigers R, Fardell JE. Neurobiological Basis of Chemotherapy-Induced Cognitive Impairment: A Review of Rodent Research. *Neurosci Biobehav Rev* (2011) 35(3):729–41. doi: 10.1016/j.neubiorev.2010.09.006
30. Pyter LM, Pineros V, Galang JA, McClintock MK, Prendergast BJ. Peripheral Tumors Induce Depressive-Like Behaviors and Cytokine Production and Alter Hypothalamic-Pituitary-Adrenal Axis Regulation. *Proc Natl Acad Sci U S A* (2009) 106(22):9069–74. doi: 10.1073/pnas.0811949106
31. Bianchi-Frias D, Vakar-Lopez F, Coleman IM, Plymate SR, Reed MJ, Nelson PS. The Effects of Aging on the Molecular and Cellular Composition of the Prostate Microenvironment. *PLoS One* (2010) 5(9):e12501. doi: 10.1371/journal.pone.0012501
32. McDowell KL, Begley LA, Mor-Vaknin N, Markovitz DM, Macoska JA. Leukocytic Promotion of Prostate Cellular Proliferation. *Prostate* (2010) 70(4):377–89. doi: 10.1002/pros.21071
33. Ishii K, Takahashi S, Sugimura Y, Watanabe M. Role of Stromal Paracrine Signals in Proliferative Diseases of the Aging Human Prostate. *J Clin Med* (2018) 7(4):pii: E68. doi: 10.3390/jcm7040068

34. Levesque C, Nelson PS. Cellular Constituents of the Prostate Stroma: Key Contributors to PCa Progression and Therapy Resistance. *Cold Spring Harb Perspect Med* (2018) 8(8):pii: a030510. doi: 10.1101/cshperspect.a030510
35. Bianchi-Frias D, Damodarasamy M, Hernandez SA, Gil da Costa RM, Vakar-Lopez F, Coleman IM, et al. The Aged Microenvironment Influences the Tumorigenic Potential of Malignant Prostate Epithelial Cells. *Mol Cancer Res* (2019) 17(1):321–31. doi: 10.1158/1541-7786.MCR-18-0522
36. Thompson TC, Southgate J, Kitchener G, Land H. Multistage Carcinogenesis Induced by Ras and Myc Oncogenes in a Reconstituted Organ. *Cell* (1989) 56(6):917–30. doi: 10.1016/0092-8674(89)90625-9
37. Zhang S, Zhang HS, Reuter VE, Slovin SF, Sher HI, Livingston PO. Expression of Potential Target Antigens for Immunotherapy on Primary and Metastatic Prostate Cancers. *Clin Cancer Res* (1998) 4(2):295–302.
38. Thompson TC, Timme TL, Park SH, Yang G, Ren C. Mouse Prostate Reconstitution Model System: A Series of *In Vivo* and *In Vitro* Models for Benign and Malignant Prostatic Disease. *Prostate* (2000) 43(4):248–54. doi: 10.1002/1097-0045(20000601)43:4<248::aid-pros3>3.0.co;2-p
39. Dalvi A, Lucki I. Murine Models of Depression. *Psychopharmacol (Berl)* (1999) 147(1):14–6. doi: 10.1007/s002130051131
40. Crawley JN. *What's Wrong With My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice*. New York: Wiley-Liss (2000).
41. Brönimann S, Pradere B, Karakiewicz P, Abufaraj M, Briganti A, Shariat SF. An Overview of Current and Emerging Diagnostic, Staging and Prognostic Markers for Prostate Cancer. *Expert Rev Mol Diagn* (2020) 20(8):841–50. doi: 10.1080/14737159.2020.1785288
42. Ozerdem U, Wojcik EM, Duan X, Erşahin Ç, Barkan GA. Prognostic Utility of Quantitative Image Analysis of Microvascular Density in Prostate Cancer. *Pathol Int* (2013) 63(5):277–82. doi: 10.1111/pin.12056
43. Melegh Z, Oltean S. Targeting Angiogenesis in PCa. *Int J Mol Sci* (2019) 20 (11):2676. doi: 10.3390/ijms20112676
44. Dawes RP, Burke KA, Byun DK, Xu Z, Stastka P, Chan L, et al. Chronic Stress Exposure Suppresses Mammary Tumor Growth and Reduces Circulating Exosome TGF-β Content *via* α-Adrenergic Receptor Signaling in MMTV-PyMT Mice. *Breast Cancer (Auckl)* (2020) 14:1178223420931511. doi: 10.1177/1178223420931511
45. Gonzalez-Moreno O, Lecanda J, Green JE, Segura V, Catena R, Serrano D, et al. VEGF Elicits Epithelial-Mesenchymal Transition (EMT in Prostate Intraepithelial Neoplasia (PIN)-Like Cells *via* an Autocrine Loop. *Exp Cell Res* (2010) 316(4):554–67. doi: 10.1016/j.yexcr.2009.11.020
46. Diener KR, Need EF, Buchanan G, Hayball JD. TGF-Beta Signalling and Immunity in Prostate Tumourigenesis. *Expert Opin Ther Targets* (2010) 14 (2):179–92. doi: 10.1517/14728220903544507
47. Darrington E, Zhong M, Vo BH, Khan SA. Vascular Endothelial Growth Factor A, Secreted in Response to Transforming Growth Factor-β1 Under Hypoxic Conditions, Induces Autocrine Effects on Migration of PCa Cells. *Asian J Androl* (2012) 14(5):745–51. doi: 10.1038/aja.2011.197
48. Sánchez Ramírez J, Morera Díaz Y, Bequet-Romero M, Hernández-Bernal F, Selman-Housein Bernal KH, de la Torre Santos A, et al. Characteristics of the Specific Humoral Response in Patients With Advanced Solid Tumors After Active Immunotherapy With a VEGF Vaccine, at Different Antigen Doses and Using Two Distinct Adjuvants. *BMC Immunol* (2017) 18(1):39. doi: 10.1186/s12865-017-0222-z
49. Tang J, Li Z, Lu L, Cho CH. β-Adrenergic System, a Backstage Manipulator Regulating Tumour Progression and Drug Target in Cancer Therapy. *Semin Cancer Biol* (2013) 23(6 Pt B):533–42. doi: 10.1016/j.semcancer.2013.08.009
50. Prell RA, Gearin L, Simmons A, Vanroey M, Jooss K. The Anti-Tumor Efficacy of a GM-CSF-Secreting Tumor Cell Vaccine Is Not Inhibited by Docetaxel Administration. *Cancer Immunol Immunother* (2006) 55 (10):1285–93. doi: 10.1007/s00262-005-0116-4
51. Dobrenis K, Gauthier LR, Barroca V, Magnon C. Granulocyte Colony-Stimulating Factor Off-Target Effect on Nerve Outgrowth Promotes Prostate Cancer Development. *Int J Cancer* (2015) 136(4):982–8. doi: 10.1002/ijc.29046
52. Begley LA, Kasina S, MacDonald J, Macoska JA. The Inflammatory Microenvironment of the Aging Prostate Facilitates Cellular Proliferation and Hypertrophy. *Cytokine* (2008) 43(2):194–9. doi: 10.1016/j.cyto.2008.05.012
53. Loberg RD, Ying C, Craig M, Yan L, Snyder LA, Pienta KJ. CCL2 as an Important Mediator of Prostate Cancer Growth *In Vivo* Through the Regulation of Macrophage Infiltration. *Neoplasia* (2007) 9(7):556–2. doi: 10.1593/neo.07307
54. Lu Y, Chen Q, Corey E, Xie W, Fan J, Mizokami A, et al. Activation of MCP-1/CCR2 Axis Promotes PCa Growth in Bone. *Clin Exp Metastasis* (2009) 26 (2):161–9. doi: 10.1007/s10585-008-9226-7
55. Yoshimura T. The Chemokine MCP-1 (CCL2) in the Host Interaction With Cancer: A Foe or Ally? *Cell Mol Immunol* (2018) 15(4):335–45. doi: 10.1038/cmi.2017.135
56. Nagpal ML, Davis J, Lin T. Overexpression of CXCL10 in Human Prostate LNCaP Cells Activates its Receptor (CXCR3) Expression and Inhibits Cell Proliferation. *Biochim Biophys Acta* (2006) 1762(9):811–8. doi: 10.1016/j.bbadis.2006.06.017
57. Zhao R, Bei X, Yang B, Wang X, Jiang C, Shi F, et al. Endothelial Cells Promote Metastasis of Prostate Cancer by Enhancing Autophagy. *J Exp Clin Cancer Res* (2018) 37(1):221. doi: 10.1186/s13046-018-0884-2
58. Elsässer-Beile U, Przytulski B, Gierschner D, Grussenmeyer T, Katzenwadel A, Leiber C, et al. Comparison of the Activation Status of Tumor Infiltrating and Peripheral Lymphocytes of Patients With Adenocarcinomas and Benign Hyperplasia of the Prostate. *Prostate* (2000) 45(1):1–7. doi: 10.1002/1097-0045(20000915)45:1<1::aid-pros1>3.0.co;2-v
59. Maolake A, Izumi K, Natsagdorj A, Iwamoto H, Kadomoto S, Makino T, et al. Tumor Necrosis Factor-α Induces PCa Cell Migration in Lymphatic Metastasis Through CCR7 Upregulation. *Cancer Sci* (2018) 109(5):1524–31. doi: 10.1111/cas.13586
60. Ashok A, Keener R, Rubenstein M, Stookey S, Bajpai S, Hicks J, et al. Consequences of Interleukin 1β-Triggered Chronic Inflammation in the Mouse Prostate Gland: Altered Architecture Associated With Prolonged CD4(+) Infiltration Mimics Human Proliferative Inflammatory Atrophy. *Prostate* (2019) 79(7):732–45. doi: 10.1002/pros.23784
61. Mandai M, Hamanishi J, Abiko K, Matsumura N, Baba T, Konishi I. Dual Faces of Ifnγ in Cancer Progression: A Role of PD-L1 Induction in the Determination of Pro- and Antitumor Immunity. *Clin Cancer Res* (2016) 22 (10):2329–34. doi: 10.1158/1078-0432.CCR-16-0224
62. Untergasser G, Plas E, Pfister G, Heinrich E, Berger P. Interferon-Gamma Induces Neuroendocrine-Like Differentiation of Human Prostate Basal-Epithelial Cells. *Prostate* (2005) 64(4):419–29. doi: 10.1002/pros.20261
63. Kundu M, Roy A, Pahan K. Selective Neutralization of IL-12 P40 Monomer Induces Death in PCa Cells *via* IL-12-IFN-γ. *Proc Natl Acad Sci USA* (2017) 114(43):11482–7. doi: 10.1073/pnas.1705536114
64. Nguyen DP, Li J, Tewari AK. Inflammation and PCa: The Role of Interleukin 6 (IL-6). *BJU Int* (2014) 113(6):986–92. doi: 10.1111/bju.12452
65. Hu B, Qiu-Lan H, Lei RE, Shi C, Jiang HX, Qin SY. Interleukin-9 Promotes Pancreatic Cancer Cells Proliferation and Migration *via* the miR-200a/Beta-Catenin Axis. *BioMed Res Int* (2017) 2017:2831056. doi: 10.1155/2017/2831056
66. You FP, Zhang J, Cui T, Zhu R, Lv CQ, Tang HT, et al. Th9 Cells Promote Antitumor Immunity *via* IL-9 and IL-21 and Demonstrate Atypical Cytokine Expression in Breast Cancer. *Int Immunopharmacol* (2017) 52:163–7. doi: 10.1016/j.intimp.2017.08.031
67. Goswami R, Kaplan MH. A Brief History of IL-9. *J Immunol* (2011) 186 (6):3283–8. doi: 10.4049/jimmunol.1003049
68. Yu D, Zhong Y, Li X, Li Y, Li X, Cao J, et al. ILs-3, 6 and 11 Increase, But ILs-10 and 24 Decrease Stemness of Human PCa Cells *In Vitro*. *Oncotarget* (2015) 6(40):42687–703. doi: 10.18632/oncotarget.5883
69. Zhang Q, Liu S, Ge D, Zhang Q, Xue Y, Xiong Z, et al. Interleukin-17 Promotes Formation and Growth of Prostate Adenocarcinoma in Mouse Models. *Cancer Res* (2012) 72(10):2589–99. doi: 10.1158/0008-5472.CAN-11-3795
70. Nelson JB, Nguyen SH, Wu-Wong JR, Opgenorth TJ, Dixon DB, Chung LW, et al. New Bone Formation in an Osteoblastic Tumor Model Is Increased by Endothelin-1 Overexpression and Decreased by Endothelin A Receptor Blockade. *Urology* (1999) 53(5):1063–9. doi: 10.1016/s0090-4295(98)00658-x
71. Lopez DM, Voigt W. Adenocarcinoma R-3327 of the Copenhagen Rat as a Suitable Model for Immunological Studies of PCa. *Cancer Res* (1977) 37(7 Pt 1):2057–61.
72. Ravindranathan S, Nguyen KG, Kurtz SL, Frazier HN, Smith SG, Koppolu BP, et al. Tumor-Derived Granulocyte Colony-Stimulating Factor Diminishes Efficacy of Breast Tumor Cell Vaccines. *Breast Cancer Res* (2018) 20(1):126. doi: 10.1186/s13058-018-1054-3

73. Hassan S, Karpova Y, Flores A, D'Agostino RJr, Kulik G. Surgical Stress Delays Prostate Involution in Mice. *PLoS One* (2013) 8(11):e78175. doi: 10.1371/journal.pone.0078175
74. Dantzer R, Bluthé R-M, Castanon N, Chauvet N, Capuron L, Goodall G, et al. Cytokine Effects on Behavior. In: Ader R, Felten DL, Cohen N, editors. *Psychoneuroimmunology*, vol. 1. San Diego: Academic Press (2001). p. 703–27.
75. Vegas O, Beitia G, Sánchez-Martin JR, Arregi A, Azpiroz A. Behavioral and Neurochemical Responses in Mice Bearing Tumors Submitted to Social Stress. *Behav Brain Res* (2004) 155:125–34. doi: 10.1016/j.bbr.2004.04.006
76. Makinde AY, Rizvi A, Crapo JD, Pearlstein RD, Slater JM, Gridley DS. Metalloporphyrin Antioxidant Alters Cytokine Responses After Radiation in a Prostate Tumor Model. *Radiat Res* (2010) 173:441–52. doi: 10.1667/RR1765.1
77. McBride CM, Clipp E, Peterson BL, Lipkus IM, Demark-Wahnefried W. Psychological Impact of Diagnosis and Risk Reduction Among Cancer Survivors. *Psychooncology* (2000) 9(5):418–27. doi: 10.1002/1099-1611(200009/10)9:5<418::aid-pon474>3.0.co;2-e
78. Goodwin PJ, Leszcz M, Ennis M, Koopmans J, Vincent L, Guther H, et al. The Effect of Group Psychosocial Support on Survival in Metastatic Breast Cancer. *N Engl J Med* (2001) 345:1719–26. doi: 10.1056/NEJMoa011871
79. Bartolomucci A, Palanza P, Sacerdote P, Ceresini G, Chirieleison A, Panerai AE, et al. Individual Housing Induces Altered Immune Endocrine Responses to Psychological Stress in Male Mice. *Psychoneuroendocrinology* (2003) 28:540–58. doi: 10.1016/s0306-4530(02)00039-2
80. Burgess C, Cornelius V, Love S, Graham J, Richards M, Ramirez A. Depression and Anxiety in Women With Early Breast Cancer: Five Year Observational Cohort Study. *BMJ* (2005) 330:702–5. doi: 10.1136/bmj.38343.670868.D3
81. Hamer M, Chida Y, Molloy GJ. Psychological Distress and Cancer Mortality. *J Psychosom Res* (2009) 66(3):255–8. doi: 10.1016/j.jpsychores.2008.11.002
82. Chida Y, Hamer M, Wardle J, Steptoe A. Do Stress-Related Psychosocial Factors Contribute to Cancer Incidence and Survival? *Nat Clin Pract Oncol* (2008) 5(8):466–75. doi: 10.1038/ncponc1134
83. Reed MJ, Karres N, Eyman D, Cruz A, Brekken RA, Plymate S. The Effects of Aging on Tumor Growth and Angiogenesis are Tumor-Cell Dependent. *Int J Cancer* (2007) 120(4):753–60. doi: 10.1002/ijc.22351
84. Ershler WB, Stewart JA, Hacker MP, Moore AL, Tindle BH. B16 Murine Melanoma and Aging: Slower Growth and Longer Survival in Old Mice. *J Natl Cancer Inst* (1984) 72(1):161–4. doi: 10.1093/jnci/72.1.161
85. Pili R, Guo Y, Chang J, Nakanishi H, Martin GR, Passaniti A. Altered Angiogenesis Underlying Age-Dependent Changes in Tumor Growth. *J Natl Cancer Inst* (1994) 86(17):1303–14. doi: 10.1093/jnci/86.17.1303
86. Itzhaki O, Kaptzan T, Skutelsky E, Sinai J, Michowitz M, Siegal A, et al. Age-Adjusted Antitumoral Therapy Based on the Demonstration of Increased Apoptosis as a Mechanism Underlying the Reduced Malignancy of Tumors in the Aged. *Biochim Biophys Acta* (2004) 1688(2):145–59. doi: 10.1016/j.bbadis.2003.11.009
87. Rohena-Rivera K, Sánchez-Vázquez MM, Aponte-Colón DA, Forestier-Román IS, Quintero-Aguiló ME, Martínez-Ferreret M. CCL-4 Enhances Prostate Cancer Migration and Invasion by Modulating Integrin Expression. *Int J Clin Exp Med* (2016) 9(3):5426–38.
88. Fang LY, Izumi K, Lai KP, Liang L, Li L, Miyamoto H, et al. Infiltrating Macrophages Promote Prostate Tumorigenesis *via* Modulating Androgen Receptor-Mediated CCL4-STAT3 Signaling. *Cancer Res* (2013) 73(18):5633–46. doi: 10.1158/0008-5472.CAN-12-3228
89. Kogan-Sakin I, Cohen M, Paland N, Madar S, Solomon H, Molchadsky A, et al. Prostate Stromal Cells Produce CXCL-1, CXCL-2, CXCL-3 and IL-8 in Response to Epithelia-Secreted IL-1. *Carcinogenesis* (2009) 30(4):698–705. doi: 10.1093/carcin/bgp043
90. Liao YY, Tsai HC, Chou PY, Wang SW, Chen HT, Lin YM, et al. CCL3 Promotes Angiogenesis by Dysregulation of miR-374b/VEGF-A Axis in Human Osteosarcoma Cells. *Oncotarget* (2016) 7(4):4310–25. doi: 10.18632/oncotarget.6708
91. Marinho A, Soares R, Ferro J, Lacerda M, Schmitt FC. Angiogenesis in Breast Cancer is Related to Age But Not to Other Prognostic Parameters. *Pathol Res Pract* (1997) 193(4):267–73. doi: 10.1016/S0344-0338(97)80003-9
92. Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, et al. Adaptive Immunity Maintains Occult Cancer in an Equilibrium State. *Nature* (2007) 450(7171):903–7. doi: 10.1038/nature06309
93. Yaqub S, Aandahl EM. Inflammation Versus Adaptive Immunity in Cancer Pathogenesis. *Crit Rev Oncog* (2009) 15(1-2):43–63. doi: 10.1615/critrevoncog.v15.i1-2.20
94. Nasu Y, Bangma CH, Hull GW, Lee HM, Hu J, Wang J, et al. Adenovirus-Mediated Interleukin-12 Gene Therapy for Prostate Cancer: Suppression of Orthotopic Tumor Growth and Pre-Established Lung Metastases in an Orthotopic Model. *Gene Ther* (1999) 6(3):338–49. doi: 10.1038/sj.gt.3300834
95. Nasu Y, Bangma CH, Hull GW, Yang G, Wang J, Shimura S, et al. Combination Gene Therapy With Adenoviral Vector-Mediated HSV-Tk +GCV and IL-12 in an Orthotopic Mouse Model for Prostate Cancer. *Prostate Cancer Prostatic Dis* (2001) 4(1):44–55. doi: 10.1038/sj.pcan.4500494
96. Qualls JE, Murray PJ. Tumor Macrophages Protective and Pathogenic Roles in Cancer Development. *Curr Top Dev Biol* (2011) 94:309–28. doi: 10.1016/B978-0-12-380916-2.00010-3
97. Erlandsson A, Carlsson J, Lundholm M, Fält A, Andersson SO, Andrén O, et al. M2 Macrophages and Regulatory T Cells in Lethal Prostate Cancer. *Prostate* (2019) 79(4):363–9. doi: 10.1002/pros.23742
98. Smith CM, Wilson NS, Waithman J, Villadangos JA, Carbone FR, Heath WR, et al. Cognate CD4(+) T Cell Licensing of Dendritic Cells in CD8(+) T Cell Immunity. *Nat Immunol* (2004) 5(11):1143–8. doi: 10.1038/ni1129
99. Nesbeth YC, Martinez DG, Toraya S, Scarlett UK, Cubillos-Ruiz JR, Rutkowski MR, et al. CD4+ T Cells Elicit Host Immune Responses to MHC Class II-Negative Ovarian Cancer Through CCL5 Secretion and CD40-Mediated Licensing of Dendritic Cells. *J Immunol* (2010) 184(10):5654–62. doi: 10.4049/jimmunol.0903247
100. Sepah SC, Bower JE. Positive Affect and Inflammation During Radiation Treatment for Breast and Prostate Cancer. *Brain Behav Immun* (2009) 23(8):1068–72. doi: 10.1016/j.bbi.2009.06.149
101. Carlson LE, Speca M, Patel KD, Goodey E. Mindfulness-Based Stress Reduction in Relation to Quality of Life, Mood, Symptoms of Stress, and Immune Parameters in Breast and Prostate Cancer Outpatients. *Psychosom Med* (2003) 65(4):571–81. doi: 10.1097/01.PSY.0000074003.35911.41
102. Carlson LE, Speca M, Faris P, Patel KD. One Year Pre-Post Intervention Follow-Up of Psychological, Immune, Endocrine and Blood Pressure Outcomes of Mindfulness-Based Stress Reduction (MBSR) in Breast and Prostate Cancer Outpatients. *Brain Behav Immun* (2007) 21(8):1038–49. doi: 10.1016/j.bbi.2007.04.002

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

SYSTEMATIC REVIEW
published: 14 October 2021
doi: 10.3389/fonc.2021.739263

Balancing the Risk-Benefit Ratio of Immune Checkpoint Inhibitor and Anti-VEGF Combination Therapy in Renal Cell Carcinoma: A Systematic Review and Meta-Analysis

Li Tao[1†], *Huiyun Zhang*[2†], *Guangyu An*[2], *Haoning Lan*[1], *Yaoqi Xu*[1], *Yang Ge*[2*] and *Jiannan Yao*[2*]

[1] Beijing Chaoyang Hospital, Capital Medical University, Beijing, China, [2] Department of Oncology, Beijing Chaoyang Hospital, Capital Medical University, Beijing, China

OPEN ACCESS

Edited by:
Sanja Štifter,
Skejby Sygehus, Denmark

Reviewed by:
Eric Lehrer,
Icahn School of Medicine at Mount Sinai, United States
Ignacio Duran,
Marqués de Valdecilla University Hospital, Spain

***Correspondence:**
Jiannan Yao
silversand1986@sina.com
Yang Ge
Interna-1@163.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Genitourinary Oncology, a section of the journal Frontiers in Oncology

Received: 10 July 2021
Accepted: 20 September 2021
Published: 14 October 2021

Citation:
Tao L, Zhang H, An G, Lan H, Xu Y, Ge Y and Yao J (2021) Balancing the Risk-Benefit Ratio of Immune Checkpoint Inhibitor and Anti-VEGF Combination Therapy in Renal Cell Carcinoma: A Systematic Review and Meta-Analysis. Front. Oncol. 11:739263. doi: 10.3389/fonc.2021.739263

Background: Although immune checkpoint inhibitors (ICIs) combined with vascular endothelial growth factor receptor (VEGFR)-targeted therapy and sunitinib monotherapy have been widely applied to metastatic renal cell carcinoma (mRCC), effectiveness and safety data are still lacking. To optimize clinical decision-making, we conducted a systematic review and meta-analysis of published randomized clinical trials to characterize the efficacy and the risk of adverse events (AEs) in patients treated with ICIs plus anti-VEGF therapy.

Materials and Methods: We used PubMed, EMBASE, and the Cochrane Library to retrieve randomized controlled trials (RCTs) published before March 27, 2021. The efficacy outcomes were progression-free survival (PFS), overall survival (OS), and objective response rate (ORR). The pooled risk ratio (RR) and 95% confidence intervals (CI) of AEs were calculated in the safety analysis.

Results: Six RCTs involving 4,227 patients were identified after a systematic search. For OS, ICI and anti-VEGF combination therapy decreased mortality approximately 30% in the intention-to-treat population (ITT) (hazard ratio (HR) = 0.70, 95% CI: 0.57–0.87), but there was no statistical difference in patients evaluated as "favorable" by the International Metastatic Renal-Cell Carcinoma Database Consortium (IMDC) criteria compared with monotherapy (HR = 0.90, 95% CI: 0.55–1.46, $p = 0.66$). In terms of PFS, the progression risk for all participants declined 35% (HR = 0.65, 95% CI: 0.50–0.83) and patients evaluated as "poor" by IMDC benefited further (HR = 0.46, 95% CI: 0.36–0.58). No evident divergence was found in age and sex subgroups. The RRs of all-grade hypertension, arthralgia, rash, proteinuria, high-grade (grades 3–5) arthralgia, and proteinuria developed after combination therapy were increased compared with sunitinib. The risk of high-grade hypertension and rash showed no statistical difference. However, the risk of hand-foot skin reaction (HFSR), stomatitis, and dysgeusia decreased in combination therapy groups.

Conclusions: Compared with sunitinib, OS, PFS, and ORR were significantly improved in patients receiving ICI and anti-VEGF combination therapy at the expense of increased specific AEs. More attention should be paid to individualized application of these combination therapies to achieve the best benefit-risk ratio in the clinic.

Systematic Review Registration: [https://inplasy.com/] INPLASY: 202130104.

Keywords: combination therapy, renal cell carcinoma (RCC), immune checkpoint inhibitors (ICI), efficacy, safety, VEGF targeted therapy

1 INTRODUCTION

Renal cell carcinoma (RCC) is the most common renal neoplasm (1) and approximately 30% of patients present with metastatic disease (2), thus, aggravating the mortality of RCC. In the last decade, medical treatment for RCC has laid great emphasis on vascular endothelial growth factor receptor (VEGFR) monoclonal antibody and tyrosine kinase inhibitors (TKI) (3). These VEGF targeted therapies have improved clinical outcomes by suppressing endothelial cell proliferation and reforming carcinoma vasculature. Depending on treatment type, metastatic RCC response rates can reach 30%, and median overall survival can reach up to 2 years (4). Extensive clinical research has shown that sunitinib, a widely used VEGF inhibitor, is associated with drug resistance and numerous adverse events which may lead to frequent treatment withdrawal (4–6). Additionally, 63% of patients receiving sunitinib reported grade 3 or higher adverse events (AEs) including hypertension, rash, fatigue, and hand-foot skin syndrome (HFSR) (7).

Recently, the development and approval of immune checkpoint inhibitors (ICIs) has altered the treatment paradigm for RCC (8). Agents that target cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), programmed cell death receptor-1 (PD-1), and programmed cell death ligand-1 (PD-L1) are the most widely studied and recognized (9). However, these agents are broadly associated with ill-defined AEs, referred to as immune-related adverse events (irAEs), and characterized by clinical manifestations similar to autoimmunity disorders (10). Moreover, long-term exposure to ICIs can cause primary or secondary resistance (11). The leading underlying mechanisms for resistance include neoantigen loss, defect of antigen presentation, alternative immune checkpoints, and defective interferon signaling.

In order to address these concerns, multiple research groups are actively seeking effective treatments for RCC, as evidenced by 321 clinical trials listed at ClinicalTrials.gov as of February 10, 2020, including combination therapy of anti-VEGF and ICIs (8). Antiangiogenics (such as cabozantinib and axitinib) with pleiotropic immunomodulating properties, combined with immunotherapies, are preferred to traditional monotherapy (4). In RCC, the von Hippel-Lindau (VHL) gene is often silenced or lost, which drives the development of a highly vascularized pathology. Notably, PD-1 and its ligands are reported to be expressed on kidney macrophages, dendritic cells, lymphocytes, and renal proximal tubule epithelial cells (12). These two factors contribute to the immune-suppressive microenvironment. Thus, the combination of ICIs and VEGF targeted therapy offers synergistic improvements (7). However, the optimal combination regimen and sequence of treatments will likely continue to evolve as novel therapeutic agents and combinations gain FDA approval (13). To date, both combination therapy with ICIs and anti-VEGF or sunitinib monotherapy have been recommended in the revised National Comprehensive Cancer Network (NCCN) guidelines.

Despite the demonstrated success of combination therapy, several important questions remain unresolved. Will the combination of ICIs and anti-VEGF improve the prognosis at a cost of increased toxicity? Are there any clinical factors that could guide decision making in order to prolong effective treatment and maintain patient quality of life (QoL)? Based on the remarkable efficacy shown previously and the recent findings of the randomized controlled trials with combination therapy, we performed a systematic review and meta-analysis to further evaluate the impact of ICIs and anti-VEGF combination therapy on the clinical outcomes of RCC patients. Our findings catalog the frequency and severity of the most common AEs, including hypertension, arthralgia, rash, proteinuria, HFSR, stomatitis, and dysgeusia, which might lead to treatment withdrawal and severe clinical consequences (14–22).

2 METHODS

2.1 Search Strategy

We performed a systematic search for associated studies published before March 27, 2021, in Pubmed, Embase, and the Cochrane Library. The search terms were as follows: "renal carcinoma/exp" and "randomized controlled trial/exp" and ("vasculartropin/exp" or "anti-angiogenesis/exp" or "angiogenesis inhibitor/exp") and ("immune checkpoint inhibitor/exp" or "programmed cell death protein 1/exp" or "programmed cell death ligand protein 1/exp" or "cytotoxic T-lymphocyte-associated protein 4/exp") and "human/exp". No language limitation was applied, and all adopted studies were screened manually from the reference list and other relevant articles. Two reviewers (LT and HZ) independently searched and assessed the content and quality. Any disagreement was resolved by the corresponding author. The PRISMA statement is displayed in **Supplementary Table 1**.

2.2 Study Selection

The inclusion criteria were as follows: (1) Patients who were diagnosed with RCC or had untreated advanced RCC with a clear-cell component and at least one measurable lesion according to the Response Evaluation Criteria in Solid Tumors (RECIST); (2) Karnofsky performance status score of at least 70 (scores range from 0 to 100, with lower scores indicating greater disability); (3) Adults (18 years old or older); (4) adequately controlled blood pressure, with or without medications; and adequate organ function; (5) patients without previous systemic therapy for advanced disease; (6) studies reported with efficacy, including overall survival (OS), progression-free survival (PFS), objective response rate (ORR), and associated AEs; (7) randomized controlled trial studies; and (8) when results from an RCT were reported and analyzed more than once, the primary data were included.

The exclusion criteria were as follows: (1) not related to RCC; (2) reviews, meta-analysis, case reports, letters, or expert opinions; (3) single arm; (4) insufficient data; (5) experimental group did not receive combination therapy of ICIs and anti-VEGF; (6) duplicates; (7) studies that enrolled patients younger than 18 years old or animals; and (8) not RCTs.

2.3 Data Extraction and Risk of Bias Assessment

Two reviewers (LT and HZ) independently extracted the data, and any disagreement was settled through discussion. The following data from eligible studies were collected: National Clinical Trial (NCT) number, first author, treatment arms, control arms, the overall number of patients, publication year, enrollment criteria, characteristics of patients, outcomes, study methods, and number of selected adverse events. The risk of bias was assessed by the Cochrane Collaboration and was classified as "low", "unclear", or "high" in several areas.

2.4 Outcome Measures

Outcomes for efficacy were evaluated by PFS, OS, and ORR (defined by the Response Evaluation Criteria in Solid Tumors), and safety was evaluated by events of selected AEs. The severity of AEs was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0.

2.5 Statistical Analysis

The hazard ratios (HR) were represented with 95% confidence intervals (CI) for generic inverse variance outcomes, and risk ratios (RR) were shown with 95% confidence intervals for outcomes. We adopted mean values for continuous outcomes.

Statistical heterogeneity across trials or subgroups was tested using the I^2 testing. As six of the trials were multicenter, the random effects model was adopted in all analyses to balance the effect of each study, and all included studies were equally weighted (23). The Inverse-Variance (I-V) pooling model was applied to analyze OS, PFS, and ORR, while the Mantel-Haenszel (M-H) pooling model was adopted in the analysis of adverse events. An I^2 >50% implied significant heterogeneity (24). Subgroup analysis and sensitivity analysis were performed where appropriate. Subgroup analysis was conducted for the primary outcomes: (1) subgroups with different evaluations from the IMDC; (2) PD-L1-positive or PD-L1-negative subgroups; (3) age subgroup (divided by the age of 65); and (4) sex subgroup.

3 RESULTS

A total of 3,042 studies were identified, of which 1,006 were duplicates. We scanned titles and abstracts and excluded 1,897 articles for not meeting the inclusion criteria. Having obtained full-text articles for 139 citations, we excluded 133 for non-RCT. Finally, six articles involving 4,227 participants were adopted in this systematic review and meta-analysis. The selection flow diagram is shown in **Figure 1A**.

3.1 Study Characteristics

The final analysis included six RCTs published between 2018 and 2021, all with sunitinib as the control arm. All the patients in these trials had never received any systematic anticancer therapy for RCC. Atezolizumab plus bevacizumab was applied as the treatment arms in NCT01984242 and NCT02420821. In other RCTs, different treatment combinations were adopted. Six trials researched the influence of PD-L1 expression on PFS. Five trials explored the impact of PD-L1 expression on OS. Overall, 4,227 participants were available for PFS and ORR and 4,025 for OS. Characteristics of included studies are shown in **Table 1**.

3.2 Risk and Bias

All six trials had an unclear risk of performance bias because their design was open label **(Figure 1B)**. Due to the absence of allocation design and independent assessment institution results in one trial (NCT01984242), the selection and detection bias were determined to be unclear. Publication bias was evaluated by constructing a funnel plot in the meta-analysis of the all-grade adverse events. Begg's test standardizes the effect size by subtracting the weighted mean and dividing it by the standard error, and then verifies whether the effect size is correlated with the standard error by correcting the rank correlation analysis (31). Begg's test was assessed by funnel plot asymmetry, and $p < 0.05$ was defined as significant publication bias **(Supplementary Figure 1)**. Egger's regression test uses linear regression to measure the symmetry of inverted funnel plot according to the natural log of ratio, and the intercept of the line represents the degree of asymmetry (32). If $p > 0.05$, there is no publication offset **(Supplementary Figure 2)**. Only the p-value of Egger's test for HFSR ($p>|t| = 0.045$) showed obvious publication bias, likely owing to the inadequate included articles ($n < 10$). Review Manager Version 5.2 (Cochrane IMS, Oxford, UK) and Stata/SE 16.0 was used to conduct statistical analysis.

3.3 Efficacy

3.3.1 Overall Survival

Five studies that included 2006 participants from the combination group and 2019 participants from the sunitinib groups examined the overall survival by HR. Combination

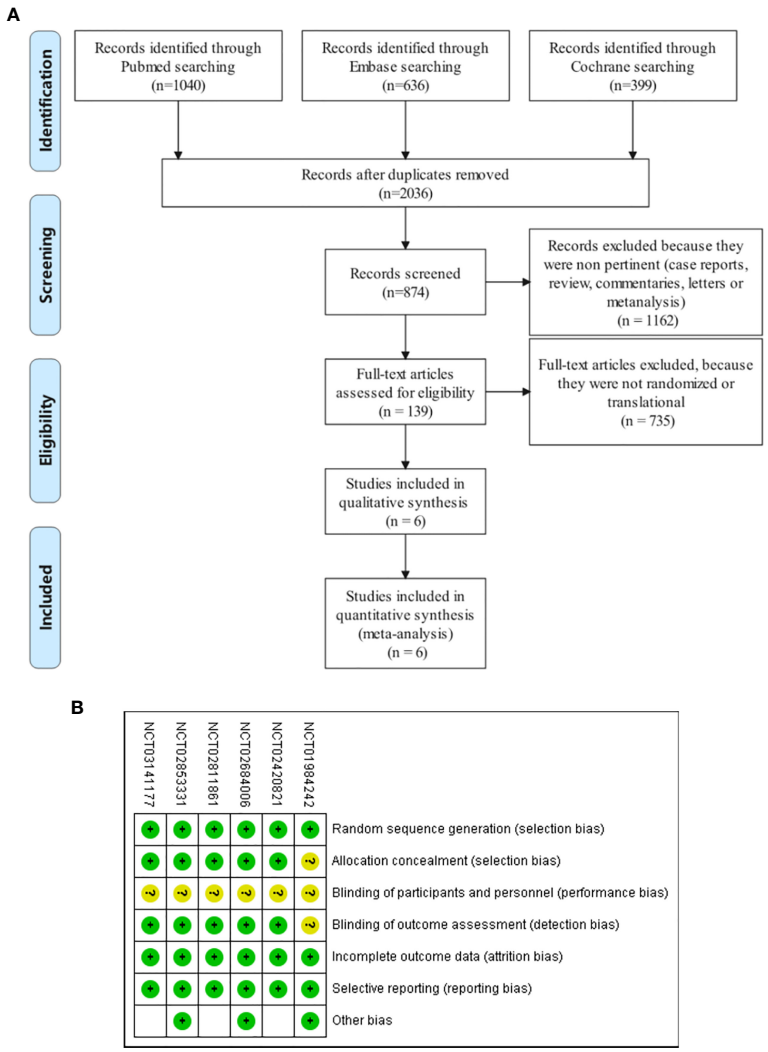


FIGURE 1 | **(A)** Flow diagram of study selection. Database searching was based on PubMed, EMBASE, and the Cochrane Library. **(B)** Quality assessment for six included studies. Quality of trials was categorized into three grades: low risk of bias (+), high risk of bias (–), and unclear (?).

therapy of ICIs and anti-VEGF decreased the risk of death relative to sunitinib alone by 30% (HR = 0.70, 95% CI: 0.57–0.87, p = 0.001; I^2 = 62%) (**Figure 2A**). We performed the subgroup analysis in four dimensions to further investigate the potential factors contributing to the outcomes.

3.3.1.1 OS in Patients With PD-L1-Positive Expression (≥1%)

Five articles were adopted to analyze the OS in the patients with PD-L1-positive expression (≥1%). The risk of death in combination therapy was decreased by 25% compared with sunitinib monotherapy (HR = 0.75, 95% CI: 0.62–0.91, p = 0.003; I^2 = 0%) (**Figure 2B**).

3.3.1.2 Subgroup Analysis OS by Age, Sex, and IMDC

Three trials were enrolled for IMDC evaluation concerning age and sex subgroups. No significant difference was detected in the age and sex subgroups (**Supplementary Figure S3**). For IMDC evaluation, the combination therapy showed little contribution (HR = 0.90, 95% CI: 0.55–1.46, p = 0.66; I^2 = 0%) in the favorable group, while showing decreased risk of death by 35% in the intermediate-risk subgroups (HR = 0.65, 95% CI: 0.51–0.83, p = 0.0004; I^2 = 0%) and 63% in the poor-risk subgroups (HR = 0.37, 95% CI: 0.26–0.54, p < 0.00001; I^2 = 0%) (**Figure 2C).**

3.3.2 PFS

A total of 4,227 patients from six RCTs were included to analyze HR in the intention-to-treat population (ITT) and PD-L1-positive subgroups, and four RCTs were adopted for IMDC evaluation in age and sex subgroups. Compared with sunitinib monotherapy, the combination of ICIs and anti-VEGF therapy decreased the hazard ratio for PFS by 35% (HR = 0.65, 95% CI: 0.50–0.83, p = 0.0008; I^2 = 89%) (**Figure 3A**).

TABLE 1 | Characteristics of the included studies.

NCT	NCT01984242 (25)	NCT02420821 (26)	NCT02684006 (27)	NCT02811861 (28)	NCT02853331 (29)	NCT03141177 (30)
Study	Immotion150	Immotion151	Javelin Renal 101	—	Keynote-426	CheckMate 9ER
Year	2018	2019	2020	2021	2019	2021
Author	McDermott, D. F.	Rini, B. I.	Motzer, R. J.	Motzer, R. J.	Rini, B. I.	Choueiri, T. K.
Treatment arms	Atezolizumab+ Bevacizumab*	Atezolizumab+ Bevacizumab	Avelumab+ Axitinib	Pembrolizumab+ Levatinib**	Pembrolizumab+ Axitinib	Nivolumab+ Cabozantinib***
Control	Sunitinib	Sunitinib	Sunitinib	Sunitinib	Sunitinib	Sunitinib
Number of patients	101 vs. 101	454 vs. 461	442 vs. 444	355 vs. 357	432 vs. 429	323 vs. 328
Median age(years)	62 vs. 61	62 vs. 60	62 vs. 61	64 vs. 62	62 vs. 61	62 vs. 61
Sex (male% / female%)	73/27 vs. 78/22	70/30 vs. 76/24	71/29 vs. 77/23	72/28 vs. 77/23	71/29 vs. 75/25	77/23 vs. 71/29
PD-L1 +(% of patients)	50 vs. 59	49 vs. 40	55 vs. 25	30 vs. 33	59 vs. 62	26 vs. 25
Prognostic model	MSKCC	MSKCC	IMDC	IMDC**** and MSKCC	IMDC	IMDC
Favorable risk %	30 vs. 21	20 vs. 20	21 vs. 22	31 vs. 35	32 vs. 30	23 vs. 22
Intermediate risk %	61 vs. 69	69 vs. 69	61 vs. 62	59 vs. 54	55 vs. 57	58 vs. 57
Poor risk %	9 vs. 10	11 vs. 11	16 vs. 16	9 vs. 10	13 vs. 12	19 vs. 21
Primary endpoints	PFS	OS, PFS	OS, PFS	OS, PFS	OS, PFS	OS, PFS
Median PFS (months)	11.7 vs. 8.4	11.2 vs. 8.4	13.8 vs. 8.4	23.9 vs. 9.2	15.1 vs. 11.1	16.6 vs. 8.3
Median OS (months)	NR	33.6 vs. 34.9	NR	NR	NR	NR
ORR	NR	151/454 vs. 144/460	227/442 vs. 114/444	252/355 vs. 129/357	256/432 vs. 153/429	180/323 vs. 89/328

NR not reported, PFS progression-free survival, OS overall survival, ORR objective response ratio.

** Atezolizumab alone arm was not considered.*

***Levatinib and Everolimus combination arm was not considered.*

****Nivolumab, Ipilimumab and Cabozantinib combination arm was not considered.*

*****Only IMDC was adopted in our analysis*

3.3.2.1 PFS in Patients With PD-L1-Positive Expression (≥1%)

In terms of the subgroups of PD-L1 expression, positive expression was associated with a steady 41% decrease in the hazard ratio (HR = 0.59, 95% CI: 0.50–0.70, $p < 0.00001$; $I^2 = 39\%$), while negative expression did not show a statistically significant decrease (HR = 0.73, 95% CI: 0.51–1.03, $p = 0.07$; $I^2 = 78\%$) **(Figure 3B)**.

3.3.2.2 Subgroup Analysis PFS by Age, Sex, and IMDC

Similarly, no significant differences were detected in PFS for age and sex subgroups **(Supplementary Figure S4)**, and the hazard ratio decreased when the IMDC evaluation worsened. Moreover, combination therapy decreased the risk of progression by 40% (HR = 0.60, 95% CI: 0.44–0.81, $p = 0.001$; $I^2 = 49\%$), 42% (HR = 0.58, 95% CI: 0.44–0.76, $p < 0.0001$; $I^2 = 75\%$), and 54% (HR = 0.46, 95% CI: 0.36–0.58, $p < 0.00001$; $I^2 = 15\%$) compared with sunitinib monotherapy in the favorable-, intermediate-, and poor-risk subgroups, respectively **(Figure 3C).**

3.3.3 ORR

Six studies were included to analyze the ORR. Compared with sunitinib, combination therapy increased the ORR by 111% (ORR = 2.11, 95% CI: 1.44–3.08, $p = 0.0001$; $I^2 = 88\%$) **(Figure 4)**.

3.4 Safety

Six randomized studies were adopted to calculate the RR of all- and high-grade (grades 3 to 5) AEs. According to previous research, some specific adverse events (e.g., proteinuria, arthralgia, rash, hypertension, diarrhea, stomatitis, HFSR, and dysgeusia) are monitored in RCC treatment and their presentation may lead to drug withdrawal. Therefore, we laid greater emphasis on these AEs and performed further meta-analysis to research the safety of combination therapy with ICIs and anti-VEGF versus sunitinib monotherapy. Except for proteinuria, all six RCTs were enrolled in the analysis of all-grade AEs and five RCTs were adopted in high-grade situations.

3.4.1 Proteinuria

Five studies were included in the analysis of all-grade, while four studies were included in high-grade proteinuria. Compared with sunitinib monotherapy, patients who received ICIs plus anti-VEGF therapy had significantly increased risk for all-grade proteinuria (RR = 2.27, 95% CI: 1.55–3.32, $p < 0.0001$; $I^2 = 75\%$). The same trend was observed for high-grade proteinuria (RR = 2.34, 95% CI: 1.33–4.12, $p = 0.003$; $I^2 = 27\%$) **(Figure 5)**.

3.4.2 Arthralgia

The combination of ICIs and anti-VEGF therapy increased the risk of both all-grade (RR = 2.14, 95% CI: 1.76–2.61, $p < 0.00001$; $I^2 = 32\%$) and high-grade arthralgia (RR = 2.48, 95% CI: 1.06–5.10, $p = 0.04$; $I^2 = 0\%$) compared with sunitinib monotherapy **(Figure 6)**.

3.4.3 Rash

All six studies demonstrated a significantly increased risk for all-grade rash when comparing combination therapy and sunitinib monotherapy (RR = 1.61, 95% CI: 1.27–2.04, $p < 0.0001$; $I^2 = 57\%$), but this trend was not statistically significant for high-grade rash (RR = 2.26, 95% CI: 0.77–6.68, $p = 0.14$; $I^2 = 32\%$) **(Figure 7).**

3.4.4 Hypertension

The comparison between patients treated with combination therapy in all-grade (six studies included) (RR = 1.17, 95% CI: 0.87–1.58, $p = 0.30$; $I^2 = 93\%$) and high-grade hypertension (five studies included) (RR = 1.17, 95% CI: 0.93–1.46, $p = 0.18$; $I^2 = 65\%$) did not reveal any significantly increased risk, respectively **(Figure 8)**.

