Volume 21, No 2 | International Journal of Radiation Research, April 2023

Stereotactic radiotherapy and cytokines: preliminary analysis in oligometastatic Non-Small-Cell lung cancer

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► ***Original article***

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Received: March 2022
Final revised: September 2022
Accepted: November 2022

Int. J. Radiat. Res., April 2023; 21(2): 247-254

DOI: 10.52547/ijrr.21.2.10

Keywords: *Stereotactic radiotherapy, cytokines, oligometastatic, lung cancer.*

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ABSTRACT

***Background*:** Cytokines have a recognized role in the physiopathology of cancer disease and could be involved also in the "abscopal effect". Aim of this work is the preliminary analysis of inflammatory mediators in patients with oligometastatic non-small-cell lung cancer (NSCLC) undergoing stereotactic radiotherapy (SRT). ***Materials and Methods*:** This was a feasibility multi-institutional study that prospectively included oligometastatic NSCLC patients undergoing SRT from June 2018 until August 2018 and healthy controls. Blood samples were collected at three different time points (1-5 days before SRT, 1-5 days after SRT and 28-35 days after SRT). A commercially available kit was used for quantitative analysis of 44 inflammation molecules. Nine patients and four healthy controls were enrolled. ***Results*:** Several cytokines (54.5%) resulted undetectable in a significant percentage of the samples and were not further analyzed. Levels of seven inflammatory molecules (bDNF, MIP-1b, PDGF-bb, PIGF-1, RANTES, SDF1-a, and bNGF) showed significant variations after SRT in the NSCLC patients cohort. ***Conclusion*:** Significant plasmatic concentration changes after SRT were reported for a relevant proportion of the evaluated molecules. The results of this study will contribute to define a selection of cytokines and chemokines that will be analyzed in a prospective trial with a larger sample of patients.

INTRODUCTION

In the last years, systemic treatment of Non-Small Cell Lung Cancer (NSCLC) underwent a revolution due to the advances in the field of immunotherapy, as demonstrated by the impact of immune-checkpoint inhibitors on patients' prognosis both in the metastatic and in locally advanced setting [1, 2]. Simultaneously, the technological advance in image guidance, dose delivery and organ motion control led to the development of highly conformal radiotherapy techniques such as Stereotactic Radiation Therapy (SRT) that delivers high doses per-fraction to the tumor with steep gradient, thus sparing the surrounding healthy tissues. The clinical effectiveness of SRT is confirmed by the very high rates of local control both in early disease and in metastatic setting [1,2], although the underlying biological mechanisms have been only partially elucidated and likely involve vascular damage and immunostimulation leading to indirect tumor cell death [3].

Tumor cells either directly or indirectly killed by SRT release antigens that might induce cellular expression of Major Histocompatibility Complex class 1 (MHC-1), adhesion, costimulatory and immunomodulatory molecules, thus promoting antigen-presenting cells (APCs) activity with consequent priming of the lymphocytes and induction of anti-tumor adaptive immunity [3, 4]. This could explain the so-called "abscopal effect", a systemic response characterized by the regression of neoplastic sites outside of the irradiated volume, and support the intriguing hypothesis that radiotherapy could be used not only as a local treatment, but also as an anti-tumor vaccine [5]. Cytokines and chemokines play a pivotal role in the signal coordinating the immune system and are involved in multiple aspects of tumor biology, since their elaborate network mediates both antitumoral