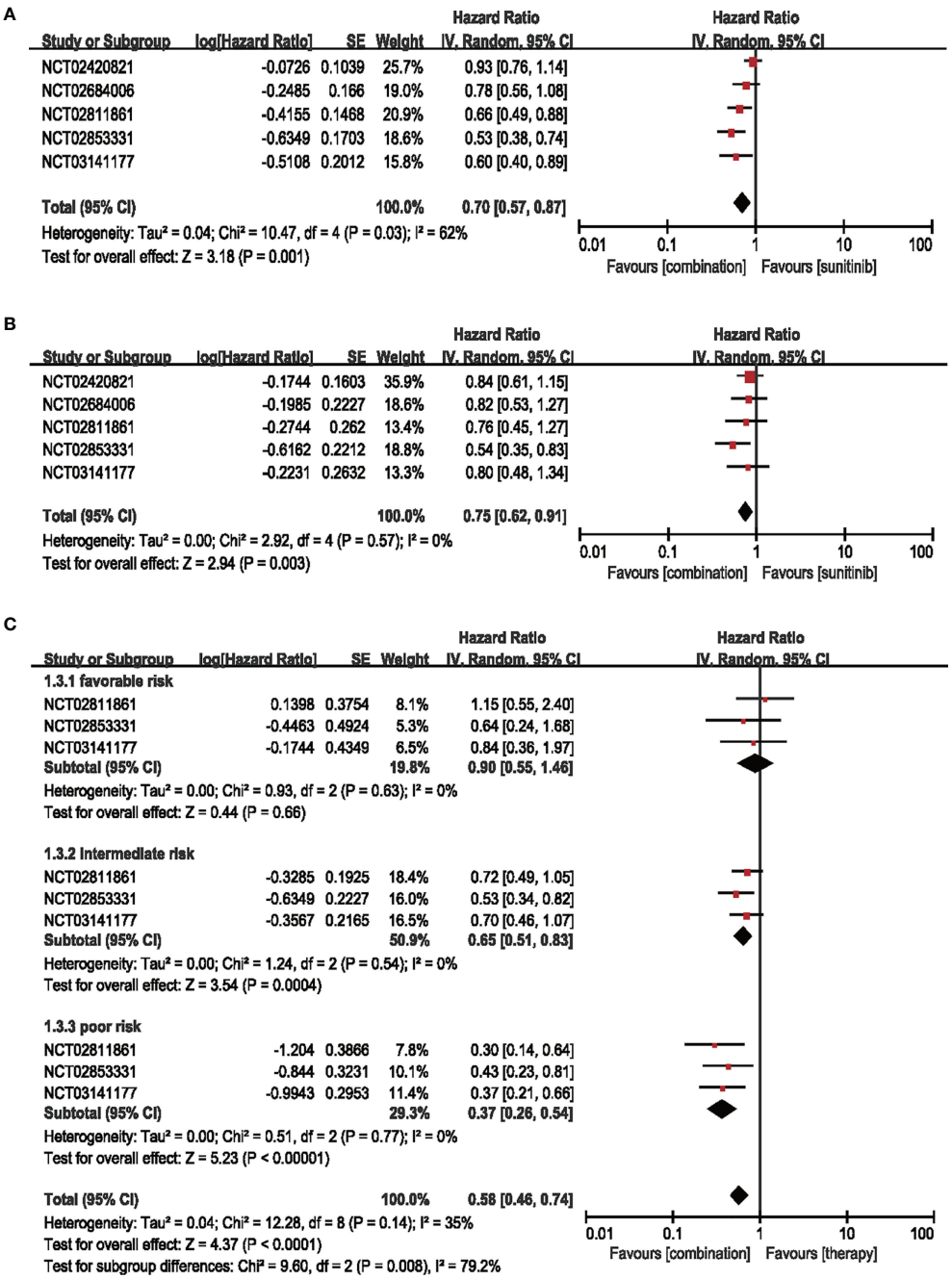


FIGURE 2 | **(A)** Forest plot of OS in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy. **(B)** Forest plot of PD-L1-positive patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy. **(C)** Forest plot of different IMDC-evaluated patients treated with combination therapy of ICI and anti-VEGF *vs.* sunitinib monotherapy.

3.4.5 Diarrhea

Compared with sunitinib monotherapy, no evident difference was shown in the analysis of the all-grade (RR = 0.94, 95% CI: 0.68–1.30, p = 0.72; I^2 = 96%) or high-grade (RR = 1.46, 95% CI: 0.86–2.48, p = 0.16; I^2 = 71%) diarrhea (**Figure 9**).

3.4.6 Stomatitis

Patients treated with combination therapy of ICIs and anti-VEGF showed a decreased risk (RR = 0.71, 95% CI: 0.56–0.91, p = 0.008; I^2 = 76%) of all-grade stomatitis, while no significant benefit (RR = 0.72, 95% CI: 0.34-1.54, p = 0.40; I^2 = 50%) was obtained in high-grade stomatitis (**Figure 10**).

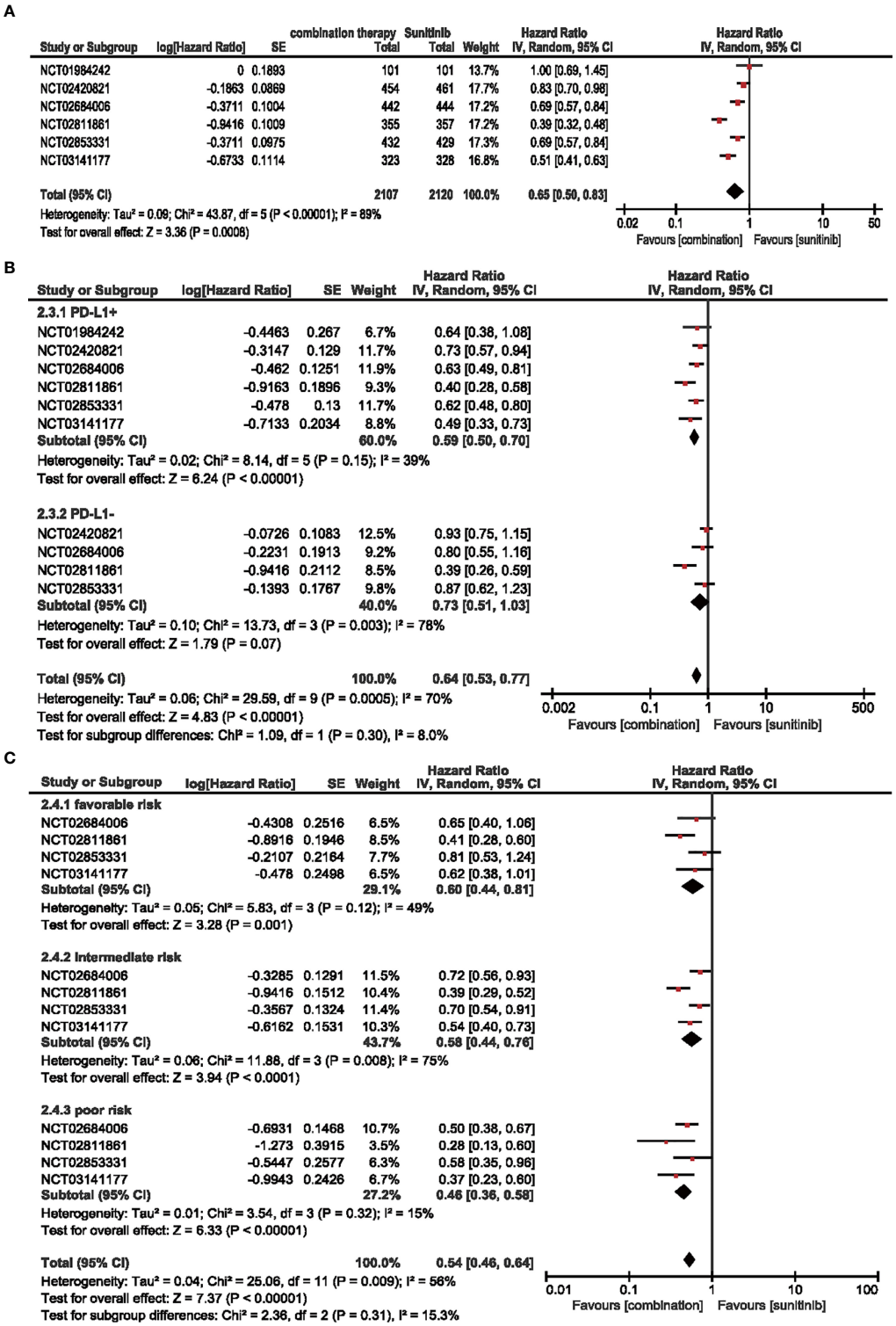


FIGURE 3 | **(A)** Forest plot of PFS in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy. **(B)** Forest plot of PD-L1-positive and PD-L1-negative patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy. **(C)** Forest plot of different IMDC-evaluated patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy.

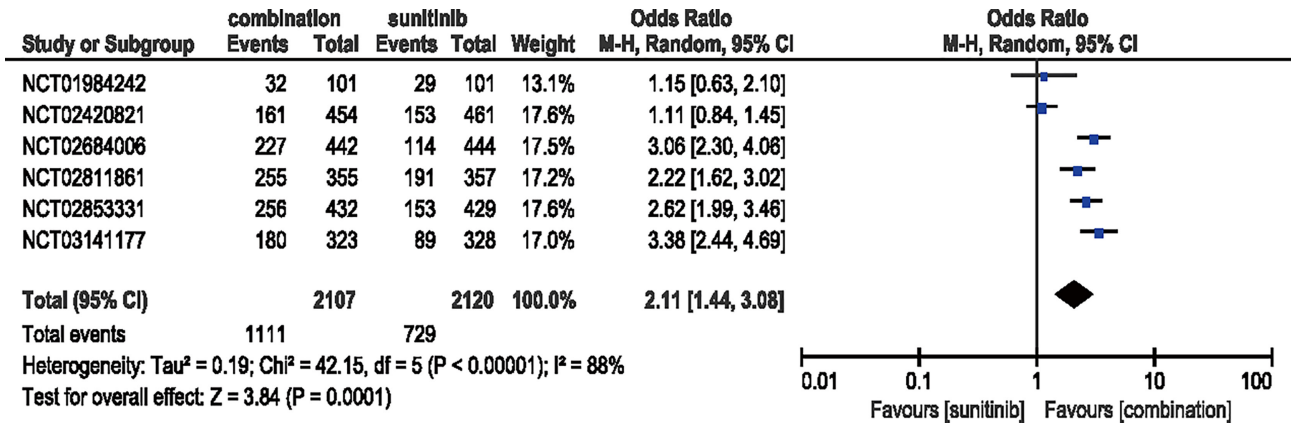


FIGURE 4 | Forest plot of ORR in patients treated with combination therapy of ICIs and anti-VEFR *vs.* sunitinib monotherapy.

3.4.7 HFSR

The risk of all-grade HFSR decreased (RR = 0.47, 95% CI: 0.28–0.79, $p = 0.004$; $I^2 = 96\%$) with the combination of ICIs and anti-VEGF therapy compared with sunitinib. However, no significant difference (RR = 0.93, 95% CI: 0.46–1.86, $p = 0.83$; $I^2 = 77\%$) was detected in the same analysis of high-grade HFSR (**Figure 11**).

3.4.8 Dysgeusia

In the analysis of all-grade dysgeusia, the RR decreased (RR = 0.42, 95% CI: 0.26–0.68, $p = 0.0004$; $I^2 = 91\%$) with the treatment of ICIs plus anti-VEGF therapy compared with sunitinib alone. However, the high-grade situation failed to support the same trend which may be due to inadequate incidence data (RR = 0.98, 95% CI: 0.17–5.65, $p = 0.98$; $I^2 = 0\%$) (**Figure 12**).

4 DISCUSSION

Although considerable progress has been made in deducing the molecular mechanism of advanced RCC and relevant targeting drugs, the overall efficiency of these therapies is not yet

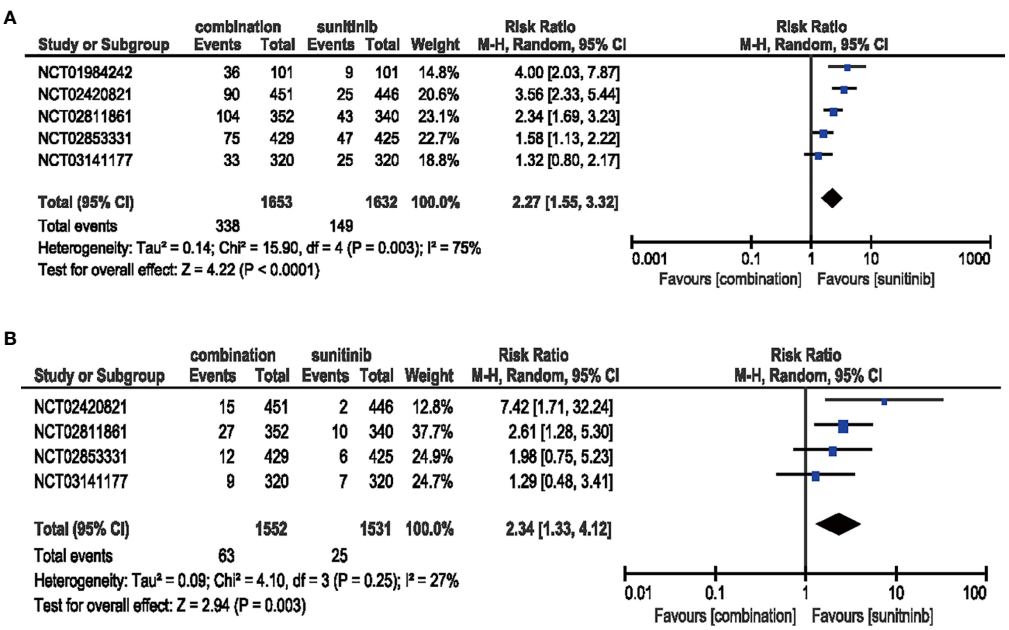


FIGURE 5 | **(A)** Forest plot of all-grade proteinuria in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy. **(B)** Forest plot of high-grade proteinuria in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy.

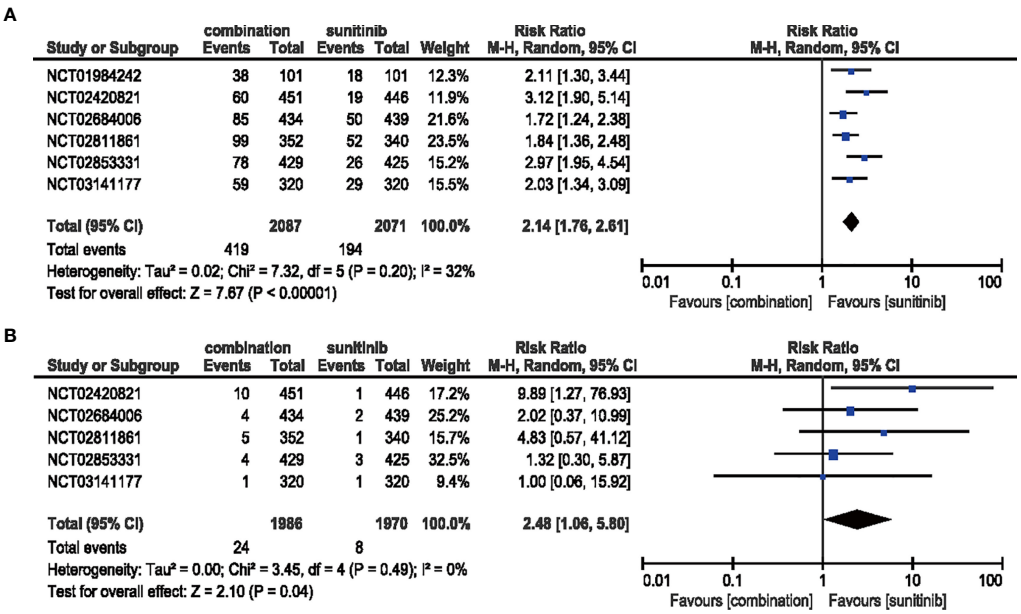


FIGURE 6 | **(A)** Forest plot of all-grade arthralgia in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy. **(B)** Forest plot of high-grade arthralgia in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy.

satisfactory (33). Recently, the combination of targeting agents and immune checkpoint inhibitors to treat advanced RCC has been the top priority, owing to its potential additive or synergistic effects due to the high-level blockade of aberrant signaling (16, 34). Therefore, the current meta-analysis was performed to evaluate the therapeutic effect and associated AEs of combination therapy of ICIs and anti-VEGF versus sunitinib for first-line treatment of advanced RCC.

Combination therapy demonstrated tremendous efficacy compared with the traditional strategy of sunitinib

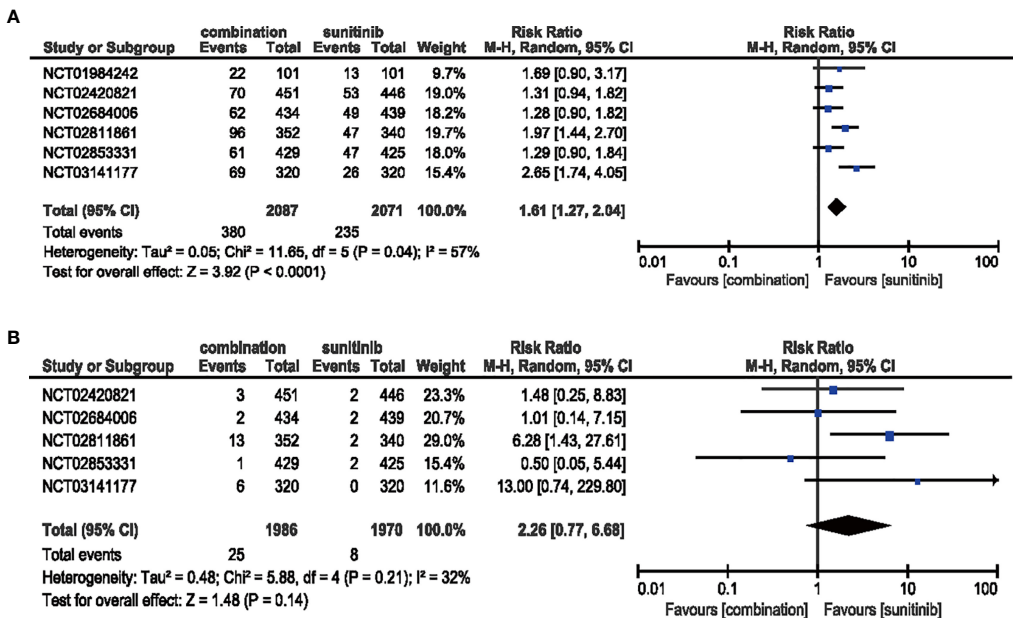


FIGURE 7 | **(A)** Forest plot of all-grade rash in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy. **(B)** Forest plot of high-grade rash in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy.

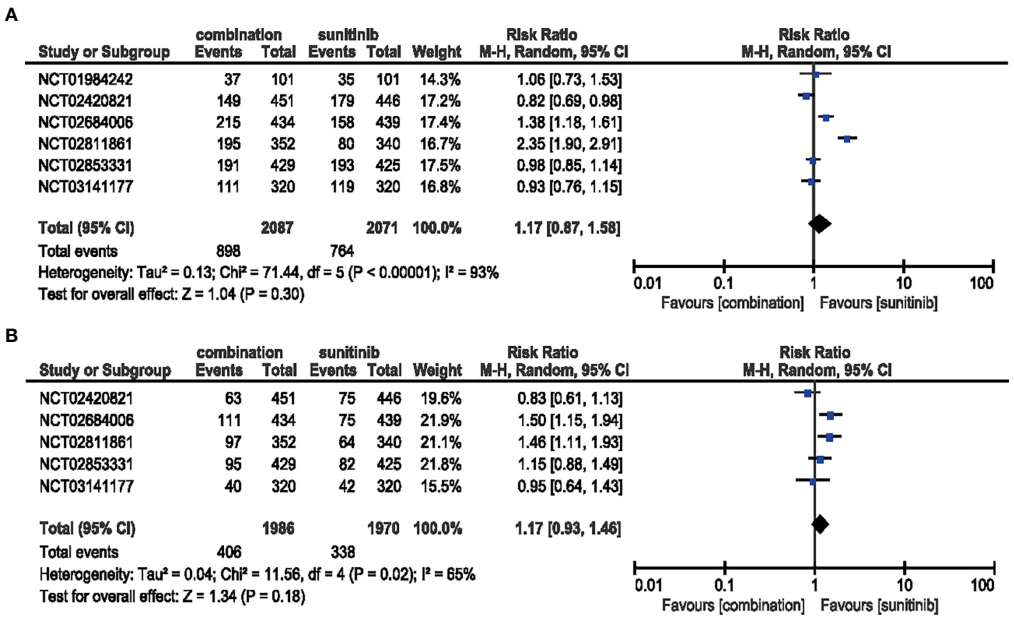


FIGURE 8 | **(A)** Forest plot of all-grade hypertension in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy. **(B)** Forest plot of high-grade hypertension in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy.

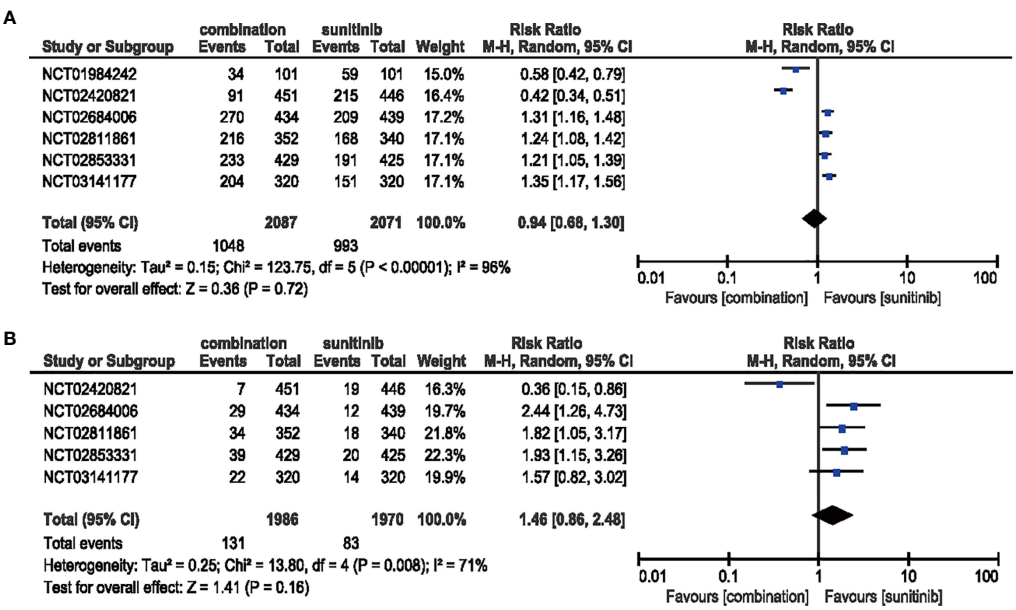


FIGURE 9 | **(A)** Forest plot of all-grade diarrhea in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy. **(B)** Forest plot of high-grade diarrhea in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy.

monotherapy. According to our systematic analysis, the HRs were decreased in OS and PFS, and ORR was improved markedly. RCC is strongly linked to loss-of-function mutation in the *VHL* gene (35), which in turn, plays a vital role in reforming the tumor microenvironment (TME) with angiogenesis, pH regulation, and glucose transportation to suppress the chemotaxis and maturity of immune cells, thereby contributing to cancer survival. According to a previous study,

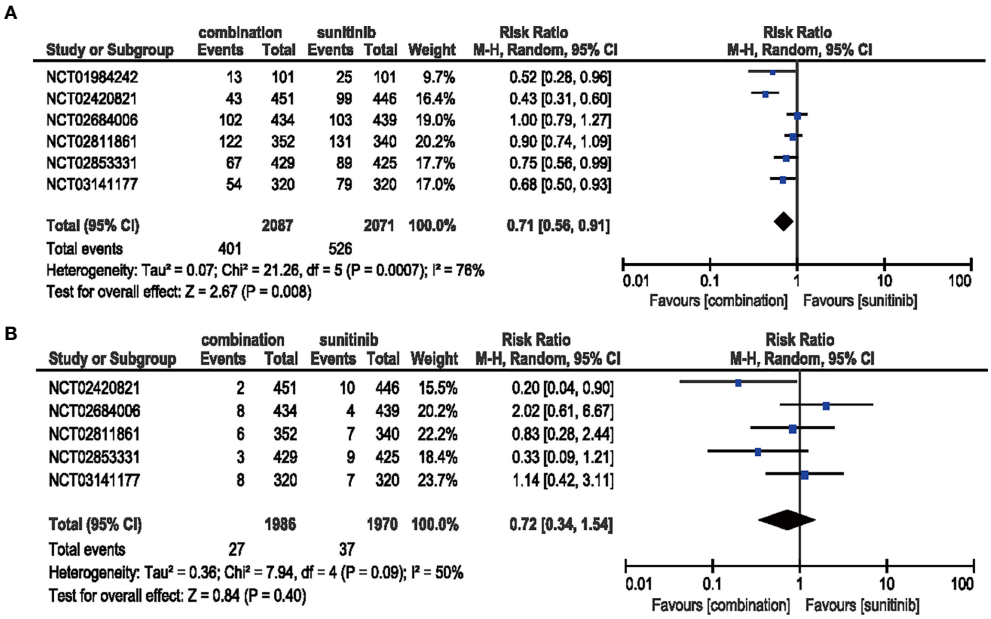


FIGURE 10 | **(A)** Forest plot of all-grade stomatitis in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy. **(B)** Forest plot of high-grade stomatitis in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy.

the outstanding performance of the combination strategy can be attributed to normalizing the TME in the presence of cancer-derived VEGF and enhancing the function of cluster of differentiation eith positive T ($CD8^+T$) cells to eliminate the cancer cells (36).

As mentioned before, the combination of ICIs and anti-VEGF therapy improves efficacy along with an increase in side effects. Hence, it is important to identify biomarkers to predict efficacy to further individualized precision therapy and balance the risk-benefit ratio. In the current study, the assumption that patients with PD-L1 expression could receive more benefits from ICI and anti-VEGF combination therapy was born out by the analysis of PFS and OS in the PD-L1-positive subgroup, while the HR of PFS in PD-L1-negative subgroup failed to reach statistical significance. Unfortunately, the HR of OS in the PD-L1-negative subgroup could not be calculated due to insufficient data. Other studies from Sun (37) and Buti (38) support the present conclusion that patients with PD-L1-positive expression might benefit more from combination therapy. However, we cannot assess whether PD-L1-negative expression is an obstructive factor to combination therapy-associated improvements in survival. Therefore, cautiousness is necessary regarding the use of PD-L1 expression level as a predictive factor for advanced outcomes (15). Indeed, PD-L1-negative patients might benefit from the combination therapy, and the heterogeneity of PD-L1 assessment criteria cannot be neglected (38). Moreover, PD-L1 expression levels would be needed to determine the specific threshold of the most effective combination therapy in the future. Furthermore, no obvious difference was detected in sex or age in the PFS subgroups. The slight discrepancy in the HR of OS was acceptable in the age subgroup because of a prolonged survival time for the younger population.

Another subgroup analysis according to IMDC evaluation was conducted. We found that the HR of the IMDC poor-risk population decreased (HR = 0.37; 95% CI: 0.26–0.54) compared with the intermediate-risk (HR = 0.65, 95% CI: 0.51–0.83) population in OS, while the favorable-risk population did not significantly benefit from combination therapy (RR = 0.90, 95% CI: 0.55–1.46). The latest NCCN guidelines recommend ICIs and anti-VEGF combination therapy including axitinib + pembrolizumab, cabozantinib + nivolumab, and lenvatinib + pembrolizumab as first-line therapy for RCC patients with relapse or stage IV disease, regardless of IMDC score (39). Similarly, the European Association of Urology (EAU) guidelines offer three combination regimens as mentioned above for treatment-naive patients with clear-cell metastatic RCC, without considering IMDC risk. In our study, PFS appears to benefit each IMDC subgroup but failed to convert to the prolonged OS. This might occur for several reasons. Firstly, the IMDC evaluation system is based on clinical features, while renal cell carcinoma is known for its high heterogeneity (40), which requires more precise molecular features to identify dominant tumor subtypes. Motzer et al. (41) identified seven molecular subsets associated with differential clinical outcomes to angiogenesis blockade alone or with a checkpoint inhibitor among 823 tumors from the IMmotion151 trial. In their study, tumors from favorable-risk patients were enriched in the angiogenic/stromal (No. 1) and angiogenic (No. 2) clusters, which exhibited higher expression of

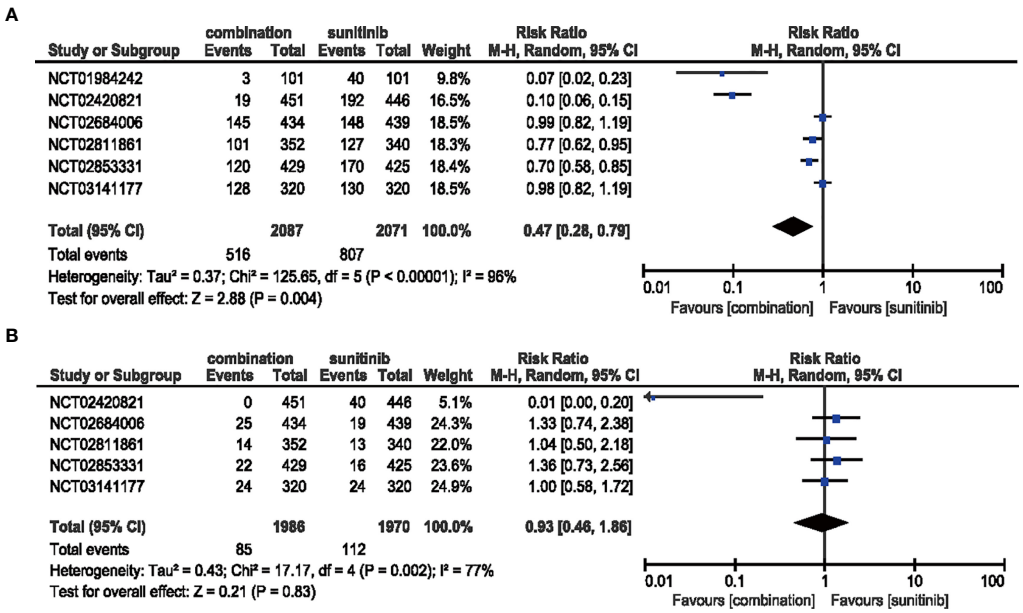


FIGURE 11 | **(A)** Forest plot of all-grade HFSR in patients treated with combination therapy of ICIs and anti-VEGF *vs*. sunitinib monotherapy. **(B)** Forest plot of high-grade HFSR in patients treated with combination therapy of ICIs and anti-VEGF *vs*. sunitinib monotherapy.

genes associated with the VEGF pathway. These findings provide a molecular explanation for the nonsignificant clinical outcomes to sunitinib monotherapy versus combined ICI + VEGF inhibition. Secondly, the baseline PD-L1 expression was lower in favorable-risk patients compared with the intermediate/poor-risk patients in the Checkmate 214 trial (42), in which IMDC favorable-risk patients failed to benefit from nivolumab plus ipilimumab combination therapy in contrast with sunitinib monotherapy. This might be an explanation for why the favorable-risk subgroup did not benefit more significantly from combination therapy compared with sunitinib in our study. Thirdly, extended follow-up from the Keynote 426 trial suggests that PFS in the favorable IMDC subgroup began to separate after 12 months and 70% of patients with favorable-risk disease in the pembrolizumab plus axitinib arm achieved an objective response compared with 50% of patients in the sunitinib group (43). Due to slow progress in favorable risk tumors, an overall survival benefit from the combination of immunotherapy and anti-VEGF therapy might require extended follow-up to present the "long tail" phenomenon of immunotherapy features. In summary, for IMDC favorable-risk patients, more molecular biomarkers besides PD-L1 are needed to select specific populations to guide clinical strategies better, and the existing data support combination therapy as more beneficial to IMDC intermediate- and poor-risk subgroups.

Proteinuria and hypertension are the most common AEs occurring in targeted therapy (15). Proteinuria is closely related to glomerular barrier dysfunction (44, 45). Since renal disorder is an independent risk factor for cardiovascular disease (46), combination therapy may increase the burden on the kidney, resulting in direct damage to renal tubules because podocytes and tubular cells widely express VEGF, leading to continuous drug accumulation and hypertension (15, 47). A significantly increased risk of developing hypertension was detected among RCC patients with continuous daily dosing compared with the intermittent dosing schedule (48, 49). Recently, anti-VEGF treatment was recognized as a potential trigger for an increased incidence of cardiovascular toxicity (50). Nonetheless, the management of hypertension remains controversial. Both enalapril and candesartan (angiotensin-converting enzyme inhibitor and angiotensin receptor blocker, respectively) were reported to inhibit myocardial angiogenesis induced by VEGF, while nifedipine (calcium channel blockers)-induced VEGF secretion (15, 51). Thus, the selection of medications may require a balance between side-effects and toxicity in conjunction with anti-VEGF.

Concerning arthralgia, the RR was increased by the combination therapy of ICIs and anti-VEGF therapy in both all-grade (RR = 2.14, 95% CI: 1.76–2.61) and high-grade AEs (RR = 2.48, 95% CI: 1.06–5.80). A previous study indicated that single nucleotide polymorphisms in the PD-1 gene were associated with susceptibility to rheumatoid arthritis, predisposing these patients to immune-mediated arthralgia (21). The clinical outcomes suggest that the rheumatoid factor should be verified and measures should be taken to prevent arthralgia.

Since the RR of any- and high-grade diarrhea did not reach statistical significance, it seems that the combination therapy did not increase the risk of developing diarrhea. However, the addition of ICIs increases the risk of diarrhea compared with chemotherapy alone (18). A further evaluation of toxicity is required as the underlying pathogenesis for sunitinib- or

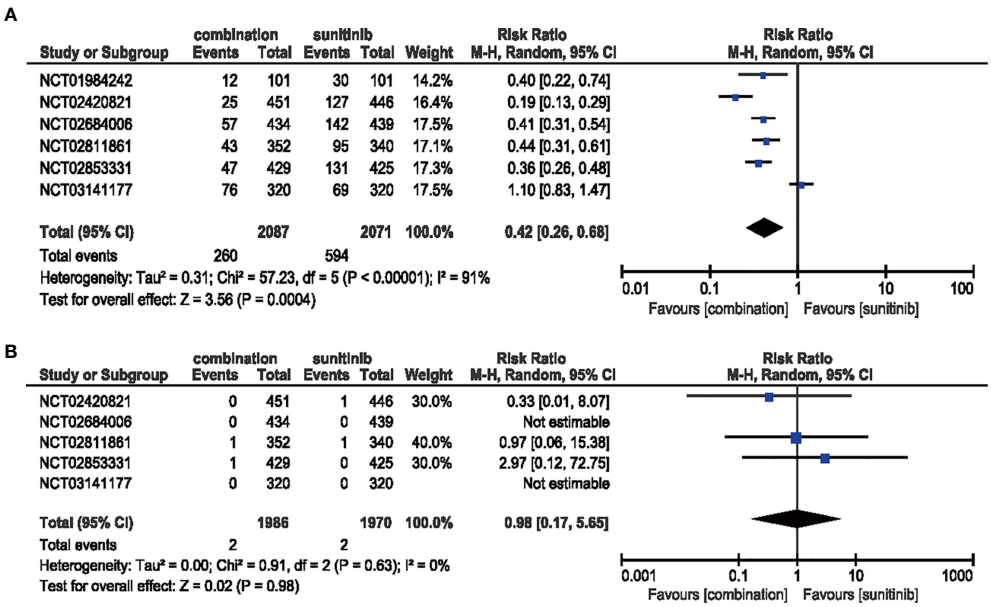


FIGURE 12 | **(A)** Forest plot of all-grade dysgeusia in patients treated with combination therapy of ICIs and anti-VEGF *vs.* sunitinib monotherapy. **(B)** Forest plot of high-grade dysgeusia in patients treated with combination therapy of ICIs and anti-VEGFR *vs.* sunitinib monotherapy.

ICI-induced diarrhea is still unknown. Measures to cope with diverse AEs have been shown to exert a positive role in preventing the symptoms, including rehydration, electrolyte replacements, and loperamide (52). Interestingly, immunotherapy can be rechallenged after symptoms are resolved.

Rash and HFSR are the common AEs resulting from ICI monotherapy, and surprisingly, combination therapy had a reversal effect on them. The results of the current analysis suggested that the risk ratio of all-grade rash increased to 1.61 (95% CI: 1.27–2.04, $p < 0.0001$), while the combination therapy dramatically decreased the risk of all-grade HFSR (RR = 0.47, 95% CI: 0.28–0.79, $p = 0.004$). Reportedly, the application of TKI is strongly associated with all-grade HFSR. We also found that a specific combination therapy strategy (atezolizumab plus bevacizumab) held a tremendous potential to decrease the HFSR risk without TKI. Moreover, skin toxicity stands out among all types of AEs when patients are treated with ICIs (53). The blockade of PD-1 receptor by ICIs such as pembrolizumab, nivolumab, and avelumab triggers similar dermatological AEs (54). Further investigation is required to understand whether the decreased incidence of HFSR is attributed to the absence of TKI or different strategies of combination therapy. In order to improve QoL, a recent study highlighted adequate monitoring to maintain dose strength and prevent the worsening of lesions, including prescription of oral antihistamines and topical steroids with high potency (19).

Oral adverse events (OAEs) associated with TKIs and ICIs, are often overlooked (55). These events lead to significant consequences and disabilities, such as difficulty chewing and swallowing food (potentially leading to low QoL), dose modification, drug withdrawal induced by difficulty in administering oral medications, and a high risk of local and systemic infections. In the current study, the combination of ICIs and anti-VEGF decreased the risk of all-grade dysgeusia and stomatitis, contributing to continuous drug application. Dysgeusia and stomatitis were improved rapidly at untreated intervals and systematic management. However, these might recur with additional doses of the target agent. Since the discontinuation of treatment-induced OAEs has been studied sparsely, the development of systematic management can ensure safety outcomes (18).

Taking the current analysis into consideration, we have identified a series of problems that remain to be solved. Firstly, it is still unclear how ICIs and anti-VEGF can be best applied and combined in systematic therapy. Notably, the specific combination of atezolizumab and bevacizumab described in the NCT01984242 and NCT02420821 trials showed outstanding performance in managing all-grade AEs (stomatitis, diarrhea, and HFSR), indicating that specific medication combination may exert a positive impact on the safety. However, due to the limited quantity and quality of the included studies, we could not perform subgroup analysis on the influence of a specific combination of ICIs or anti-VEGF medication. Secondly, it is unclear whether the survival benefits outweigh the potentially increased risk for AEs with concurrent or sequential therapy in RCC patients. Thirdly, in combination therapy, discontinuation is usually caused by high-grade or severe AEs. Standardized solutions should be studied and adopted to minimize the negative impact of some common AEs. A series of dermatological suggestions in a previous study (19) indicated the potential to overcome AEs and sustain continuous administration. Finally, since all patients may benefit from

combination therapy, precise biomarkers are required to optimize the clinical efficacy between combination and monotherapy.

5 STRENGTHS AND LIMITATIONS

The strengths of this meta-analysis are as follows: to the best of our knowledge, this is the most comprehensive study to evaluate the efficacy and safety of ICIs combined with anti-VEGF therapy. Moreover, all identified studies were RCTs with high quality and low-to-moderate risk of bias. The current meta-analysis delved into the differences between PD-L1-positive/negative and ITT subsets to determine the optimal population for progressive or metastatic RCC based on PD-L1 expression. The optimal clinical decisions were based on the common, specific, and representative AEs; the differences in severity were also explored.

Nonetheless, as only six RCTs were included in the current meta-analysis, data were insufficient for specific subgroup analysis. Therefore, excluding the influence of drug classification and identifying optimal patients benefiting from combination agents requires further study, and the observed heterogeneity cannot be explained. The random-effects model might minimize some of these issues and balance the weight of various sample sizes in the trials. Additionally, PD-L1 expression scores were divided into positive and negative expression to investigate the optimal benefit of combination therapy. However, it was not sufficient for a primary conclusion due to the absence of cutoff values in PD-L1 expression.

6 CONCLUSION

The current analysis showed that ICIs combined with anti-VEGF improved the prognosis in patients with RCC. However, for OS, existing evidence failed to prove a better prognosis for favorable-risk patients evaluated by IMDC. However, the incidence of specific AEs increased obviously compared with monotherapy. The contradictory performance for different AEs is a serious issue that may prevent standardized clinical administration. Thus, we cautiously conclude that combination therapy can be widely utilized in the future with the development of optimal administration and systemic AE management. Additionally, individualized therapy should be intensively studied to achieve the best benefit-risk ratio in clinical application.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JY, LT, and HZ contributed to conception and design of the study. LT organized the database, performed the statistical analysis, and wrote the first draft of the manuscript. LT, JY, and HZ wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was supported by a grant from the College Students Science Innovation Project of Capital Medical University (XSKY2021247).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.739263/full#supplementary-material.