[DOI: 10.52547/ijrr.21.2.10]

immune response and processes promoting carcinogenesis (such as chronic inflammation and immunotolerance) [6]. A deeper comprehension of the patterns of expression and activity of these molecules, although hindered by the redundancy and pleiotropism that characterize their activities and interactions, could offer a precious weapon to potentiate the effect of immunotherapy. Despite the large number of ongoing clinical trials assessing the link between immunotherapy and radiotherapy, the real physiology of the interaction is still largely unknown. Only a few studies analyzed variations in the plasma concentration of cytokines and inflammatory molecules induced by radiotherapy [7]. To the best of our knowledge, up to date no paper has been published on this topic focusing on metastatic NSCLC patients. The purpose of this study is to conduct a preliminary analysis of the plasmatic concentrations of several inflammatory mediators in patients with oligometastatic NSCLC undergoing SRT in order to identify eventual patterns induced by radiotherapy and possible correlations with already known pathophysiological mechanisms. The originality and novelty of this study consists in addressing this issue by weighing the attention mainly on feasibility and methodological problems. For these reasons, potential pitfalls regarding procedures, timing and inclusion criteria have been addressed in order to design a future prospective study.

MATERIALS AND METHODS

Patients' population

Patients affected by oligometastatic/ oligorecurrent NSCLC were prospectively enrolled in the present study. Inclusion criteria were as follows: performance status according to ECOG 0 or 1, normal renal and hepatic functions, white blood cell count ≥2,500/mm3, haemoglobin levels ≥9 g/dl, platelet cell count ≥100,000/mm3, age>18 years, histological diagnosis of NSCLC (adenocarcinoma or squamous cell carcinoma), synchronous or metachronous oligometastatic or oligoprogressive disease, defined as no more than five metastatic lesions involving a maximum of three organs (respectively at diagnosis, after or during the systemic therapy). Written informed consent concerning treatment risk and biological monitoring was obtained from each patient. SRT was permitted for any localization of disease (bone, brain, visceral metastases). The total dose and the fractionation was chosen according to the Institutional Policy: up to 6 fractions with a dose per fraction ≥ 6 Gy. Treatments were planned with Intensity Modulated Radiation Therapy (IMRT) or Volumetric Modulated Arc Therapy (VMAT) techniques.

The patients were 5 males and 4 females, with a median age of 72 years (mean 67, range 49-80 years). Only three patients were on systemic therapy (1 chemotherapy with Pemetrexed 500 mg/m^2, q21, 1 tyrosine kinase inhibitor with Gefitinib and 1 immunotherapy with Nivolumab) at the time of SRT. Sites of SRT were lung (4 cases 4 lesions), bone (2 cases), bone (2 cases, 3 lesions) and liver (1 case). At the last follow up examinations, two of the patients were dead. Healthy controls, on the other hand, were two males and two females. In order to evaluate the physiological modifications of the cytokines, healthy controls were also included, with a ratio of 2 cases: 1 control.

Characteristics of patients and RT treatment are summarized in table 1. This study was approved by Spedali Civili of Brescia Ethic Board (Registration number: NP 3553, Registration date: 16 dec 2019). The study was conducted in agreement with the Declaration of Helsinky and all patients provided written informed consent before enrollment.

Table 1. Patients' main characteristics; TKI (Tyrosine Kinase Inhibitor).

Patients' characteristics	
Histology	Adenocarcinoma 88.9%, squamous cell carcinoma 11.1%
Sex	Male 5 (55.6%); female 4 (44.4%)
Age	median 72 years (mean 67, range 49 -80 years)
Mutational status	EGFR positive 11.1%, negative 88.9%; ALK positive 11.1%, negative 88.9%
PD-L1 expression	<1% 11.1%; 1%-50% 22.2%; >50% 33.3%; not assessable/not performed 33.3%
Disease presentation	syncronous oligo-metastatic 33.3%; 55.6% metacronous oligo-metastatic; 11.1% oligo-progressive metastatic
Ongoing systemic treatment at time of SBRT	None 66.7%, immunotherapy 11.1%, TKI 11.1%, chemotherapy 11.1%
Previous chemotherapy	yes 44.4% no 55.6% (mean number of previous lines 0.56 for the whole population, 1.25 for patients that underwent chemotherapy)
Previous immunotherapy	yes 22.2% no 77.8%
Previous TKI	yes 11.1% no 88.9%
RT site	lung 44.4% bone 33.3% brain 22.2% liver 11.1% (one patient treated on two sites, lung and bone)
RT schedule	lung lesions 55Gy/5fr (3 lesions) or 60Gy/8fr (1 lesion); bone lesions 36Gy/6fr (1 lesion), 30Gy/3fr (1 lesion), 18 Gy/3fr (1 lesion); brain lesions 21Gy/1fr (1 lesion), 16Gy/1fr (1 lesion); liver lesion 60Gy/3fr