REFERENCES

1. Schanza LM, Seles M, Stotz M, Fosselteder J, Hutterer GC, Pichler M, et al. MicroRNAs Associated With Von Hippel-Lindau Pathway in Renal Cell Carcinoma: A Comprehensive Review. *Int J Mol Sci* (2017) 18(11). doi: 10.3390/ijms18112495
2. Zheng S, Zhang M, Bai H, He M, Dong L, Cai L, et al. Preparation of AS1411 Aptamer Modified Mn-MoS(2) QDs for Targeted MR Imaging and Fluorescence Labelling of Renal Cell Carcinoma. *Int J Nanomedicine.* (2019) 14:9513–24. doi: 10.2147/IJN.S215883
3. Soares A, Monteiro F, Maluf FC, Bastos DA, Jardim DL, Sasse AD, et al. Advanced Renal Cell Carcinoma (RCC) Management: An Expert Panel Recommendation From the Latin American Cooperative Oncology Group (LACOG) and the Latin American Renal Cancer Group (LARCG). *J Cancer Res Clin Oncol* (2020) 146:1829–45. doi: 10.1007/s00432-020-03236-4
4. Claps M, Stellato M, Zattarin E, Mennitto A, Sepe P, Guadalupi V, et al. Current Understanding of Urachal Adenocarcinoma and Management Strategy. *Curr Oncol Rep* (2020) 22:9. doi: 10.1007/s11912-020-0878-z
5. Chen CH, Bhasin S, Khanna P, Joshi M, Joslin PM, Saxena R, et al. Study of Cathepsin B Inhibition in VEGFR TKI Treated Human Renal Cell Carcinoma Xenografts. *Oncogenesis* (2019) 8:15. doi: 10.1038/s41389-019-0121-7
6. Qu L, Ding J, Chen C, Wu ZJ, Liu B, Gao Y, et al. Exosome-Transmitted lncARSR Promotes Sunitinib Resistance in Renal Cancer by Acting as a Competing Endogenous RNA. *Cancer Cell* (2016) 29:653–68. doi: 10.1016/j.ccell.2016.03.004
7. Tacconi E, Tuthill M, Protheroe A. Review of Adjuvant Therapies in Renal Cell Carcinoma: Evidence to Date. *Onco Targets Ther* (2020) 13:12301–16. doi: 10.2147/OTT.S174149
8. Chau V, Bilusic M. Pembrolizumab in Combination With Axitinib as First-Line Treatment for Patients With Renal Cell Carcinoma (RCC): Evidence to Date. *Cancer Manag Res* (2020) 12:7321–30. doi: 10.2147/CMAR.S216605
9. Marin-Acevedo JA, Soyano AE, Dholaria B, Knutson KL, Lou Y. Cancer Immunotherapy Beyond Immune Checkpoint Inhibitors. *J Hematol Oncol* (2018) 11:8. doi: 10.1186/s13045-017-0552-6
10. Franzin R, Netti GS, Spadaccino F, Porta C, Gesualdo L, Stallone G, et al. The Use of Immune Checkpoint Inhibitors in Oncology and the Occurrence of AKI: Where Do We Stand. *Front Immunol* (2020) 11:574271. doi: 10.3389/fimmu.2020.574271

11. Deleuze A, Saout J, Dugay F, Peyronnet B, Mathieu R, Verhoest G, et al. Immunotherapy in Renal Cell Carcinoma: The Future Is Now. *Int J Mol Sci* (2020) 21:2532. doi: 10.3390/ijms21072532
12. Curran CS, Kopp JB. PD-1 Immunobiology in Glomerulonephritis and Renal Cell Carcinoma. *BMC Nephrol.* (2021) 22:80. doi: 10.1186/s12882-021-02257-6
13. Khetani VV, Portal DE, Shah MR, Mayer T, Singer EA. Combination Drug Regimens for Metastatic Clear Cell Renal Cell Carcinoma. *World J Clin Oncol* (2020) 11:541–62. doi: 10.5306/wjco.v11.i8.541
14. Chu D, Lacouture ME, Weiner E, Wu S. Risk of Hand-Foot Skin Reaction With the Multitargeted Kinase Inhibitor Sunitinib in Patients With Renal Cell and Non-Renal Cell Carcinoma: A Meta-Analysis. *Clin Genitourin Cancer* (2009) 7:11–9. doi: 10.3816/CGC.2009.n.002
15. Zhu X, Stergiopoulos K, Wu S. Risk of Hypertension and Renal Dysfunction With an Angiogenesis Inhibitor Sunitinib: Systematic Review and Meta-Analysis. *Acta Oncol* (2009) 48:9–17. doi: 10.1080/02841860802314720
16. Wang L, Ma L, Wang X, Li B, Guo S, Qiao Q. Therapeutic Effects and Associated Adverse Events of First-Line Treatments of Advanced Renal Cell Carcinoma (RCC): A Meta-Analysis. *Int Urol Nephrol* (2015) 47:617–24. doi: 10.1007/s11255-015-0932-1
17. Fernández-Pello S, Hofmann F, Tahbaz R, Marconi L, Lam TB, Albiges L, et al. A Systematic Review and Meta-Analysis Comparing the Effectiveness and Adverse Effects of Different Systemic Treatments for Non-Clear Cell Renal Cell Carcinoma. *Eur Urol* (2017) 71:426–36. doi: 10.1016/j.eururo.2016.11.020
18. Santoni M, Conti A, De Giorgi U, Iacovelli R, Pantano F, Burattini L, et al. Risk of Gastrointestinal Events With Sorafenib, Sunitinib and Pazopanib in Patients With Solid Tumors: A Systematic Review and Meta-Analysis of Clinical Trials. *Int J Cancer* (2014) 135:763–73. doi: 10.1002/ijc.28544
19. Rosenbaum SE, Wu S, Newman MA, West DP, Kuzel T, Lacouture ME. Dermatological Reactions to the Multitargeted Tyrosine Kinase Inhibitor Sunitinib. *Support Care Cancer* (2008) 16:557–66. doi: 10.1007/s00520-008-0409-1
20. Ibrahim EM, Kazkaz GA, Abouelkhair KM, Bayer AM, Elmasri OA. Sunitinib Adverse Events in Metastatic Renal Cell Carcinoma: A Meta-Analysis. *Int J Clin Oncol* (2013) 18:1060–9. doi: 10.1007/s10147-012-0497-2
21. Khoja L, Day D, Wei-Wu Chen T, Siu LL, Hansen AR. Tumour- and Class-Specific Patterns of Immune-Related Adverse Events of Immune Checkpoint Inhibitors: A Systematic Review. *Ann Oncol* (2017) 28:2377–85. doi: 10.1093/annonc/mdx286
22. Brown LC, Desai K, Zhang T, Ornstein MC. The Immunotherapy Landscape in Renal Cell Carcinoma. *BioDrugs* (2020) 34:733–48. doi: 10.1007/s40259-020-00449-4
23. Tufanaru C, Munn Z, Stephenson M, Aromataris E. Fixed or Random Effects Meta-Analysis? Common Methodological Issues in Systematic Reviews of Effectiveness. *Int J Evid Based Healthc* (2015) 13:196–207. doi: 10.1097/XEB.0000000000000065
24. Huedo-Medina TB, Sánchez-Meca J, Marín-Martínez F, Botella J. Assessing Heterogeneity in Meta-Analysis: Q Statistic or I2 Index. *Psychol Methods* (2006) 11:193–206. doi: 10.1037/1082-989X.11.2.193
25. McDermott DF, Huseni MA, Atkins MB, Motzer RJ, Rini BI, Escudier B, et al. Clinical Activity and Molecular Correlates of Response to Atezolizumab Alone or in Combination With Bevacizumab Versus Sunitinib in Renal Cell Carcinoma. *Nat Med* (2018) 24:749–57. doi: 10.1038/s41591-018-0053-3
26. Rini BI, Powles T, Atkins MB, Escudier B, McDermott DF, Suarez C, et al. Atezolizumab Plus Bevacizumab Versus Sunitinib in Patients With Previously Untreated Metastatic Renal Cell Carcinoma (IMmotion151): A Multicentre, Open-Label, Phase 3, Randomised Controlled Trial. *Lancet* (2019) 393:2404–15. doi: 10.1016/S0140-6736(19)30723-8
27. Motzer RJ, Penkov K, Haanen J, Rini B, Albiges L, Campbell MT, et al. Avelumab Plus Axitinib Versus Sunitinib for Advanced Renal-Cell Carcinoma. *N Engl J Med* (2019) 380:1103–15. doi: 10.1056/NEJMoa1816047
28. Motzer R, Alekseev B, Rha SY, Porta C, Eto M, Powles T, et al. Lenvatinib Plus Pembrolizumab or Everolimus for Advanced Renal Cell Carcinoma. *N Engl J Med* (2021) 384:1289–300. doi: 10.1056/NEJMoa2035716
29. Rini BI, Plimack ER, Stus V, Gafanov R, Hawkins R, Nosov D, et al. Pembrolizumab Plus Axitinib Versus Sunitinib for Advanced Renal-Cell Carcinoma. *N Engl J Med* (2019) 380:1116–27. doi: 10.1056/NEJMoa1816714
30. Choueiri TK, Powles T, Burotto M, Escudier B, Bourlon MT, Zurawski B, et al. Nivolumab Plus Cabozantinib Versus Sunitinib for Advanced Renal-Cell Carcinoma. *N Engl J Med* (2021) 384:829–41. doi: 10.1056/NEJMoa2026982
31. Begg CB, Mazumdar M. Operating Characteristics of a Rank Correlation Test for Publication Bias. *Biometrics* (1994) 50:1088–101. doi: 10.2307/2533446
32. Egger M, Davey Smith G, Schneider M, Minder C. Bias in Meta-Analysis Detected by a Simple, Graphical Test. *BMJ* (1997) 315:629–34. doi: 10.1136/bmj.315.7109.629
33. Wang HT, Xia M. A Meta-Analysis of Efficacy and Safety of Sorafenib Versus Other Targeted Agents for Metastatic Renal Cell Carcinoma. *Med (Baltimore)* (2019) 98:e13779. doi: 10.1097/MD.0000000000013779
34. Hutson TE. Targeted Therapies for the Treatment of Metastatic Renal Cell Carcinoma: Clinical Evidence. *Oncologist* (2011) 16 Suppl 2:14–22. doi: 10.1634/theoncologist.2011-S2-14
35. Teixeira AL, Dias F, Gomes M, Fernandes M, Medeiros R. Circulating Biomarkers in Renal Cell Carcinoma: The Link Between microRNAs and Extracellular Vesicles, Where are We Now. *J Kidney Cancer VHL* (2014) 1:84–98. doi: 10.15586/jkcvhl.2014.19
36. Adams VR, Leggas M. Sunitinib Malate for the Treatment of Metastatic Renal Cell Carcinoma and Gastrointestinal Stromal Tumors. *Clin Ther* (2007) 29:1338–53. doi: 10.1016/j.clinthera.2007.07.022
37. Sun L, Zhang L, Yu J, Zhou Y, Shi C, Wasan HS, et al. To be or Not to be: Whether Anti-Angiogenic Agent Combined With Immune Checkpoint Inhibitoris Necessary in the Treatment of Advanced or Metastatic Renal Cell Carcinoma. *Med Oncol* (2020) 37:15. doi: 10.1007/s12032-020-1340-7
38. Buti S, Petrelli F, Ghidini A, Vavassori I, Maestroni U, Bersanelli M. Immunotherapy-Based Combinations Versus Standard First-Line Treatment for Metastatic Clear Cell Renal Cell Carcinoma: A Systematic Review and Meta-Analysis. *Clin Transl Oncol* (2020) 22:1657–63. doi: 10.1007/s12094-020-02292-z
39. Motzer RJ, Jonasch E, Boyle S, Carlo MI, Manley B, Agarwal N, et al. NCCN Guidelines Insights: Kidney Cancer, Version 1.2021. *J Natl Compr Canc Netw* (2020) 18:1160–70. doi: 10.6004/jnccn.2020.0043
40. Gerlinger M, Rowan AJ, Horswell S, Math M, Larkin J, Endesfelder D, et al. Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. *N Engl J Med* (2012) 366:883–92. doi: 10.1056/NEJMoa1113205
41. Motzer RJ, Banchereau R, Hamidi H, Powles T, McDermott D, Atkins MB, et al. Molecular Subsets in Renal Cancer Determine Outcome to Checkpoint and Angiogenesis Blockade. *Cancer Cell* (2020) 38:803–17.e4. doi: 10.1016/j.ccell.2020.10.011
42. Motzer RJ, Tannir NM, McDermott DF, Arén Frontera O, Melichar B, Choueiri TK, et al. Nivolumab Plus Ipilimumab Versus Sunitinib in Advanced Renal-Cell Carcinoma. *N Engl J Med* (2018) 378:1277–90. doi: 10.1056/NEJMoa1712126
43. Powles T, Plimack ER, Soulières D, Waddell T, Stus V, Gafanov R, et al. Pembrolizumab Plus Axitinib Versus Sunitinib Monotherapy as First-Line Treatment of Advanced Renal Cell Carcinoma (KEYNOTE-426): Extended Follow-Up From a Randomised, Open-Label, Phase 3 Trial. *Lancet Oncol* (2020) 21:1563–73. doi: 10.1016/S1470-2045(20)30436-8
44. Liu H, Xu H, Shen C, Wu C. Effect of the Best Compatibility of Components in Corni Fructus on WT1 Expression in Glomerular Podocytes of Type 2 Diabetic Rats With Early Nephropathy. *Am J Chin Med* (2012) 40:537–49. doi: 10.1142/S0192415X12500413
45. Li LL, Chen ZQ, Wang YH, Zhang JH, Yin ZW, Li LL, et al. Relationship Between Urinary Nephrin and Urinary Albumin Changes in Diabetic Rats and Effects of Yiqiyangyinhuayutongluo Recipe. *J Tradit Chin Med* (2012) 32:278–82. doi: 10.1016/s0254-6272(13)60025-4
46. Razavian M, Heeley EL, Perkovic V, Zoungas S, Weekes A, Patel AA, et al. Cardiovascular Risk Management in Chronic Kidney Disease in General Practice (the AusHEART Study). *Nephrol Dial Transplant* (2012) 27:1396–402. doi: 10.1093/ndt/gfr599
47. Imai E, Isaka Y. Targeting Growth Factors to the Kidney: Myth or Reality. *Curr Opin Nephrol Hypertens* (2002) 11:49–57. doi: 10.1097/00041552-200201000-00008
48. Qi WX, Shen Z, Lin F, Sun YJ, Min DL, Tang LN, et al. Incidence and Risk of Hypertension With Vandetanib in Cancer Patients: A Systematic Review and Meta-Analysis of Clinical Trials. *Br J Clin Pharmacol* (2013) 75:919–30. doi: 10.1111/j.1365-2125.2012.04417.x

49. Deng H, Li M, Wu Q, Wang L, Hong Z, Yi F, et al. A 2/1 Sunitinib Dosing Schedule Provides Superior Antitumor Effectiveness and Less Toxicity Than a 4/2 Schedule for Metastatic Renal Cell Carcinoma: A Systematic Review and Meta-Analysis. *Front Oncol* (2020) 10:313. doi: 10.3389/fonc.2020.00313
50. González León T, Morera Pérez M. Renal Cancer in the Elderly. *Curr Urol Rep* (2016) 17:6. doi: 10.1007/s11934-015-0562-2
51. León-Mateos L, Mosquera J, Antón Aparicio L. Treatment of Sunitinib-Induced Hypertension in Solid Tumor by Nitric Oxide Donors. *Redox Biol* (2015) 6:421–5. doi: 10.1016/j.redox.2015.09.007
52. Rapoport BL, van Eeden R, Sibaud V, Epstein JB, Klastersky J, Aapro M, et al. Supportive Care for Patients Undergoing Immunotherapy. *Support Care Cancer.* (2017) 25:3017–30. doi: 10.1007/s00520-017-3802-9
53. Sibaud V, Meyer N, Lamant L, Vigarios E, Mazieres J, Delord JP. Dermatologic Complications of Anti-PD-1/PD-L1 Immune Checkpoint Antibodies. *Curr Opin Oncol* (2016) 28:254–63. doi: 10.1097/CCO.0000000000000290
54. Belum VR, Benhuri B, Postow MA, Hellmann MD, Lesokhin AM, Segal NH, et al. Characterisation and Management of Dermatologic Adverse Events to Agents Targeting the PD-1 Receptor. *Eur J Cancer* (2016) 60:12–25. doi: 10.1016/j.ejca.2016.02.010
55. Boers-Doets CB, Epstein JB, Raber-Durlacher JE, Ouwerkerk J, Logan RM, Brakenhoff JA, et al. Oral Adverse Events Associated With Tyrosine Kinase and Mammalian Target of Rapamycin Inhibitors in Renal Cell Carcinoma: A Structured Literature Review. *Oncologist* (2012) 17:135–44. doi: 10.1634/theoncologist.2011-0111

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Oncology

ORIGINAL RESEARCH
published: 20 October 2021
doi: 10.3389/fonc.2021.727317

Plasmatic Exosome Number and Size Distinguish Prostate Cancer Patients From Healthy Individuals: A Prospective Clinical Study

Mariantonia Logozzi[1†], Davide Mizzoni[1†], Rossella Di Raimo[1†], Alessandro Giuliani[2], Martina Maggi[3], Alessandro Sciarra[3] and Stefano Fais[1]*

[1] Department of Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy, [2] Environment and Health Department, Istituto Superiore di Sanità, Rome, Italy, [3] Department of Urology, Policlinico Umberto I, Università La Sapienza, Rome, Italy

OPEN ACCESS

Edited by:
Sakthivel Muniyan, University of Nebraska Medical Center, United States

Reviewed by:
Gilda Alves Brown, Rio de Janeiro State University, Brazil
Faisal Kunnathodi, Prince Sultan Military Medical City, Saudi Arabia

***Correspondence:**
Stefano Fais
stefano.fais@iss.it

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Genitourinary Oncology, a section of the journal Frontiers in Oncology

Received: *18 June 2021*
Accepted: *28 September 2021*
Published: *20 October 2021*

Citation:
Logozzi M, Mizzoni D, Di Raimo R, Giuliani A, Maggi M, Sciarra A and Fais S (2021) Plasmatic Exosome Number and Size Distinguish Prostate Cancer Patients From Healthy Individuals: A Prospective Clinical Study. Front. Oncol. 11:727317. doi: 10.3389/fonc.2021.727317

There is a urgent need for valuable strategy in early and less invasive diagnosis for cancer. Preliminary data have shown that the plasmatic levels of exosomes increase in cancer condition. This study investigates the relevance of plasmatic levels and size distribution of exosomes in 42 individuals with no signs of urological disease (CTR) as compared to 65 prostate cancer patients (PCa). It was used Nanoparticle Tracking Analysis (NTA), a highly reliable and sensitive method for exosomes characterization and quantification. The relation structure among the NTA-derived parameters was assessed by means of Principal Component Analysis, which allowed detecting the global discriminant power of NTA test in terms of Receiver Operating Characteristic (ROC) curve and the selection of cut-off thresholds. The results showed that PCa had significantly higher plasmatic levels of exosomes and that the exosomes were smaller in size as compared to the CTR; the values reached 89% sensitivity and 71% specificity, in distinguishing PCa from CTR. These results propose a new exosome-based non-invasive clinical approach for the clinical follow-up of prostate cancer undergoing surgical treatment; in addition this method may be developed as a new screening test for prostate cancer's early diagnosis. While this clinical study was performed in prostate cancer, it may represent a proof of concept extendable to virtually all cancers, as it is suggested by both pre-clinical evidence and clinical data obtained with different technical approaches.

Keywords: plasmatic exosomes, prostate cancer, NTA, screening test, liquid biopsy

INTRODUCTION

Among the common phenotypes characterizing malignant tumors, hypoxia, low nutrient supply, extracellular acidosis are by far the most relevant. Recent evidence has suggested that the increased number of exosomes may well implement this list (1–4). Exosomes received considerable attention in the last decade for their peculiar structure, biophysical properties and function in a plethora of biological processes in which they are involved (5–7). Exosomes are extracellular nanovesicles (40–180 nm) released by virtually all cell types under normal and pathological conditions (5, 6, 8–10). Thanks to the ability to transmit their cargo of lipids, proteins, DNAs, mRNAs, miRNAs, and other metabolites into the target cells, exosomes play a pivotal role in intercellular communication. Indeed, exosomes can modulate both physiological and pathological processes, including tumor progression, elimination of toxic substances, as well as drug and therapeutic antibodies delivery (5, 6, 8, 10–15). For these reasons they have been investigated for a clinical application as well, including both diagnosis and therapy.

In fact, due to their ability to deliver a broad range of molecules, exosomes are considered the ideal source of new and more specific tumor biomarkers (5–7, 16–26), including fully active molecules (e.g. CAIX) (27). The few clinical studies have shown that exosomes are detectable in many biological fluids where they have been investigated in both normal and disease conditions (5, 28–31). Exosomes continuously travel the body and an interest is growing to their ability to protect delivered molecules by packaging them within lipid vesicles (32).

However, to date, notwithstanding the increasing preclinical evidence, the data supporting the presence of specific tumor markers in exosomes from either plasma or other body fluids samples are still inconclusive. On the other hand, a critical role of plasmatic levels of exosomes in the clinical follow up of tumor patients has been hypothesized (18). The first evidence supporting the potential use of exosome levels in human body fluids as a tumor progression marker was in melanoma patients with advanced disease (28). Melanoma patients showed significantly increased level of plasmatic exosomes as compared to healthy donors (28). Pre-clinical evidence has also shown that the increased levels of plasmatic exosomes were directly related to the presence of a tumor mass (28). More recent reports have shown that the surgical treatment of the primary tumor led to a dramatic reduction of the plasmatic exosome levels (33, 34). Preclinical investigation has shown that the microenvironmental acidity induces a marked increase in exosome release by tumor cells, independently from the tumor histotype, thus providing a possible etiopathogenetic role of paracrine factors for the increased plasmatic levels observed in cancer patients (9, 35). Pre-clinical investigation has also shown that tumor microenvironmental acidity is responsible for the release of smaller exosomes with a more homogeneous distribution as compared to the exosomes released at buffered conditions (9, 35). Thus, it appears conceivable to hypothesize that in tumor patients microenvironmental acidity may have a pivotal role in determining both the increase of circulating exosomes and their size reduction (10). Together with influencing exosome number and size, tumor acidity induces over-expression of known tumor biomarkers such as PSA (Prostate Specific Antigen) in exosome from prostate cancer patients (35). A recent clinical study has shown that the expression of PSA on plasmatic exosomes distinguished prostate cancer patients from both Benign Prostate Hyperplasia (BPH) and healthy subject (36). Tumor microenvironmental acidity influenced also the expression of proteins, such as CAIX, that on exosomes exert their full enzymatic activity (27, 37). Moreover, CA IX expression and activity were correlated to the exosome intraluminal acidic pH, showing for the first time that plasmatic exosomes from tumor patients are acidic (27).

Some clinical studies have also shown that the number of plasmatic exosomes may represent a valuable new tool for monitoring cancer patients, while obtained with two different techniques and in different cancer hystotypes, such as prostate cancer (35) and oral cancers (34). On the basis of these two very preliminary studies we decided to carry on with a clinical study aimed at assessing the clinical relevance of both the number and size distribution of plasmatic exosomes independently from the potential presence of known or unknown molecular biomarkers. To this purpose we compared a cohort of prostate cancer patients (PCa), to individuals with no signs of urological disease (CTR). The technological approach was to exploit the Nanoparticle Tracking Analysis (NTA), following the repeated rounds of ultracentrifugation, for exosomes characterization and quantification since it is considered a reliable, efficient, and objective technique for the study of exosomes (9, 28, 35, 36, 38–40). This assay was performed in plasma samples from 65 PCa and 42 CTR, providing detailed information on both the exosomes plasmatic levels (particles/ml) and the size distribution (nm). The results showed that the number and size of plasmatic exosomes significantly distinguished PCa patients from the CTR group with high sensitivity and specificity. We consider our results of great importance in providing a non-invasive new tool allowing to distinguish prostate cancer patients from healthy subjects, but also exploitable for early screening, diagnosis, and clinical follow-up of all malignant tumors.

Abbreviations: CTR, Individuals with no signs of urological disease (Control group); PCa, Prostate Cancer patients; PSA, Prostate Specific Antigen; DRE, Digital Rectal Examination; NSFC-exo, PSA-expressing exosomes analyzed by nanoscale flow-cytometry (NSFC); NTA, Nanoparticle Tracking Analysis; PCA, Principal Component Analysis; PCnano1, exosomes size component; PCnano2, exosomes concentration component; CanVar, Canonical Variate; ROC, Receiver Operating Characteristic; AUC, Area Under the Curve.

MATERIALS AND METHODS

Population

The review board of each participating institution approved the trial, which was conducted in accordance with the current International Conference on Harmonisation guidelines for Good Clinical Practice and the principles of the Declaration of Helsinki. The study was approved by the Istituto Superiore di

Sanità Ethics Committee on 18/04/2017 (Rif. Prot. PRE-275/17). Written informed consent was obtained from all subjects involved in the study.

All authors assume responsibility for the completeness and accuracy of the data and analyses and for the fidelity of the trial to the protocol. All the authors had full access to the data, drafted the manuscript, reviewed and approved the manuscript before submission, and made the decision to submit the manuscript for publication. No sponsor provided funding for the study.

Eligible cases were divided in 2 groups: control cases (CTR) and prostate cancer cases (PCa). All cases were consecutively included in the study as out-patients referred to Department of Urology on the basis of the inclusion criteria. Patients were correctly informed, accepted to be included in the study, and signed an informed consensus prior to each procedure. Human plasma samples were collected from EDTA-treated whole blood, 5 mL into BD Vacutainer® K3-EDTA-coated collection tubes (Beckton Dickinson, Franklin Lakes, NJ, USA), from department of Urological Sciences, Policlinico Umberto I, Sapienza University of Rome, Italy. Once collected, the samples were labeled by the clinical center with an identification code and were manipulated anonymously and blinded in the testing phase with the code assigned by the clinical center.

This is an experimental observational clinical research study in which no additional and/or administered drug tests and/or modified therapy are performed. The aim of this study was to compare a population of males without signs of urological disease to prostate cancer patients that were pooled between individuals with different Gleason score.

More in details:

CTR. The control group consisted of 42 male individuals consecutively referred to our department with the following inclusion criteria: age from 18 to 50 years; no clinical evidence of BPH or PCa [digital rectal examination (DRE) and ultrasonography (US)]; prostate volume less than 30 cc; total PSA level less than 1.4 ng/mL; no familiarity for PCa; no therapies that can influence PSA determination; no acute prostatic inflammation; no prostatitis.

PCa. The PCa group consisted of 65 male individuals from 51 to 80 years consecutively referred to our department with a histologically confirmed diagnosis of prostate adenocarcinoma (prostate biopsy). Total PSA (ng/mL) were from 1.8 to 100.0. None of cases was submitted to androgen deprivation therapies, chemotherapies, new generation hormone therapies or other therapies that can influence PSA determination. No acute prostatic inflammation, no prostatitis. All cases were stratified in risk classes (low, intermediate or high according to EAU classification) based on total PSA levels, Gleason score [6 (3 + 3), 7 (3 + 4), 7 (4 + 3), 8-10], and clinical stage (T1-T2 N0 M0, T3 N0 M0 or N1).

Preparation of Exosomes From Plasma of CTR and PCa

To obtain plasma from blood samples, EDTA-treated blood from PCa patients and CTR were centrifuged at 400 x g for 20 min. Plasma was then collected and stored at −80°C until analysis. Upon thawing, 1 mL of plasma underwent the centrifugal procedure as previously described (6, 41) in order to eliminate cell debris, organelles and microvesicles, and pellet exosomes. In the last step, plasma samples were centrifuged for 1 h 30 min at 110,000 x g using a Fiberlite™ F50L-24 x 1.5 Fixed-Angle Rotor, K-Factor: 33 (Thermo Fisher Scientific, Waltham, MA, USA) in the Sorvall WX Ultracentrifuge Series (Thermo Fisher Scientific), to obtain the exosomal pellet, which was then washed in PBS and resuspended in the appropriate buffer for subsequent analyzes. In particular, the exosomal pellet was resuspended in PBS for Nanoparticle Tracking Analysis and Flow Cytometry Analysis, and in CHAPS buffer 1x for western blot analysis.

Nanoparticle Tracking Analysis

Nanoparticle Tracking Analysis (NTA) from Malvern (NanoSight NS300, Worcestershire, UK) was used for the measurement of size distribution and concentration of exosomes samples in liquid suspension in the range from 10 – 1000 nm based on the analysis of Brownian motion (35). Following laser beam illumination, the light scattering allowed to visualize, record and track the particles with a CCD or CMOS camera. Five videos of typically 60 s duration were taken. Data were analyzed using the NTA 3.0 software (Malvern Instruments) which was optimized to first detect and then track each particle on a frame-by-frame basis. NTA is based on the phenomenon of the random movement (diffusion) of small particles when they are dispersed in a liquid, allowing direct and precise measurement of the concentration and size of the particles. The Brownian motion of each particle was tracked using the Stokes–Einstein equation: $D° = kT/6\pi\eta r$, where D° is the diffusion coefficient, $kT/6\pi\eta r = f_0$ is the frictional coefficient of the particle, for the special case of a spherical particle of radius r moving with uniform velocity in a continuous fluid of viscosity η, k is Boltzmann's constant, and T is the absolute temperature.

The evaluation of the Particle Size Distribution (PSD) was performed through the parameters Mean, Mode, SD, D10, D50 (Median) and D90 which indicate respectively the average, most frequent particle class size, standard deviation, and the 10%, 50% and 90% percentiles of the analyzed particles. Specifically, D10, D50 and D90 indicate the size below which 10%, 50% and 90% respectively of total number of exosomes is included, mean and mode point to the average particle size and the most represented size value respectively, while SD is the standard deviation (average distance from the mean) of the distribution.

Western Blot Analysis

For the two groups (CTR and PCa), 4 mL of plasma was pooled and Size Exclusion Chromatography (SEC) was performed for the isolation of plasma-derived exosomes, as described previously (42).

Exosomes from plasma of CTR and PCa patients were lysed in CHAPS buffer 1x containing Tris 10 mM pH 7.4, $MgCl_2$ 1 mM, ethyleneglycoltetraacetic acid (EGTA) 1 mM, CHAPS 0.5%, glycerol 10%, phenylmethylsulfonyl fluoride (PMSF) 1 mM and protease inhibitor cocktail (1 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 μg/mL aprotinin, and PMSF 1 mM). Protein

concentration was determined using the Bradford protein assay (Bio-Rad Laboratories, Inc, Hercules, CA, USA). Thirty micrograms of exosomal lysates were resolved on 10% acrylamide gel and transferred to a Protran BA85 nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA).

Nonspecific binding sites were blocked by incubation in PBS containing 0.05% Tween 20 and 5% milk powder. Blotting was performed using anti-Tsg 101 (C-2, Santa CruzBiotechnology, Dallas, TX, USA), anti-CD81 (B-11, Santa Cruz Biotechnology, Dallas, TX, USA), and anti-Alix (3A9, Thermo Fisher Scientific, Waltham, MA, USA) monoclonal antibodies, for 18 h at 4°C. After incubation with appropriate anti-mouse peroxidase-conjugated secondary antibody (IgG; Amersham Biosciences, Milan, Italy) for 1 h at room temperature, membranes were revealed by enhanced chemiluminescent (ECL) substrate (Thermo Fisher Scientific,Waltham, MA, USA).

Flow Cytometry Analysis of Exosomes

Exosomes purified from plasma were diluted in PBS in a final volume of 50 µL. Anti-human CD81 allophycocyanin (APC) conjugated (Beckman Coulter, Brea, CA, USA) and anti-human PSA fluorescein (FITC) conjugated (clone 5A6, Abcam, Cambridge, UK) or anti-human IgG2a APC conjugated and anti-human IgG1 FITC conjugated (Beckman Coulter) were added to the exosome preparation at optimal pre-titered concentrations and left for 20 min at RT. The same procedure was performed for the analysis of anti-human CD9 phycoerythrin (PE) conjugated (M-L13, RUO (GMP) BD Biosciences, USA) and anti-human PSA fluorescein (FITC) conjugated (clone 5A6, Abcam, Cambridge, UK), using anti-human IgG1 (PE) conjugated and anti-human IgG2a (FITC) conjugated as isotype controls, respectively.

500 µL of PBS were added to samples before the acquisition on the CytoFLEX flow cytometer (Beckman Coulter).

The cytometer was calibrated using a mixture of non-fluorescent silica beads and fluorescent (green) latex beads with sizes ranging from 110 nm to 1300 nm. This calibration step enables the determination of the sensitivity and resolution of the flow cytometer (fluorescent latex beads) and the size of extracellular vesicles (silica beads). All samples were acquired at low flow rate for the same amount of time in order to obtain an estimate of absolute counts of exosomes comparable between various samples. The analysis of the data was performed with FlowJo software (FlowJo, LLC; Ashland, Oregon, USA) (35, 36).

Statistical Analysis

The inferential statistics was based upon the t-test over the above described parameters of PSD distribution, adopting the Satterwaithe correction when in presence of a statistically significant difference in the standard deviation of the two groups.

The relation structure among the NTA-derived parameters was assessed by means of Principal Component Analysis: the first two extracted components (PCnano1, PCnano2) were used to calculate a canonical variate by which assess the global discriminant power of NTA test in terms of Receiver Operating Characteristic (ROC) curve. ROC strategy allowed the estimation of both the global discriminant ability of the test (area under the roc curve, AUROC) and the selection of cut-off thresholds maximizing sensitivity (percentage of correctly diagnosed PCa patients) and specificity (percentage of negative result in CTR individuals) (43). In order to eliminate the suspect of an effect of disease/age necessary link, we checked the possible confounding role of different ages in the two groups by computing the Pearson correlation between both exosome concentration and size with age separately in the two CTR and PCa classes. No statistically significant correlation was scored (**Supplementary Table S1**). The lack of any statistically significant correlation between age and exosome descriptors, albeit indirectly, rules out any possible effect of age on the results. The statistical analysis of the results obtained was being performed with the SAS System program 9.4 version. The analysis of the ROC curves was performed by Sigma Plot 11.2 version.

RESULTS

Characterization and Distribution of Plasma Exosomes Between PCa Patients and Individuals With No Signs of Urological Disease (CTR) by Nanoparticle Tracking Analysis (NTA)

Plasma exosomes from PCa and CTR were characterized for number and size distribution by NTA (**Figure 1**), for the expression of exosome housekeeping markers by Western blot analysis (CD81, Tsg 101 and Alix, **Supplementary Figure S1**) (35, 36), and for the contemporary expression of exosome housekeeping markers (CD9 and CD81) and PSA by Nanoscale Flow Cytometry (**Supplementary Figure S2**) (35, 36).

We first compared PCa and CTR groups in terms of both size and number (concentration) by NTA (**Figure 1**). **Figure 1** shows a representative NTA distribution of exosome samples by either CTR (**Figure 1A**) or PCa (**Figure 1B**), as far as either size (nm, abscissa axis) or number (particles/ml, ordinate axis) are concerned.

The statistical analysis showed a significant difference between CTR and PCa exosome plasma samples (**Table 1**) for both the concentration and the size parameters (with the only exception of SD). In particular the difference in terms of the exosome number between PCa patients and CTR was highly significant ($p<0.0001$).

It is worth noting a significant increase in number of exosomes in PCa as well as the shrinking in their size as registered by all the size descriptors. On the other hand, the SD of the size distributions relative to PCa and CTR are substantially identical, suggesting a general ('rigid') shift of distribution going from CTR to PCa.

In detail, the graphs in **Figures 2** and **3** represent the NTA variables distribution of PCa and CTR included within the 25th and 75th percentiles, discriminating PCa from CTR. PCa exosomes were not only more numerous, but also smaller than the CTR exosomes. In fact, all the dimensional distribution

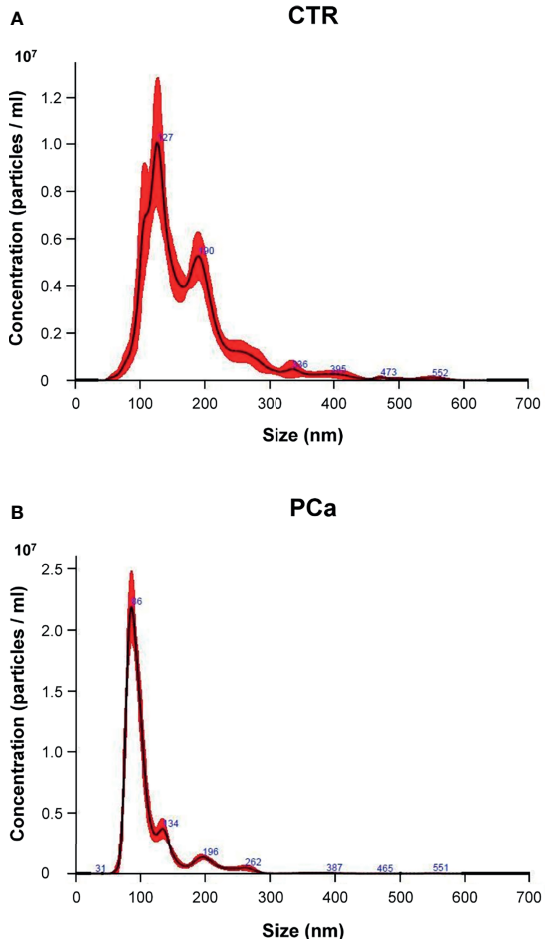


FIGURE 1 | Representative NTA distribution of plasma exosomes in CTR individuals **(A)** and PCa patients **(B)**.

parameters analyzed (such as mean, mode, D10, D50 and D90) were significantly different between the two groups (**Table 1**; **Figures 2** and **3**). Mean (nm) and mode (nm) are parameters useful for describing the set of size of exosomes and their frequency distribution in each plasma sample. D10, D50 and D90 are dimensional parameter that indicate that 10%, 50% and 90% respectively of the exosomes are included below the corresponding nanometers, indicating the spread of exosomes sizes within the sample.

After, the number of PSA-expressing exosomes (NSFC-exo) was acquired using the Nanoscale Flow Cytometry (NSFC) technique and underwent the same statistical analysis adopted for assessing the between group differences relative to the global exosome population. Interestingly, the PSA-specific index (NSFC-exo) showed an almost perfect separation between the groups for the virtual absence of PSA-carrying nanoparticles in healthy subjects (data not shown), supporting our previous results (36).

The aim of the current study was primarily to compare the NSFC-exo in terms of exosomes number and size, between PCa

TABLE 1 | Descriptive and inferential statistics or the two patient groups (PCa = Prostate Cancer, CTR = individuals with no signs of urological disease) as for the entire set of NTA derived descriptors.

Variable	Group	Mean	Std. Dev.	p (t-test)
Number (concentration, particles/mL)	PCa	2.88×10^9	1.43×10^9	<0.0001
	CTR	1.56×10^9	0.57×10^9	
Mean (size, nm)	PCa	131.4	21.44	<0.0005
	CTR	145.9	18.82	
Mode (size, nm)	PCa	89.53	13.77	<0.003
	CTR	97.44	12.26	
D10 (size, nm)	PCa	80.02	10.99	<0.0001
	CTR	88.10	9.09	
D50 (size, nm)	PCa	109.8	19.06	<0.0001
	CTR	124.6	18.01	
D90 (size, nm)	PCa	210.96	35.96	0.0081
	CTR	228.57	30.77	
SD (size variability, nm)	PCa	64.42	11.70	NS
	CTR	68.05	10.16	

NS, Not Significant.

patients and CTR. The analysis of PSA-specific index must be intended as instrumental for checking the hypothesis if the aspecific exosome distribution approach was guided by PSA-containing sub-population of vesicles or if it carried autonomous information as we discuss in the following.

Mutual Relation Between the Size and Number of PCa and CTR Plasma Exosomes

The following statistical analysis was aimed at investigating the mutual relationships between size and number of plasmatic exosomes. For this purpose, we computed a Principal Component Analysis (PCA) over the original data set having the participants (both CTR and PCa) as statistical units and the NTA-derived indexes as variables (**Table 2**). Three principal components explain the totality of the variance (96.7%), but the first two components (PCnano1, PCnano2) account for the by far the most relevant part of information. As a matter of fact the relative proportion of variance explained by the two main components was: PC1 (PCnano1): 71.7%, PC2 (PCnano2) = 15.0% with a cumulative proportion of explained variance equal to 86.7%.

The inspection of the loading matrix (loadings are the correlation coefficients between original variables and components, bolded the most relevant correlations) is reported in **Table 2** allows us to immediately discover the mutual independence of size (PCnano1) and number (PCnano2) of exosomes (principal components are each other orthogonal by construction). Both size (PCnano1) and concentration (PCnano2) allow for a clear separation of PCa and CTR patients (**Figure 4**).

Assuming that the principal components are each other orthogonal by construction, the mutual independence of the size and number of exosomes suggests that the PCa *vs.* CTR separation obtained by these two components results from two independent mechanisms even if both related to cancer condition. This is evident in **Table 3** reporting the descriptive and inferential statistics for PCnano1 and PCnano3 in the two groups. It is worth noting that principal component scores have by

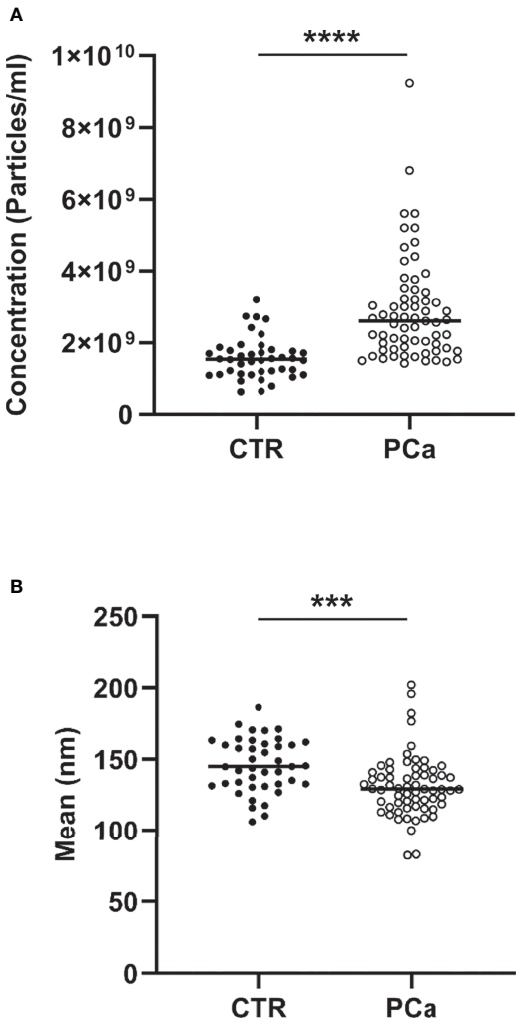


FIGURE 2 | NTA distribution and quantification of CTR and PCa plasmatic exosomes by concentration **(A)** and size **(B)** parameters included within the 25th and 75th percentiles.***$p< 0.001$, ****$p< 0.0001$.

construction zero mean and unit standard deviation on the entire data set (PCa + CTR) and are each other mutually orthogonal. Both size and concentration components show a neat statistical significance as for PCa *vs* CTR comparison, their mutual linear independence allows us to hypothesize that shrinkage and concentration increase of exosomes derive from two different mechanisms, even if both related to cancer condition.

The plot in **Figure 4** reports the distribution of exosomes in the PCnano1 (size) and PCnano2 (concentration), highlighting a clear shift of tumor samples on the top left part of the graph (high number/small size).

ROC Curve Between PCa Patients and CTR

While the above reported analyses re-assure us of the biological relevance of both exosome concentration and size in cancer, they do not allow to assess the prognostic and diagnostic relevance of 'aspecific' exosome descriptors. In a previous study (36) we already assessed the ability of a specific (PSA-carrying) exosome sub-population in discriminating prostate cancer from healthy donors. The results of the above study actually provided a new approach in distinguishing not only prostate cancer from individuals without a cancer, but also prostate cancer patients from patients with prostate benign hypertrophy (BPH), that is considered a benign inflammatory condition, but with some signs that too often may lead to a cancer over diagnosis, such as the serum PSA levels. However, we were also very curious to extend our previous very preliminary observation showing higher levels of plasmatic exosomes in prostate cancer patients, while with small numbers. With the results of the present study we do not want to suggest a generalized shift from a specific to an aspecific approach; rather a complementary use of the aspecific approach (i.e. plasmatic exosome levels) in a 'primary screening for the presence of a cancer disease' made by the Nanoparticle Tracking Analysis technology that can provide a precise analysis of both number and size of plasmatic exosome, while with no direct relation to their specific content, in terms of molecular biomarkers.

Here we pursue a much more ambitious goal: to use only exosome-related information with no reference to a specific biomarker for cancer screening. As a consequence, we do expect a decreased predictive power with respect to the specific approach. This decrease in predictive power is in any case balanced by the much easier (and less costly) procedure and by the promise the simple evaluation of exosome concentration and size could be a warning signal of the presence of a cancer, independently of its particular biotype. We faced this task by a canonical discriminant analysis having as X variables PCnano1 and PCnano2 and as Y variable the healthy/patient categorization. The goal of canonical analysis is to generate a pair (canonical variates) of linear combinations of X and Y variables endowed with maximal mutual correlation (44). In the particular case of canonical discriminant analysis there is only one Y variable expressed in categorical (in this case binary) values. Thus implies we are looking for the linear combination of PCnano1 and PCnano2 that allows for the best separation of PCa and CTR subjects. The procedure generated a pair of canonical variates endowed with a statistically significant correlation (Canonical Correlation = 0.58, F-value = 27.05 $p < 0.0001$). The formula of linear combination of the canonical variate relative to PCnano1 and PCnano2 was CanVar1 = -0.68 *PCnano1 + 1.02*PCnano2.

As expected, the coefficient for the number of exosomes component (PCnano2) was higher than the one for exosomes size due to the higher discriminant ability of exosome number with respect to their size. Under the same heading, the coefficients for the two components have an opposite sign reminiscent of the 'increase in number' and 'decrease in size' effect of cancer (**Figure 5A**).

The ROC analysis performed on the canonical variate (CanVar1) gave rise to a statistically significant discrimination (AUROC = 0.86, $p < 0.0001$) and maximal sensitivity/specificity

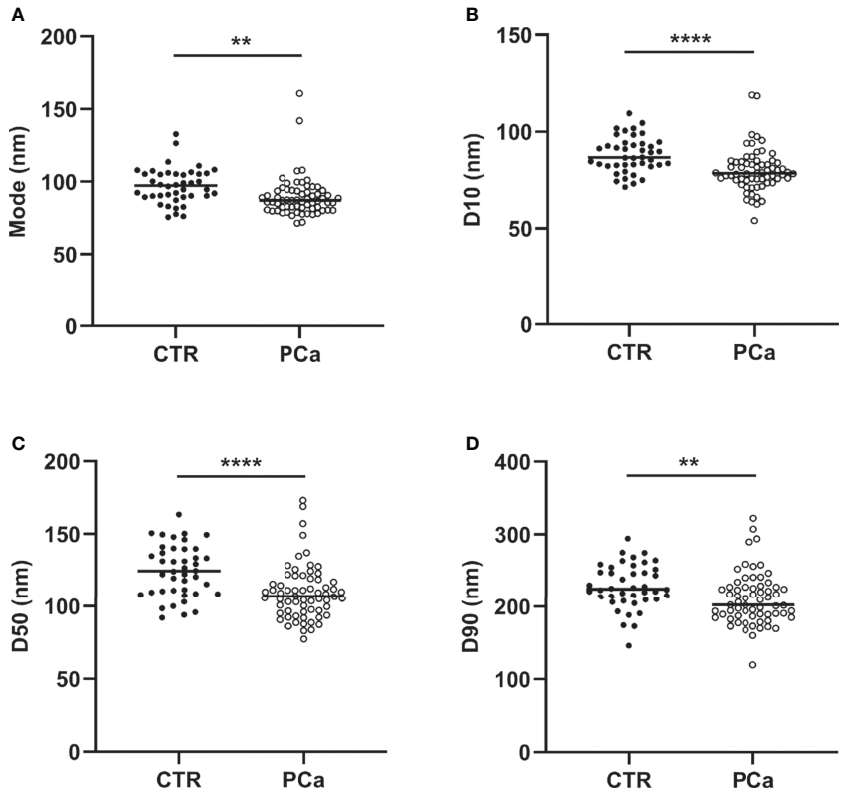


FIGURE 3 | NTA distribution parameters (mode, D10, D50 and D90), correspondent to **(A)**, **(B)**, **(C)** and **(D)** panels respectively of CTR and PCa plasmatic exosomes included within the 25th and 75th percentiles. $^{**}p < 0.01$, $^{****}p < 0.0001$.

TABLE 2 | Component Loadings.

Loading pattern			
Variable	**PCnano1**	**PCnano2**	**PCnano3**
Concentration	-0.00776	**0.97176**	0.22901
Mean	**0.99266**	-0.02747	-0.04256
Mode	**0.86521**	-0.01709	0.41626
SD	0.77564	0.23181	-0.56827
D10	**0.92206**	-0.15468	0.28347
D50	**0.96473**	-0.10536	0.10432
D90	**0.94959**	0.12012	-0.24998

The bold values correspond to the original variables with higher correlation with extracted components (Component Loading).

at cut-off = -0.544 (canonical variate is a z-score with mean zero and unit standard deviation) reaching 89% sensitivity and 71% specificity (**Figure 5B**).

Non-Specific Predictivity of Cancer Risk With Exosome Concentration and Size

Despite the "non-specific" (no consideration of PSA expression) predictivity is lower than the specific one of PSA-expressing exosomes, it allows for a very considerable predictive power that could be useful for a future 'first-level screening' of general cancer risk or cancer staging. To this aim, it is interesting to check the relation structure among non-specific and specific exosome-based

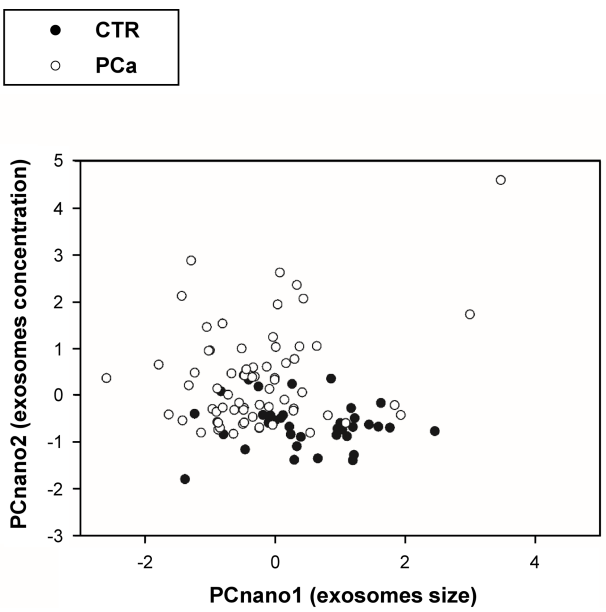


FIGURE 4 | Projection (component scores) of participants in the bi-dimensional space spanned by the two principal components (PCnano1 = exosome size component, and PCnano2 = exosomes number component).

TABLE 3 | Descriptive and inferential statistics of the two patient groups (PCa and CTR) as for the two main principal components of NTA derived descriptors.

Variable	Group	Mean	Std. Dev.	P (t-test)
PCnano1	PCa	-0.258	1.00	<0.0007
(size)	CTR	0.400	0.870	
PCnano2	PCa	0.390	1.05	<0.0001
(concentration)	CTR	-0.604	0.490	

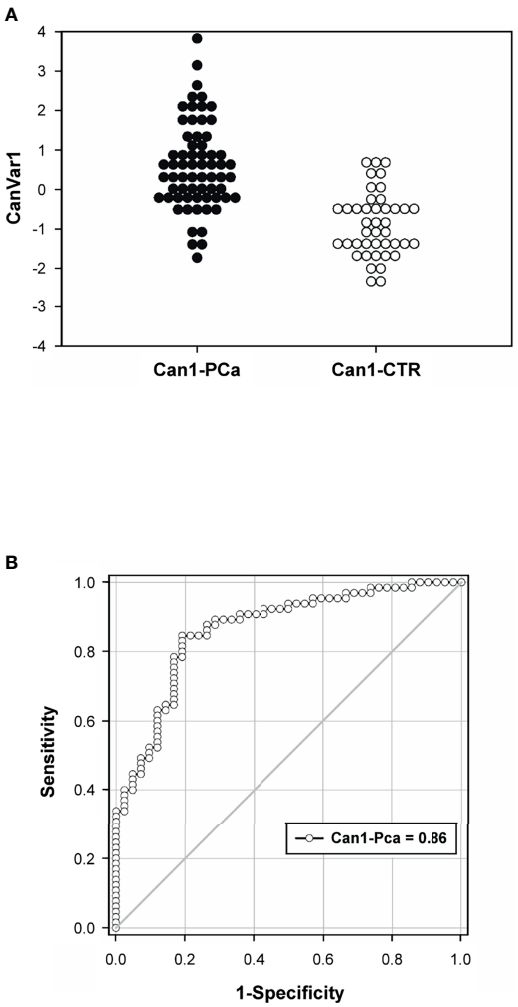


FIGURE 5 | Canonical Variate distribution (CanVar1) **(A)** and Receiving Operating Characteristics (ROC) curve **(B)** relative to cancer-healthy discrimination based on CanVar1.

biomarkers. The demonstration of a certain degree of independence of 'aspecific' (PCnano1, PCnano2) exosome descriptors from PSA specific (NSFC-exo) one, points to the fact cancer-healthy discrimination obtained by exosome size and number builds upon biological features not strictly related to prostate cancer specificity and thus could be used for 'general' cancer screening.

Table 4 reports the pairwise correlation between the above mentioned aspecific and specific scores. The specific biomarker (NSFC-exo) only has a statistically significant (but relatively weak)

TABLE 4 | Pearson correlation coefficients between PCnano1, PCnano2 and NSFC exo.

Pearson correlation coefficientsProb > |r| under H_0: Rho=0Number of observations			
Variable	**PCnano1**	**PCnano2**	**NSFC-exo**
PCnano1	1.00000	0.00000	-0.04868
		1.0000	0.6742
	107	107	77
PCnano2	0.00000	1.00000	**0.38477**
	1.0000		**0.0006**
	107	107	77
NSFC-exo	-0.04868	0.38477	1.00000
	0.6742	0.0006	
	77	77	77

The bold values show the statistically significant correlations of the specific biomarker (NSFC-exo), since NSFC-exo only has a statistically significant (but relatively weak) correlation with PCnano2.

correlation with PCnano2, PCnano1 and PCnano2 are mutually orthogonal by construction and PCnano1 is completely independent (near zero correlation) with NSFC-exo. This result indicates that the 'cancer-related information' exploited by both the size and number of exosomes is widely independent to the specific (prostate) cancer type. This result is particularly promising for the future use of size and concentration of exosomes as a general cancer biomarker.

DISCUSSION

Although research efforts, the burden of cancer keeps on increasing without showing stop signs or forthcoming hopes that the trend will reverse (45–54). The need to detect new and more effective prevention and therapeutic strategies has been finding promising new hopes in exosomes in recent years because of their unique properties as well as their involvement in several physiological and pathological processes. Previous investigation have shown that these nanovesicles can be purified from body fluids, including plasma, and there characterized and quantified (28, 34, 35). This, study was set up to show through a really objective assay, i.e. Nanoparticle Tracking Analysis (NTA), that physical parameters, such as the number and the size of plasmatic exosomes, could distinguish cancer patients from healthy subjects, with the ultimate goal to provide a new non-invasive tool based on quantification of circulating exosomes for diagnosis and clinical follow up of prostate cancer. We used NTA, a highly reliable and sensitive method of exosomes characterization and quantification (9, 35, 36, 38–40). In this kind of study, when a population of patients with a prostate cancer diagnosis before surgery and medical therapy was compared to a healthy males'group we have been obliged to use "individuals with no signs of urological disease", as control group; in turn meaning males under 50 years old while the PCa was of over 50 aged males. This allowed us to compare the plasmatic levels of exosomes between healthy males and cancer patients. We preliminary showed that there was no correlation in terms of age between the two groups (**Supplementary Table S1**). Thus, we analysed the data comparing the two groups in term of either number and size. The results showed that plasmatic exosomes from PCa patients were significantly more numerous and smaller as

compared to plasmatic exosomes from the group of CTR. Indeed, all analysed variables (concentration, mean, mode, D10, D50, D90) were significantly different between CTR and PCa subjects. Among these non-specific indices, the most discriminating variable was the number of exosomes ($p<0.0001$). Then, through computing Principal Component Analysis (PCA), we showed that both size (PCnano1) and plasmatic concentration (PCnano2) of exosomes caused significant discrimination between PCa and CTR individuals, but through two independent mechanisms. The mutual independence between size and number of exosomes further was validated through the computation of the canonical variate coefficient. The ROC analysis performed on the combination of the size and number of plasmatic exosomes (canonical variate 1, CanVar1) showed a maximal sensitivity (89%) and specificity (71%) at cut-off = -0.544. This method allows us to discriminate in a statistically significant manner (AUROC = 0.86, $p < 0.0001$) PCa patients from CTR. Finally, we analyzed the correlation between non-specific and specific exosome-based biomarkers. The specific biomarker based on PSA-expressing exosomes (NSFC-exo) had a statistically significant (but relatively weak) correlation with exosomes number (PCnano2) only, suggesting that the kind of 'cancer-related' information provided by both size and number of exosomes is widely independent to the specific (prostate) cancer type. Despite the "non-specific" (no consideration of PSA expression) predictivity is lower than the specific one of PSA-exosome, it allows for a very considerable predictive power that could be useful for a future 'first-level screening' of general cancer risk or cancer staging or even in predicting after surgery recurrence.

The "liquid biopsy" based on circulating tumor exosomes is a promising and reliable tool for the diagnosis, monitoring, and prognosis of diseases, including tumors, allowing a better sensitivity and specificity of traditional diagnostic techniques, as well as a reduced use of more invasive methodologies (7, 20, 55–59). A high level of circulating exosomes and their miRNA cargos could be useful as potential diagnostic biomarkers, as was observed for alcoholic hepatitis (60).The enrichment of specific markers makes exosomes valuable tools to investigate new biomarker sources useful for tumor diagnosis and prognosis (1, 5, 18, 26, 31). In men with high PSA levels, exosome gene expression in urine was associated with a better ability to distinguish patients with higher-grade prostate cancer, with the consequent reduction of unnecessary biopsies (61, 62). Based on this scenario, in previous papers, we showed increased plasmatic levels of PSA-expressing exosomes in PCa patients compared to BPH and CTR subjects, supporting the clinical relevance of exosomes as tumor biomarkers (35, 36). These studies have prompted a significant boost regarding the clinical utility of exosomes. We thus focused our attention on physical characteristics of the exosomes, such as their number and size, in order to verify whether they could represent signs of malignancy that allowed to clearly distinguishing the healthy subjects from the tumor patients, regardless to the presence of tumor specific biomarkers, whose identification is of course a primary endpoint (33, 63–66).

Although the existence of an open debate in the extracellular vesicles community (6, 67), there is a common agreement on the use of ultracentrifugation to obtain a the most reliable and useful purification of extracellular vesicles from either cell culture supernatant or body fluids. Thus, we used ultracentrifugation for exosome purification and NTA for quantification and size distribution of exosomes in a plasma volume. As detailed above the results showed that prostate cancer patients had significantly higher exosome levels and a reduced size as compared to healthy individuals, thus supporting the clinical use of this approach and its potential use in screening test for prostate cancer early diagnosis.

In summarizing the novelty of this approach includes: a) the demonstration that the measurement of exosome levels and their size in the plasma of human beings, while apparently aspecific, may be helpful in a 'general screening' for the presence of a 'cancer pathology'. Clearly this is only a 'preliminary finding' that in any case represents a warning signal to be followed by more specific investigations; b) the NTA analysis of plasmatic exosomes number and size may be helpful in the follow up of cancer patients underwent either medical or surgical treatment, and we want to emphasize with very reduced costs and no invasiveness, as compared to the current diagnostic equipment.

In conclusion, these results express a high clinical impact, strongly suggesting that the concentration and size of circulating exosomes may implement the equipment of cancer biomarkers, particularly for prostate cancer, thus providing a promising new tool for early-stage cancer detection. The results of our study have shown high level of sensitivity and specificity of both exosome number and size in distinguishing prostate cancer patients from a group of individuals with no sign of urological disease, making this approach potentially useful for screening, diagnosis and follow-up of prostate cancer patients. Accordingly, it is reasonable to speculate to exploit in other cancers the clinical potential of the exosome-based approach with the ultimate and ambitious aim of identifying a universal screening test, which remains currently not available. Furthermore, since resection of the primary tumor has been observed to greatly reduce the level of exosomes in oral cancers (34), and the plasmatic levels of exosomes were related to the presence of a primary tymor, in either melanoma (28) or brain tumors (68), monitoring the number of exosomes could also be a winning strategy to control recurrence following tumor resection and to evaluate the effectiveness of the response to anticancer therapy on the tumor mass. From a pathogenetic point of view it appears highly reasonable that the increased plasmatic levels of exosomes in tumor patients may be due to both the hostile microenvironmental condition, such as acidity (9) and the tumor mass (28, 34). Of course the measurements of exosome plasmatic levels needs a clinical validation in terms of platform technology, but the results of our study strongly support the use of ultracentrifugation and NTA as a reliable technical approach.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Istituto Superiore di Sanità (protocol code Rif. Prot. PRE-275/17 on 18/04/2017). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ML and SF conceived, designed and supervised the study project. DM and RDR performed the experiments. MM and AS collected blood samples from study participants, written informed consent and relevant clinical information of participants, describing the characteristics of the population studied. DM, ML, AG, RDR, and MM collected the data. AG performed and explained the statistical analysis of the data. DM, ML, AG, and SF wrote the original draft of the manuscript. SF, DM, AG, RDR, and ML revised and edited the last version of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

ACKNOWLEDGMENTS

We would like to thank Dr. Daniela F. Angelini from the Neuroimmunology Unit, IRCCS Santa Lucia Foundation, 00179 Rome, Italy for the exosome characterization with housekeeping markers (CD81 and CD9) and PSA using the flow cytometer. Furthermore, we thank Dr Roberto Santoliquido from Alfatest Srl, 00166 Rome, Italy, for the technical support concerning the Nanoparticle Tracking Analysis (NTA).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.727317/full#supplementary-material

REFERENCES

1. Fais S, Venturi G, Gatenby B. Microenvironmental Acidosis in Carcinogenesis and Metastases: New Strategies in Prevention and Therapy. *Cancer Metastasis Rev* (2014) 33(4):1095–108. doi: 10.1007/s10555-014-9531-3
2. Fais S, Overholtzer M. Cell-In-Cell Phenomena in Cancer. *Nat Rev Cancer* (2018) 18(12):758–66. doi: 10.1038/s41568-018-0073-9
3. Pillai SR, Damaghi M, Marunaka Y, Spugnini EP, Fais S, Gillies RJ. Causes, Consequences, and Therapy of Tumors Acidosis. *Cancer Metastasis Rev* (2019) 38(1–2):205–22. doi: 10.1007/s10555-019-09792-7
4. Gillies RJ, Pilot C, Marunaka Y, Fais S. Targeting Acidity in Cancer and Diabetes. *Biochim Biophys Acta Rev Cancer* (2019) 1871(2):273–80. doi: 10.1016/j.bbcan.2019.01.003
5. Yáñez-Mó M, Siljander PR-M, Andreu Z, Bedina Zavec A, Borràs FE, Buzas EI, et al. Biological Properties of Extracellular Vesicles and Their Physiological Functions. *J Extracell Vesicles* (2015) 4(1):27066. doi: 10.3402/jev.v4.27066
6. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): A Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J Extracell Vesicles* (2018) 7(1):1535750. doi: 10.1080/20013078.2018.1535750
7. Fais S, O'Driscoll L, Borras FE, Buzas E, Camussi G, Cappello F, et al. Evidence-Based Clinical Use of Nanoscale Extracellular Vesicles in Nanomedicine. *ACS Nano* (2016) 10(4):3886–99. doi: 10.1021/acsnano.5b08015
8. Spugnini E, Logozzi M, Di Raimo R, Mizzoni D, Fais S. A Role of Tumor-Released Exosomes in Paracrine Dissemination and Metastasis. *Int J Mol Sci* (2018) 19(12):3968. doi: 10.3390/ijms19123968
9. Logozzi M, Mizzoni D, Angelini D, Di Raimo R, Falchi M, Battistini L, et al. Microenvironmental pH and Exosome Levels Interplay in Human Cancer Cell Lines of Different Histotypes. *Cancers* (2018) 10(10):370. doi: 10.3390/cancers10100370
10. Logozzi M, Spugnini E, Mizzoni D, Di Raimo R, Fais S. Extracellular Acidity and Increased Exosome Release as Key Phenotypes of Malignant Tumors. *Cancer Metastasis Rev* (2019) 38(1–2):93–101. doi: 10.1007/s10555-019-09783-8
11. Federici C, Petrucci F, Caimi S, Cesolini A, Logozzi M, Borghi M, et al. Exosome Release and Low pH Belong to a Framework of Resistance of Human Melanoma Cells to Cisplatin. Lebedeva IV, Editor. *PLoS One* (2014) 9(2):e88193. doi: 10.1371/journal.pone.0088193
12. Iessi E, Logozzi M, Lugini L, Azzarito T, Federici C, Spugnini EP, et al. Acridine Orange/exosomes Increase the Delivery and the Effectiveness of Acridine Orange in Human Melanoma Cells: A New Prototype for Theranostics of Tumors. *J Enzyme Inhib Med Chem* (2017) 32(1):648–57. doi: 10.1080/14756366.2017.1292263
13. Zhao H, Achreja A, Iessi E, Logozzi M, Mizzoni D, Di Raimo R, et al. The Key Role of Extracellular Vesicles in the Metastatic Process. *Biochim Biophys Acta Rev Cancer* (2018) 1869(1):64–77. doi: 10.1016/j.bbcan.2017.11.005
14. Logozzi M, Mizzoni D, Bocca B, Di Raimo R, Petrucci F, Caimi S, et al. Human Primary Macrophages Scavenge AuNPs and Eliminate It Through Exosomes. A Natural Shuttling for Nanomaterials. *Eur J Pharm Biopharm* (2019) 137:23–36. doi: 10.1016/j.ejpb.2019.02.014
15. Logozzi M, Di Raimo R, Properzi F, Barca S, Angelini DF, Mizzoni D, et al. Nanovesicles Released by OKT3 Hybridoma Express Fully Active Antibodies. *J Enzyme Inhib Med Chem* (2021) 36(1):175–82. doi: 10.1080/14756366.2020.1852401
16. Zocco D, Ferruzzi P, Cappello F, Kuo WP, Fais S. Extracellular Vesicles as Shuttles of Tumor Biomarkers and Anti-Tumor Drugs. *Front Oncol* (2014) 4:267/abstract. doi: 10.3389/fonc.2014.00267/abstract
17. Sumrin A, Moazzam S, Khan AA, Ramzan I, Batool Z, Kaleem S, et al. Exosomes as Biomarker of Cancer. *Braz Arch Biol Technol* (2018) 61(0):e18160730. doi: 10.1590/1678-4324-2018160730
18. Cappello F, Logozzi M, Campanella C, Bavisotto CC, Marcilla A, Properzi F, et al. Exosome Levels in Human Body Fluids: A Tumor Marker by Themselves? *Eur J Pharm Sci* (2017) 96:93–8. doi: 10.1016/j.ejps.2016.09.010
19. Li A, Zhang T, Zheng M, Liu Y, Chen Z. Exosomal Proteins as Potential Markers of Tumor Diagnosis. *J Hematol Oncol* (2017) 10(1):175. doi: 10.1186/s13045-017-0542-8
20. Huang T, Deng C-X. Current Progresses of Exosomes as Cancer Diagnostic and Prognostic Biomarkers. *Int J Biol Sci* (2019) 15(1):1–11. doi: 10.7150/ijbs.27796
21. Meng Y, Sun J, Wang X, Hu T, Ma Y, Kong C, et al. Exosomes: A Promising Avenue for the Diagnosis of Breast Cancer. *Technol Cancer Res Treat* (2019) 18:153303381882142. doi: 10.1177/1533033818821421
22. Campanella C, Caruso Bavisotto C, Logozzi M, Marino Gammazza A, Mizzoni D, Cappello F, et al. On the Choice of the Extracellular Vesicles for Therapeutic Purposes. *Int J Mol Sci* (2019) 20(2):236. doi: 10.3390/ijms20020236

23. Soung Y, Ford S, Zhang V, Chung J. Exosomes in Cancer Diagnostics. *Cancers* (2017) 9(12):8. doi: 10.3390/cancers9010008
24. Vafaei S, Fattahi F, Ebrahimi M, Janani L, Shariftabrizi A, Madjd Z. Common Molecular Markers Between Circulating Tumor Cells and Blood Exosomes in Colorectal Cancer: A Systematic and Analytical Review. *Cancer Manag Res* (2019) 11:8669–98. doi: 10.2147/CMAR.S219699
25. Wong C-H, Chen Y-C. Clinical Significance of Exosomes as Potential Biomarkers in Cancer. *World J Clin Cases* (2019) 7(2):171–90. doi: 10.12998/wjcc.v7.i2.171
26. Logozzi M, Mizzoni D, Di Raimo R, Fais S. Exosomes: A Source for New and Old Biomarkers in Cancer. *Cancers* (2020) 12(9):2566. doi: 10.3390/cancers12092566
27. Logozzi M, Mizzoni D, Capasso C, Del Prete S, Di Raimo R, Falchi M, et al. Plasmatic Exosomes From Prostate Cancer Patients Show Increased Carbonic Anhydrase IX Expression and Activity and Low Ph. *J Enzyme Inhib Med Chem* (2020) 35(1):280–8. doi: 10.1080/14756366.2019.1697249
28. Logozzi M, De Milito A, Lugini L, Borghi M, Calabrò L, Spada M, et al. High Levels of Exosomes Expressing CD63 and Caveolin-1 in Plasma of Melanoma Patients. Cao Y, Editor. *PLoS One* (2009) 4(4):e5219. doi: 10.1371/journal.pone.0005219
29. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJA. Delivery of siRNA to the Mouse Brain by Systemic Injection of Targeted Exosomes. *Nat Biotechnol* (2011) 29(4):341–5. doi: 10.1038/nbt.1807
30. Fais S, Logozzi M, Lugini L, Federici C, Azzarito T, Zarovni N, et al. Exosomes: The Ideal Nanovectors for Biodelivery. *Biol Chem* (2013) 394(1):1–15. doi: 10.1515/hsz-2012-0236
31. Properzi F, Logozzi M, Fais S. Exosomes: The Future of Biomarkers in Medicine. *Biomark Med* (2013) 7(5):769–78. doi: 10.2217/bmm.13.63
32. O'Driscoll L, Stoorvogel W, Théry C, Buzas E, Nazarenko I, Siljander P, et al. European Network on Microvesicles and Exosomes in Health and Disease (ME-HaD). *Eur J Pharm Sci* (2017) 98:1–3. doi: 10.1016/j.ejps.2017.01.003
33. Campanella C, Rappa F, Sciumè C, Marino Gammazza A, Barone R, Bucchieri F, et al. Heat Shock Protein 60 Levels in Tissue and Circulating Exosomes in Human Large Bowel Cancer Before and After Ablative Surgery: Exosomal Hsp60 in Large Bowel Cancer. *Cancer* (2015) 121(18):3230–9. doi: 10.1002/cncr.29499
34. Rodríguez Zorrilla S, Pérez-Sayans M, Fais S, Logozzi M, Gallas Torreira M, García García A. A Pilot Clinical Study on the Prognostic Relevance of Plasmatic Exosomes Levels in Oral Squamous Cell Carcinoma Patients. *Cancers* (2019) 11(3):429. doi: 10.3390/cancers11030429
35. Logozzi M, Angelini DF, Iessi E, Mizzoni D, Di Raimo R, Federici C, et al. Increased PSA Expression on Prostate Cancer Exosomes in *In Vitro* Condition and in Cancer Patients. *Cancer Lett* (2017) 403:318–29. doi: 10.1016/j.canlet.2017.06.036
36. Logozzi M, Angelini DF, Giuliani A, Mizzoni D, Di Raimo R, Maggi M, et al. Increased Plasmatic Levels of PSA-Expressing Exosomes Distinguish Prostate Cancer Patients From Benign Prostatic Hyperplasia: A Prospective Study. *Cancers* (2019) 11(10):1449. doi: 10.3390/cancers11101449
37. Logozzi M, Capasso C, Di Raimo R, Del Prete S, Mizzoni D, Falchi M, et al. Prostate Cancer Cells and Exosomes in Acidic Condition Show Increased Carbonic Anhydrase IX Expression and Activity. *J Enzyme Inhib Med Chem* (2019) 34(1):272–8. doi: 10.1080/14756366.2018.1538980
38. Gercel-Taylor C, Atay S, Tullis RH, Kesimer M, Taylor DD. Nanoparticle Analysis of Circulating Cell-Derived Vesicles in Ovarian Cancer Patients. *Anal Biochem* (2012) 428(1):44–53. doi: 10.1016/j.ab.2012.06.004
39. Oosthuyzen W, Sime NEL, Ivy JR, Turtle EJ, Street JM, Pound J, et al. Quantification of Human Urinary Exosomes by Nanoparticle Tracking Analysis: Nanoparticle Tracking Analysis and Exosomes. *J Physiol* (2013) 591(23):5833–42. doi: 10.1113/jphysiol.2013.264069
40. Zhang W, Peng P, Kuang Y, Yang J, Cao D, You Y, et al. Characterization of Exosomes Derived From Ovarian Cancer Cells and Normal Ovarian Epithelial Cells by Nanoparticle Tracking Analysis. *Tumor Biol* (2016) 37(3):4213–21. doi: 10.1007/s13277-015-4105-8
41. Caby M-P, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-Like Vesicles Are Present in Human Blood Plasma. *Int Immunol* (2005) 17(7):879–87. doi: 10.1093/intimm/dxh267
42. Baranyai T, Herczeg K, Onódi Z, Voszka I, Módos K, Marton N, et al. Isolation of Exosomes From Blood Plasma: Qualitative and Quantitative Comparison of Ultracentrifugation and Size Exclusion Chromatography Methods. Rito-Palomares M, Editor. *PLoS One* (2015) 10(12):e0145686. doi: 10.1371/journal.pone.0145686
43. Hanley JA, McNeil BJ. The Meaning and Use of the Area Under a Receiver Operating Characteristic (ROC) Curve. *Radiology* (1982) 143(1):29–36. doi: 10.1148/radiology.143.1.7063747
44. Härdle WK, Simar L. Canonical Correlation Analysis. In: *Applied Multivariate Statistical Analysis, 4th ed*. Berlin Heidelberg: Springer-Verlag (2015). p. 443–54. doi: 10.1007/978-3-662-45171-7
45. *Global Cancer Observatory* . Available at: https://gco.iarc.fr/.
46. Schröder FH, Hugosson J, Roobol MJ, Tammela TLJ, Zappa M, Nelen V, et al. Screening and Prostate Cancer Mortality: Results of the European Randomised Study of Screening for Prostate Cancer (ERSPC) at 13 Years of Follow-Up. *Lancet Lond Engl* (2014) 384(9959):2027–35. doi: 10.1016/S0140-6736(14)60525-0
47. Chou R, Croswell JM, Dana T, Bougatsos C, Blazina I, Fu R, et al. Screening for Prostate Cancer: A Review of the Evidence for the U.S. Preventive Services Task Force. *Ann Intern Med* (2011) 155(11):762. doi: 10.7326/0003-4819-155-11-201112060-00375
48. Carroll PR. Early Stage Prostate Cancer–do We Have a Problem With Over-Detection, Overtreatment or Both? *J Urol* (2005) 173(4):1061–2. doi: 10.1097/01.ju.0000156838.67623.10
49. Fowler FJ, Barry MJ, Walker-Corkery B, Caubet J-F, Bates DW, Lee JM, et al. The Impact of a Suspicious Prostate Biopsy on Patients' Psychological, Socio-Behavioral, and Medical Care Outcomes. *J Gen Intern Med* (2006) 21(7):715–21. doi: 10.1111/j.1525-1497.2006.00464.x
50. Loeb S, Vellekoop A, Ahmed HU, Catto J, Emberton M, Nam R, et al. Systematic Review of Complications of Prostate Biopsy. *Eur Urol* (2013) 64(6):876–92. doi: 10.1016/j.eururo.2013.05.049
51. Moyer VA. Screening for Prostate Cancer: U.S. Preventive Services Task Force Recommendation Statement. *Ann Intern Med* (2012) 157(2):120. doi: 10.7326/0003-4819-157-2-201207170-00459
52. Draisma G, Etzioni R, Tsodikov A, Mariotto A, Wever E, Gulati R, et al. Lead Time and Overdiagnosis in Prostate-Specific Antigen Screening: Importance of Methods and Context. *J Natl Cancer Inst* (2009) 101(6):374–83. doi: 10.1093/jnci/djp001
53. Schröder FH, Hugosson J, Roobol MJ, Tammela TLJ, Ciatto S, Nelen V, et al. Screening and Prostate-Cancer Mortality in a Randomized European Study. *N Engl J Med* (2009) 360(13):1320–8. doi: 10.1056/NEJMoa0810084
54. Hoffman RM. Screening for Prostate Cancer. *N Engl J Med* (2011) 365(21):2013–9. doi: 10.1056/NEJMcp1103642
55. Choi D-S, Lee J, Go G, Kim Y-K, Gho YS. Circulating Extracellular Vesicles in Cancer Diagnosis and Monitoring: An Appraisal of Clinical Potential. *Mol Diagn Ther* (2013) 17(5):265–71. doi: 10.1007/s40291-013-0042-7
56. Cui S, Cheng Z, Qin W, Jiang L. Exosomes as a Liquid Biopsy for Lung Cancer. *Lung Cancer Amst Neth* (2018) 116:46–54. doi: 10.1016/j.lungcan.2017.12.012
57. Silva J, Garcia V, Rodriguez M, Compte M, Cisneros E, Veguillas P, et al. Analysis of Exosome Release and its Prognostic Value in Human Colorectal Cancer. *Genes Chromosomes Cancer* (2012) 51(4):409–18. doi: 10.1002/gcc.21926
58. Nawaz M, Camussi G, Valadi H, Nazarenko I, Ekström K, Wang X, et al. The Emerging Role of Extracellular Vesicles as Biomarkers for Urogenital Cancers. *Nat Rev Urol* (2014) 11(12):688–701. doi: 10.1038/nrurol.2014.301
59. Duijvesz D, Versluis CYL, van der Fels CAM, Vredenbregt-van den Berg MS, Leivo J, Peltola MT, et al. Immuno-Based Detection of Extracellular Vesicles in Urine as Diagnostic Marker for Prostate Cancer: Extracellular Vesicles as Marker for PCa. *Int J Cancer* (2015) 137(12):2869–78. doi: 10.1002/ijc.29664
60. Momen-Heravi F, Saha B, Kodys K, Catalano D, Satishchandran A, Szabo G. Increased Number of Circulating Exosomes and Their microRNA Cargos Are Potential Novel Biomarkers in Alcoholic Hepatitis. *J Transl Med* (2015) 13(1):261. doi: 10.1186/s12967-015-0623-9
61. McKiernan J, Donovan MJ, O'Neill V, Bentink S, Noerholm M, Belzer S, et al. A Novel Urine Exosome Gene Expression Assay to Predict High-Grade Prostate Cancer at Initial Biopsy. *JAMA Oncol* (2016) 2(7):882. doi: 10.1001/jamaoncol.2016.0097
62. McKiernan J, Donovan MJ, Margolis E, Partin A, Carter B, Brown G, et al. A Prospective Adaptive Utility Trial to Validate Performance of a Novel Urine Exosome Gene Expression Assay to Predict High-Grade Prostate Cancer in Patients With Prostate-Specific Antigen 2–10 Ng/Ml at Initial Biopsy. *Eur Urol* (2018) 74(6):731–8. doi: 10.1016/j.eururo.2018.08.019

63. Moon P-G, Lee J-E, Cho Y-E, Lee SJ, Chae YS, Jung JH, et al. Fibronectin on Circulating Extracellular Vesicles as a Liquid Biopsy to Detect Breast Cancer. *Oncotarget* (2016) 7(26):40189–99. doi: 10.18632/oncotarget.9561
64. Lee SJ, Lee J, Jung JH, Park HY, Lee C-H, Moon P-G, et al. Exosomal Del-1 as a Potent Diagnostic Marker for Breast Cancer: A Prospective Cohort Study. *J Clin Oncol* (2017) 35(15_suppl):11548–8. doi: 10.1200/JCO.2017.35.15_suppl.11548
65. Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, et al. Glypican-1 Identifies Cancer Exosomes and Detects Early Pancreatic Cancer. *Nature* (2015) 523(7559):177–82. doi: 10.1038/nature14581
66. Huber V, Fais S, Iero M, Lugini L, Canese P, Squarcina P, et al. Human Colorectal Cancer Cells Induce T-Cell Death Through Release of Proapoptotic Microvesicles: Role in Immune Escape. *Gastroenterology* (2005) 128(7):1796–804. doi: 10.1053/j.gastro.2005.03.045
67. Konoshenko MYU, Lekchnov EA, Vlassov AV, Laktionov PP. Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. *BioMed Res Int* (2018) 2018:1–27. doi: 10.1155/2018/8545347
68. Osti D, Del Bene M, Rappa G, Santos M, Matafora V, Richichi C, et al. Clinical Significance of Extracellular Vesicles in Plasma From Glioblastoma Patients. *Clin Cancer Res* (2019) 25(1):266–76. doi: 10.1158/1078-0432.CCR-18-1941

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Oncology

ORIGINAL RESEARCH
published: 17 November 2021
doi: 10.3389/fonc.2021.778729

Mixed-Beam Approach for High-Risk Prostate Cancer Carbon-Ion Boost Followed by Photon Intensity-Modulated Radiotherapy: Preliminary Results of Phase II Trial AIRC-IG-14300

Giulia Marvaso[1,2‡], Barbara Vischioni[3‡], Matteo Pepa[1‡], Mattia Zaffaroni[1], Stefania Volpe[1,2], Filippo Patti[1,3†], Federica Bellerba[4], Sara Gandini[4], Stefania Comi[5], Giulia Corrao[1,2], Dario Zerini[1], Matteo Augugliaro[1], Cristiana Fodor[1], Stefania Russo[3], Silvia Molinelli[3], Mario Ciocca[3], Rosalinda Ricotti[3], Francesca Valvo[3], Tommaso Giandini[6], Barbara Avuzzi[7], Riccardo Valdagni[2,7], Ottavio De Cobelli[2,8], Federica Cattani[5], Ester Orlandi[3], Barbara Alicja Jereczek-Fossa[1,2§] and Roberto Orecchia[9§]*

[1] Division of Radiotherapy, Istituto Europeo di Oncologia (IEO), European Institute of Oncology Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Milan, Italy, [2] Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy, [3] Clinical Department, National Center for Oncological Hadrontherapy (CNAO), Pavia, Italy, [4] Department of Experimental Oncology, Istituto Europeo di Oncologia (IEO), European Institute of Oncology Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Milan, Italy, [5] Medical Physics Unit, Istituto Europeo di Oncologia (IEO), European Institute of Oncology Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Milan, Italy, [6] Medical Physics Unit, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Istituto Nazionale dei Tumori, Milan, Italy, [7] Department of Radiation Oncology 1, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Istituto Nazionale dei Tumori, Milan, Italy, [8] Division of Urology, Istituto Europeo di Oncologia (IEO), European Institute of Oncology Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Milan, Italy, [9] Scientific Directorate, Istituto Europeo di Oncologia (IEO), European Institute of Oncology Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Milan, Italy

Purpose: This study represents a descriptive analysis of preliminary results of a Phase II trial on a novel mixed beam radiotherapy (RT) approach, consisting of carbon ions RT (CIRT) followed by intensity-modulated photon RT, in combination with hormonal therapy, for high-risk prostate cancer (HR PCa) with a special focus on acute toxicity.

Methods: Primary endpoint was the evaluation of safety in terms of acute toxicity. Secondary endpoints were early and long-term tolerability of treatment, quality of life (QoL), and efficacy. Data on acute and late toxicities were collected according to RTOG/EORTC. QoL of enrolled patients was assessed by IPSS, EORTC QLQ-C30, EORTC QLQ-PR25, and sexual activity by IIEF-5.

Results: Twenty-six patients were enrolled in the study, but only 15 completed so far the RT course and were included. Immediately after CIRT, no patients experienced GI/GU toxicity. At 1 and 3 months from the whole course RT completion, no GI/GU toxicities greater than grade 2 were observed. QoL scores were overall satisfactory.

OPEN ACCESS

Edited by:
Fabio Campodonico,
Ente Ospedaliero Ospedali Galliera, Italy

Reviewed by:
Matteo Droghetti,
University of Bologna, Italy
Concetta Schiavone,
Ente Ospedaliero Ospedali Galliera, Italy

***Correspondence:**
Stefania Volpe
stefania.volpe@ieo.it

†Present address:
Filippo Patti,
Clinical Department, National Center for Oncological Hadrontherapy (CNAO), Pavia, Italy

‡These authors share first authorship

§These authors share last authorship

Specialty section:
This article was submitted to Genitourinary Oncology, a section of the journal Frontiers in Oncology

Received: 17 September 2021
Accepted: 25 October 2021
Published: 17 November 2021

Citation:
Marvaso G, Vischioni B, Pepa M, Zaffaroni M, Volpe S, Patti F, Bellerba F, Gandini S, Comi S, Corrao G, Zerini D, Augugliaro M, Fodor C, Russo S, Molinelli S, Ciocca M, Ricotti R, Valvo F, Giandini T, Avuzzi B, Valdagni R, De Cobelli O, Cattani F, Orlandi E, Jereczek-Fossa BA and Orecchia R (2021) Mixed-Beam Approach for High-Risk Prostate Cancer Carbon-Ion Boost Followed by Photon Intensity-Modulated Radiotherapy: Preliminary Results of Phase II Trial AIRC-IG-14300. Front. Oncol. 11:778729. doi: 10.3389/fonc.2021.778729

Conclusions: The feasibility of the proposed mixed treatment schedule was assessed, and an excellent acute toxicity profile was recorded. Such findings instil confidence in the continuation of this mixed approach, with evaluation of long-term tolerability and efficacy.

Keywords: carbon-ion radiotherapy, intensity modulated radiotherapy, high-risk prostate cancer, phase II study, mixed-beam approach

INTRODUCTION

Prostate cancer (PCa) is the most common solid organ malignancy in men, and radiotherapy (RT) plays a significant role in the treatment of organ-confined or locally advanced disease (1). Although low- and intermediate-risk PCa show excellent outcomes with surgery or RT, high-risk (HR) disease PCa continues to have a high rate of recurrence and progression, both locally and distantly, making research necessary for escalation or combined strategies.

At least 17–31% of these men present with HR localized or locally advanced disease (2) and need a curative treatment, which includes surgery or external beam radiotherapy (EBRT) combined with androgen deprivation therapy (ADT), and an optional brachytherapy boost (3). From a RT perspective, the most peculiar biological feature of PCa is its low α/β ratio, corresponding to a relative radio-resistance, which has fostered the development through the years of different schedules with varying degrees of hypofractionation. Many of these studies (4, 5) have demonstrated to be equally if not more effective in terms of local control and less likely to cause side effects. However, the role of hypofractionation with stereotactic body RT (SBRT) in HR PCa patients remains controversial, especially when it becomes necessary to perform elective pelvic nodal irradiation (6).

In parallel, dose-escalation studies, particularly with a dose boost to the dominant intraprostatic lesions (DIL), have shown an advantage in terms of local control of disease (7, 8), although limited by greater toxicity to adjacent organs such as the bladder and anterior wall of the rectum (9).

In this context, the use of heavy particles was proven to be both safe and effective. In fact, firstly, they allow to reach a steep dose gradient due to the inverted profile of in-depth dose deposition compared to photons, which permits a greater sparing of organs at risk (OARs) (10, 11). Secondly, carbon-ion radiotherapy (CIRT), already been in use in various Centers at an experimental level for more than 10 years, demonstrated a greater efficacy compared to standard radiation techniques due to its peculiar physical features. It is now widely accepted that beams of high linear energy transfer (LET) particles can offer a biological advantage for radioresistant malignancies due to their higher relative biological effectiveness (RBE) (12–14).

In the light of improving outcomes in HR PCa patients without compromising treatment safety, we explored the use of carbon ions to escalate the dose to the prostate and the addition of a standard photon treatment to the pelvic lymph nodes.

In fact, the purpose of this prospective phase II study, sponsored by the Italian Association of Cancer Research (Associazione Italiana per la Ricerca sul Cancro, AIRC), is to evaluate the feasibility of a radiation schedule that comprises a dose boost to the prostate, delivered with carbon ions, followed by a conventional course of pelvic photon RT in patients affected by HR PCa undergoing neoadjuvant and adjuvant long-term hormone therapy.

This study represents a descriptive analysis of preliminary results, with a special focus on acute toxicity. In particular, only acute toxicity events were collected due to the short patients' follow-up available. The analysis on oncological outcomes and late toxicity events will be performed when more mature data will be collected.

MATERIALS AND METHODS

Trial Characteristics

The protocol was approved by the Ethics Committee (R86/14-IEO98) of the European Institute of Oncology (IEO), coordinating center, and subsequently presented and registered to the ethics committees of the other participating centers, and has been registered at ClinicalTrials.gov (NCT02672449). The study was designed as a prospective, multicentric, phase II open-label trial. The patients have been enrolled at three radiation oncology facilities in northern Italy, namely, National Center of Oncological Hadrontherapy (Centro Nazionale di Adroterapia Oncologica, CNAO) in Pavia, National Cancer Institute (Fondazione IRCCS Istituto Nazionale dei Tumori, INT) in Milan, and European Institute of Oncology IRCCS (Istituto Europeo di Oncologia, IEO) in Milan.

The trial was supposed to enrol 65 consecutive patients (15); however, due to delays in authorizations and to the emergence of competitive surgical trials, it recruited a total of 26 patients.

Abbreviations: ADT, androgen deprivation therapy; AIRC, Associazione Italiana per la Ricerca sul Cancro (Italian Association for Cancer Research); CIRT, carbon ion radiotherapy ; CNAO, Centro Nazionale di Adroterapia Oncologica (National Center of Oncological Hadrontherapy); CT, computed tomography ; CTV, clinical target volume ; DIL, dominant intraprostatic lesion; EBRT, external beam radiotherapy; ECOG, Eastern Cooperative Oncology Group; EORTC, European Organisation for Research and Treatment of Cancer; G, grade; GG, grade group; GI, gastrointestinal; GS, Gleason score; GU, genitourinary; HR, high risk; IIEF-15, International Index of Erectile Function; IEO, Istituto Europeo di Oncologia (European Institute of Oncology); IMRT, intensity-modulated radiotherapy; INT, Istituto Nazionale dei Tumori (National Cancer Institute); iPSA, initial PSA; IPSS, International Prostatic Symptoms Score; IQR, interquartile range; LET, linear energy transfer; NCCN, National Comprehensive Cancer Network; NIRS, National Institute of Radiological Sciences; PCa, prostate cancer; PCSM, prostate cancer specific mortality; PSA, prostate-specific antigen ; PTV, planning target volume ; QLQ-C30, Quality of Life Questionnaire-Core 30; QLQ-PR25, Quality of Life Questionnaire PRostate specific; QoL, quality of life; RBE, relative biological effectiveness; RT, radiotherapy; RTOG, Radiation Therapy Oncology Group; SBRT, stereotactic body radiotherapy; T, tumour; WPRT, whole pelvis radiotherapy.

Sample size has been recalculated accordingly, by considering the actual accrual. All enrolled patients signed an informed consent before starting treatment. The study protocol has been previously published (NCT02672449); therefore, patients' selection, treatment delivery, outcomes of the study, and statistical analyses will be only briefly described hereafter (15).

Patients Selection

This study included patients affected by HR PCa, as defined by National Comprehensive Cancer Network (NCCN) risk categories [T3a and/or prostate-specific antigen (PSA) >20 ng/ml and/or Gleason score (GS) 8–10]. Inclusion criteria are listed in **Table 1**.

Radiation Therapy Treatment Planning and Delivery

Computed tomography (CT) simulation, volumes of interest contouring, and treatment delivery were performed following the previously described methodology (15, 16).

In particular, all patients first received the CIRT boost to the prostate and to the proximal third of the seminal vesicles at CNAO. The dose prescribed to the planning target volume (PTV) boost was 16.6 Gy (RBE) in four fractions [4.15 Gy (RBE)/fraction, over 1 week]. The CIRT technique used at CNAO (17) consists of two lateral opposed beams, with the PTV receiving at least 95% of the prescribed dose. To complete the RT course, the patients received intensity-modulated RT (IMRT), with the clinical target volume (CTV) including the whole pelvis, and the PTV derived as a 5 mm CTV expansion. Total dose to the PTV pelvis ranged from 45 to 50.4 Gy in 1.8–2 Gy/fraction. Both PTV boost and PTV pelvis received at least 95% of the prescribed dose. Dose constraints to the OARs were derived by considering the plan sum, that is, CIRT + IMRT course. Further details on treatment delivery are available in the study protocol (15).

Assessment of Quality of Life and Follow–Up

The primary endpoint of this study was to evaluate the feasibility and safety of the proposed treatment through the evaluation of acute side effects by physician reported outcomes, according to the Radiation Therapy Oncology Group (RTOG)/European Organisation for Research and Treatment of Cancer (EORTC) scale. This was achieved by assessing the percentage of patients who report at least one episode of grade (G) 3 or 4 toxicity during treatment or within 1 month after RT. Side effects were also evaluated by patients' reported outcomes with dedicated questionnaires for treatment-related quality of life (QoL), according to EORTC quality of life-core 30 (QLQ-C30), international prostatic symptoms score (IPSS), and international index of erectile function (IIEF-15) questionnaires. Toxicities evaluated at 3, 12, and 24 months have been considered as secondary endpoints. During follow-up, erectile function was evaluated with the IIEF questionnaire (minimum = 1, serious; maximum = 25, optimal condition). The state of prostatic symptoms was evaluated through the IPSS questionnaire (minimum = 0, no symptoms; maximum score 25 = acute symptoms). The "Global Health Status" based on the EORTC QLQ-C30 questionnaire was described through median and interquartile range. The efficacy of treatment was investigated as secondary endpoint in terms of biochemical response, through prostate specific antigen evaluation every 3 months.

Statistical Analysis

The primary endpoint of this analysis was acute toxicity that was tested by simply counting the number of patients free from cumulative 1-month acute toxicity after RT.

Categorical variables were reported as frequencies (percentages), whereas continuous variables were summarized with the median value and interquartile range (25th–75th percentiles). We evaluated time trends of IPSS, EORTC QLQ-C30, and the IIEF-5 questionnaires. The missing IPSS scores (n=4) were replaced by the median score at the same time point.

All scales of EORTC QLQ-C30 (functioning scales: Physical Functioning, Role Functioning, Emotional Functioning, Cognitive Functioning, Social Functioning; general health status scales: Global Health Status/QoL; symptom scales: Fatigue, Nausea/Vomiting, Pain, Dyspnoea, Insomnia, Appetite loss, Constipation, Diarrhoea, Financial Problems) were built

TABLE 1 | Inclusion and exclusion criteria.

INCLUSION CRITERIA	EXCLUSION CRITERIA
• Histologically confirmed adenocarcinoma of the prostate, high- risk category according to NCCN version 1.2016 (cT3a and/or PSA >20 ng/mL and/or Gleason score of 8-10)	• Previous pelvic RT • Previous prostatectomy • Concomitant inflammatory bowel disease or other serious systemic comorbidities
• Age > 18 years • cN0 and cM0 • Eastern Cooperative Oncology Group (ECOG) Performance Status < 2 • ADT recommended ○ 3 months before RT ○ Concomitant to RT ○ up to 2 years after the end of RT • Good urinary flow (peak flow >10 mL/s) • Written informed consent	• Previous invasive cancer (within 5 years before the PCa diagnosis unless the patient has been free from disease for at least 3 years) except for nonmelanoma skin malignancies • Presence of hip prosthesis

ECOG, Eastern Cooperative Oncology Group; NCCN, National Comprehensive Cancer Network; PCa, prostate cancer; PSA, prostate-specific antigen; RT, radiotherapy.

according to the EORTC manual and transformed to 0–100 scales, with higher scores reflecting either more symptoms or higher levels of functioning or QoL.

For EORTC QLQ-C30 questionnaire, imputation of missing answers was performed as follows: if a patient answered less than half the questions in a scale, the scale was considered to be missing; if a patient answered at least half of the questions in a scale, the average score of the answered questions was calculated and imputed as the response to questions which had not been answered.

For IIEF-15 questionnaire, all observations with more than 30% missing items at a given time point were excluded from the analysis. In all other cases, a missing item was replaced with the mean score of the items from the same domain where available, and with the median value of the item at the same time point when all the items from the same domain were missing.

Within-patient score changes of IPSS, every scale of EORTC QLQ-C30 and IIEF-5 questionnaires were calculated at each time point from baseline. The baseline was defined as the time point right before RT start, meaning 3 months after ADT administration, as specified in the study protocol (15). Linear mixed models for repeated measures were used to detect a trend in the changes. All estimates were adjusted for the baseline score. Residuals from full models were checked to assess normal distribution, and boxplots of the score changes were provided for the main results.

A two-sided p value < 0.05 was considered significant for all statistical analyses.

The analyses were performed using SAS software (SAS Institute Inc., Cary, USA), version 9.4, and R software (https://www.Rproject.org), version 3.5.2.

RESULTS

Study Population

Since October 2017, 26 consecutive patients who fulfilled the inclusion criteria have been treated. Sixteen patients have completed the prescribed treatment according to protocol guidelines so far, and one patient dropped out the protocol due to non-PCa-related clinical motivations, so 15 patients were included in the analysis. A follow-up of at least 3 months was available for them. All patients underwent concomitant ADT. Patients' characteristics are listed in **Table 2**.