Blood processing and multiplex analysis

Peripheral blood was collected at the following selected times: 1-5 days before SRT (T1), within 5 days after last fraction of SRT (T2) and between 28 and 35 days after SRT (T3). Samples (10 ml) were collected in EDTA containing tubes (S-Monovette K3E Sarstedt, Germany) and within one hour after

[DOI: 10.52547/ijrr.21.2.10]

collection were centrifuged at 2000g at 4°C for 10 minutes. Subsequently, the supernatant obtained was transferred to sterile 1.5 ml tubes (Sarstedt, Germany) and stored at -80 ° C until analysis.

Serum levels of 45 inflammatory molecules were measured using Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex™ kit (Thermo Fisher Scientific, United States)-according to the manufacturers' instructions. All the evaluated molecules are summarized in table 2.

Table 2. Summary of the cytokines analyzed.

ANALYZED CYTOKINES									
GM-CSF	IL-1 (IL-1 beta; IL-1 alpha; IL-1RA)	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8 (IL-8/ CXCL 8)	IL-9	IL-10
IL-12 (IL-12 p70)	IL-13	IL-15	IL-17A;	IL-18	IL-21	IL-22	IL-23	IL-27	IL-31
BDNF	Eotaxin /CCL11	EGF	FGF-2	GRO alpha/ CXCL1	HGF	NGF beta	LIF	IFN-alfa	IFN-gamma
IP-10/ CXCL10	MCP-1/ CCL2	MIP-1 alpha/ CCL3	MIP-1 beta/ CCL4	RANTES /CCL5	SDF-1 alpha/ CXCL12	TNF alpha	TNF beta/ LTA	PDGF-BB	PLGF
SCF	VEGF-A	VEGF-D							

Briefly, a standard curve was prepared through serial dilution of antigen standards. The assay was performed on 96-wells plates provided with the kit. Premixed magnetic microspheres conjugated to specific antibodies were added to each well and subsequent washed twice with diluted wash buffer. Standards and undiluted samples were added to the wells with shaking for 30 minutes at 500 rpm at room temperature and then incubated overnight at 4°C. At the end of the incubation, excess material was removed with two washes and 25 µl of detection biotinylated antibodies were added to the wells with shaking at 500 rpm for 30 minutes. Wells were washed 2 times and incubated with 50 µl of Streptavidin-Ficoerythrin with shaking at 500 rpm for 30 minutes. After further washing, 120 µl of Reading Buffer were added under with shaking at 500 rpm for 5 minutes. Subsequently, the samples were reading using the Bio-Plex tool MAGPIX Multiplier Reader (BIO-RAD Laboratories, United States) and data analysis was performed using Luminex software (BIO-RAD Laboratories).

Endpoints and statistical analysis

The endpoint of this study was to measure the variation of inflammatory molecules concentrations after SRT at predetermined time points. Wilcoxon signed rank test was used to compare concentrations at two time points (T1-T2, T2-T3, T1-T3) and W Randall test among the three time points (T 1-2-3); significance was set at a p-value <0,05. All statistical analysis was performed using SPSS v.23.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Study population and SRT treatment

Nine consecutive NSCLC patients treated between June and August 2018 with SRT at two institutions (Brescia University and Modena University Radiation Oncology Departments) were included in the study, as well as 4 healthy controls (2 male and 2 female volunteers, with no associated medical conditions).

It should be noted that 24 molecules (54.5%) resulted below the detectable levels in more than 75% of the samples. Quantitative analysis was therefore performed on the 20 detectable cytokines and inflammatory molecules, as summarized in table 