Toxicity Outcomes

Overall, patients' tolerance to treatment was acceptable. After the CIRT boost, no patients experienced gastrointestinal (GI)/genitourinary (GU) toxicity. At 1 and 3 months from RT completion (CIRT followed by IMRT), no GI or GU toxicities greater than grade (G) 2 were observed. In details, considering acute GU toxicity, eight patients have not reported any toxicity. Concerning GI, five patients presented G1 acute toxicity and two of them G2 (**Table 3**). Longer follow-up (12 months) was available for seven patients, with one patient presenting GU toxicity classified as G1 and one patient presenting GU toxicity reported as G2.

TABLE 2 | Statistics of patients, tumour, and treatment characteristic*.

VARIABLES	CATEGORIES	STATISTICS
Age, Median (IQR)		74 (59-83)
iPSA, Median (IQR)		12.3 (3.3-63.1)
PSA preRT, median (IQR)		1.2 (0.13-23.57)
T, n (%)	cT1a-c	3 (15)
	cT2a-c	10 (50)
	cT3a	7 (35)
Total GS, n (%)	6 (GG = 1)	1 (5)
	3+4 (GG = 2)	1 (5)
	4+3 (GG = 3)	3 (15)
	8 (GG = 4)	12 (60)
	9 (GG = 5)	3 (15)

GG, grade group; GS, Gleason score; iPSA, initial prostate-specific antigen; IQR, interquartile range; RT, radiotherapy; T, tumour.
**Data available for 20 patients.*

TABLE 3 | Induced acute and late toxicity.

Variable		Grade	Number of patients (%*)
Acute toxicity	GU	0	8 (53.3%)
		1	5 (33.3%)
	GI	0	12 (80.0%)
		1	1 (6.7%)
Late toxicity[a]	GU	0	5 (53.3%)
		1	0 (0%)
		2	1 (6.7%)
	GI	0	5 (33.3%)
		1	1 (6.7%)

GI, gastrointestinal; GU, genitourinary.
[a]Missing data for one patient.
**Percentage refers to the whole cohort of patients (15).*

Quality of Life Scores

QLQ-c30 QoL was analysed to evaluate the urinary function, and revealed an overall improvement from baseline at 1 month, even if not statistically significant, which kept constant at the following time points (**Figure 1A**). These results are consistent with those obtained from IPSS analysis (**Figures 2A, B**). Change of IPSS/QoL score was positive across the considered time points, as a statistically significant marked improvement from baseline was observed ($p = 0.04$) (**Figure 2A**). Similar findings, despite not statistically significant ($p = 0.10$), were observed considering the IPSS questionnaire, with no significant deterioration from baseline at all the considered time points. In particular, no IPSS score changes were observed at 1 month after baseline and after RT completion (**Figure 2B**). Considering the QLQ-c30 fatigue score change, a trend towards improvement from baseline was observed, especially at 1 month, even if not statistical significant ($p = 0.71$) and maintained across the considered time points (**Figure 1B**). The same considerations hold for gastrointestinal diarrhoea, as evaluated by QLQ-c30, with an improvement from baseline (**Figure 1C**). The analysis of IIEF-5 did not show any significant change of erectile function from baseline ($p = 0.90$), although a worsening of function

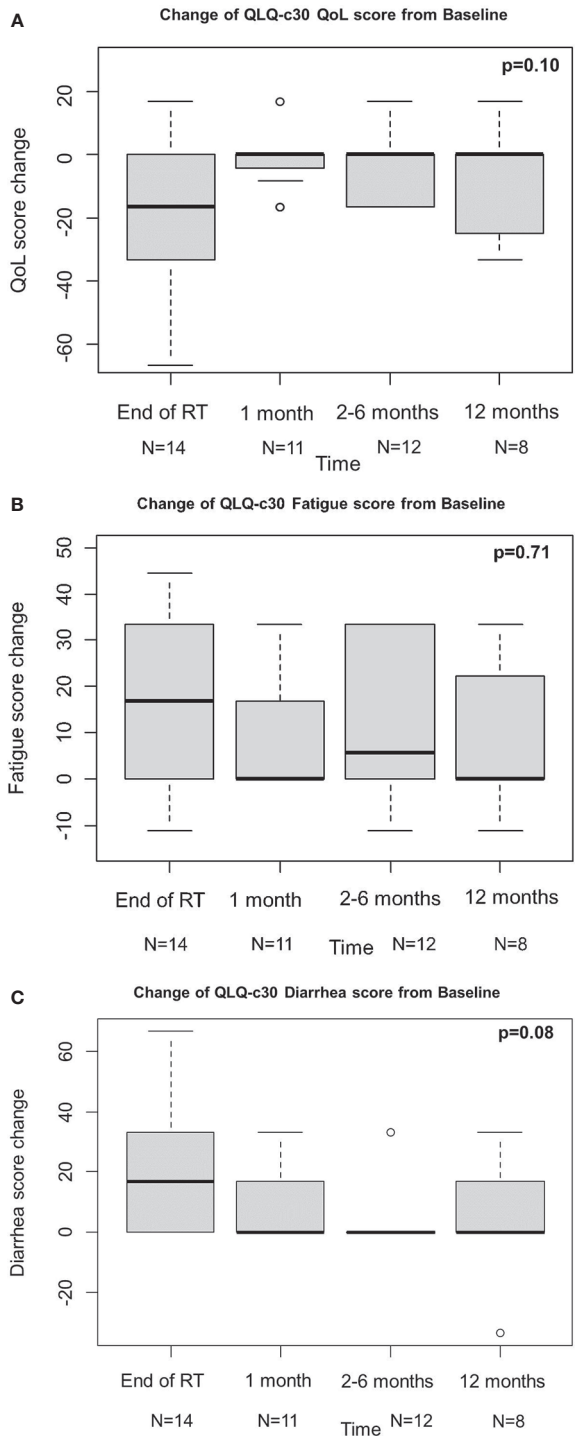


FIGURE 1 | Change of QLQ-c30 QoL score **(A)**, fatigue score **(B)**, diarrhea score **(C)** from baseline.

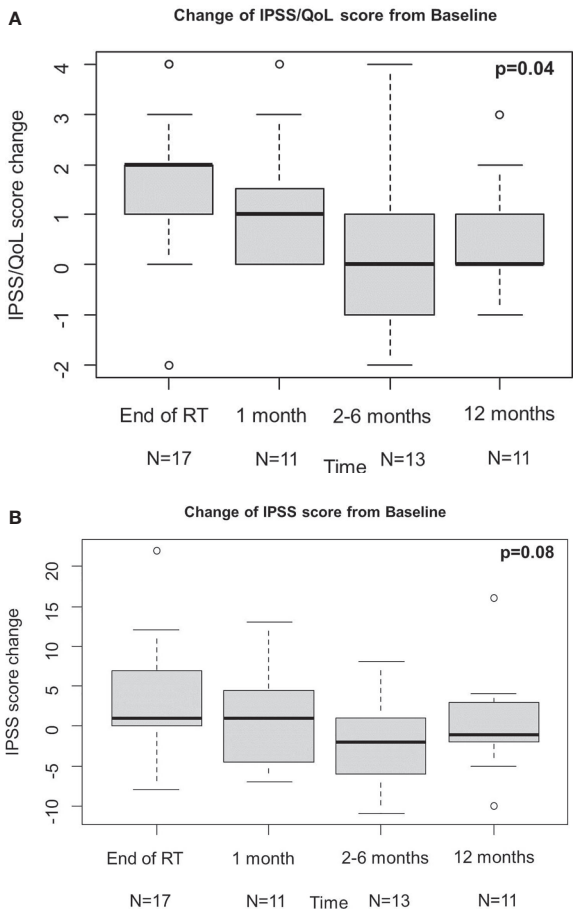


FIGURE 2 | Results of IPSS/QoL **(A)** and IPSS **(B)**. IPSS, international prostatic symptoms score; QoL, quality of life.

was observed at the end of RT, which gradually improved at the following time points. A worsening of erectile function was also observed after 12 months, but only six score changes from baseline were available at that time point (**Figure 3**).

Oncological Outcomes

As stated above, a complete evaluation of oncological outcomes will be object of a separate investigation; however, preliminary results are reported here. As of July 2021, no patients experienced biochemical recurrence. Median PSA value at diagnosis was 12.37 ng/ml [interquartile range (IQR) 8.38–25.00 ng/ml]. After 3 months of hormone therapy and before radiation treatment, median PSA was 1.2 ng/ml (IQR 0.49–5.5 ng/ml). At last follow-up (median 6 months, IQR 3–12 months), median PSA was 0.08 ng/ml (IQR 0.02–0.15 ng/ml).

DISCUSSION

The present investigation aimed at evaluating preliminary outcomes of a novel mixed-beam approach for HR PCa CIRT followed by photon IMRT on prostate and pelvic lymph nodes, with a focus on acute GI and GU toxicity and QoL. At the current state, the mixed treatment schedule proposed herein shows an optimal 1-month acute toxicity profile. This reflects on the

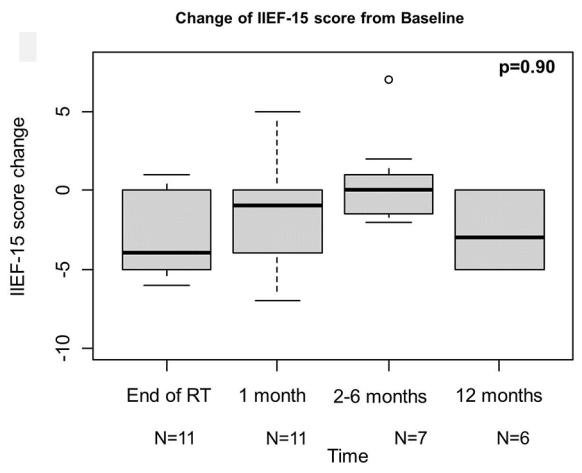


FIGURE 3 | Results of IIEF-15. IIEF-15, international index of erectile function.

patient-reported QoL scores, which were overall satisfactory, as the changes with respect to baseline values showed an improvement or at least a non-worsening.

The optimal management of locally advanced HR PCa is still a matter of debate, with a rate of recurrence that still remains high (55% at 10 years), even when ADT is administered concomitantly (18, 19). As of today, it is difficult to compare the efficacy of different radical treatment strategies (i.e., surgery, RT, new agents added to standard ADT and RT) in HR PCa, due to the scarcity of randomized controlled trials. Among the most recent experiences that are currently investigating the use of second-generation ADT in combination with local approaches, results are awaited from STAMPEDE (abiraterone) (20), ENZARAD (NCT02446444, enzalutamide), and ATLAS (21), ARNEO (22), PROTEUS (NCT03767244, apalutamide) trials.

Modern RT, including IMRT and hypofractionation, has a central role among the available treatment options. However, currently, there is no level 1 evidence on the survival advantages of brachytherapy, SBRT, or protons over another form of radiation therapy (23). In particular, CHHiP (24) and HYPRO30 trials (25) demonstrated that hypofractionated schemes, exploiting the low α/β ratio of PCa, constitute a valid treatment option for HR patients. However, the number of studies involving extreme hypofractionation is relatively low, and a direct comparison of different hypofractionation schemes is still lacking. Therefore, despite being cited in clinical practice guidelines next to moderate hypofractionation schemes, the current level of evidence is too low to implement extreme hypofractionation as a standard of care. In particular, one of the limits consists in the fact that ultra-hypofractionated regimens in HR PCa lead to a risk of higher toxicity in case of prophylactic whole pelvis radiotherapy (WPRT).

Particle therapy has been gaining interest due to the unique physical and radiobiological properties of protons and other heavy ions, including carbon ions, compared to photons. Specifically, the use of carbon ions as a boost is motivated by their sharper dose gradient that ensures a better OARs sparing and by their high relative biological effectiveness (RBE), with a therapeutic effect up to three times higher with respect to photons and protons (10, 11). In addition, carbon ions might make radioresistant clusters more sensitive to subsequent photon therapy. Such unique physical and biological advantages make them a valuable candidate in the treatment of PCa.

Safety and effectiveness evidence on carbon ions in the treatment of PCa mainly derive from the Japanese experience. The first clinical trial employing CIRT in PCa was activated at the National Institute of Radiological Sciences (NIRS) and dates back to 1994 (26). At the same institute, three additional phase I/II trials over a 13-year time frame and two phase II trials demonstrated the high potential of CIRT in the treatment of PCa. A study by Nomiya et al. (27), aiming at evaluating the feasibility of a 3-week CIRT treatment schedule for PCa, reported G0 and G1 GU acute toxicity events in 10 (22%) and 34 (74%) of patients, respectively, and acute G2 urinary frequency in only two patients (4%). Similar findings were reported by Akakura et al. (12), who analysed the outcomes of a series of 96 patients treated with CIRT +/− ADT in adjuvant or neoadjuvant settings, reported no patients exhibiting G3 or higher acute radiation-induced toxicity. The first prospective observational study conducted outside NIRS was the one by Kawamura et al. (28), which reported low GU and GI toxicities as well as an acceptable biochemical control during the first 5 years following moderately hypofractionated CIRT for localized PCa. These results are in line with those obtained in the present study, with acute GU G0 and G1 toxicity events occurring in 8/15 (53.3%) and 5/15 (33.3%) patients, respectively. Notably, the risk of toxicity in our protocol was slightly higher considering the larger irradiated volume (prostate + pelvis).

No patients experienced biochemical recurrence. However, follow-up is too short to derive robust results, which can be also affected by the fact that some patients are still receiving hormonal therapy. It is expected that these results will be in line with available evidence, highlighting that this treatment modality has an excellent efficacy profile. Indeed, regarding oncological outcomes, the study by Nomiya et al. (27) reported no biochemical failures or distant metastases at last follow-up, with PSA showing a good response in most patients (94%). At 5-year, Akakura et al. (12) reported an overall, cause-specific, clinical recurrence-free, and biochemical recurrence-free survival rates of 87.7, 94.9, 90, 82.6%, respectively. Local control was achieved in all patients but one. Analogously, a study by Kasuya et al. (29), who analysed the treatment outcomes of HR localized PCa treated with CIRT + ADT compared with standard treatment modalities, demonstrated that the association of these two treatments yielded quite favourable treatment outcomes, with biochemical recurrence occurring in 90 out 608 (14.8%) patients, with a median follow-up of 88.4 months. The 5/10-year rates of PCa specific mortality (PCSM) and overall mortality, including PCSM and non-prostate cancer specific mortality, were 1.5 and 5.0%, respectively. Analogously, Kawamura et al. (28) reported a 5-year biochemical relapse-free rate of 92% in the HR group.

The efficacy of dose-escalated EBRT to the prostate alone in patients with HR disease might be limited by the increased likelihood of occult pelvic lymph node metastases outside of the radiation field. The advantage of such mixed beam approach is to irradiate the whole pelvis with a prophylactic intent and, at the same time, to escalate the dose to the prostate. This approach is expected to increase locoregional control by eradicating micrometastatic lesions in the pelvis without jeopardising OARs sparing.

As mentioned above, patient- and physician-reported outcomes were overall satisfactory. The only exception was represented by the erectile function, whose worsening was observed at the end of RT and at 12 months. However, these findings need to be interpreted carefully. To start with, it is important to consider that erectile function was assessed only on six patients, as they were the only ones having a follow-up of at least 12 months at the time of the study. Additionally, it should be taken into account that such observed worsening might be the result of cumulative side effects of ADT and RT. More mature results about erectile function and more in general on all the considered patient's and physician's reported outcomes will be available after all patients will have completed the hormonal therapy course (i.e., up to 2 years after ADT beginning) and will shed light on the actual impact of ADT on toxicity outcomes.

As stated above, this paper mainly analyses acute toxicity events. The analysis on oncological outcomes and late toxicities will be performed when more mature data will be available and will be object of a separate publication. As of today, no patients experienced biochemical recurrence. However, follow-up is too short to derive robust results, which can be also affected by the fact that some patients are still receiving hormonal therapy. It is expected that these results will be in line with available evidence, highlighting that this treatment modality has an excellent efficacy profile.

This study, which explores the combination of different RT approaches in the treatment of HR PCa, represents a novelty in the modern RT scenario. This experience proved the feasibility of this novel RT workflow, including safe sharing of medical imaging data between centres *via* trusted channels, effective RT plans sum for dosimetric considerations, as well as an acceptable overall treatment duration for the enrolled patients.

However, the study suffers from some limitations. First of all, the accrual was scarce and lower than expected, due to the fact that most patients with HR PCa undergo surgery. This is mainly due to the fact that the indication for surgery moved from low-risk patients, who are candidate for active surveillance according to most recent guidelines, to patients with HR disease. Therefore, the number of patients to carry out the analysis was low and the follow-up was short. However, the absence of severe toxicity encourages further investigations in this setting.

CONCLUSIONS

In conclusion, results from this preliminary analysis demonstrated the overall safety of such combined treatment modality, due to the low incidence of acute GU/GI toxicities and promising QoL scores following CIRT for PCa. These findings confirm the available evidence on CIRT safety, even with larger irradiated volumes. Therefore, available data on efficacy about CIRT in HR setting seem encouraging and could confirm a new role of carbon ions in this clinical setting.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee (R86/14-IEO98) of the European Institute of Oncology (IEO). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, GM, BV, RV, MC, RR, FV, TG, FC, EO, BJ-F, and RO. Methodology, GM, BV, FP, SC, GC, DZ, MA, SR, and SM. Validation, GM, MP, MZ, and FP. Formal analysis, FB and SG. Investigation, GM, BV, RV, MC, RR, FV, TG, EO, BJ-F, and RO. Data curation, GM, MP MZ, and FP. Writing—original draft preparation, GM, MP, MZ, FP, FB, and SG. Writing—review and editing, BV, SC, GC, DZ, MA, CF, SR, SM, MC, RR, FV, TG, BA, RV, OC, FC, EO, BJ-F, and RO. Supervision, BJ-F. Project administration, RO. Funding acquisition, RO. All authors contributed to the article and approved the submitted version.

FUNDING

The study was fully funded by the University of Milan with APC funds. FP was partially supported by Associazione Italiana per la Ricerca sul Cancro (AIRC), project IG-14300 "Carbon ions boost followed by pelvic photon intensity modulated radiotherapy for high-risk prostate cancer," registered at ClinicalTrials.gov (NCT02672449), approved by IEO R86/14-IEO 98. MZ received a research fellowship by the European Institute of Oncology-Cardiologic Center Monzino Foundation (FIEO-CCM), with a project entitled "Proton therapy vs photon-based IMRT for parotid gland tumors: A model based approach with Normal Tissue Complication Probability (NTCP)" outside the current study. SV and FB are PhD students within the European School of Molecular Medicine (SEMM), Milan, Italy. This work was also partially supported

by the Italian Ministry of Health with Ricerca Corrente and 5x1000 funds. The sponsors did not play any role in the study design, collection, analysis, and interpretation of data, nor in the writing of the manuscript, nor in the decision to submit the manuscript for publication.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.778729/full#supplementary-material

REFERENCES

1. Dall'Era MA, Albertsen PC, Bangma C, Carroll PR, Carter HB, Cooperberg MR, et al. Active Surveillance for Prostate Cancer: A Systematic Review of the Literature. *Eur Urol* (2012) 62:976–83. doi: 10.1016/j.eururo.2012.05.072
2. Cooperberg MR, Cowan J, Broering JM, Carroll PR. High-Risk Prostate Cancer in the United States, 1990-2007. *World J Urol* (2008) 26:211–8. doi: 10.1007/s00345-008-0250-7
3. Schaeffer E, Srinivas S, Antonarakis ES, Armstrong AJ, Bekelman JE, Cheng H, et al. NCCN Prostate Cancer Guidelines. *J Natl Compr Canc Netw* (2021) 19(2):134–43. doi: 10.6004/jnccn.2021.0008
4. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, et al. Cancer Incidence and Mortality Patterns in Europe: Estimates for 40 Countries in 2012. *Eur J Cancer Oxf Engl 1990* (2013) 49:1374–403. doi: 10.1016/j.ejca.2012.12.027
5. Arnold M, Karim-Kos HE, Coebergh JW, Byrnes G, Antilla A, Ferlay J, et al. Recent Trends in Incidence of Five Common Cancers in 26 European Countries Since 1988: Analysis of the European Cancer Observatory. *Eur J Cancer Oxf Engl 1990* (2015) 51:1164–87. doi: 10.1016/j.ejca.2013.09.002
6. Lawton CA, DeSilvio M, Roach M 3rd, Uhl V, Kirsch R, Seider M, et al. An Update of the Phase III Trial Comparing Whole Pelvic to Prostate Only Radiotherapy and Neoadjuvant to Adjuvant Total Androgen Suppression: Updated Analysis of RTOG 94-13, With Emphasis on Unexpected Hormone/Radiation Interactions. *Int J Radiat Oncol Biol Phys* (2007) 69:646–55. doi: 10.1016/j.ijrobp.2007.04.003
7. Henderson DR, Tree AC, van As NJ. Stereotactic Body Radiotherapy for Prostate Cancer. *Clin Oncol R Coll Radiol G B* (2015) 27:270–9. doi: 10.1016/j.clon.2015.01.011
8. Viani GA, Stefano EJ, Afonso SL. Higher-Than-Conventional Radiation Doses in Localized Prostate Cancer Treatment: A Meta-Analysis of Randomized, Controlled Trials. *Int J Radiat Oncol Biol Phys* (2009) 74:1405–18. doi: 10.1016/j.ijrobp.2008.10.091
9. Lips IM, van der Heide UA, Haustermans K, van Lin EN, Pos F, Franken SP, et al. Single Blind Randomized Phase III Trial to Investigate the Benefit of a Focal Lesion Ablative Microboost in Prostate Cancer (FLAME-Trial): Study Protocol for a Randomized Controlled Trial. *Trials* (2011) 12:255. doi: 10.1186/1745-6215-12-255
10. Held KD, Kawamura H, Kaminuma T, Paz AE, Yoshida Y, Liu Q, et al. Effects of Charged Particles on Human Tumor Cells. *Front Oncol* (2016) 6:23. doi: 10.3389/fonc.2016.00023
11. Durante M, Orecchia R, Loeffler JS. Charged-Particle Therapy in Cancer: Clinical Uses and Future Perspectives. *Nat Rev Clin Oncol* (2017) 14:483–95. doi: 10.1038/nrclinonc.2017.30
12. Akakura K, Tsujii H, Morita S, Tsuji H, Yagishita T, Isaka S, et al. Phase I/II Clinical Trials of Carbon Ion Therapy for Prostate Cancer. *Prostate* (2004) 58:252–8. doi: 10.1002/pros.10328
13. Ishikawa H, Tsuji H, Kamada T, Yanagi T, Mizoe JE, Kanai T, et al. Carbon Ion Radiation Therapy for Prostate Cancer: Results of a Prospective Phase II Study. *Radiother Oncol J Eur Soc Ther Radiol Oncol* (2006) 81:57–64. doi: 10.1016/j.radonc.2006.08.015
14. Zietman AL, Bae K, Slater JD, Shipley WU, Efstathiou JA, Coen JJ, et al. Randomized Trial Comparing Conventional-Dose With High-Dose Conformal Radiation Therapy in Early-Stage Adenocarcinoma of the Prostate: Long-Term Results From Proton Radiation Oncology Group/American College of Radiology 95-09. *J Clin Oncol Off J Am Soc Clin Oncol* (2010) 28:1106–11. doi: 10.1200/JCO.2009.25.8475
15. Marvaso G, Jereczek-Fossa BA, Vischioni B, Ciardo D, Giandini T, Hasegawa A, et al. Phase II Multi-Institutional Clinical Trial on a New Mixed Beam RT Scheme of IMRT on Pelvis Combined With a Carbon Ion Boost for High-Risk Prostate Cancer Patients. *Tumori* (2017) 103:314–8. doi: 10.5301/tj.5000587
16. Gugliandolo SG, Marvaso G, Comi S, Pepa M, Romanò C, Zerini D, et al. Mixed-Beam Approach for High-Risk Prostate Cancer: Carbon-Ion Boost Followed by Photon Intensity-Modulated Radiotherapy. Dosimetric and Geometric Evaluations (AIRC IG-14300). *Phys Med PM Int J Devoted Appl Phys Med Biol Off J Ital Assoc Biomed Phys AIFB* (2020) 76:327–36. doi: 10.1016/j.ejmp.2020.07.012
17. Rossi S. The National Centre for Oncological Hadrontherapy (CNAO): Status and Perspectives. *Phys Med PM Int J Devoted Appl Phys Med Biol Off J Ital Assoc Biomed Phys AIFB* (2015) 31:333–51. doi: 10.1016/j.ejmp.2015.03.001
18. Zelefsky MJ, Pei X, Chou JF, Schechter M, Kollmeier M, Cox B, et al. Dose Escalation for Prostate Cancer Radiotherapy: Predictors of Long-Term Biochemical Tumor Control and Distant Metastases-Free Survival Outcomes. *Eur Urol* (2011) 60:1133–9. doi: 10.1016/j.eururo.2011.08.029
19. Arcangeli S, Strigari L, Gomellini S, Saracino B, Petrongari MG, Pinnarò P, et al. Updated Results and Patterns of Failure in a Randomized Hypofractionation Trial for High-Risk Prostate Cancer. *Int J Radiat Oncol Biol Phys* (2012) 84:1172–8. doi: 10.1016/j.ijrobp.2012.02.049
20. James ND, de Bono JS, Spears MR, Clarke NW, Mason MD, Dearnaley DP, et al. Abiraterone for Prostate Cancer Not Previously Treated With Hormone Therapy. *N Engl J Med* (2017) 377:338–51. doi: 10.1056/NEJMoa1702900
21. Sandler HM, Mckenzie MR, Tombal BF, Baskin-Bey E, Freedland SJ, Roach M, et al. ATLAS: A Randomized, Double-Blind, Placebo-Controlled, Phase 3 Trial of Apalutamide (ARN-509) in Patients With High-Risk Localized or Locally Advanced Prostate Cancer Receiving Primary Radiation Therapy. *J Clin Oncol* (2016) 34:TPS5087–TPS5087. doi: 10.1200/JCO.2016.34.15_suppl.TPS5087
22. Tosco L, Laenen A, Gevaert T, Salmon I, Decaestecker C, Davicioni E, et al. Neoadjuvant Degarelix With or Without Apalutamide Followed by Radical Prostatectomy for Intermediate and High-Risk Prostate Cancer: ARNEO, a Randomized, Double Blind, Placebo-Controlled Trial. *BMC Cancer* (2018) 18:354. doi: 10.1186/s12885-018-4275-z
23. Choudhury A, Henry A, Mitin T, Chen R, Joseph N, Spratt DE. Photons, Protons, SBRT, Brachytherapy-What Is Leading the Charge for the Management of Prostate Cancer? A Perspective From the GU Editorial Team. *Int J Radiat Oncol Biol Phys* (2021) 110:1114–21. doi: 10.1016/j.ijrobp.2021.01.003
24. Dearnaley D, Syndikus I, Mossop H, Khoo V, Birtle A, Bloomfield D, et al. Conventional Versus Hypofractionated High-Dose Intensity-Modulated Radiotherapy for Prostate Cancer: 5-Year Outcomes of the Randomised, non-Inferiority, Phase 3 CHHiP Trial. *Lancet Oncol* (2016) 17:1047–60. doi: 10.1016/S1470-2045(16)30102-4
25. Incrocci L, Wortel RC, Alemayehu WG, Aluwini S, Schimmel E, Krol S, et al. Hypofractionated Versus Conventionally Fractionated Radiotherapy for Patients With Localised Prostate Cancer (HYPRO): Final Efficacy Results From a Randomised, Multicentre, Open-Label, Phase 3 Trial. *Lancet Oncol* (2016) 17:1061–9. doi: 10.1016/S1470-2045(16)30070-5
26. Ishikawa H, Tsuji H, Kamada T, Akakura K, Suzuki H, Shimazaki J, et al. Carbon-Ion Radiation Therapy for Prostate Cancer. *Int J Urol Off J Jpn Urol Assoc* (2012) 19:296–305. doi: 10.1111/j.1442-2042.2012.02961.x
27. Nomiya T, Tsuji H, Maruyama K, Toyama S, Suzuki H, Akakura K, et al. Phase I/II Trial of Definitive Carbon Ion Radiotherapy for Prostate Cancer: Evaluation of Shortening of Treatment Period to 3 Weeks. *Br J Cancer* (2014) 110:2389–95. doi: 10.1038/bjc.2014.191
28. Kawamura H, Kubo N, Sato H, Mizukami T, Katoh H, Ishikawa H, et al. Moderately Hypofractionated Carbon Ion Radiotherapy for Prostate Cancer; a Prospective Observational Study 'Gunma0702'. *BMC Cancer* (2020) 20:75. doi: 10.1186/s12885-020-6570-8

29. Kasuya G, Ishikawa H, Tsuji H, Haruyama Y, Kobashi G, Ebner DK, et al. Cancer-Specific Mortality of High-Risk Prostate Cancer After Carbon-Ion Radiotherapy Plus Long-Term Androgen Deprivation Therapy. *Cancer Sci* (2017) 108:2422–9. doi: 10.1111/cas.13402

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Oncology

ORIGINAL RESEARCH
published: 11 January 2022
doi: 10.3389/fonc.2021.781121

Cost and Toxicity Comparisons of Two IMRT Techniques for Prostate Cancer: A Micro-Costing Study and Weighted Propensity Score Analysis Based on a Prospective Study

Ingrid Masson[1†], Martine Bellanger[2,3†], Geneviève Perrocheau[2], Marc-André Mahé[1,4], David Azria[5], Pascal Pommier[6], Nathalie Mesgouez-Nebout[7], Philippe Giraud[8], Didier Peiffert[9], Bruno Chauvet[10], Philippe Dudouet[11], Naji Salem[12], Georges Noël[13], Jonathan Khalifa[14], Igor Latorzeff[15], Catherine Guérin-Charbonnel[16,17] and Stéphane Supiot[1]*

[1] Department of Radiation Oncology, Institut de Cancérologie de l'Ouest René Gauducheau, Saint-Herblain, France, [2] Department of Human and Social Sciences, Institut de Cancérologie de l'Ouest René Gauducheau, Saint-Herblain, France, [3] UMR CNRS6051, EHESP (Ecole des Hautes Etudes en Santé Publique - School of Public Health), University of Rennes, Rennes, France, [4] Department of Radiation Oncology, François Baclesse Cancer Center, Caen, France, [5] Fédération Universitaire d'Oncologie Radiothérapie (FOROM), Institut Régional du Cancer Montpellier (ICM), Université de Montpellier, Institut de Recherche en Cancérologie de Montpellier (IRCM), Montpellier, France, [6] Department of Radiation Oncology, Léon Bérard Center, Lyon, France, [7] Department of Radiation Oncology, Institut de Cancérologie de l'Ouest Paul Papin, Angers, France, [8] Department of Radiation Oncology, Georges Pompidou European Hospital, Paris, France, [9] Department of Radiation Oncology, Lorraine Cancer Institute, Vandœuvre-lès-Nancy, France, [10] Department of Radiation Oncology, Sainte Catherine Institute, Avignon, France, [11] Department of Radiation Oncology, Pont de Chaume Clinic, Montauban, France, [12] Department of Radiation Oncology, Paoli-Calmettes Institute, Marseille, France, [13] Department of Radiation Oncology, Cancerology Institute of Strasbourg-Europe, Strasbourg, France, [14] Department of Radiation Oncology, Institut Universitaire du Cancer de Toulouse-Oncopole (IUCT-Oncopole), Toulouse, France, [15] Department of Radiation Oncology, Pasteur Clinic, Toulouse, France, [16] Clinical Trial Sponsor Unit/Biometry, Institut de Cancérologie de l'Ouest René Gauducheau, Saint-Herblain, France, [17] Centre de Recherche en Cancérologie et Immunologie Nantes Angers - Center for Research in Cancerology and Immunology Nantes-Angers (CRCINA), Institut National de la Santé et de la Recherche Médicale - National Institute for Health and Medical Research (INSERM) UMR1232, Centre National de la Recherche Scientifique - National Center for Scientific Research (CNRS) ERL6001, University of Nantes, Nantes, France*

OPEN ACCESS

Edited by:
Gustavo Cardoso Guimaraes, Beneficência Portuguesa de São Paulo, Brazil

Reviewed by:
Yoichiro Okubo, Kanagawa Cancer Center, Japan
Giuliano Aita, A.C. Camargo Cancer Center, Brazil

***Correspondence:**
Martine Bellanger
martine.bellanger@ico.unicancer.fr

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Genitourinary Oncology, a section of the journal Frontiers in Oncology

Received: *22 September 2021*
Accepted: *10 December 2021*
Published: *11 January 2022*

Citation:
Masson I, Bellanger M, Perrocheau G, Mahé M-A, Azria D, Pommier P, Mesgouez-Nebout N, Giraud P, Peiffert D, Chauvet B, Dudouet P, Salem N, Noël G, Khalifa J, Latorzeff I, Guérin-Charbonnel C and Supiot S (2022) Cost and Toxicity Comparisons of Two IMRT Techniques for Prostate Cancer: A Micro-Costing Study and Weighted Propensity Score Analysis Based on a Prospective Study. Front. Oncol. 11:781121. doi: 10.3389/fonc.2021.781121

Background: Intensity modulated radiation therapy (IMRT) combined with androgen deprivation therapy (ADT) has become the standard treatment for patients with high-risk prostate cancer. Two techniques of rotational IMRT are commonly used in this indication: Volumetric Modulated Arc Therapy (VMAT) and helical tomotherapy (HT). To the best of our knowledge, no study has compared their related costs and clinical effectiveness and/or toxicity in prostate cancer. We aimed to assess differences in costs and toxicity between VMAT and HT in patients with high-risk prostate cancer with pelvic irradiation.

Material and Methods: We used data from the "RCMI pelvis" prospective multicenter study (NCT01325961) including 155 patients. We used a micro-costing methodology to identify cost differences between VMAT and HT. To assess the effects of the two techniques on total actual costs per patient and on toxicity we used stabilized inverse probability of treatment weighting.

Results: The mean total cost for HT, €2019 3,069 (95% CI, 2,885–3,285) was significantly higher than the mean cost for VMAT €2019 2,544 (95% CI, 2,443–2,651)

(p <.0001). The mean ± SD labor and accelerator cost for HT was €2880 (± 583) and €1978 (± 475) for VMAT, with 81 and 76% for accelerator, respectively. Acute GI and GU toxicity were more frequent in VMAT than in HT (p = .021 and p = .042, respectively). Late toxicity no longer differed between the two groups up to 24 months after completion of treatment.

Conclusion: Use of VMAT was associated with lower costs for IMRT planning and treatment than HT. Similar stabilized long-term toxicity was reported in both groups after higher acute GI and GU toxicity in VMAT. The estimates provided can benefit future modeling work like cost-effectiveness analysis.

Keywords: Volumetric Arc Therapy (VMAT), helical tomotherapy (HT), high risk prostate cancers, micro-costing, inverse probability of treatment weighting (IPTW), toxicity, France

INTRODUCTION

Intensity modulated radiation therapy (IMRT) combined with androgen deprivation therapy (ADT) for 2–3 years has become the standard treatment for patients with high-risk prostate cancer (1–6). The further technological improvement with rotational modulated radiotherapy makes it possible to achieve dose escalation in the primary tumor while sparing normal tissues or organs. Two techniques of rotational IMRT are commonly used: Volumetric Modulated Arc Therapy (VMAT RapidArc™ Varian Medical Systems and VMAT® Elekta) and helical tomotherapy (HT: Tomotherapy® Accuray).

Several studies have suggested more favorable dosimetric parameters with VMAT when compared with step-and-shoot or conventional IMRT in patients with prostate cancers, including high-risk prostate cancers (7–12). VMAT results in improved sparing of organs at risk (OARs) and delivery efficiency, combined with significantly lower treatment time and reduced monitor units (MUs) (7–12). There was no worsening clinical effectiveness or toxicity reported (7).

When comparing VMAT techniques and helical tomotherapy (HT) only small significant dosimetric differences in prostate cancer were observed (13) including high-risk prostate cancer with pelvic nodal irradiation (14). Both accelerators exhibited the same plan quality (13), but with more homogeneous dose distribution for HT (14) and in some studies, better OAR sparing for HT (15, 16), whereas VMAT was associated with shorter treatment time and lower MUs (13, 14, 16). There was no comparative analysis of clinical effectiveness or toxicity of VMAT versus HT in high-risk prostate cancer, whether the radiotherapy was to the prostate alone or to the prostate and pelvic lymph nodes.

Among attempts to estimate costs and effectiveness associated with these advanced technologies, we retrieved only one in prostate cancer that compared VMAT to IMRT and found it "cost-effective" (7). In a recent systematic review on cost-effectiveness of prostate cancer radiotherapy, stereotactic body radiotherapy (SBRT) appeared the least expensive from a societal perspective followed by IMRT. Proton beam therapy (PBT) was both the most expensive and the least effective (17). Nevertheless, lack of evidence in cost estimates and "uncertainty surrounding improvements in outcomes" of new technologies (e.g., disease progression, adverse events) make cost-effectiveness analysis of "new versus traditional technologies of radiotherapy" in prostate cancer challenging (18).

To the best of our knowledge, no study has compared costs and clinical effectiveness and/or toxicity of VMAT versus HT in prostate cancer. Only two studies (19, 20) from a French prospective multicenter study evaluated the clinical outcomes and costs associated with these techniques in patients with head and neck cancer.

The present study aimed to assess differences in costs and toxicity between VMAT and helical tomotherapy in patients with high-risk prostate cancer. For this study, we considered no differences between VMAT RapidArc™ and VMAT® Elekta, as done in the literature, since this is the same VMAT technology developed by two manufacturers. We referred to them both as VMAT, when appropriate.

MATERIALS AND METHODS

Study Design

We used data from the "Intensity modulated Radiation Therapy in pelvic lymph node irradiation" prospective multicenter study (*so called in French "RCMI pelvis")*, which aimed to compare costs and clinical outcomes of IMRT with VMAT (Rapid'Arc™ and VMAT® Elekta) versus helical tomotherapy (HT) (Tomotherapy®) in prostate, cervical, and anal canal cancers, with pelvic lymph node irradiation (https://www.clinicaltrials.gov/, NCT01325961). The "RCMI pelvis" study had the same protocol as the 'ART-ORL' study on head and neck cancer, and both studies were carried out jointly by fourteen French academic cancer centers (**Supplementary Table A1**) (19, 20). Patients were assigned to one of the accelerators based on their availability in each center or at the discretion of the investigators. No randomization between treatments was thus possible. The expected number of patients to be included was 20 anal canal cancers, 50 cervical cancers, and 150 prostate cancers. However,

to obtain a homogenous population, for the current study we only selected patients with prostate cancer. The inclusion criteria were patients aged ≥18 years with histologically proven high-risk prostate cancer according to d'Amico's classification (21) and the Eastern Cooperative Oncology Group (ECOG) Performance Status ≤2, and who received pelvic lymph node irradiation and androgen deprivation therapy (ADT) for 3 years. Patients included in the GETUG 18 trial were also eligible for the RCMI pelvis study (22). The exclusion criteria were lombo-aortic irradiation, salvage radiotherapy after radical prostatectomy, and re-irradiation. Enrolled patients had an abdominal, pelvic, and thoracic CT Scan and/or Positron Emission Tomography–Computed Tomography (PET-CT) and Prostate specific antigen (PSA) test. Delineation followed guidelines for high-risk prostate cancer (22). Clinical Target Volume (CTV) 1 included pelvic lymph node areas, prostate, and seminal vesicles. CTV 2 was limited to the prostate. Planning Target Volume (PTV) 1 and 2 were respectively defined by: CTV1 + 1 cm, CTV2 + 1 cm (+0.5 cm posterior). A moderate hypofractionated Simultaneous Integrated Boost (SIB) plan was delivered to 34 fractions: 54.4 Gy to PTV1 and 74.8 Gy to PTV2. For GETUG 18 trial patients only, a normo-fractionated sequential plan was delivered in 40 fractions: 46 Gy to PTV 1 then 34 Gy to PTV2. Dose prescription followed the International Commission on Radiation Units 83 (23). All patients provided signed informed consent to participate in the study. The study was approved by the National Ethics Committee (Ref: 11/03, 4 January 2011) and the National Committee for protection of personal data (DR-2011-277 N °911317).

Micro-Costing Study

The micro-costing methodology is considered the most accurate approach for costing hospital services (24). It makes it possible to identify all the resources used within hospital production processes and per patient (24). In our micro-costing study, we defined the production process as being from treatment planning to the last radiation therapy (RT) session. Of note, ADT was not included in the costing analysis, since this is a standardized procedure. To measure and value the resources used, we followed, and adapted when necessary, the previously described method of Perrier et al. (19). For this reason, we only identified use of resources that was likely to vary between VMAT and HT to estimate actual cost differences between the two strategies. Our study assumed differences in the resources used in all planning stages (i.e., image registration and contouring, inverse planning, patient-specific quality control (QC), and pre-treatment patient setup verification), along with treatment delivery, accelerator QC for IMRT, and preventive internal and external maintenance. CT scan planning was supposed to be similar in both techniques. We did not include Record and Verify Systems (RVSs) as Tomotherapy treatments were already integrated into the RVSs from the other manufacturers. Patient-specific Quality Assurance (QA) software, independent of the machine, was excluded accordingly. Last, we considered standard radiation bunker that may accommodate any accelerator currently used (19) and also any change in radiotherapy advancements over its life span. We therefore assumed similar construction costs for the two techniques studied.

Detailed data on resource utilization of labor and equipment were collected using chronometers. They included all personnel time spent and equipment mobilization time for each patient over their course of treatment. For example, for a treatment session, this ranged from a patient's admission to discharge from the irradiation room. We thus included not only the time of treatment delivery, but also image control time and any potential interruption time. Labor included radiation therapist, dosimetrist, medical physicist, radiation oncologist, resident and biomedical technician. We assigned values of time resources used to estimate costs, as follows. Unit costs of labor were based on the average annual full gross wages (i.e., annual payroll taxes included) divided by the number of workable yearly hours. We estimated the full gross wage on a 15-year experience basis and on a collective labor agreement of UNICANCER, a consortium of the 18 French cancer centers. For all cost estimates, we used the same method as described in Perrier et al. (19), except for the costs of QC and internal maintenance (IM), given in Equation (1) below and for which resources were collected from questionnaires.

$$\text{QC and IM cost} = \Sigma_{\text{labor}=1}^{2} \text{hourly full wage} * \text{QC and IM Nhs} \quad (1)$$

Where Nhs = annual number of dedicated hours, and labor = medical physicist or biomedical technician.

We obtained data on capital resources, such as equipment (i.e., accelerator and Treatment Planning Systems—TPS—for VMAT only, as for HT accelerator cost includes TPS) from standardized questionnaires, as previously described (19). Equipment was valued using replacement and maintenance costs with a life expectancy of 12 years for the accelerators and 5 years for the TPS, based on data available from the literature (25, 26). We estimated the costs of accelerators, annual external maintenance contracts and TPS directly from the catalog prices of the manufacturers.

We estimated total actual costs per patient that vary between VMAT and HT from the hospital perspective. All estimates were in Euro 2019, all taxes included, but without discounting as the analytic time horizon for costs was less than 3 months. It is worth noting that we could not estimate the resources used for treatments associated with acute toxicity, since all treatment types, but hospitalization, were not collected in the frame of the study.

Toxicity

We analyzed acute toxicity (≤3 months) and late toxicity [up to 24 months, based on previously published studies (27, 28)], that were scored using the National Cancer Institute Common

Terminology Criteria for Adverse Events (CTCAE) version 3.0. Toxicity was reported as follows: no toxicity (grade 0), grade 1, grade 2, and grades 3–4. Acute adverse events (serious adverse events with hospitalizations included) that were clinical outcomes within the same time horizon as the estimated costs were reported on electronic Case report forms (CRF). Besides, all late adverse events were collected as well. For the analysis, we reported Gastrointestinal (GI) toxicity, Genito-urinary (GU) toxicity, and sexual toxicity. Lastly, patient reported outcomes such as Quality of Life (QoL) were not investigated in this study.

Statistical Analysis

We described patients' characteristics using mean and standard deviation (SD) for continuous variables, or count and percentage for categorical variables. In addition, we assessed differences between treatment groups, from the original datasets, using logistic regression, and multinomial logistic regression for binary variables and tumor stage (cT) respectively, and linear regression for continuous variables. We compared differences in hours spent for treatment, QC, and internal maintenance between the two techniques using a non-parametric Wilcoxon test and we used a paired Wilcoxon test for comparing differences in duration of treatment session. We tested uncertainty on the total cost of each technique by running a one-way sensitivity analysis over the range of plausible parameters and illustrating with a Tornado plot showing the impact of increasing and decreasing each of the parameters by 20%, a range suggested in the methods of sensitivity analysis in economic evaluation (29). In addition, we used probabilistic sensitivity analyses with a non-parametric bootstrap method, with 1,000 iterations, to obtain the 95% confidence intervals (CI) of total actual costs (30).

Similarly to Bibault et al. (20), we used propensity scores to obtain balancing covariates at enrollment in the VMAT and HT groups to address potential selection bias due to non-randomization (31). We used the stabilized inverse probability of treatment weighting (IPTW) (32). We estimated standardized differences to compare the balance in measured baseline covariates between the two treatment groups. Due to the study design, we could not include cancer center as a covariate, though it was likely to be a confounding factor. As mentioned above, investigators assigned patients to one of the accelerators based on their availability in each cancer center or at their discretion. We estimated the propensity score (PS) using a logistic regression in which treatment assignment was regressed (32). With ITPW, each patient was weighted by the inverse probability of receiving the treatment they actually received (i.e., 1/PS in the HT group and 1/(1 − PS) in the VMAT group). Finally, we applied the stabilized IPTW, to preserve the size of the pseudo-population as well as to reduce the variance of the treatment effect estimates (32).

To assess the impact of the two techniques on total costs and toxicity we ran linear regressions (costs) and ordered logistic regressions (toxicity) adjusting for sequential versus SIB plans. All regressions were performed before and after having introduced IPTW.

We performed all analyses using R software version 3.6.1. We used the stdidff package (https://CRAN.R-project.org/package=stddiff) to compute the standardized differences for all categorical variables with more than two levels.

RESULTS

Patient Characteristics

Two hundred and fifteen patients were recruited between April 2011 and January 2015: 155 with prostate cancer, 30 with cervix cancer and 30 with anal cancer (**Supplementary Figure A1**). Of the 155 patients with prostate cancer, 106 patients were treated with VMAT and 49 with HT. We had information on resource use for all patients (155); and for toxicity, information was available for 147 patients (i.e., 98/106 in the VMAT group; 49/49 in the HT group).

At baseline, age, performance status, cT and cN stages, and PSA varied between the two techniques, (with significant differences for N stage). After IPTW, we observed almost no differences between the VMAT and HT groups (**Table 1**). For the Gleason scores, when checking the balance between groups we found no significant difference before or after weighting ($p > 0.5$).

Resource Use

We observed similar median annual treatment times (Interquartile range—IQR) spent with VMAT and helical tomotherapy (HT), with 2,513 h (IQR, 92.5) and 2,520 h (IQR, 216), respectively ($P = .7$). In addition, we did not find any statistical differences in median annual time spent for quality control and internal preventive maintenance, 194.5 h (IQR, 81.75) were spent with VMAT and 144 h (IQR, 56) with HT ($p = .28$). We found that the median time for the first session was 18 min per patient for HT and 20 min for VMAT ($p = .31$) (**Supplementary Figure A2**). Treatment times then decreased across sessions for VMAT (15 min) while keeping the same for HT (18 min) ($p = .002$ and $p < .001$), up to the fourth and more sessions, for which 13 and 17 min ($p < .001$) were reported for VMAT and HT, respectively. This demonstrates a learning process effect on time spent per patient.

Total Actual Costs per Patient

Table 2 presents the total actual costs per patient as well as major cost components. After inverse probability of treatment weighting and control for covariates, the mean cost for helical tomotherapy (HT), estimated at € 3,069 (95% CI, 2,885–3,285) was significantly higher than the mean cost for VMAT €2,544 (95% CI, 2,443–2,651) ($p < .0001$). We found a substantial variation in session costs (i.e., accelerator + labor costs), accounting for 94% of HT costs and only 78% of VMAT costs ($p < .001$) (**Table 2**). As shown in **Table 2**, only the planning phase was more costly for VMAT than for HT, with €566 (292), and €189 (117) mean costs (SD), respectively ($p < .0001$)

TABLE 1 | Patient characteristics.

	Unweighted				Weighted		
	VMAT (n = 106)	**HT (n = 49)**	**P-value**	**d**	**VMAT (Pseudo-data)**	**HT (Pseudo-data)**	**d**
Age (years)			.3084	0.171			–0.002
mean (SD)	68 (7)	70 (9)			69 (7)	69 (9)	
Performance status			.1078	0.271			–0.010
0	93 (87.7%)	38 (77.6%)			84.7%	85.1%	
1 or 2	13 (12.3%)	11 (22.4%)			15.3%	14.9%	
cT stage				0.356			0.048
cT1	14 (13.2%)	9 (18.4%)			15.0%	15.9%	
cT2	35 (33.0%)	10 (20.4%)	.1460		28.2%	26.2%	
cT3	55 (51.9%)	27 (55.1%)	.5802		53.7%	54.6%	
cT4	2 (1.9%)	3 (6.1%)	.4005		3.1%	3.3%	
N stage			.0322	0.361			0.002
cN0	96 (90.6%)	38 (77.6%)			85.5%	85.5%	
cN1	10 (9.4%)	11 (22.4%)			14.5%	14.5%	
PSA (ng/ml); capped values			.2652	0.194			0.024
mean (SD)	15 (13)	18 (13)			17 (14)	17 (12)	

d, Standardized difference; IPTW, Inverse probability of treatment weighting; HT, helical tomotherapy.

(**Table 2**). This yielded a mean cost difference of €525 in the HT group compared with the VMAT group.

The Tornado diagram (**Figure 1**) shows the most influential parameters (i.e., prices, cost, time with ±20% variation) on estimated costs. The latter were highly sensitive to the annual operating time as well as to the accelerator immobilization time (**Figure 1**). The costs decreased by 12.1 and 15.5% following a 20% increase in the annual operating time and a 20% decrease in the accelerator immobilization time, for VMAT and for HT, respectively. To a lesser extent, costs were sensitive to time spent by the radiation therapist (**Figure 1**).

For each technique, the sequential plan exhibited significantly higher costs than the SIB plan (p <.001), with €2,659 (95% CI, 2,504–2,834) versus €2,498 (95% CI, 2376–2657) for VMAT, and €3,902 (95% CI, 3473–4357) versus €2,895 (95% CI, 2754–3076) for helical tomotherapy. The variation was mainly due to the irradiation phase, where accelerator immobilization time and labor time were more expensive in the sequential process than in the SIB process (**Supplementary Figure A3**).

Acute and Late Toxicity

As shown in **Table 3A**, the proportion of patients with acute grade 3–4 toxicities was very low in both groups. This went from 0 to 1% of patients having Gastrointestinal (GI) toxicity (p = .033) to 4% with genitourinary (GU) toxicity in both groups (p = .145). After IPTW and control for covariates, we observed significantly higher frequency in acute GI and GU toxicities for VMAT than for HT (p = .021 and p = .042, respectively) but similar sexual toxicities in the two groups (p = .236).

TABLE 2 | Total actual cost per patient (€2019) after Inverse probability of treatment weighting (IPTW).

Cost	**VMAT mean (SD)**	**HT mean (SD)**	**P-value**
Image registration, contouring—Labor	109.10 (76.47)	38.75 (56.59)	–
Image registration, contouring—TPS	95.98 (65.79)	0.00 (0.00)	–
Image registration, contouring—Total	205.07 (139.93)	38.75 (56.59)	<.0001
Inverse planning and validation—Labor	141.47 (104.02)	67.49 (66.39)	–
Inverse planning and validation—TPS	133.48 (90.17)	0.00 (0.00)	–
Inverse planning and validation—Total	274.96 (191.66)	67.49 (66.39)	<.0001
Patient quality control—Labor	19.96 (12.12)	24.00 (10.43)	.0574
Position verification D0*—Labor	22.94 (30.40)	12.21 (7.66)	.0272
Setup verification D0* Accelerator	43.27 (56.24)	46.19 (25.85)	.6200
Setup verification D0*—Total	66.21 (84.83)	58.40 (32.78)	.6529
Planning Cost	**566.19 (292.71)**	**188.64 (116.58)**	**<.0001**
Session—Labor	484.52 (135.28)	546.84 (120.39)	.0050
Session—Accelerator	1,493.66 (362.39)	2,333.30 (466.52)	<.0001
Session Cost	**1,978.18 (475.14)**	**2,880.13 (582.64)**	**<.0001**
Total actual cost	**2,544.37**	**3,068.77**	**<.0001**
CI 95%**	**[2,442.88; 2,651.11].**	**[2,885.34; 3,285.04]**	

**D0 pre-treatment patient setup verification.*

***95% confidence intervals (CI) of total actual costs computed based on probabilistic sensitivy analyses with a non-parametric bootstrap method, with 1,000 iterations excluding (25/1,000) 2.5% values at either end of the estimated distribution (30).*

HT, helical tomotherapy.

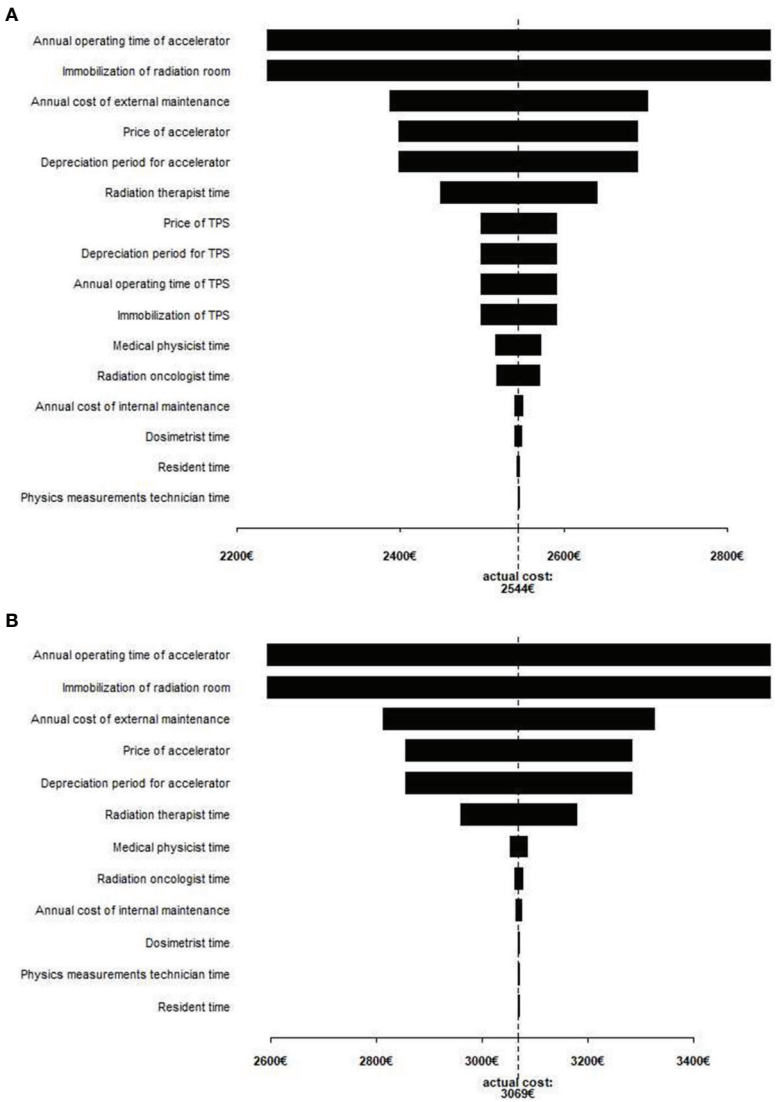


FIGURE 1 | Tornado diagram for VMAT **(A)** and helical tomotherapy **(B)** total actual costs. Tornado diagram shows the graphical output of the one- way sensitivity analysis. On the x-axis, the value of total cost, and the vertical line represents the total cost with all parameter baseline values. Each parameter has its own bar and the length of each bar shows how much impact that parameter can have total cost when varied 20% more of less than its baseline value is. The bars are arranged in descending order of length, so that the diagram exhibits from the most to the least sensitive factors.

In the long-term period, in both unweighted and weighted populations, the proportions of patients for whom physicians reported late grade 3–4 toxicities remained dramatically low (with no more than 4% of patients experiencing grade 3–4 GI, GU and sexual toxicity), whatever the machine (**Table 3B**). Overall, VMAT and HT did not differ significantly in late GI, GU and sexual toxicity either ($p = .162$; $p = .669$; and $p = .062$ respectively after IPTW) (**Table 3B**).

There was no difference in serious adverse event related hospitalization, nor any permanent discontinuation of treatment reported in the study. In acute toxicity, we observed only one hospitalization due to dysuria in the HT group. In late toxicity, three hospitalizations were noted for dysuria on stenosis, hematuria, and rectal bleeding on radiation proctitis in the VMAT group and one for rectal bleeding on radiation proctitis in the HT group.

DISCUSSION

The analysis based on the "RCMI pelvis" prospective multicenter study found that each hospital spent on average an additional €525 per patient when they used helical tomotherapy (HT) rather than VMAT for IMRT preparation and delivery in high-risk prostate cancer patients with pelvic lymph node irradiation. Considering health outcomes, although acute GI

TABLE 3 | Acute and late toxicity.

TABLE 3A. Acute Toxicity*

Toxicity	Grade*	Unweighted			Weighted		
		VMAT (n = 98)	HT (n = 49)	P-value	VMAT	HT	P-value
GI toxicity	0	17 (17.3%)	14 (28.6%)	**.0339**	16.6%	29.2%	**.0215**
	1	48 (49.0%)	26 (53.1%)		47.3%	51.6%	
	2	32 (32.7%)	9 (18.4%)		34.9%	19.3%	
	3–4	1 (1.0%)	0 (0.0%)		1.2%	0.0%	
GU toxicity	0	10 (10.2%)	7 (14.3%)	.1446	9.5%	16.1%	**.0420**
	1	34 (34.7%)	24 (49.0%)		34.5%	50.9%	
	2	50 (51.0%)	16 (32.7%)		52.2%	29.7%	
	3–4	4 (4.1%)	2 (4.1%)		3.9%	3.3%	
Sexual toxicity	0	49 (50.0%)	33 (67.3%)	.2628	48.6%	66.2%	.2369
	1	21 (21.4%)	1 (2.0%)		21.5%	3.4%	
	2	27 (27.6%)	15 (30.6%)		28.7%	30.3%	
	3–4	1 (1.0%)	0 (0.0%)		1.2%	0.0%	

GI, gastro-intestinal; GU, genito-urinary; HT, helical tomotherapy.
**Acute toxicity: worse grade during three months of follow up.*

TABLE 3B | Late toxicity*.

Toxicity	Grade*	Unweighted			Weighted		
		VMAT (n = 98)	HT (n = 49)	P-value	VMAT	HT	P-value
GI toxicity	0	41 (41.8%)	27 (55.1%)	.2964	41.4%	59.2%	.1627
	1	38 (38.8%)	13 (26.5%)		39.3%	21.4%	
	2	18 (18.4%)	7 (14.3%)		18.4%	16.2%	
	3-4	1 (1.0%)	2 (4.1%)		0.9%	3.2%	
GU toxicity	0	26 (26.5%)	14 (28.6%)	.4797	25.3%	35.3%	.6696
	1	50 (51.0%)	20 (40.8%)		49.7%	38.4%	
	2	20 (20.4%)	14 (28.6%)		23.2%	23.3%	
	3-4	2 (2.0%)	1 (2.0%)		1.8%	3.0%	
Sexual toxicity	0	38 (38.8%)	15 (30.6%)	**.0214**	37.7%	32.0%	.0628
	1	19 (19.4%)	1 (2.0%)		19.1%	3.7%	
	2	37 (37.8%)	33 (67.3%)		39.2%	64.3%	
	3-4	4 (4.1%)	0 (0.0%)		4.0%	0.0%	

GI, gastro-intestinal; GU, genito-urinary; HT, helical tomotherapy.
**Late toxicity: worse grade from 6 months onwards.*

and GU toxicity were significantly higher for VMAT than for HT, the late toxicity outcomes (up to 24 months after completion of treatment) no longer differed between the two groups.

The economic results are consistent with the data on head and neck cancers published by Perrier et al., which show an excess in mean costs for HT over VMAT (19). In the two studies, the findings were explained by differences in the price of accelerators and in costs of external maintenance, along with differences in treatment times. Helical tomotherapy has remained more expensive than VMAT accelerators, despite a reduction in price variation between 2013 and 2019, the two years of estimation. In addition, longer RT sessions result in increased times spent by personnel and accelerators. The results from our study are also consistent with existing evidence on treatment delivery as a major cost component of IMRT (33–35). In line with our estimates, and for prostate cancer, HT was associated with a longer average treatment delivery time than VMAT (15, 36). Of note, this finding holds true, whether time was measured only for irradiation (15, 36) or for the whole treatment delivery as we and Perrier et al. (19) did. For VMAT mostly, we observed a learning effect, which was previously demonstrated during the implementation process of new techniques (37). The micro-costing methodology makes possible time assessment that is closest to clinical practice. However, our evaluation differs from Perrier et al., as we estimated all times spent (such as operating time, QC time of treatment machines and internal maintenance) from standardized questionnaires completed by the cancer centers included in the study. This resulted in findings in line with the Health Economics in Radiation (ESTRO/HERO) project for which annual QC time estimated was 150 to 200 h, based on a time-driven, activity-based, costing methodology (TD-ABC) (25). Finally, the study observed lower costs for SIB hypofractionated IMRT compared to sequential IMRT in prostate cancers, and this, regardless of the accelerators, as in Hulstaert et al. who found lower average costs in hypofractionated schemes in lung and breast cancers compared to standard fractionation schemes (35). In the latter, treatment

delivery was the major cost driver, a factor of cost variation that we also observed in the sensitivity analysis we carried out.

In our micro-costing study, we were able to refer to the Diagnosis-Related Group (DRG) related tariff for a given procedure, and that the national health insurance system (NHIS) reimburses hospitals. As reported in a recent European study, a higher reimbursement is made in France for treatments with helical tomotherapy when comparing to other VMAT machines (38). The current tariff for a HT session is twice that of a VMAT session when our actual estimations showed a 20% incremental cost (i.e., a € 525 (95% CI, 442–634) difference between the two techniques).

In terms of toxicity, comparative evidence between VMAT and helical tomotherapy is limited. Our study found significant higher frequency in acute GI and GU in VMAT than in HT, but no worse significant outcomes in late toxicity. Our results are similar to those of Bibault et al. in head and neck cancers (20), who after IPTW adjustment found significantly more acute salivary disorders in the VMAT group, but no difference between HT and VMAT in all toxicities evaluated in the long term (more than 15), except for salivary function (20). Stabilized long term symptoms after significant differences in acute symptoms associated with IMRT have been reported elsewhere (39).

Strengths and Limitations

This study used rigorous methods to assess the costs and toxicity associated with helical tomotherapy (HT) and VMAT IMRT in prostate cancers. The large variation in baseline characteristics between the two techniques demonstrated the importance of using the propensity score as it makes appropriate adjustment possible for reducing confounding bias when estimating treatment effects. In the economic analysis of radiotherapy, micro-costing represents an accurate method for collecting details of the resources used for costing procedures. To our knowledge, this study is the first of its kind to estimate the costs and toxicity of VMAT and HT in high-risk prostate cancers with pelvic lymph node irradiation with long follow-up time. The identification of treatment delivery as a major cost driver and source of difference between the techniques used is a result of particular interest, given cancer centers' efforts to introduce new radiotherapy techniques. Applying micro-costing methodology contributes to a broader European (EU) effort to improve knowledge of the resources allocated to radiotherapy within the overall resources for treating cancer patients (26, 38, 40–43), and ultimately to improve the quality of care. However, one should keep in mind that our evaluation was based on French cancer center labor costs and payment schemes, which differ from other EU and international oncology centers (38). This should be considered carefully before generalizing our findings.

This study has certain limitations. The first relate to the data collection. Collecting data in real-world clinical practice has some advantages, but it can also be challenging on both the clinical and the funding sides. This has resulted in variability in patients recruited across cancer centers and in a small population sample. In addition, randomization between treatment groups was not possible. We note that the propensity scores we used do not account for the effect of unmeasured covariates (44, 45). Because patients were assigned to only one technique in each cancer center, based on their availability (only 4 centers out of 14 had both VMAT and HT accelerators), or on investigators' discretion, as earlier mentioned, we could not take the cancer center effect into account, although we expect that it is a confounding factor. Another limitation of the study is the difference in time horizon between the cost analysis and the toxicity assessment, due to data collected. We were not able to estimate the costs of treatments used for acute adverse events. This has resulted in underestimating short term costs, and has made impossible to relate the differences in cost to the differences in outcomes, a requirement in cost-effectiveness analysis. Moreover, as in any economic evaluation, and especially when conducting a micro-costing study, we made assumptions and simplifications that Perrier et al. have already reported (19) and that may have an impact on our final estimates. We thus advise caution before generalizing our results. Finally, our model does not apply to low- or intermediate risk prostate cancer patients where pelvic irradiation is not routinely recommended (46), where ADT is usually limited to unfavorable intermediate-risk tumors (5), and SBRT or proton therapy to the prostate gland only is a therapeutic option (46).

CONCLUSION

Assessing the effectiveness and value associated with the IMRT treatment of prostate cancer with pelvic lymph node irradiation is of particular importance. The study found evidence to support the hypothesis of cost differences between VMAT and HT for IMRT preparation and delivery in favor of VMAT, and no toxicity differences in the long term after more acute GI and GU toxicity for VMAT than for HT. Our findings have already the potential to help clinicians' decision-making. For the payer, the French NIHS, these findings support an increase in the DRG reimbursement of a VMAT session. Our approach paved the way to a cost-effectiveness model that will combine the long-term health impacts of the two techniques, already identified, to the quality of life reported by patients to be assessed along with long-term costs of VMAT and HT.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the last author (SS).

ETHICS STATEMENT

The study was approved by the National Ethics Committee (Ref: 11/03, 4 January 2011) and the National Committee for protection of personal data (DR-2011-277 N°911317). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, M-AM and GP. Methodology, IM, MB, GP, CG-C, and SS. Software, MB and CG-C. Validation, IM, MB, GP, CG-C, and SS. Formal analysis, IM, MB, GP, and SS. Investigation, DA, PP, NM-N, PG, DP, BC, PD, NS, GN, JK, IL, and SS. Resources, IM, MB, and GP. Data curation, IM, GP, and CG-C. Writing—Original draft preparation, IM, MB, and SS. Writing—Review and editing, IM, MB, and SS. Visualization, IM, MB, and SS. Supervision, MB and SS. Project administration, GP. Funding acquisition, M-AM. All authors contributed to the article and approved the submitted version.

FUNDING

The three manufacturers (Accuray, Inc., Varian Medical Systems, and Elekta AB) financially supported the RCMI pelvis study. However, the RCMI pelvis is an independent study and the ideas and opinions expressed in this work are those of the authors and do not necessarily represent those of the manufacturers. The funders (Accuray, Inc.; Varian Medical Systems; Elekta AB) had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

ACKNOWLEDGMENTS

The authors are grateful to Lionel Perrier and Jean-Emmanuel Bibault for their inputs in previous studies on IMRT. We thank Philippe Tessier and Yohann Foucher for their help in improving the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.781121/full#supplementary-material

REFERENCES

1. Bolla M, Van Tienhoven G, Warde P, Dubois JB, Mirimanoff R-O, Storme G, et al. External Irradiation With or Without Long-Term Androgen Suppression for Prostate Cancer With High Metastatic Risk: 10-Year Results of an EORTC Randomised Study. *Lancet Oncol* (2010) 11 (11):1066–73. doi: 10.1016/S1470-2045(10)70223-0
2. Bolla M, de Reijke TM, Van Tienhoven G, Van den Bergh ACM, Oddens J, Poortmans PMP, et al. Duration of Androgen Suppression in the Treatment of Prostate Cancer. *N Engl J Med* (2009) 360(24):2516–27. doi: 10.1056/NEJMoa0810095
3. Pilepich MV, Winter K, Lawton CA, Krisch RE, Wolkov HB, Movsas B, et al. Androgen Suppression Adjuvant to Definitive Radiotherapy in Prostate Carcinoma–Long-Term Results of Phase III RTOG 85-31. *Int J Radiat Oncol Biol Phys* (2005) 61(5):1285–90. doi: 10.1016/j.ijrobp.2004.08.047
4. Mottet N, van den Bergh RCN, Briers E, Van den Broeck T, Cumberbatch MG, De Santis M, et al. EAU-EANM-ESTRO-ESUR-SIOG Guidelines on Prostate Cancer-2020 Update. Part 1: Screening, Diagnosis, and Local Treatment With Curative Intent. *Eur Urol* (2021) 79(2):243–62. doi: 10.1016/j.eururo.2020.09.042
5. Martin JM, Supiot S, Berthold DR. Pharmacotherapeutic Management of Locally Advanced Prostate Cancer: Current Status. *Drugs* (2011) 71(8):1019–41. doi: 10.2165/11591500-000000000-00000
6. Parker C, Castro E, Fizazi K, Heidenreich A, Ost P, Procopio G, et al. Prostate Cancer: ESMO Clinical Practice Guidelines for Diagnosis, Treatment and Follow-Up†. *Ann Oncol* (2020) 31(9):1119–34. doi: 10.1016/j.annonc.2020.06.011
7. Fogarty GB, Ng D, Liu G, Haydu LE, Bhandari N. Volumetric Modulated Arc Therapy is Superior to Conventional Intensity Modulated Radiotherapy–a Comparison Among Prostate Cancer Patients Treated in an Australian Centre. *Radiat Oncol* (2011) 6:108. doi: 10.1186/1748-717X-6-108
8. Ren W, Sun C, Lu N, Xu Y, Han F, Liu YP, et al. Dosimetric Comparison of Intensity-Modulated Radiotherapy and Volumetric-Modulated Arc Radiotherapy in Patients With Prostate Cancer: A Meta-Analysis. *J Appl Clin Med Phys* (2016) 17(6):254–62. doi: 10.1120/jacmp.v17i6.6464
9. Aznar MC, Petersen PM, Logadottir A, Lindberg H, Korreman SS, Kjær-Kristoffersen F, et al. Rotational Radiotherapy for Prostate Cancer in Clinical Practice. *Radiother Oncol* (2010) 97(3):480–4. doi: 10.1016/j.radonc.2010.09.014
10. Hardcastle N, Tomé WA, Foo K, Miller A, Carolan M, Metcalfe P. Comparison of Prostate IMRT and VMAT Biologically Optimised Treatment Plans. *Med Dosim* (2011) 36(3):292–8. doi: 10.1016/j.meddos.2010.06.001
11. Palma D, Vollans E, James K, Nakano S, Moiseenko V, Shaffer R, et al. Volumetric Modulated Arc Therapy for Delivery of Prostate Radiotherapy: Comparison With Intensity-Modulated Radiotherapy and Three-Dimensional Conformal Radiotherapy. *Int J Radiat OncologyBiologyPhysics* (2008) 72(4):996–1001. doi: 10.1016/j.ijrobp.2008.02.047
12. Palma D, Vollans E, James K, Nakano S, Moiseenko V, Shaffer R, et al. Volumetric Modulated Arc Therapy (VMAT) for Delivery of Prostate Radiotherapy: Reduction in Treatment Time and Monitor Unit Requirements Compared to Intensity Modulated Radiotherapy. *Int J Radiat Oncol Biol Phys* (2008) 72(1):S312. doi: 10.1016/j.ijrobp.2008.06.1083
13. Rao M, Yang W, Chen F, Sheng K, Ye J, Mehta V, et al. Comparison of Elekta VMAT With Helical Tomotherapy and Fixed Field IMRT: Plan Quality, Delivery Efficiency and Accuracy. *Med Phys* (2010) 37(3):1350–9. doi: 10.1118/1.3326965
14. Pasquier D, Cavillon F, Lacornerie T, Touzeau C, Tresch E, Lartigau E. A Dosimetric Comparison of Tomotherapy and Volumetric Modulated Arc Therapy in the Treatment of High-Risk Prostate Cancer With Pelvic Nodal Radiation Therapy. *Int J Radiat Oncol Biol Phys* (2013) 85(2):549–54. doi: 10.1016/j.ijrobp.2012.03.046
15. Wolff D, Stieler F, Welzel G, Lorenz F, Abo-Madyan Y, Mai S, et al. Volumetric Modulated Arc Therapy (VMAT) vs. Serial Tomotherapy, Step-and-Shoot IMRT and 3D-Conformal RT for Treatment of Prostate Cancer. *Radiother Oncol* (2009) 93(2):226–33. doi: 10.1016/j.radonc.2009.08.011
16. Tsai C-L, Wu J-K, Chao H-L, Tsai Y-C, Cheng JC-H. Treatment and Dosimetric Advantages Between VMAT, IMRT, and Helical Tomotherapy in Prostate Cancer. *Med Dosim* (2011) 36(3):264–71. doi: 10.1016/j.meddos.2010.05.001
17. Konski A. Cost Effectiveness of Prostate Cancer Radiotherapy. *Transl Androl Urol* (2018) 7(3):371–7. doi: 10.21037/tau.2017.12.38
18. Schroeck FR, Jacobs BL, Bhayani SB, Nguyen PL, Penson D, Hu J. Cost of New Technologies in Prostate Cancer Treatment: Systematic Review of Costs and Cost Effectiveness of Robotic-Assisted Laparoscopic Prostatectomy, Intensity-Modulated Radiotherapy, and Proton Beam Therapy. *Eur Urol* (2017) 72 (5):712–35. doi: 10.1016/j.eururo.2017.03.028
19. Perrier L, Morelle M, Pommier P, Boisselier P, Coche-Dequeant B, Gallocher O, et al. Cost Analysis of Complex Radiation Therapy for Patients With Head and Neck Cancer. *Int J Radiat OncologyBiologyPhysics* (2016) 95(2):654–62. doi: 10.1016/j.ijrobp.2016.02.013
20. Bibault J-E, Dussart S, Pommier P, Morelle M, Huguet M, Boisselier P, et al. Clinical Outcomes of Several IMRT Techniques for Patients With Head and Neck Cancer: A Propensity Score-Weighted Analysis. *Int J Radiat Oncol Biol Phys* (2017) 99(4):929–37. doi: 10.1016/j.ijrobp.2017.06.2456

21. D'Amico AV, Whittington R, Malkowicz SB, Schultz D, Blank K, Broderick GA, et al. Biochemical Outcome After Radical Prostatectomy, External Beam Radiation Therapy, or Interstitial Radiation Therapy for Clinically Localized Prostate Cancer. *JAMA* (1998) 280(11):969–74. doi: 10.1001/jama.280.11.969
22. Sargos P, Guerif S, Latorzeff I, Hennequin C, Pommier P, Lagrange J-L, et al. Definition of Lymph Node Areas for Radiotherapy of Prostate Cancer: A Critical Literature Review by the French Genito-Urinary Group and the French Association of Urology (GETUG-AFU). *Cancer Treat Rev* (2015) 41 (10):814–20. doi: 10.1016/j.ctrv.2015.10.005
23. Gregoire V, Mackie TR. Dose Prescription, Reporting and Recording in Intensity-Modulated Radiation Therapy: A Digest of the ICRU Report 83. *Imaging Med* (2011) 3(3):367–73. doi: 10.2217/iim.11.22
24. Tan SS, van Putten E, Nijdam WM, Hanssens P, Beute GN, Nowak PJ, et al. A Microcosting Study of Microsurgery, LINAC Radiosurgery, and Gamma Knife Radiosurgery in Meningioma Patients. *J Neurooncol* (2011) 101 (2):237–45. doi: 10.1007/s11060-010-0243-4
25. Defourny N, Perrier L, Borras J-M, Coffey M, Corral J, Hoozée S, et al. National Costs and Resource Requirements of External Beam Radiotherapy: A Time-Driven Activity-Based Costing Model From the ESTRO-HERO Project. *Radiother Oncol* (2019) 138:187–94. doi: 10.1016/j.radonc.2019.06.015
26. Dunscombe P, Grau C, Defourny N, Malicki J, Borras JM, Coffey M, et al. Guidelines for Equipment and Staffing of Radiotherapy Facilities in the European Countries: Final Results of the ESTRO-HERO Survey. *Radiother Oncol* (2014) 112(2):165–77. doi: 10.1016/j.radonc.2014.08.032
27. Goineau A, Marchand V, Rigaud J, Bourdin S, Rio E, Campion L, et al. Prospective Evaluation of Quality of Life 54 Months After High-Dose Intensity-Modulated Radiotherapy for Localized Prostate Cancer. *Radiat Oncol* (2013) 8(1):53. doi: 10.1186/1748-717X-8-53
28. Pederson AW, Fricano J, Correa D, Pelizzari CA, Liauw SL. Late Toxicity After Intensity-Modulated Radiation Therapy for Localized Prostate Cancer: An Exploration of Dose–Volume Histogram Parameters to Limit Genitourinary and Gastrointestinal Toxicity. *Int J Radiat OncologyBiologyPhysics* (2012) 82(1):235–41. doi: 10.1016/j.ijrobp.2010.09.058
29. Briggs A, Sculpher M, Buxton M. Uncertainty in the Economic Evaluation of Health Care Technologies: The Role of Sensitivity Analysis. *Health Econ* (1994) 3(2):95–104. doi: 10.1002/hec.4730030206
30. Briggs AH, Wonderling DE, Mooney CZ. Pulling Cost-Effectiveness Analysis Up by its Bootstraps: A non-Parametric Approach to Confidence Interval Estimation. *Health Econ* (1997) 6(4):327–40. doi: 10.1002/(SICI)1099-1050(199707)6:4<327::AID-HEC282>3.0.CO;2-W
31. Rosenbaum PR, Rubin DB. Reducing Bias in Observational Studies Using Subclassification on the Propensity Score. *J Am Stat Assoc* (1984) 79(367):9. doi: 10.1080/01621459.1984.10478078
32. Austin PC, Stuart EA. Moving Towards Best Practice When Using Inverse Probability of Treatment Weighting (IPTW) Using the Propensity Score to Estimate Causal Treatment Effects in Observational Studies. *Stat Med* (2015) 34(28):3661–79. doi: 10.1002/sim.6607
33. Defourny N, Monten C, Grau C, Lievens Y, Perrier L. Critical Review and Quality-Assessment of Cost Analyses in Radiotherapy: How Reliable are the Data? *Radiother Oncol* (2019) 141:14–26. doi: 10.1016/j.radonc.2019.09.020
34. Van de Werf E, Verstraete J, Lievens Y. The Cost of Radiotherapy in a Decade of Technology Evolution. *Radiother Oncol* (2012) 102(1):148–53. doi: 10.1016/j.radonc.2011.07.033
35. Hulstaert F, Mertens A-S, Obyn C, Van Halewyck D, van der Straten B, Lievens Y. *Innovative Radiotherapy Techniques: A Multicentre Time-Driven Activity-Based Costing Study*. Health Technology Assessment (HTA) Brussels: Belgian Health Care Knowledge Centre (KCE (2013). KCE Reports 198C. D/2013/10.273/9.
36. Oliver M, Ansbacher W, Beckham WA. Comparing Planning Time, Delivery Time and Plan Quality for IMRT, RapidArc and Tomotherapy. *J Appl Clin Med Phys* (2009) 10(4):117–31. doi: 10.1120/jacmp.v10i4.3068
37. Bonastre J, Noël E, Chevalier J, Gerard JP, Lefkopoulos D, Bourhis J, et al. Implications of Learning Effects for Hospital Costs of New Health Technologies: The Case of Intensity Modulated Radiation Therapy. *Int J Technol Assess Health Care* (2007) 23(2):248–54. doi: 10.1017/S026646230707033X
38. Lievens Y, Defourny N, Corral J, Gasparotto C, Grau C, Borras JM, et al. How Public Health Services Pay for Radiotherapy in Europe: An ESTRO–HERO Analysis of Reimbursement. *Lancet Oncol* (2020) 21(1):e42–54. doi: 10.1016/S1470-2045(19)30794-6
39. Fransson P, Nilsson P, Gunnlaugsson A, Beckman L, Tavelin B, Norman D, et al. Ultra-Hypofractionated Versus Conventionally Fractionated Radiotherapy for Prostate Cancer (HYPO-RT-PC): Patient-Reported Quality-of-Life Outcomes of a Randomised, Controlled, non-Inferiority, Phase 3 Trial. *Lancet Oncol* (2021) 22(2):235–45. doi: 10.1016/S1470-2045(20)30581-7
40. Lievens Y, Dunscombe P, Defourny N, Gasparotto C, Borras JM, Grau C. HERO (Health Economics in Radiation Oncology): A Pan-European Project on Radiotherapy Resources and Needs. *Clin Oncol* (2015) 27(2):115–24. doi: 10.1016/j.clon.2014.10.010
41. Grau C, Defourny N, Malicki J, Dunscombe P, Borras JM, Coffey M, et al. Radiotherapy Equipment and Departments in the European Countries: Final Results From the ESTRO-HERO Survey. *Radiother Oncol* (2014) 112(2):155–64. doi: 10.1016/j.radonc.2014.08.029
42. Lievens Y, Grau C. Health Economics in Radiation Oncology: Introducing the ESTRO HERO Project. *Radiother Oncol* (2012) 103(1):109–12. doi: 10.1016/j.radonc.2011.12.026
43. Defourny N, Dunscombe P, Perrier L, Grau C, Lievens Y. Cost Evaluations of Radiotherapy: What do We Know? An ESTRO-HERO Analysis. *Radiother Oncol* (2016) 121(3):468–74. doi: 10.1016/j.radonc.2016.12.002
44. Austin PC, Grootendorst P, Anderson GM. A Comparison of the Ability of Different Propensity Score Models to Balance Measured Variables Between Treated and Untreated Subjects: A Monte Carlo Study. *Stat Med* (2007) 26 (4):734–53. doi: 10.1002/sim.2580
45. Dehejia RH, Wahba S. Propensity Score-Matching Methods for Nonexperimental Causal Studies. *Rev Econ Stat* (2002) 84(1):151–61. doi: 10.1162/003465302317331982
46. National Comprehensive Cancer Network. *NCCN Guidelines Version 2.2021 Prostate Cancer* (2021). Available at: https://www.nccn.org/professionals/physician_gls/pdf/prostate.pdf.

Conflict of Interest: DA declares conflict of interest with Novagray, which has nothing to do with this study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Oncology

ORIGINAL RESEARCH
published: 12 April 2022
doi: 10.3389/fonc.2022.855546

Quality-of-Life Outcomes in Female Patients With Ileal Conduit or Orthotopic Neobladder Urinary Diversion: 6-Month Results of a Multicenter Prospective Study

Salvatore Siracusano[1], Agustina Zaka[2], Pierfrancesco Bassi[3], Paolo Gontero[4], Ettore Mearini[5], Ciro Imbimbo[6], Alchiede Simonato[7], Fabrizio Dal Moro[8], Gianluca Giannarini[9], Claudio Valotto[9], Francesco Montorsi[10], Renzo Colombo[10], Francesco Porpiglia[11], Riccardo Bartoletti[12], Marco Vella[7], Andrea Minervini[13], Antonio Benito Porcaro[14], Federico Romantini[1], Carlo Vicentini[1], Renato Talamini[15], Vincenzo Ficarra[16] and Cristina Lonardi[17]*

[1] Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila, Italy, [2] Department of Surgery, Dentistry, Pediatrics and Gynaecology, University of Verona, Verona, Italy, [3] Department of Medicine and Surgery, Rome Catholic University, Rome, Italy, [4] Department of Surgical Science, Turin University, Turin, Italy, [5] Department of Medicine and Surgery, Perugia University, Perugia, Italy, [6] Department of Urology, Federico II Naples University, Naples, Italy, [7] Department of Urology, Palermo University, Palermo, Italy, [8] Department of Urology, Padova University, Padova, Italy, [9] Department of Urology, Udine Hospital, Udine, Italy, [10] Department of Urology, San Raffaele Hospital, Vita e Salute University, Milan, Italy, [11] Department of Urology, Orbassano Hospital, Turin University, Turin, Italy, [12] Department of Urology, Pisa University, Pisa, Italy, [13] Department of Urology, Florence University, Florence, Italy, [14] School of Medicine and Surgery, Verona University, Verona, Italy, [15] Department of Epidemiology, Aviano Oncology Center, Aviano, Italy, [16] Department of Urology, University of Messina, Messina, Italy, [17] Department of Human Sciences, Verona University, Verona, Italy

OPEN ACCESS

Edited by:
Sanja Štifter, Skejby Sygehus, Denmark

Reviewed by:
Clemens Mathias Rosenbaum, Asklepios Klinik Barmbek, Germany
Roman Sosnowski, Maria Sklodowska-Curie National Research Institute of Oncology, Poland

***Correspondence:**
Salvatore Siracusano
salvatore.siracusano@univaq.it

Specialty section:
This article was submitted to Genitourinary Oncology, a section of the journal Frontiers in Oncology

Received: *15 January 2022*
Accepted: *14 March 2022*
Published: *12 April 2022*

Citation:
Siracusano S, Zaka A, Bassi P, Gontero P, Mearini E, Imbimbo C, Simonato A, Dal Moro F, Giannarini G, Valotto C, Montorsi F, Colombo R, Porpiglia F, Bartoletti R, Vella M, Minervini A, Porcaro AB, Romantini F, Vicentini C, Talamini R, Ficarra V and Lonardi C (2022) Quality-of-Life Outcomes in Female Patients With Ileal Conduit or Orthotopic Neobladder Urinary Diversion: 6-Month Results of a Multicenter Prospective Study. Front. Oncol. 12:855546. doi: 10.3389/fonc.2022.855546

Radical cystectomy (RC) often affects patients' life as this surgery is a traumatic and invasive event for the patients, with drawbacks on their daily, social, working, and sex life. Such changes in the quality of life (QoL) of patients are commonly studied through retrospective clinical evaluations and rarely with longitudinal studies. To date, studies focusing on functional outcomes, sexual function, and health-related QoL for female patients are lacking. We evaluated 37 patients using EORTC QLQ-C30 (QLQ-30) and Short-Form 36 (SF-36) questionnaires, before and after surgery, at 3 and 6 months of follow-up. The mean values for the emotional functioning in QLQ-C30 as well as the mental health in SF-36 were significantly higher in the ONB group compared to the IC group at 3 months of follow-up. These differences were not significant at 6 months of follow-up. At 6 months of follow-up, the ONB group showed a higher mean score in the physical and role functioning than the IC group. Although there was a statistically significant age difference at baseline of the two groups, none of the results are correlated with age, as demonstrated by Spearman's analysis. The ONB seems to represent the most advantageous solution compared to the IC in terms of QOL at the 6-month follow-up.

Keywords: bladder cancer, female, radical cystectomy, HRQOL, urinary diversion

INTRODUCTION

Bladder cancer (BC) is one of the most frequent cancers in men and women, counting 81,190 new estimated cases in 2018. Radical cystectomy (RC) with lymph node dissection with ileal conduit (IC) or orthotopic neobladder (ONB) urinary diversion is the standard treatment, recommended by the European Association of Urology (EAU) guidelines, for localized muscle-invasive bladder cancer (1). However, RC is also a recommended treatment for high-risk non muscle-invasive bladder cancer (NMIBC) when non-responsive to standard treatments. In this context, it is well known that RC often deeply affects patients' life, as this surgery is a traumatic and invasive event for the patients, with drawbacks on their daily, social, working, and sex life (2, 3). Such changes and quality of life (QoL) of patients are commonly studied through retrospective clinical evaluations and rarely with longitudinal studies. To date, studies focusing on functional outcomes, sexual function, and health-related QoL for female patients are lacking especially in the short term, although as a whole statistically significant worse overall survival, recurrence-free survival, and cancer-specific survival are reported in comparison to male patients (4). The aim of this study was to evaluate the QoL in female patients in the first 6 months postoperatively with IC or ONB utilizing EORTC QLQ-C30 (QLQ-30) and Short-Form 36 (SF-36) questionnaires in a prospective longitudinal fashion.

PATIENTS AND METHODS

This longitudinal study involved 37 consecutive female patients, out of a total of 188 patients of which 151 were males, that had undergone RC and urinary diversion (UD) for urothelial BC in thirteen Italian academic urological centers between September 2019 and July 2020. All patients were older than 18 and were affected by either muscle-invasive BC or by non-responder high-grade non-muscle-invasive BC, according to EAU Guidelines (1). They had all undergone pelvic and iliac lymph node dissection with radical en bloc cystectomy as described by Skinner and Lieskovsky (5) followed by UD by either IC or ONB with Vescica Ileale Padovana (VIP) as previously described by Pagano et al. (6). QoL was measured using the QLQ-C30 and the SF-36 questionnaires before surgery and at 3 and 6 months postoperatively. Baseline characteristics, including demographic profile, body mass index (BMI), Charlson Comorbidity Index (CCI), modified frailty index (m-FI), pathological tumor stage, 90-day complications (7), and neo-adjuvant chemotherapy were collected and compared. To rule out the possible effects of disease-related factors or of the psychological burden of a recent surgical procedure, patients with cancer recurrence or with less than 6 months of follow-up were excluded from the analysis. Patients unable to understand or fill out the questionnaire due to cognitive impairment or insufficient command of the Italian language (four patients) were also excluded. All patients provided written informed consent. The study was approved by the Ethics Committees of Verona and Rovigo (protocol number UQOL1Y) and was conducted in accordance with the principles of research involving human subjects as expressed in the Declaration of Helsinki and the Good Clinical Practice guidelines.

QoL Questionnaires

All patients were evaluated using the QLQ-C30 and SF-36 questionnaires. The QLQ-C30 is a modular 30-item questionnaire developed and copyrighted by the EORTC as an integrated tool designed to assess the QoL of cancer patients participating in clinical trials. This tool has been translated into 81 languages and used in more than 3,000 studies worldwide. Its cross-cultural validity and reliability have been established. The questionnaire is composed of nine multi-item scales: five functional scales (physical, role, cognitive, emotional and social), three symptom scales (fatigue, pain, nausea/vomiting), a global health and QoL scale, and items assessing the perceived financial burden of cancer and other symptoms frequently reported by cancer patients, such as constipation, diarrhea, dyspnea, loss of appetite, and sleep disturbance (8).

The SF-36 measures different health concepts selected from those used by the Medical Outcomes Study. Through 36 multiple-choice questions, the data are aggregated into 8 scales that investigate physical activity, role and physical health, physical pain, health in general, vitality, social activities, role and emotional state, and mental health (9). There is also a question about the change in health status over the last year. We used the Italian version of SF-36 and of the QLQ-30. In the QLQ-30, the majority of questions were assigned a score from 1 to 4 (1 = not at all, 2 = a little, 3 = quite a bit, 4 = very much). For two questions were assigned a score from 1 to 7 (very poor to excellent). As suggested to the EORTC Manual scoring, we linearly transformed all variables to a 0–100 scale. This manual contains scoring procedures for the QLQ-C30, and it also contains summary information about supplementary modules (EORTC Data Center). The principle for scoring these scales is the same in all cases: (1) estimate the average of the items that contribute to the scale—this is the raw score; and (2) use a linear transformation to standardize the raw score, so that scores range from 0 to 100. For the functional items, the higher score represents a higher level of functioning. For the symptoms/single items, a higher score means a higher level of symptomatology/problems. Data were collected from each of the patients through an individual interview, conducted in the outpatient clinic in the course of a follow-up evaluation visit.

Statistical Analysis

Mean values with standard deviations (± SD) were computed and reported for continuous variables (i.e., age, BMI, Charlson comorbidity, and modified frailty index), and for all items included in the QLQ-C30 and SF-36. The Wilcoxon two-sample test was used to verify differences between continuous variables, whereas the chi-square test was used to compare categorical variables (i.e., gender, levels of education, pathological tumor stage, Clavien–Dindo grade and neoadjuvant chemotherapy).

Spearman correlation analysis was used to determine the correlation between age and baseline QoL score. Statistical significance was achieved when the two-sided p-value was 0.05 or less. Statistical analyses were conducted using SAS version 9.3 software (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Thirty-seven female patients undergoing RC and UD were included in the study. Urinary diversion following RC was IC in 75.6% (28/37) of the population and ONB in 24.3% (9/37). Patients in the ONB group were significantly younger than those in the IC group (mean age 62.8 and 70.2 years, respectively; p = 0.03). Barring that, the two groups did not present statistically significant differences with regard to degree of education, BMI, Charlson comorbidity index, modified frailty index, pathological tumor stage, Clavien–Dindo grade, and neoadjuvant therapy (**Table 1**). In all patients, the QoL was assessed before surgery and 3 and 6 months postoperatively. As far as QoL is concerned and reported in **Table 2**, we found that the mean values for only the emotional functioning in QLQ-C30 as well as the mental health in SF-36 were significantly higher in the ONB group compared to the IC group at 3 months of follow-up. For emotional functioning, the means (± SD) were 90.6 (± 15.7) and 71.1 (± 24.2) respectively (p = 0.02), and for mental health the means were 72.0 (± 15.1) and 54.9 (± 19.7), respectively (p = 0.02). These differences were not significant at the 6-month follow-up. At the 6-month follow-up, we found that the ONB group compared with the IC group had a higher mean score in the physical and role functioning (QLQ-C30). For physical functioning, the means (± SD) were 88.2 (± 19.1) and 71.7 (± 25.1) respectively (p = 0.05), and for role functioning the means were 90.7 (± 12.1) and 62.5 (± 33.8), respectively (p = 0.03). A significant lower body pain (SF36) was found in the ONB group compared with the IC group: 84.6 (± 21.5) and 61.1 (± 30.9), respectively (p = 0.05). Other items did not yield statistically significant results at 6 months (**Table 2**). Although there was a baseline age difference between the two groups, Spearman correlation analysis showed that none of the significant parameters abovementioned (i.e., physical and role functioning, body pain) were correlated with age (**Table 3**).

DISCUSSION

According to EAU guidelines, radical cystectomy remains the gold standard treatment for muscle-invasive bladder cancer. The urological literature emphasizes the importance of HRQOL in patients undergoing RC and urinary diversion; however, the information available in this regard is always based on retrospective and non-prospective studies. In this regard, it is known that cross-sectional retrospective studies show various biases that may not reflect the real quality of life of patients. In this context, it is therefore clear that the optimal way is to carry out longitudinal studies possibly randomized with the use of questionnaires validated in the patients' original language. Currently, the use of prospective studies to evaluate the QoL in patients undergoing RC is not very widespread, as only a few authors have undertaken this methodological approach (3, 10).

In this setting, it is not yet clear whether the HRQOL between patients with continent urinary diversion and incontinent urinary diversion is comparable even if a meta-analysis in this regard would confirm it (11). In our study, we focused our attention on the female population as the data in this regard are particularly lacking due to the lower incidence of BC in female compared to male. We decided to measure the QoL in the first 6 months because scoring data in this postoperative period showed

TABLE 1 | Demographic and pathological characteristics of female patients with RC for localized MIBC according to different urinary diversions: prospective multicenter study in Italy.

Characteristics	ONB	IC	p-value[1]
Female, N	*9*	*28*	
Age (years), mean (± SD)	*62.8 (± 6.0)*	*70.2 (± 9.0)*	***0.03***
Education (years), N (%)			
5–8	*2 (22.2)*	*15 (53.6)*	
9–11	*2 (22.2)*	*4 (14.3)*	
≥12	*5 (55.6)*	*9 (32.1)*	*0.38*
BMI (kg/m^2), mean (± SD)	*24.8 (± 4.7)*	*23.5 (± 5.2)*	*0.40*
Charlson comorbidity index, mean (± SD)	*1.4 (± 1.6)*	*2.6 (± 2.1)*	*0.19*
Modified frailty index, mean (± SD)	*0.8 (± 1.0)*	*1.1 (± 1.6)*	*0.65*
Pathological tumor stage, N (%)			
Organ confined: ≤pT2, pN0	*5 (55.6)*	*13 (46.4)*	
Non-organ confined: pT3–pT4, pN0	*3 (33.3)*	*7 (25.0)*	
Lymph node-positive: pN+	*1 (11.1)*	*8 (28.6)*	*0.56*
Clavien–Dindo grade, N (%)			
I	*7 (77.8)*	*21 (75.0)*	
II	*2 (22.2)*	*4 (14.3)*	
III–IV	*–*	*3 (10.7)*	*0.54*
Neoadjuvant chemotherapy, N (%)			
No	*8 (88.9)*	*24 (85.7)*	
Yes	*1 (11.1)*	*4 (14.3)*	*0.81*

Bold value is statistically significant.

TABLE 2 | Mean and standard deviation (± SD) of EORTC QLQ-C30 and short form 36 scale preoperatively and at each follow-up of female patients with RC for localized MIBC according to different urinary diversion: prospective multicenter study in Italy.

	Preoperatively (baseline)			*3 months of follow-up*			*6 months of follow-up*		
	ONB	***IC***	***p*[1]**	***ONB***	***IC***	***p*[1]**	***ONB***	***IC***	***p*[1]**
EORTC QLQ-C30									
Global health status	*63.0 (± 19.6)*	*59.6 (± 27.2)*	*0.84*	*71.9 (± 14.7)*	*58.0 (± 24.2)*	*0.14*	*71.3 (± 17.2)*	*55.7 (± 27.3)*	*0.11*
Function scale									
Physical	*85.2 (± 15.2)*	*81.4 (± 18.4)*	*0.65*	*80.8 (± 19.3)*	*70.7 (± 25.6)*	*0.34*	*88.2 (± 19.1)*	*71.7 (± 25.1)*	***0.05***
Role	*79.6 (± 16.2)*	*78.6 (± 26.4)*	*0.78*	*83.3 (± 23.6)*	*76.3 (± 26.7)*	*0.60*	*90.7 (± 12.1)*	*62.5 (± 33.8)*	***0.03***
Emotional	*69.4 (± 20.0)*	*65.1 (± 24.8)*	*0.70*	*90.6 (± 15.7)*	*71.1 (± 24.2)*	***0.02***	*83.3 (± 17.7)*	*72.0 (± 24.3)*	*0.21*
Cognitive	*85.2 (± 10.0)*	*84.5 (± 14.3)*	*0.98*	*93.8 (± 12.4)*	*89.3 (± 15.9)*	*0.48*	*92.6 (± 16.9)*	*83.3 (± 22.2)*	*0.14*
Social	*81.5 (± 25.6)*	*83.9 (± 23.3)*	*0.80*	*91.7 (± 23.6)*	*78.4 (± 25.2)*	*0.14*	*81.5 (± 29.4)*	*76.8 (± 29.2)*	*0.57*
Symptom scale									
Fatigue	*33.3 (± 11.1)*	*41.3 (± 22.3)*	*0.51*	*33.3 (± 7.9)*	*42.6 (± 22.9)*	*0.62*	*17.3 (± 20.9)*	*37.3 (± 27.1)*	*0.06*
Nausea-vomiting	*16.7 (± 0.0)*	*31.5 (± 26.9)*	*0.33*	*16.7 (± 0.0)*	*35.2 (± 29.4)*	*0.55*	*3.7 (± 7.4)*	*9.5 (± 16.0)*	*0.38*
Pain	*40.5 (± 21.2)*	*47.1 (± 27.2)*	*0.67*	*25.0 (± 13.9)*	*41.0 (± 24.2)*	*0.15*	*13.0 (± 18.2)*	*25.0 (± 31.6)*	*0.39*
Dyspnea	*33.3 (± 0.0)*	*33.3 (± 0.0)*	*1.00*	*33.3 (± 0.0)*	*33.3 (± 0.0)*	*1.00*	*11.1 (± 16.7)*	*11.9 (± 22.6)*	*0.86*
Insomnia	*44.4 (± 17.2)*	*60.0 (± 29.8)*	*0.30*	*44.4 (± 19.3)*	*63.6 (± 23.4)*	*0.23*	*18.5 (± 33.8)*	*31.0 (± 33.9)*	*0.25*
Appetite loss	*33.3 (± 0.0)*	*55.6 (± 29.6*	*0.33*	*33.3 (± 0.0)*	*56.7 (± 22.5)*	*0.38*	*7.4 (± 14.7)*	*14.3 (± 24.7)*	*0.84*
Constipation	*58.3 (± 31.9)*	*55.6 (± 33.3)*	*0.86*	*44.4 (± 19.3)*	*61.5 (± 30.0)*	*0.42*	*18.5 (± 24.2)*	*28.6 (± 31.1)*	*0.44*
Diarrhea	*(±)*	*33.3 (± 0.0)*		*33.3 (± 0.0)*	*33.3 (± 0.0)*	*1.00*	*3.7 (± 11.1)*	*11.9 (± 24.4)*	*0.37*
Financial difficulties	*33.3 (± 0.0)*	*52.4 (± 26.2)*	*0.38*	*50.0 (± 23.6)*	*48.5 (± 27.3)*	*0.81*	*14.8 (± 24.2)*	*14.3 (± 23.0)*	*1.00*
Short Form 36									
Physical functioning	*68.6 (± 35.4)*	*72.7 (± 29.8)*	*0.79*	*80.0 (± 18.9)*	*63.6 (± 25.9)*	*0.21*	*68.9 (± 36.3)*	*59.5 (± 30.0)*	*0.37*
Role physical	*85.0 (± 33.5)*	*70.6 (± 30.9)*	*0.36*	*100.0 (± 0.0)*	*91.1 (± 23.2)*	*0.43*	*61.1 (± 48.6)*	*43.8 (± 46.0)*	*0.36*
Body pain	*55.9 (± 27.2)*	*69.0 (± 29.2)*	*0.25*	*68.0 (± 24.0)*	*71.1 (± 28.8)*	*0.62*	*84.6 (± 21.5)*	*61.1 (± 30.9)*	***0.05***
General health	*45.3 (± 21.0)*	*50.5 (± 23.7)*	*0.80*	*51.9 (± 8.6)*	*48.0 (± 10.2)*	*0.32*	*50.0 (± 7.6)*	*46.5 (± 10.9)*	*0.33*
Vitality	*55.0 (± 20.0)*	*45.7 (± 22.0)*	*0.28*	*65.0 (± 19.6)*	*48.9 (± 20.4)*	*0.06*	*55.6 (± 19.3)*	*47.5 (± 22.8)*	*0.31*
Social functioning	*51.4 (± 25.3)*	*65.6 (± 25.6)*	*0.16*	*65.6 (± 20.9)*	*63.8 (± 28.9)*	*0.98*	*70.8 (± 30.0)*	*58.5 (± 29.3)*	*0.25*
Role emotion	*83.3 (± 33.3)*	*72.9 (± 30.4)*	*0.53*	*88.9 (± 27.2)*	*74.1 (± 29.3)*	*0.24*	*63.0 (± 48.4)*	*39.3 (± 42.6)*	*0.24*
Mental health	*52.0 (± 18.6)*	*49.3 (± 23.1)*	*0.75*	*72.0 (± 15.1)*	*54.9 (± 19.7)*	***0.02***	*61.8 (± 18.9)*	*56.0 (± 23.4)*	*0.46*

Bold values are statistically significant.

that the main changes on HRQOL following RC would begin at this time of follow-up. In our study, all patients completed the EORTC QLQ-C30 and Short Form 36 questionnaires preoperatively and at 3 and 6 months of follow-up. In particular, at baseline all patients had similar characteristics concerning education, BMI, pathological tumor stage, and Clavien–Dindo grade except for age. In particular, at the 6-month follow-up the global health status does not differ in the two groups of patients although it seems that the ONB group scored significantly higher than in the IC group in the physical and role function scales. Similarly, we observed that the ONB group had a significant lower body pain perception than the IC group. These data were age-independent as shown by Spearman's analysis, suggesting that at 6 months the QoL begins to be particularly affected in the IC group probably due to the awareness of the care needed for the management of the urinary stoma, possible urinary leak from the bag and urinary odor limiting the ability to partake in social, recreational, and professional activities. In contrast, the ONB group showed a better overall scoring than the IC group most likely related to the presence of a urinary continence allowing to continue an acceptable relationship life. In this way, a previous prospective comparison of HRQOL between patients undergoing RC and IC by Singh (3) showed, unlike what we have found, that global health status is higher in the ONB group than in the IC group and that financial difficulties were significantly greater among patients with IC. Certainly, these two data can be related to the fact that in Singh's study the prospective analysis involved both sexes overall and that on the other hand the costs and financing of the health system between India and Italy are profoundly different since in Italy any medical expense necessary following the surgical operation would be covered by the national health system. Recently, Clements published the results of a large prospective study showing a detriment of HRQOL at 3 and 6 months with a recovery and stabilization of the same HRQOL at 12 months of follow-up. In this study, the authors found better physical functioning in patients with continent urinary derivation compared to the group with ileal conduit. In our opinion, these results are partially comparable with those reported by us because the Clements study has a follow-up of up to 24 months and because it does not evaluate HRQOL exclusively in women. However, if we analyze the results of Kern, we observe that in the long term and in a perspective way there are no significant differences in HRQOL among non-continent, continent cutaneous, and continent orthotopic urinary diversion groups, which can serve as an important point of consideration during patient counseling (2).

In this context, therefore, we believe that a distinction should be made between HRQOL in the male and female cystectomized patients. In this regard, there are only few studies that try to review recent data on gender differences in oncologic and functional outcomes in women comparing them to male patients. QoL was significantly worse for women than for men (12). A review by Saighian et al. concluded that gender

TABLE 3 | Spearman correlation analysis (r) of correlation between age and preoperative (baseline) 102EORTC QLQ-C30 and short form 36 scale.

QoL scale	ONB		IC	
	r	p	r	p
EORTC QLQ-C30				
Global health status	*-0.52*	*0.15*	*0.29*	*0.15*
Function scale				
Physical	*-0.23*	*0.55*	*-0.35*	*0.07*
Role	*-0.22*	*0.56*	*-0.07*	*0.73*
Emotional	*-0.77*	***0.02***	*0.21*	*0.30*
Cognitive	*-0.20*	*0.61*	*0.13*	*0.51*
Social	*-0.64*	*0.06*	*0.03*	*0.89*
Symptom scale				
Fatigue	*0.54*	*0,21*	*-0.02*	*0.92*
Nausea-vomiting	–	–	*-0.75*	***0.02***
Pain	*0.51*	*0.24*	*-0.03*	*0.89*
Dyspnea	–	–	–	–
Insomnia	*-0.21*	*0.69*	*0.18*	*0.45*
Appetite loss	–	**–**	*-0.42*	*0.17*
Constipation	*0.21*	*0.79*	*-0.37*	*0.33*
Diarrhea	–	**–**	–	–
Financial difficulties	–	–	*-0.18*	*0.70*
Short Form 36				
Physical functioning	*-0.39*	*0.35*	*-0.17*	*0.40*
Role physical	*-0.35*	*0.56*	*0.06*	*0.82*
Body pain	*-013*	*0.75*	*0.09*	*0.67*
General health	*-0.70*	***0.04***	*-0.05*	*0.80*
Vitality	*-0.63*	*0.09*	*0.16*	*0.43*
Social functioning	*-0.40*	*0.29*	*-0.12*	*0.55*
Role emotion	*-0.26*	*0.74*	*-0.01*	*0.95*
Mental health	*-0.29*	*0.48*	*0.15*	*0.46*

Bold values are statistically significant.

negatively affects oncologic outcome after treatment for bladder cancer, with women being the weaker factor of the equation. In this setting, varying socioeconomic circumstances and biological differences in cancer initiation, as well as response to therapy, seem to be responsible for overall poorer quality of life in bladder cancer female patients, when compared to their male counterpart (13). However, in any case, our study can be considered as the first that exclusively analyzes in a longitudinal way the HRQOL of women undergoing RC with ONB or IC. In this way, it is also important to evaluate the impact of urinary incontinence that may be due to problems related to external appliances. In fact, in cases involving both sexes, leakage with conduit diversions is most commonly due to poor external appliance adherence or suboptimal stoma placement. Among ileal conduit patients, urinary leakage rates during daytime and nighttime have been reported to be as high as 40% (14, 15). Improvements in stomal creation and care, particularly with dedicated enterostomal nurse education, have largely mitigated many of these problems (16), and most patients get to a state of good functional control with minimum urinary leaks after a few months of directed education and gaining hands-on experience changing the urostomy appliance themselves. Although continent urinary diversions are used to preserve normal anatomic urinary function and volitional voiding, urinary incontinence rates and urine leakage are still relatively high. Although most patients regain control during awake hours, nighttime incontinence can affect 40% to 50% of neobladder patients (17, 18). In this context, leakage and lack of control of urinary function can negatively affect HRQOL.

In this context, our study constitutes a contribution to a small body of research addressing an important clinical question on HRQOL for which the ONB seems to represent the most advantageous solution compared to the IC in terms of HRQOL at the 6-month follow-up.

This result can be considered certainly new since to date the retrospective studies conducted in women undergoing RC with ONB or IC have not shown significant differences on HRQOL in the short and mid terms (19); however, about that, it should be borne in mind that QoL results based on interview data may suffer from potential sources of bias that risk inducing responders to report relatively "optimistic" QoLs as patients for example may respond according to what they believe their interviewer wishes to hear.

In conclusion, we can affirm that our study shows some limitations such as the small number of patients enrolled and secondarily that the study was not randomized. These limitations in our case may also be justified by the fact that usually the women who undergo RC are extremely small in number compared to the male population and finally that the randomization in these patients is not feasible because the choice of one or another type of urinary diversion is related to the overall condition of the disease and the general condition of the patient. Further large longitudinal studies will be needed to help the clinicians to understand the real HRQOL of female patients in relation to one or another type of urinary diversion.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committees of Verona and Rovigo (protocol number UQOL1Y). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SS contributed to the conception and design of the study. AZ organized the database. RT and BP performed the statistical analysis. SS and FR wrote the first draft of the manuscript. PB, PG, EM, CI, AS, FD, GG, CV, FM, RC, FP, RB, MV, AM, CV, VF and CL wrote sections of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

REFERENCES

1. Witjes JA, Bruins HM, Carrión A, Cathomas R, Compérat E, Efstathiou JA, et al. *EAU Guidelines Office*. Arnhem, The Netherlands. Available at: http://uroweb.org/guidelines/compilations-of-all-guidelines/.
2. Kern SQ, Speir RW, Tong Y, Kaimakliotis H, Masterson TA, Bihrle R, et al. Longitudinal Health Related Quality of Life After Open Radical Cystectomy: Comparison of Ileal Conduit, Indiana Pouch, and Orthotopic Neobladder. *Urology* (2021) 152:184–9. doi: 10.1016/j.urology.2020.12.036
3. Singh V, Yadav R, Sinha RJ, Gupta DK. Prospective Comparison of Quality-of-Life Outcomes Between Ileal Conduit Urinary Diversion and Orthotopic Neobladder Reconstruction After Radical Cystectomy: A Statistical Model. *BJU Int* (2014) 113(5):726–32. doi: 10.1111/bju.12440
4. Siracusano S, Zaka A, Romantini F, Porcaro AB, Vicentini C, Lonardi C. Quality of Life in Female Patients Following Ileal Neobladder and Ileal Conduit: Where Are We? *J Clin Med* (2021) 10(14):3042. doi: 10.3390/jcm10143042
5. Lerner SP, Skinner DG, Lieskovsky G, Boyd SD, Groshen SL, Ziogas A, et al. The Rationale for En Bloc Pelvic Lymph Node Dissection for Bladder Cancer Patients With Nodal Metastases: Long-Term Results. *J Urol* (1993) 149 (4):758–64; discussion 764-5. doi: 10.1016/s0022-5347(17)36200-6
6. Pagano F, Artibani W, Ligato P, Piazza R, Garbeglio A, Passerini G. Vescica Ileale Padovana: A Technique for Total Bladder Replacement. *Eur Urol* (1990) 17(2):149–54. doi: 10.1159/000464024
7. Clavien PA, Sanabria JR, Strasberg SM. Proposed Classification of Complications of Surgery With Examples of Utility in Cholecystectomy. *Surgery* (1992) 111(5):518–26.
8. Fayers P, Bottomley AEORTC Quality of Life Group; Quality of Life Unit. Quality of Life Research Within the EORTC-The EORTC QLQ-C30. European Organisation for Research and Treatment of Cancer. *Eur J Cancer* (2002) 38(Suppl 4):S125–33. doi: 10.1016/s0959
9. Lins L, Carvalho FM. SF-36 Total Score as a Single Measure of Health-Related Quality of Life: Scoping Review. *SAGE Open Med* (2016) 4:2050312116671725. doi: 10.1177/2050312116671725
10. McGregor B, O'Donnell PH, Balar A, Petrylak D, Rosenberg J, Yu EY, et al. Health-Related Quality of Life of Patients With Locally Advanced or Metastatic Urothelial Cancer Treated With Enfortumab Vedotin After Platinum and PD-1/PD-L1 Inhibitor Therapy: Results From Cohort 1 of the Phase 2 EV-201 Clinical Trial. *Eur Urol* (2022) S0302-2838(22):00072–0. doi: 10.1016/j.eururo.2022.01.032. S0302-2838(22)00072-0.
11. Yang LS, Shan BL, Shan LL, Chin P, Murray S, Ahmadi N, et al. A Systematic Review and Meta-Analysis of Quality of Life Outcomes After Radical Cystectomy for Bladder Cancer. *Surg Oncol* (2016) 25(3):281–97. doi: 10.1016/j.suronc.2016.05.027
12. Siracusano S, D'Elia C, Cerruto MA, Saleh O, Serni S, Gacci M, et al. Quality of Life in Patients With Bladder Cancer Undergoing Ileal Conduit: A Comparison of Women Versus Men. *In Vivo* (2018) 32(1):139–43. doi: 10.21873/invivo.11216
13. Sadighian M, Porten S. Gender Differences in Oncologic and Functional Outcomes in Patients With Bladder Cancer Undergoing Radical Cystectomy With Urinary Diversion. *Curr Opin Urol* (2019) 29(5):542–7. doi: 10.1097/MOU.0000000000000660
14. Protogerou V, Moschou M, Antoniou N, Varkarakis J, Bamias A, Deliveliotis C. Modified S-Pouch Neobladder vs Ileal Conduit and a Matched Control Population: A Quality-of-Life Survey. *BJU Int* (2004) 94(3):350–4. doi: 10.1111/j.1464-410X.2004.04932.x
15. Philip J, Manikandan R, Venugopal S, Desouza J, Javlé PM. Orthotopic Neobladder Versus Ileal Conduit Urinary Diversion After Cystectomy–A Quality-of-Life Based Comparison. *Ann R Coll Surg Engl* (2009) 91(7):565–9. doi: 10.1308/003588409X432293
16. Li X, Fang Q, Ji H, Pan J, Zhang J, Li Z, et al. Use of Urostomy Bags in the Management of Perioperative Urine Leakage After Radical Cystectomy. *Cancer Nurs* (2014) 37(3):170–4. doi: 10.1097/NCC.0b013e318277db29
17. Gilbert SM, Wood DP, Dunn RL, Weizer AZ, Lee CT, Montie JE, et al. Measuring Health-Related Quality of Life Outcomes in Bladder Cancer Patients Using the Bladder Cancer Index (BCI). *Cancer* (2007) 109(9):1756–62. doi: 10.1002/cncr.22556
18. El Bahnasawy MS, Osman Y, Gomha MA, Shaaban AA, Ashamallah A, Ghoneim MA. Nocturnal Enuresis in Men With an Orthotopic Ileal Reservoir: Urodynamic Evaluation. *J Urol* (2000) 164(1):10–3. doi: 10.1097/00005392-200007000-00003
19. Siracusano S, D'Elia C, Cerruto MA, Gacci M, Ciciliato S, Simonato A, et al. Quality of Life Following Urinary Diversion: Orthotopic Ileal Neobladder Versus Ileal Conduit. A Multicentre Study Among Long-Term, Female Bladder Cancer Survivors. *Eur J Surg Oncol* (2019) 45(3):477–81. doi: 10.1016/j.ejso.2018.10.061

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Oncology

ORIGINAL RESEARCH
published: 08 July 2022
doi: 10.3389/fonc.2022.866443

Prevalence and Outcomes of Unilateral Versus Bilateral Oophorectomy in Women With Ovarian Cancer: A Population-Based Study

Jiaqiang Xiong[1†], *Zhuoqun Zhang*[2†], *Yanyan Liu*[1], *Guanlan Fan*[1], *Kejia Wu*[1*] *and Wei Zhang*[1*]

[1] *Department of Obstetrics and Gynecology, Zhongnan Hospital of Wuhan University, Wuhan, China,* [2] *Department of Obstetrics and Gynecology, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China*

OPEN ACCESS

Edited by:
Sanja Štifter,
Skejby Sygehus, Denmark

Reviewed by:
Anna Myriam Perrone,
Sant'Orsola-Malpighi Polyclinic, Italy
Giorgio Bogani,
National Cancer Institute Foundation (IRCCS), Italy

***Correspondence:**
Wei Zhang
zw6676@163.com
Kejia Wu
wdzn2019@163.com

†*These authors share first authorship*

Specialty section:
This article was submitted to Genitourinary Oncology, a section of the journal Frontiers in Oncology

Received: *31 January 2022*
Accepted: *16 June 2022*
Published: *08 July 2022*

Citation:
Xiong J, Zhang Z, Liu Y, Fan G, Wu K and Zhang W (2022) Prevalence and Outcomes of Unilateral Versus Bilateral Oophorectomy in Women With Ovarian Cancer: A Population-Based Study. Front. Oncol. 12:866443. doi: 10.3389/fonc.2022.866443

Background: Unilateral oophorectomy has the benefits of preserving the ovarian function of fertility and hormone secretion, but the precise inclusion criteria for candidates for this procedure remain controversial. This study aimed to compare the prevalence and therapeutic efficiency of unilateral oophorectomy in women with ovarian cancer who underwent bilateral oophorectomy; moreover, it aimed to identify the appropriate candidates for unilateral oophorectomy.

Methods: Female patients diagnosed with stage I-III ovarian cancer between 2000 and 2017 were retrospectively identified from the Surveillance, Epidemiology, and End Results program database. Overall survival (OS) and disease-specific survival (DSS) after unilateral or bilateral (salpingo-) oophorectomy were estimated. Cumulative mortality rates (CMRs) for non-cancer comorbidities were also estimated.

Results: A total of 28,480 women with ovarian cancer were included in this study, of whom 11,517 died during the study period. Of the patients, 7.5% and 48.0% underwent unilateral and bilateral oophorectomy, respectively. Overall, for stage-Ia tumors, unilateral oophorectomy was associated with remarkably better OS and DSS than bilateral oophorectomy (OS: $p < 0.001$; DSS: $p = 0.01$). For stage-Ib and stage-Ic ovarian tumor, there was no significant difference between the OS and DSS of patients treated by unilateral oophorectomy and those treated by bilateral oophorectomy. For stage-II and stage-III ovarian cancer, unilateral oophorectomy was associated with remarkably worse OS and DSS than bilateral oophorectomy. Among the reproductive-age women younger than 50 years, the OS and DSS of patients with stage-I tumors receiving unilateral oophorectomy were comparable to those receiving bilateral oophorectomy, even for high-grade stage-Ic tumors (all $p > 0.05$). For those aged 50 years and older, OS and DSS of patients with stage-I tumor receiving unilateral oophorectomy were significantly worse than those receiving bilateral oophorectomy, even for low-grade stage-Ia ovarian tumor (OS: $p < 0.001$; DSS: $p = 0.02$).

Conclusion: Unilateral oophorectomy exhibited excellent oncological superiority and was equivalent to bilateral oophorectomy for stage-I ovarian tumors among women of reproductive age. For women of reproductive age, the criteria of unilateral oophorectomy can be appropriately broadened to high-grade stage-Ic diseases because of the better performance of unilateral oophorectomy in this population.

Keywords: ovarian cancer, prevalence, outcomes, population-based study, SEER, unilateral oophorectomy, bilateral oophorectomy

INTRODUCTION

Ovarian cancer is the sixth leading cause of global cancer deaths, accounting for over 294,000 new cancer cases and 198,000 new cancer deaths worldwide in 2019 (1). Ovarian cancer ranks fifth in cancer deaths among American women, accounting for more deaths than any other female reproductive system cancer. A woman's risk of developing ovarian cancer during her lifetime is about 1 in 78, and her lifetime chance of dying from ovarian cancer is approximately 1 in 108 (2). Although the 5-year relative-survival rate of localized ovarian cancer can reach 93%, only 16% of the cases have the opportunity to be diagnosed at an early stage (3). Of the tumors, 57% were accompanied by distant metastases at the time of cancer diagnosis, implying a particularly unfavorable prognosis with a survival rate of only 30% (3, 4). As a consequence of the low early detection, as well as the relatively high malignant potential, the overall 5-year relative survival rate generally ranges between 30% and 40% across the globe (5). Strikingly, only very modest increases have been achieved (2%–4%) since 1995 (5). Therefore, despite its significance to public health, the etiology of this lethal disease is not completely understood.

The standard treatment for ovarian cancer includes upfront surgery to accurately diagnose and stage the disease and perform maximal cytoreduction, followed by taxanes and platinum–based combination chemotherapy in most patients (6). Traditionally, surgical staging of ovarian cancer has included exploratory laparotomy with peritoneal washings, hysterectomy, salpingo-oophorectomy, omentectomy, multiple peritoneal biopsies, and potential pelvic and para-aortic lymphadenectomy.

When preservation of fertility is desired and the disease seems confined to a single ovary, preservation of the uterus and contralateral ovary is often possible (6). Compared with bilateral oophorectomy, unilateral oophorectomy can preserve the other side of the ovary to maintain the ovarian function of fertility and hormone secretion. The major concerns of unilateral oophorectomy focused on the potential risks of residual tumor, tumor recurrence, and a newly occurring tumor on the other side of the ovary (7, 8). Therefore, it is important to determine which proportion of patients are suitable and/or have the opportunity to receive unilateral oophorectomy and preserve the other side of the ovary.

This study aimed to characterize the prevalence and outcomes of unilateral oophorectomy in women with ovarian cancer and compare it with bilateral oophorectomy to distinguish the appropriate proportion of patients with ovarian cancer to be treated with unilateral oophorectomy. The results will guide researchers and clinicians in determining the optimal therapy for patients with ovarian cancer.

MATERIALS AND METHODS

Data Sources and Study Population

This retrospective cohort study used data from the Surveillance, Epidemiology, and End Results (SEER) program. The SEER database is a population-based cancer registry covering nearly 30% of the US population and collecting cancer demographics, incidence, survival, and treatment data. The SEER*Stat software version 8.3.8 was used for the analysis (9). This study was performed according to the STROCSS guidelines (Strengthening the reporting of cohort, cross-sectional and case-control studies in surgery) (10).

Female patients diagnosed with the first primary malignant ovarian cancer (site codes: C56.9) between 2000 and 2017 were extracted from the SEER 18 database (2020 submission) (11). Only patients with unilateral-originated ovarian cancer were included because patients with bilateral origin might have lost their opportunity to undergo unilateral oophorectomy. Patients diagnosed only through autopsy or death certificates were excluded. We further excluded patients without complete follow-up information, including follow-up duration and age at diagnosis. To accurately evaluate the effects of surgical operations, we further excluded patients with advanced-stage cancer or those with a cancer of unknown stage (**Figure S1**).

Since it is a publicly available database, access to the SEER data required a signed research data agreement form. The Institutional Review Board of Zhongnan Hospital of Wuhan University waived the institutional review board approval for the data obtained from the SEER database, as the study did not directly involve human subjects, and all data were anonymized. The requirement for informed consent was waived.

Definition of Variables

All patients were followed between the time of the first primary diagnosis of ovarian cancer and the time of their death, exiting the study alive, or the end of the study (December 31, 2017). Among the patients included in this study, we evaluated the following variables: age at diagnosis, race, year of diagnosis, cancer stage, American Joint Committee on Cancer Staging (AJCC) N stage, AJCC T stage, surgical therapy, cause of death, histological types, urban/rural residency at diagnosis,

median household income, follow-up time, and vital status at the end of follow-up.

As the SEER database records the survival duration in months, and a month was the shortest time interval available for analysis, survival durations shorter than 1 month were recorded as 0 months in the SEER program. Age at cancer diagnosis was divided into 4 groups for comparison: "15-39 years," "40-59 years," "60-79 years," and "80+ years". Patients aged 15-49 years were selected for specific analyses, as this proportion of women had a greater desire for fertility preservation.

For ovarian cancer, the SEER program derived TNM values of the stage from the International Federation of Gynecology and Obstetrics (FIGO) stage. Thus, FIGO information of this study was inferred from TNM-stage values. TNM-stage values were extracted from AJCC 3rd stage codes for patients diagnosed between 2000 and 2003, AJCC 6th stage codes for patients diagnosed between 2004 and 2009, AJCC 7th stage codes for patients diagnosed between 2010 and 2015, and SEER combined stage for patients diagnosed in 2016 and 2017 (12). We excluded the patients with stage IV ovarian cancer.

The SEER program provided detailed site-specific surgical information for the included patients (13–15). Surgical operations for ovarian cancer were divided into two major groups: unilateral and bilateral oophorectomy. To avoid confusion, local excision/destruction and unknown surgical operations were excluded (surgery codes: 17 and 90-99). Unilateral oophorectomy was defined as total removal of the tumor or (single) ovary, and unilateral (salpingo-) oophorectomy with or without hysterectomy (surgery codes: 25-28 and 35-37). Bilateral oophorectomy includes bilateral (salpingo-) oophorectomy with or without hysterectomy, cytoreductive surgery, and pelvic exenteration (surgery codes: 60-74). Surgical operations with unknown laterality were excluded for accuracy (surgery codes: 55-57 and 80) (13).

Causes of death of patients with ovarian cancer were classified into two major groups: death from cancer (i.e., a second primary cancer) and death from non-cancer comorbidities (i.e., deaths from any medical cause other than cancer). Causes of death were defined by the SEER cause-specific death classification variable from death certificates (15–17). Non-cancer causes were categorized into 26 major groups. These groups were further divided into seven broad categories: infectious diseases, cardiovascular diseases (CVD), respiratory diseases, gastrointestinal and liver diseases, renal diseases, external injuries, and other non-cancer causes.

Statistical Analysis

We estimated the characteristics of the patients with ovarian cancer. Trends in surgical operations were characterized by age at diagnosis and year of diagnosis. Moreover, we analyzed the overall survival (OS) and disease-specific survival (DSS) of patients using the Kaplan-Meier method. The OS rate was defined as the percentage of survivors (all causes of death) after follow-up. The DSS rate was defined as the percentage of patients who have not died from ovarian cancer (rather than from other causes) in a defined period of time (18). Cox regression models were used to assess the significance of differences in the OS and DSS analyses. The cumulative mortality rate (CMR) was estimated for non-cancer comorbidities (15).

All analyses were performed using SEER*Stat software version 8.3.8 (9) and R 3.6.3 (19). Tests were two-tailed, with a p-value of less than 0.05 considered statistically significant.

RESULTS

Baseline Characteristics

In this population-based study involving 28,480 women with stage I-III ovarian cancer, 11,517 (40.4%) deaths were recorded, with a median follow-up time of 4.1 years (range: 0–17.9 years) (**Figure S1** and **Table 1**). Most of the patients were aged 40–79 years (83.3%) and were white (82.6%). Of the cancers, 46.8% were stage-I tumors. Serous ovarian cancer accounted for the majority of tumors (51.9%), followed by endometrioid carcinoma (20.7%) (**Table 1**).

Of the patients, 95.5% (N = 27,197) had undergone surgical operations, among whom 7.9%, 50.3%, and 41.8% underwent unilateral oophorectomy, bilateral oophorectomy, and other surgical procedures, respectively (**Table S1**). Patients who underwent unilateral oophorectomy were younger. Most patients who underwent unilateral oophorectomy (70.9%) were younger than 60. Of the patients aged 15–39 years, 33.7% and 22.7% underwent unilateral oophorectomy and bilateral oophorectomy, respectively (**Table 1**). Moreover, there was a decreasing trend in the unilateral oophorectomy rate by age at cancer diagnosis (**Figure 1A**). Similarly, the unilateral oophorectomy rate decreased by FIGO stage (**Figure 1B**), especially for younger patients (**Figure 1C**). Most tumors treated by unilateral oophorectomy were in the Ia stage (53.1%), followed by Ic stage (19.4%). The Hispanic population had a higher unilateral oophorectomy rate (11.3%) than the non-Hispanic population (7.0%) (**Table 1**).

Survival Analysis of Surgical Interventions for Patients With Ovarian Cancer

The OS and DSS of patients who had undergone surgery were significantly better than those of patients who did not (all $p < 0.001$) (**Figure 2** and **Figure S2**). The prognostic superiority of surgical operation could be observed in ovarian cancer at all stages (**Figures 2B, C** and **Figure S2**).

To examine the therapeutic effects of unilateral oophorectomy, we performed survival analyses according to the type of surgical intervention (**Figure 3** and **Figure S3**). In stage-Ia tumor, unilateral oophorectomy was associated with remarkably better OS and DSS compared with bilateral oophorectomy, with a 5-year OS rate of 89.9% for unilateral oophorectomy and 87.9% for bilateral oophorectomy (OS: $p < 0.001$; DSS: $p = 0.01$) (**Figure 3A** and **Figure S3A**). For stage-Ib and stage-Ic ovarian tumor, there was no significant difference between the OS and DSS of patients treated by unilateral oophorectomy and those of patients treated by bilateral

TABLE 1 | Characteristics of patients included in this study.

Characteristics	No. of patients (%)	No. of deaths (%)	Surgical procedure	
			Unilateral oophorectomy (%)	Bilateral oophorectomy (%)
Total	28,480 (100%)	11,517 (100%)	2,145 (100%)	13,678 (100%)
Age				
15-39	2,497 (8.8%)	366 (3.2%)	842 (39.3%)	568 (4.2%)
40-59	12,174 (42.7%)	3,634 (31.6%)	678 (31.6%)	5,743 (42%)
60-79	11,549 (40.6%)	5,761 (50%)	466 (21.7%)	6,269 (45.8%)
80+	2,260 (7.9%)	1,756 (15.2%)	159 (7.4%)	1,098 (8%)
Race				
White	23,532 (82.6%)	9,716 (84.4%)	1,674 (78%)	11,417 (83.5%)
AI/AN	179 (0.6%)	74 (0.6%)	14 (0.7%)	89 (0.7%)
API	2,838 (10%)	818 (7.1%)	247 (11.5%)	1,272 (9.3%)
Black	1,806 (6.3%)	898 (7.8%)	190 (8.9%)	858 (6.3%)
Unknown	125 (0.4%)	11 (0.1%)	20 (0.9%)	42 (0.3%)
Hispanic origin				
Non-Hispanic	25,045 (87.9%)	10,385 (90.2%)	1,759 (82%)	12,101 (88.5%)
Hispanic	3,435 (12.1%)	1,132 (9.8%)	386 (18%)	1,577 (11.5%)
Year of diagnosis				
2000-2009	15,283 (53.7%)	8,025 (69.7%)	1,292 (60.2%)	7,021 (51.3%)
2010-2017	13,197 (46.3%)	3,492 (30.3%)	853 (39.8%)	6,657 (48.7%)
Rural/urban status				
Urban	3,064 (10.8%)	1,431 (12.4%)	204 (9.5%)	1,497 (10.9%)
Rural	25,392 (89.2%)	10,071 (87.4%)	1,939 (90.4%)	12,169 (89%)
Unknown	24 (0.1%)	15 (0.1%)	2 (0.1%)	12 (0.1%)
Median house-hold income[1]				
Low	370 (1.3%)	166 (1.4%)	33 (1.5%)	171 (1.3%)
Median	18,925 (66.5%)	7,859 (68.2%)	1,500 (69.9%)	9,134 (66.8%)
High	9,184 (32.2%)	3,491 (30.3%)	612 (28.5%)	4,372 (32%)
Unknown	1 (0.004%)	1 (0.009%)		1 (0%)
FIGO stage				
Stage Ia	8,314 (29.2%)	1,528 (13.3%)	1,138 (53.1%)	2,990 (21.9%)
Stage Ib	145 (0.5%)	38 (0.3%)	9 (0.4%)	57 (0.4%)
Stage Ic	4,551 (16%)	989 (8.6%)	416 (19.4%)	1,636 (12%)
Stage I, NOS	331 (1.2%)	90 (0.8%)	55 (2.6%)	103 (0.8%)
Stage IIa	1,165 (4.1%)	402 (3.5%)	68 (3.2%)	518 (3.8%)
Stage IIb	1,607 (5.6%)	599 (5.2%)	79 (3.7%)	813 (5.9%)
Stage IIc	1,308 (4.6%)	550 (4.8%)	62 (2.9%)	602 (4.4%)
Stage II, NOS	210 (0.7%)	120 (1%)	17 (0.8%)	102 (0.7%)
Stage IIIa	780 (2.7%)	386 (3.4%)	28 (1.3%)	389 (2.8%)
Stage IIIb	1,127 (4%)	617 (5.4%)	35 (1.6%)	681 (5%)
Stage IIIc	6,613 (23.2%)	4,400 (38.2%)	153 (7.1%)	4,544 (33.2%)
Stage III, NOS	2,329 (8.2%)	1,798 (15.6%)	85 (4%)	1,243 (9.1%)
Histology				
Clear cell	3,771 (13.2%)	1,138 (9.9%)	191 (8.9%)	1,575 (11.5%)
Endometrioid	5,908 (20.7%)	1,446 (12.6%)	439 (20.5%)	2,581 (18.9%)
Mucinous	4,022 (14.1%)	1,074 (9.3%)	680 (31.7%)	1,379 (10.1%)
Serous	14,779 (51.9%)	7,859 (68.2%)	835 (38.9%)	8,143 (59.5%)
Grade				
Grade I	3,808 (13.4%)	675 (5.9%)	492 (22.9%)	1,437 (10.5%)
Grade II	5,202 (18.3%)	1,740 (15.1%)	410 (19.1%)	2,272 (16.6%)
Grade III	8,511 (29.9%)	4,562 (39.6%)	372 (17.3%)	4,742 (34.7%)
Grade IV	4,448 (15.6%)	1,905 (16.5%)	155 (7.2%)	2,671 (19.5%)

AI/AN, American Indian/Alaska Native; API, Asian or Pacific Islander.

[1]Low income referred to those with a median house-hold income of less than $35,000. Median income referred to those with a median house-hold income ranged from $35,000 to $75,000. High income referred to those with a median house-hold income of more than $75,000.

oophorectomy (**stage Ib**: OS: p = 0.6; DSS: p = 0.8; **stage Ic**: OS: p = 0.06; DSS: p = 0.2) (**Figures 3B, C** and **Figures S3B, C**). For stage-IIa tumors, the OS and DSS after unilateral oophorectomy were significantly worse than those after bilateral oophorectomy (5-year OS: 50.3% vs. 72.0%, p < 0.001; 5-year DSS: 61.6% vs. 72.0%, p < 0.001) (**Figure 3D** and **Figure S3D**). For stage-IIb/IIc tumors, there was no significant difference between the OS and DSS of patients treated by unilateral oophorectomy and those of patients treated by bilateral oophorectomy (OS: p = 0.6; DSS: p = 0.6) (**Figure 3E** and **Figure S3E**). For stage-III tumors, the OS

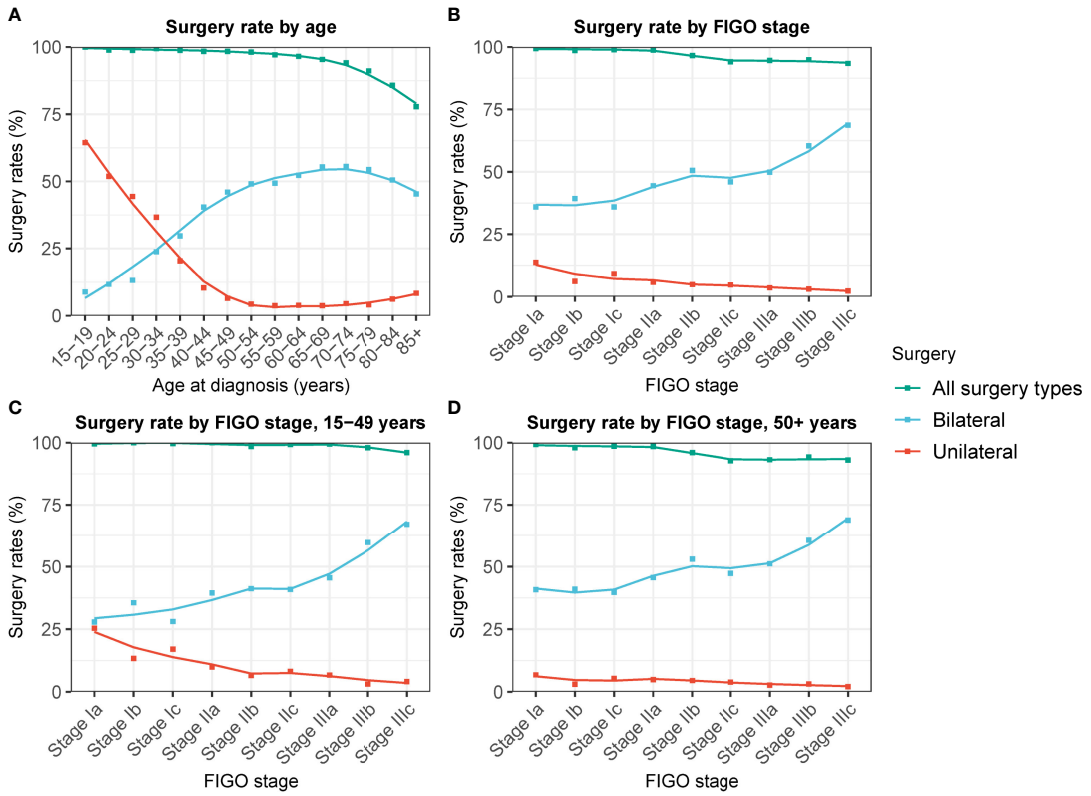


FIGURE 1 | Changes in unilateral and bilateral oophorectomy rate of patients with ovarian cancer by FIGO stage and age at cancer diagnosis. **(A)** Changes in unilateral and bilateral oophorectomy rate of patients with ovarian cancer of all stage by age at cancer diagnosis. **(B)** Changes in unilateral and bilateral oophorectomy rate of patients with ovarian cancer by FIGO stage. **(C)** Changes in unilateral and bilateral oophorectomy rate of patients aged 15-49 years with ovarian cancer by FIGO stage. **(D)** Changes in unilateral and bilateral oophorectomy rate of patients aged 50+ years with ovarian cancer by FIGO stage.

and DSS after unilateral oophorectomy were significantly worse than those after bilateral oophorectomy (OS: $p < 0.001$; DSS: $p < 0.001$) (**Figure 3F** and **Figure S3F**).

For low-grade and high-grade stage-I ovarian tumors, there was no significant difference between the OS and DSS of patients treated by unilateral oophorectomy and those of patients treated by bilateral oophorectomy (**Figures 4A–D** and **Figures S4B, C**). The OS of patients with low-grade and high-grade stage-IIa ovarian tumors undergoing unilateral oophorectomy were worse than those of patients undergoing bilateral oophorectomy (low-grade: $p = 0.03$; high-grade: $p < 0.001$) (**Figures 4E, F**). The DSS of patients with high-grade stage-IIa ovarian tumor undergoing unilateral oophorectomy were worse than those of patients undergoing bilateral oophorectomy (high-grade: $p < 0.001$) (**Figure S4F**).

We further analyzed the survival after unilateral oophorectomy or bilateral oophorectomy in patients with ovarian cancers of different histology (**Figures S5–S8**). We found that, except for high-grade stage-Ic serous ovarian cancer (Due to the inadequate cases with stage-Ib tumors, stage-Ib tumors were not included for further analyses hereafter), unilateral oophorectomy was comparable to bilateral oophorectomy in low-grade and high-grade stage-I ovarian cancer of any histology, including serous, mucinous, endometrioid, and clear cell carcinoma (**Figures S5–S8**). For high-grade stage-Ic serous ovarian cancer, the OS after unilateral oophorectomy was significantly worse than that after bilateral oophorectomy ($p = 0.03$) (**Figure S5D**).

Survival Analysis of Surgical Interventions by Stage and Age at Cancer Diagnosis

Female patients of reproductive age had a greater desire to preserve fertility; thus, we investigated the prevalence and therapeutic effects of unilateral oophorectomy in this peculiar population (**Figure 5** and **Figure S9**). Among the reproductive-age women younger than 50 years, 22.8% received unilateral oophorectomy, and 27.9% underwent bilateral oophorectomy. We found that OS and DSS of patients with low-grade and high-grade stage-I receiving unilateral oophorectomy were comparable to those of patients receiving bilateral oophorectomy (all $p > 0.05$) (**Figure 6** and **Figure S9**). For patients aged 15–59 years with high-grade stage-Ic ovarian tumor, the OS and DSS of patients receiving unilateral oophorectomy were similar to those of patients receiving bilateral oophorectomy (OS: $p = 1$; DSS: $p = 0.7$) (**Figure 5F** and **Figure S9F**).

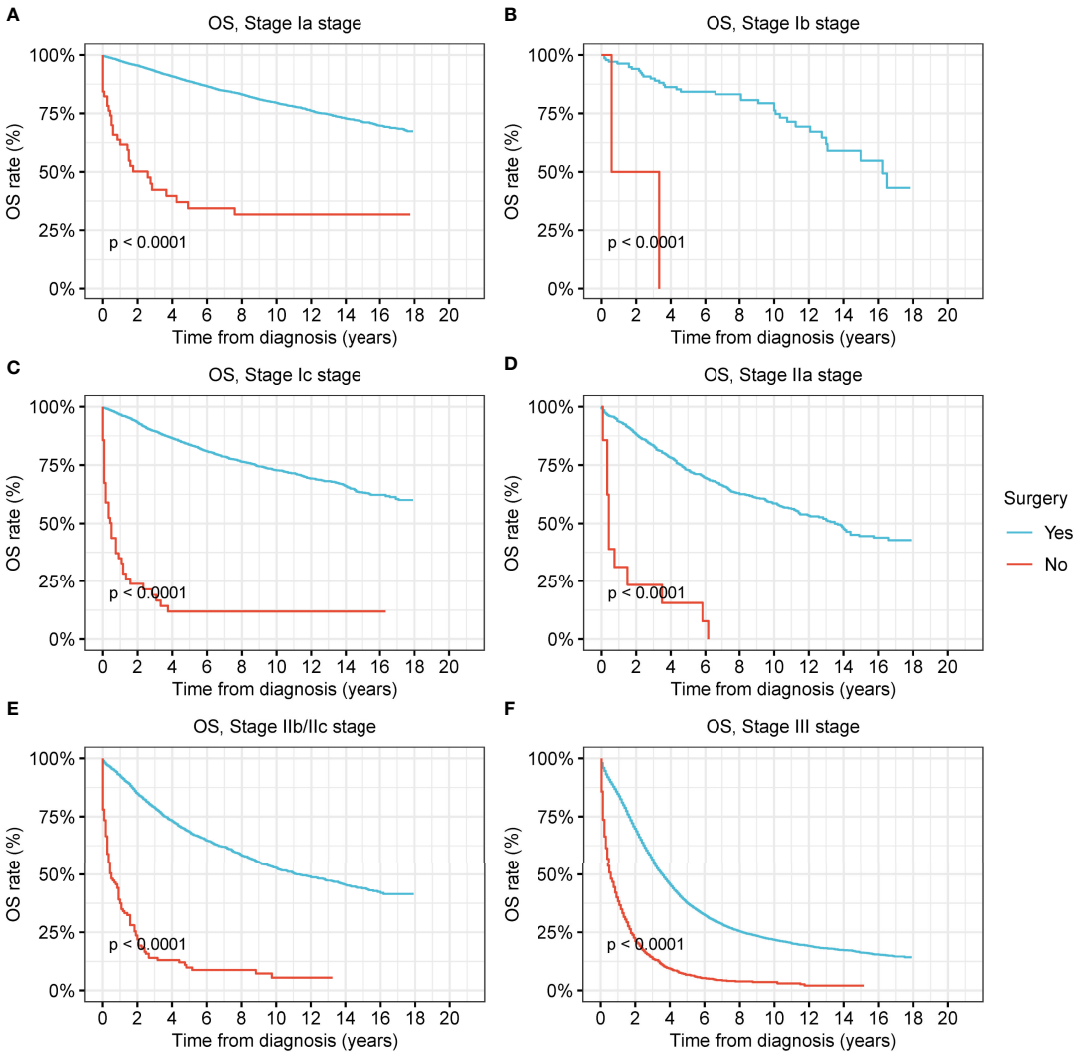


FIGURE 2 | Overall survival (OS) of patients with ovarian cancer by surgery. **(A)** OS of patients with stage-Ia ovarian cancer by surgery. **(B)** OS of patients with stage-Ib ovarian cancer by surgery. **(C)** OS of patients with stage-Ic ovarian cancer by surgery. **(D)** OS of patients with stage-IIa ovarian cancer by surgery. **(E)** OS of patients with stage-IIb/IIc ovarian cancer by surgery. **(F)** OS of patients with stage-III ovarian cancer by surgery.

For those aged 50 years and older, the OS of patients with low-grade and high-grade stage-I receiving unilateral oophorectomy was significantly worse than that of patients receiving bilateral oophorectomy (low-grade: $p < 0.001$; high-grade: $p < 0.001$) (**Figures 6A, B**). The DSS of patients aged 50 years and older with low-grade stage-I receiving unilateral oophorectomy were similar to those of patients receiving bilateral oophorectomy ($p = 0.2$) (**Figure S10A**). The DSS of patients aged 50 years and older with high-grade stage-I receiving unilateral oophorectomy were worse than that of patients receiving bilateral oophorectomy ($p < 0.001$) (**Figure S10B**). For patients aged 50 years and older with low-grade stage-Ia ovarian tumor, the OS and DSS of patients receiving unilateral oophorectomy were significantly worse than those of patients receiving bilateral oophorectomy (OS: $p < 0.001$; DSS: $p = 0.01$) (**Figure 6C** and **Figure S10C**).

Comorbidity Analysis of Patients With Ovarian Cancer Treated by Different Surgical Interventions

A comorbidity analysis was carried out on the causes of death for the patients with ovarian cancer (**Figure 7**, **Figure S11**, and **Figure S12**). In stage-I ovarian cancer, the CMR of cancer-related deaths was significantly lower in patients who underwent unilateral oophorectomy than in those who underwent bilateral oophorectomy ($p < 0.001$) (**Figure 7A**). CVDs were also remarkably decreased in patients who underwent unilateral oophorectomy (5-year CMR: unilateral oophorectomy, 1.5%; bilateral oophorectomy, 1.7%; $p = 0.04$) (**Figure 7C**). For stage-II and stage-III tumors, there were no significant differences between the CMR of unilateral and bilateral oophorectomy for both cancer-related deaths and non-cancer comorbidities (**Figure S11** and **Figure S12**).

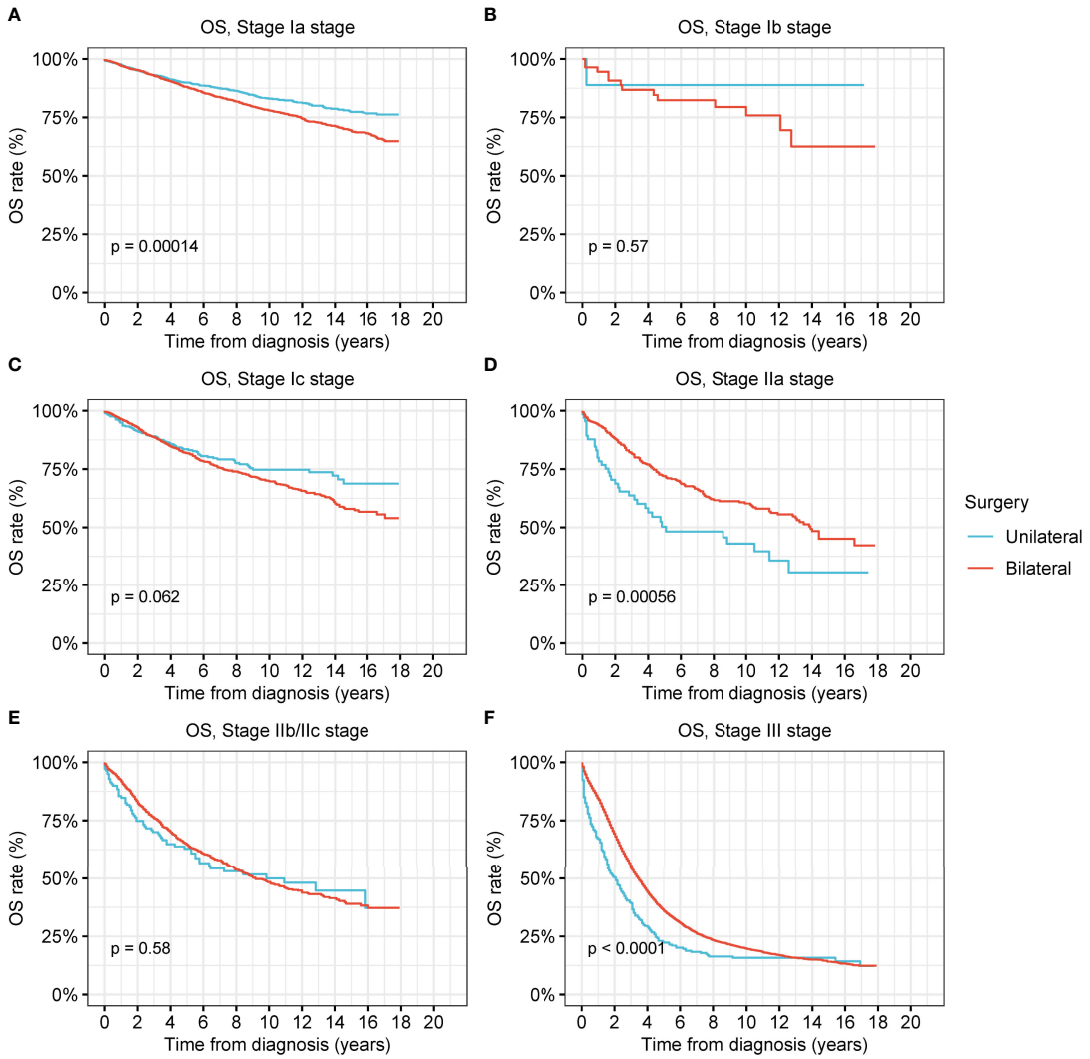


FIGURE 3 | Overall survival (OS) of patients with ovarian cancer by cancer stage and different types of surgical operation. **(A)** OS of patients with stage-Ia ovarian cancer by different types of surgical operation. **(B)** OS of patients with stage-Ib ovarian cancer by different types of surgical operation. **(C)** OS of patients with stage-Ic ovarian cancer by different types of surgical operation. **(D)** OS of patients with stage-IIa ovarian cancer by different types of surgical operation. **(E)** OS of patients with stage-IIb/IIc ovarian cancer by different types of surgical operation. **(F)** OS of patients with stage-III ovarian cancer by different types of surgical operation.

DISCUSSION

In this population-based study involving more than 28,000 women with ovarian cancer, we compared the prevalence and therapeutic efficacy of unilateral oophorectomy with bilateral oophorectomy. We found that unilateral oophorectomy exhibited excellent oncological superiority and was equivalent to bilateral oophorectomy for stage-I ovarian tumors among women of productive age; this equivalence to bilateral oophorectomy remained true for high-grade stage-Ic ovarian tumors. For patients aged 50 years and older, the performance of unilateral oophorectomy was worse than that of bilateral oophorectomy, even for low-grade stage-Ia ovarian tumors. These results indicated that unilateral oophorectomy was valuable for stage-I ovarian tumors among women of productive age.

Unilateral oophorectomy has the advantages of preserving fertility and part or full function of the ovary, while fertility is completely destroyed after bilateral oophorectomy. Fertility preservation is an important component of cervical cancer survivors' overall quality of life (20). Fertility-preserving procedures in cases of borderline ovarian tumors are now well-established because this type of lesion is often diagnosed in young women whose fertility issues are primordial (21). The status of fertility-preserving procedures in malignant ovarian cancer remains controversial. Data on the conservative management of ovarian cancer are still limited; however, the oncologic safety of fertility-sparing procedures in early ovarian

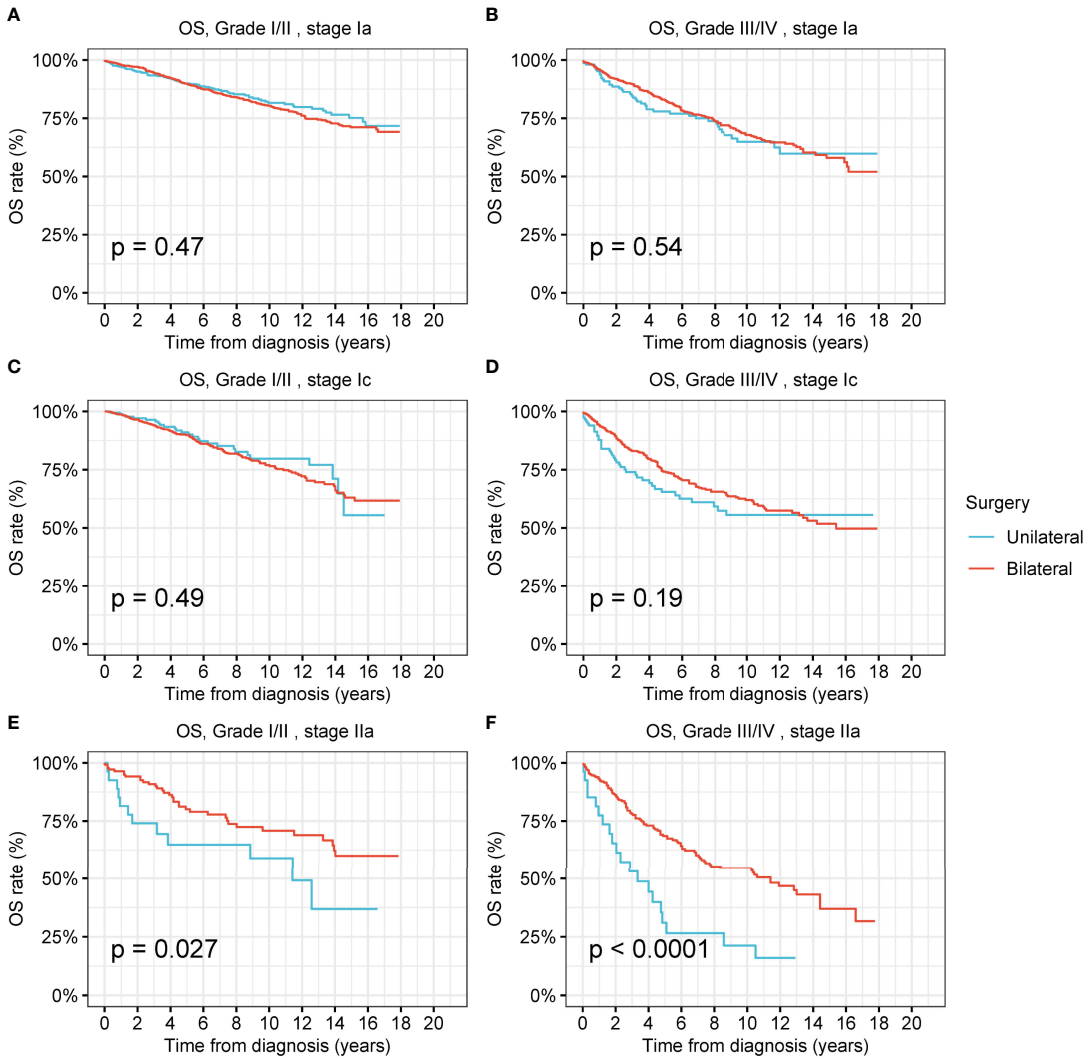


FIGURE 4 | Overall survival (OS) of patients with ovarian cancer by cancer stage, cancer grade and different types of surgical operation. **(A)** OS of patients with low-grade stage-Ia ovarian cancer by different types of surgical operation. **(B)** OS of patients with high-grade stage-Ia ovarian cancer by different types of surgical operation. **(C)** OS of patients with low-grade stage-Ic ovarian cancer by different types of surgical operation. **(D)** OS of patients with high-grade stage-Ic ovarian cancer by different types of surgical operation. **(E)** OS of patients with low-grade stage-IIa ovarian cancer by different types of surgical operation. **(F)** OS of patients with high-grade stage-IIa ovarian cancer by different types of surgical operation.

cancer has been confirmed (22–24). Researchers also proposed that high-risk disease should not be considered a contraindication to conservative surgery (23, 25). This procedure is mainly limited to women with IA grade 1 disease who wish to preserve their fertility. For some investigators, fertility-sparing procedures were found to be safe in women with more advanced-stage disease until stage IC (26). Our results further confirmed the potential candidates for this procedure. We found that age is an important factor in selecting potential candidates, as unilateral oophorectomy is valuable for stage-I ovarian tumors among women of productive age, even for high-grade stage-Ic diseases. In contrast, the performance of unilateral oophorectomy is demonstrated to be greatly weakened by age. For patients aged 50 years or older, the long-term survival after unilateral oophorectomy is worse than that after bilateral oophorectomy, even for the tumors with the lowest risk, namely, the low-grade stage-Ia tumors. Therefore, we recommend the inclusion criteria of unilateral oophorectomy be extended to high-grade stage-Ic diseases; in contrast, for those aged 50 years and older without fertility desire, unilateral oophorectomy is not recommended, and bilateral oophorectomy should be adopted as the first choice.

The major limitations of this procedure are the underlying risks of residual tumor, tumor recurrence, and possible newly occurring carcinoma in the remaining ovarian tissue. To address these concerns, postoperative chemotherapy, radiotherapy, or molecular targeted therapy should be employed for the high-risk population (27). Precise diagnosis and stage of the disease before

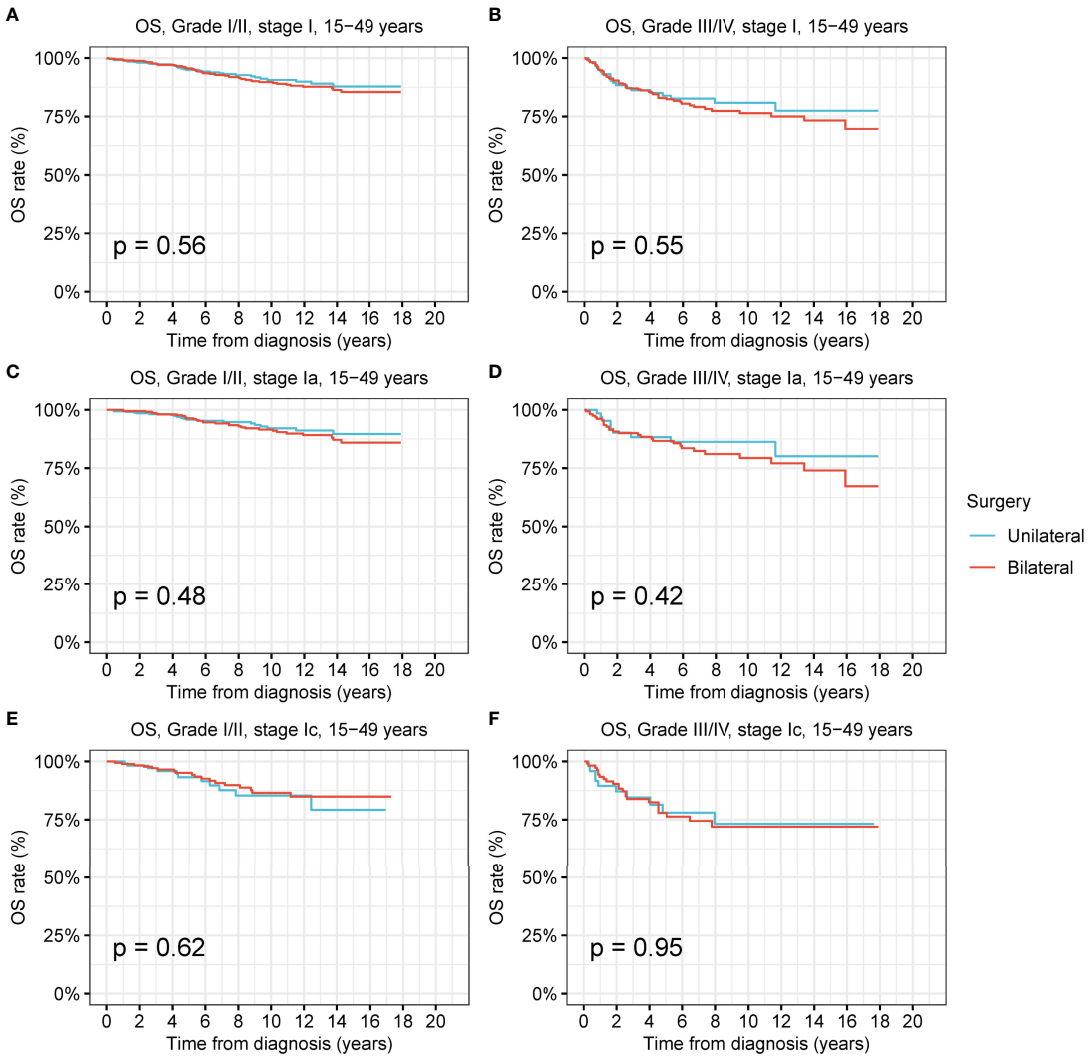


FIGURE 5 | Overall survival (OS) of patients of productive age (15-50 years) with ovarian cancer by cancer stage, cancer grade and different types of surgical operation. **(A)** OS of patients of productive age with low-grade stage-I ovarian cancer by different types of surgical operation. **(B)** OS of patients of productive age with high-grade stage-I ovarian cancer by different types of surgical operation. **(C)** OS of patients of productive age with low-grade stage-Ia ovarian cancer by different types of surgical operation. **(D)** OS of patients of productive age with high-grade stage-Ia ovarian cancer by different types of surgical operation. **(E)** OS of patients of productive age with low-grade stage-Ic ovarian cancer by different types of surgical operation. **(F)** OS of patients of productive age with high-grade stage-Ic ovarian cancer by different types of surgical operation.

the surgery are vital to guarantee the tumor clearance of surgery (28). Minimally invasive surgery, if necessary, is a viable approach to accurately diagnose and stage the tumor (29–31). Routine screening and active follow-up should be performed after this procedure to avoid future rumor recurrence or newly developed tumors.

Our results revealed that unilateral oophorectomy can decrease the long-term mortality risk of CVD; this might be a consequence of the stable hormone levels generated from the preservation of part or full ovarian function, while bilateral oophorectomy will lead to a sudden disruption in the secretion of sex hormones, mainly estrogen. In addition to its powerful roles in regulating the development and homeostasis of reproductive tissues, estrogen provides critical signaling and trophic support to a range of tissues throughout the body and across the lifespan through the activation of estrogen receptors, ERα (encoded by ESR1), ERβ (encoded by ESR2), and G-protein-coupled estrogen receptor (GPER; also known as GPR30) (32–35). Estrogens act in target tissues through estrogen receptors and G protein-coupled ERα to reduce CVD risk (33). Premenopausal women are protected from CVD relative to age-matched men (36, 37), and low levels of estrogens (i.e., hypo-estrogenemia) in young women (18–40 years) increase CVD risk (38). Moreover, early menopause (before 40 years of age) (39) is associated with accelerated atherosclerosis, a 2.6-fold increase in CVD risk (40), and an

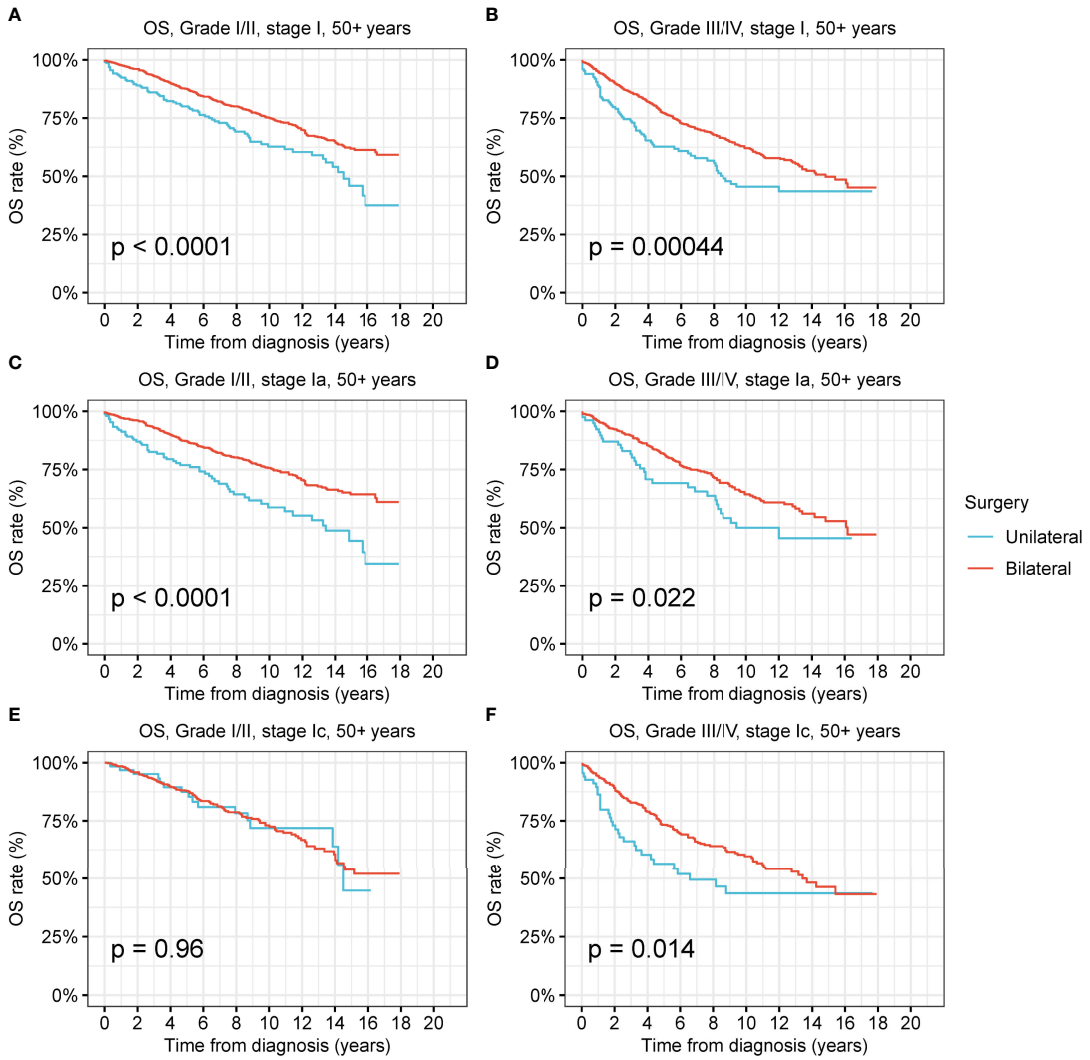


FIGURE 6 | Overall survival (OS) of patients aged 50+ years with ovarian cancer by cancer stage, cancer grade and different types of surgical operation. **(A)** OS of patients aged 50+ years with low-grade stage-I ovarian cancer by different types of surgical operation. **(B)** OS of patients aged 50+ years with high-grade stage-I ovarian cancer by different types of surgical operation. **(C)** OS of patients aged 50+ years with low-grade stage-Ia ovarian cancer by different types of surgical operation. **(D)** OS of patients aged 50+ years with high-grade stage-Ia ovarian cancer by different types of surgical operation. **(E)** OS of patients aged 50+ years with low-grade stage-Ic ovarian cancer by different types of surgical operation. **(F)** OS of patients aged 50+ years with high-grade stage-Ic ovarian cancer by different types of surgical operation.

increased risk of CVD-related mortality (41, 42). These studies supported the role of estrogens in determining CVD risk. Therefore, after bilateral oophorectomy, the destruction of ovarian function results in the demand for hormone replacement therapy (HRT), while unilateral oophorectomy, which maintains part or whole of ovarian function, does not need HRT. Furthermore, HRT may be difficult and even dangerous for some women. Endogenous estrogen from the remaining ovary after unilateral oophorectomy eliminates these difficulties and dangers.

This study had several limitations. First, given the study's descriptive and retrospective design, we could not prospectively assess the effects of surgical interventions in patients with ovarian cancer and could not draw causal inferences. Second, we could not assess the patients' physical conditions, comorbidities, and other health factors. Given the high incidence of comorbidities, cognitive impairment, frailty, functional losses, social isolation, and other factors in this population, it is important to assess these variables when proposing treatment decisions; however, the SEER program did not provide this information. Third, we could not investigate the influence of other therapies, such as radiotherapy or chemotherapy. The SEER program only provided detailed information on surgical operations.

Notwithstanding these limitations, this study may contribute to the surgical interventions and cancer surveillance literature for ovarian cancer. The strength of this study is that the data were

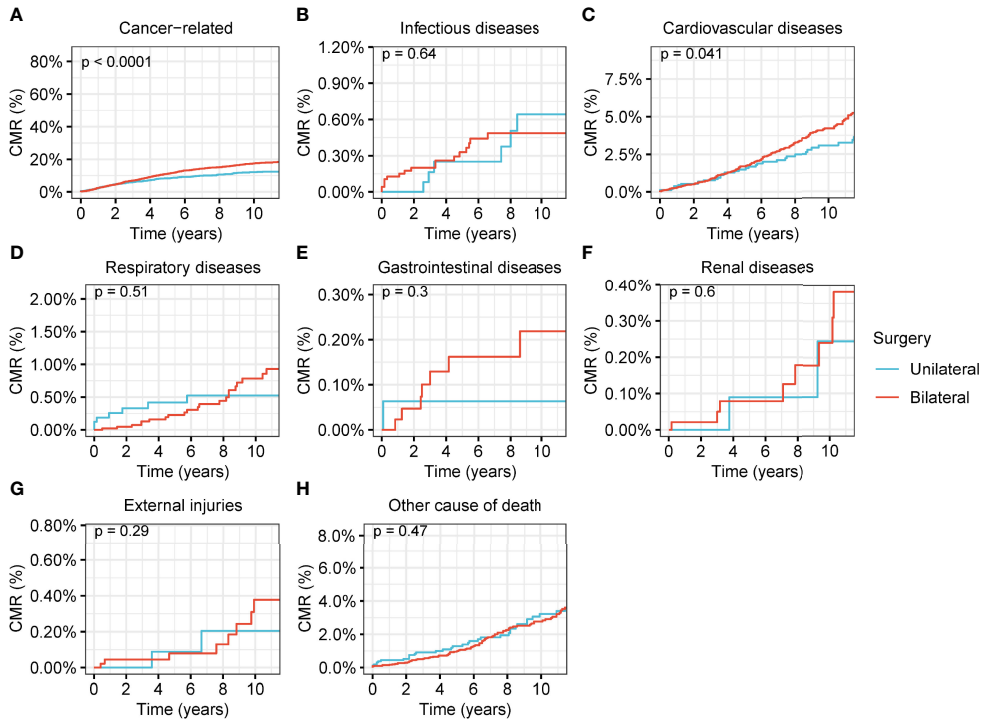


FIGURE 7 | Cumulative mortality rate (CMR) among women of productive age with stage-I ovarian cancer by different types of surgical operation. **(A)** CMR from cancer-related deaths among women of productive age with stage-I ovarian cancer by different types of surgical operation. **(B)** CMR from infectious diseases among women of productive age with stage-I ovarian cancer by different types of surgical operation. **(C)** CMR from cardiovascular diseases among women of productive age with stage-I ovarian cancer by different types of surgical operation. **(D)** CMR from respiratory diseases among women of productive age with stage-I ovarian cancer by different types of surgical operation. **(E)** CMR from gastrointestinal diseases among women of productive age with stage-I ovarian cancer by different types of surgical operation. **(F)** CMR from renal diseases among women of productive age with stage-I ovarian cancer by different types of surgical operation. **(G)** CMR from external injuries among women of productive age with stage-I ovarian cancer by different types of surgical operation. **(H)** CMR from other non-cancer causes among women of productive age with stage-I ovarian cancer by different types of surgical operation.

derived from a high-quality, population-based, real-world cancer registry. Real-world data reflect the realistic effects of different interventions in the real scenario of cancer treatment, which may avoid the limitations of clinical trials. The implications of this study are important for the development of ovarian cancer.

CONCLUSIONS

In conclusion, unilateral oophorectomy exhibited excellent oncological superiority and was equivalent to bilateral oophorectomy for stage-I ovarian tumors among women of productive age. For women of reproductive age, the criteria for unilateral oophorectomy can be appropriately broadened to high-grade stage-Ic diseases because of the comparable performance of unilateral oophorectomy in this population. For those aged 50 years and older without fertility desire, unilateral oophorectomy is not recommended, and bilateral oophorectomy should be adopted as the first choice. Moreover, unilateral oophorectomy can reduce mortality and CVD risk in women. As unilateral oophorectomy has the advantage of preserving fertility and the hormone secretion function of the ovary, guidance on selecting appropriate candidates should be developed.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://www.seer.cancer.gov.

AUTHOR CONTRIBUTIONS

WZ and KW designed the study. JX, YL, and GF performed the research. ZZ, KW, and WZ developed the typescript. All of the authors approved the typescript and agree to submit for publication.

FUNDING

This study was supported by National Key Research and Development Program of China (2021YFC2009105) and National Natural Science Foundation of China (82002760).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.866443/full#supplementary-material

REFERENCES

1. Global Burden of Disease 2019 Cancer Collaboration, Kocarnik JM, Compton K, Dean FE, Weijia F, Brian LG, et al. Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life Years for 29 Cancer Groups From 2010 to 2019: A Systematic Analysis for the Global Burden of Disease Study 2019. *JAMA Oncol* (2021) 8:420–44. doi: 10.1001/jamaoncol.2021.6987
2. American Cancer Society. *Key Statistics for Ovarian Cancer* . Available at: https://www.cancer.org/cancer/ovarian-cancer/about/key-statistics.html (Accessed January 1, 2022).
3. The SEER program. *Cancer Stat Facts: Ovarian Cancer* . Available at: https://seer.cancer.gov/statfacts/html/ovary.html (Accessed January 1, 2022).
4. N Howlader, AM Noone, M Krapcho, D Miller, A Brest, M Yu, J Ruhl, Z Tatalovich, A Mariotto, DR Lewis, HS Chen, EJ Feuer, KA Cronin eds. *SEER Cancer Statistics Review, 1975-2018*. Bethesda, MD: National Cancer Institute (2021). Available at: https://seer.cancer.gov/csr/1975_2018/.
5. Allemani C, Weir HK, Carreira H, Harewood R, Spika D, Wang XS, et al. Global Surveillance of Cancer Survival 1995-2009: Analysis of Individual Data for 25,676,887 Patients From 279 Population-Based Registries in 67 Countries (CONCORD-2). *Lancet* (2015) 385(9972):977–1010. doi: 10.1016/S0140-6736(14)62038-9
6. Nezhat FR, Pejovic T, Finger TN, Khalil SS. Role of Minimally Invasive Surgery in Ovarian Cancer. *J Minim Invasive Gynecol* (2013) 20(6):754–65. doi: 10.1016/j.jmig.2013.04.027
7. Dewilde K, Moerman P, Leunen K, Amant F, Neven P, Vergote I. Staging With Unilateral Salpingo-Oophorectomy and Expert Pathological Review Result in No Recurrences in a Series of 81 Intestinal-Type Mucinous Borderline Ovarian Tumors. *Gynecol Obstet Invest* (2018) 83(1):65–9. doi: 10.1159/000478929
8. Tsai HW, Ko CC, Yeh CC, Chen YJ, Twu NF, Chao KC, et al. Unilateral Salpingo-Oophorectomy as Fertility-Sparing Surgery for Borderline Ovarian Tumors. *J Chin Med Assoc* (2011) 74(6):250–4. doi: 10.1016/j.jcma.2011.04.003
9. Surveillance Research Program. *National Cancer Institute SEER*Stat Software Version 8.3.8* . Available at: www.seer.cancer.gov/seerstat.
10. Mathew G, Agha R, Group S. STROCSS 2021: Strengthening the Reporting of Cohort, Cross-Sectional and Case-Control Studies in Surgery. *Int J Surg* (2021) 96:106165. doi: 10.1016/j.ijsu.2021.106165
11. Surveillance, Epidemiology, and End Results (SEER) Program. SEER*Stat Database. In: *Mortality - All COD, Aggregated With State, Total U.S. (1969-2017) <Katrina/Rita Population Adjustment>, National Cancer Institute, DCCPS, Surveillance Research Program, Released December 2019. Underlying Mortality Data Provided by NCHS*. Bethesda, MD: National Cancer Institute. Available at: www.cdc.gov/nchs. Bethesda, MD National Cancer Institute
12. The SEER program. *SEER Combined/AJCC Cancer Staging* . Available at: https://seer.cancer.gov/seerstat/variables/seer/ajcc-stage/ (Accessed April 1, 2020).
13. The SEER program. *Surgery Codes of Ovary* . Available at: https://seer.cancer.gov/manuals/2022/AppendixC/Surgery_Codes_Ovary_2022.pdf (Accessed January 1, 2022).
14. Yang P, Zheng Y, Zhang L, Hou X. Endoscopic Excision for Proximal Colonic Cancer: A Challenge in Transanal Endoscopic Microsurgery - Letter to the Editor. *Int J Surg* (2021) 92:106035. doi: 10.1016/j.ijsu.2021.106035
15. Zheng Y, Yang P, Chen J, Yu K, Ye Y, Zhang L, et al. Endoscopic Excision as a Viable Alternative to Major Resection for Early Duodenal Cancers: A Population-Based Cohort Study. *Int J Surg 106644* (2022) 101:106644. doi: 10.1016/j.ijsu.2022.106644
16. Anderson C, Lund JL, Weaver MA, Wood WA, Olshan AF, Nichols HB. Noncancer Mortality Among Adolescents and Young Adults With Cancer. *Cancer* (2019) 125(12):2107–14. doi: 10.1002/cncr.32063
17. Zheng Y, Ye Y, Chen L, Ma Z, Liu L, Cheng G, et al. Prevalence and Outcomes of Focal Ablation Versus Prostatectomy for Elderly Patients With Prostate Cancer: A Population-Based Study. *J Natl Cancer Center* (2021) 2:25–32. doi: 10.1016/j.jncc.2021.11.005
18. Gschwend JE, Dahm P, Fair WR. Disease Specific Survival as Endpoint of Outcome for Bladder Cancer Patients Following Radical Cystectomy. *Eur Urol* (2002) 41(4):440–8. doi: 10.1016/S0302-2838(02)00060-X
19. R Core Team. *R: A Language and Environment for Statistical Computing* (2019). Vienna, Austria: R Foundation for Statistical Computing. Available at: https://www.R-project.org/ (Accessed December 12, 2019).
20. Martinez A, Poilblanc M, Ferron G, De Cuypere M, Jouve E, Querleu D. Fertility-Preserving Surgical Procedures, Techniques. *Best Pract Res Clin Obstet Gynaecol* (2012) 26(3):407–24. doi: 10.1016/j.bpobgyn.2012.01.009
21. Vasconcelos I, de Sousa Mendes M. Conservative Surgery in Ovarian Borderline Tumours: A Meta-Analysis With Emphasis on Recurrence Risk. *Eur J Cancer* (2015) 51(5):620–31. doi: 10.1016/j.ejca.2015.01.004
22. Morice P, Leblanc E, Rey A, Baron M, Querleu D, Blanchot J, et al. Gcclcc and Sfog: Conservative Treatment in Epithelial Ovarian Cancer: Results of a Multicentre Study of the GCCLCC (Groupe Des Chirurgiens De Centre De Lutte Contre Le Cancer) and SFOG (Societe Francaise D'oncologie Gynecologique). *Hum Reprod* (2005) 20(5):1379–85. doi: 10.1093/humrep/deh777
23. Bogani G, Ditto A, Pinelli C, Lopez S, Chiappa V, Raspagliesi F. Ten-Year Follow-Up Study of Long-Term Outcomes After Conservative Surgery for Early-Stage Ovarian Cancer. *Int J Gynaecol Obstet* (2020) 150(2):169–76. doi: 10.1002/ijgo.13199
24. Ditto A, Martinelli F, Bogani G, Lorusso D, Carcangiu M, Chiappa V, et al. Long-Term Safety of Fertility Sparing Surgery in Early Stage Ovarian Cancer: Comparison to Standard Radical Surgical Procedures. *Gynecol Oncol* (2015) 138(1):78–82. doi: 10.1016/j.ygyno.2015.05.004
25. Ditto A, Leone Roberti Maggiore U, Bogani G, Martinelli F, Chiappa V, Evangelista MT, et al. Predictive Factors of Recurrence in Patients With Early-Stage Epithelial Ovarian Cancer. *Int J Gynaecol Obstet* (2019) 145(1):28–33. doi: 10.1002/ijgo.12769
26. Park JY, Kim DY, Suh DS, Kim JH, Kim YM, Kim YT, et al. Outcomes of Fertility-Sparing Surgery for Invasive Epithelial Ovarian Cancer: Oncologic Safety and Reproductive Outcomes. *Gynecol Oncol* (2008) 110(3):345–53. doi: 10.1016/j.ygyno.2008.04.040
27. Bogani G, Ditto A, Lopez S, Bertolina F, Murgia F, Pinelli C, et al. Adjuvant Chemotherapy vs. Observation in Stage I Clear Cell Ovarian Carcinoma: A Systematic Review and Meta-Analysis. *Gynecol Oncol* (2020) 157(1):293–8. doi: 10.1016/j.ygyno.2019.12.045
28. Bogani G, Tagliabue E, Ditto A, Signorelli M, Martinelli F, Casarin J, et al. Assessing the Risk of Pelvic and Para-Aortic Nodal Involvement in Apparent Early-Stage Ovarian Cancer: A Predictors- and Nomogram-Based Analyses. *Gynecol Oncol* (2017) 147(1):61–5. doi: 10.1016/j.ygyno.2017.07.139
29. Bogani G, Borghi C, Leone Roberti Maggiore U, Ditto A, Signorelli M, Martinelli F, et al. Minimally Invasive Surgical Staging in Early-Stage Ovarian Carcinoma: A Systematic Review and Meta-Analysis. *J Minim Invasive Gynecol* (2017) 24(4):552–62. doi: 10.1016/j.jmig.2017.02.013
30. Ran X, He X, Li Z. Comparison of Laparoscopic and Open Surgery for Women With Early-Stage Epithelial Ovarian Cancer. *Front Oncol* (2022) 12:879889. doi: 10.3389/fonc.2022.879889
31. Tinelli R, Dellino M, Nappi L, Sorrentino F, D'Alterio MN, Angioni S, et al. Left External Iliac Vein Injury During Laparoscopic Pelvic Lymphadenectomy

for Early-Stage Ovarian Cancer: Our Experience and Review of Literature. *Front Surg* (2022) 9:843641. doi: 10.3389/fsurg.2022.843641
32. Jia M, Dahlman-Wright K, Gustafsson JA. Estrogen Receptor Alpha and Beta in Health and Disease. *Best Pract Res Clin Endocrinol Metab* (2015) 29(4):557–68. doi: 10.1016/j.beem.2015.04.008
33. Morselli E, Santos RS, Criollo A, Nelson MD, Palmer BF, Clegg DJ. The Effects of Oestrogens and Their Receptors on Cardiometabolic Health. *Nat Rev Endocrinol* (2017) 13(6):352–64. doi: 10.1038/nrendo.2017.12
34. Prossnitz ER, Barton M. The G-Protein-Coupled Estrogen Receptor GPER in Health and Disease. *Nat Rev Endocrinol* (2011) 7(12):715–26. doi: 10.1038/nrendo.2011.122
35. Vrtacnik P, Ostanek B, Mencej-Bedrac S, Marc J. The Many Faces of Estrogen Signaling. *Biochem Med (Zagreb)* (2014) 24(3):329–42. doi: 10.11613/BM.2014.035
36. Yang XP, Reckelhoff JF. Estrogen, Hormonal Replacement Therapy and Cardiovascular Disease. *Curr Opin Nephrol Hypertens* (2011) 20(2):133–8. doi: 10.1097/MNH.0b013e3283431921
37. Anand SS, Islam S, Rosengren A, Franzosi MG, Steyn K, Yusufali AH, et al. Risk Factors for Myocardial Infarction in Women and Men: Insights From the INTERHEART Study. *Eur Heart J* (2008) 29(7):932–40. doi: 10.1093/eurheartj/ehn018
38. Kaplan JR, Manuck SB. Ovarian Dysfunction and the Premenopausal Origins of Coronary Heart Disease. *Menopause* (2008) 15(4 Pt 1):768–76. doi: 10.1097/gme.0b013e31815eb18e
39. Archer DF. Premature Menopause Increases Cardiovascular Risk. *Climacteric* (2009) 12 Suppl:1, 26–31. doi: 10.1080/13697130903013452
40. Kannel WB, Wilson PW. Risk Factors That Attenuate the Female Coronary Disease Advantage. *Arch Intern Med* (1995) 155(1):57–61. doi: 10.1001/archinte.1995.00430010063008
41. Jacobsen BK, Nilssen S, Heuch I, Kvale G. Does Age at Natural Menopause Affect Mortality From Ischemic Heart Disease? *J Clin Epidemiol* (1997) 50 (4):475–9. doi: 10.1016/s0895-4356(96)00425-8
42. Gold EB. The Timing of the Age at Which Natural Menopause Occurs. *Obstet Gynecol Clin North Am* (2011) 38(3):425–40. doi: 10.1016/j.ogc.2011.05.002

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership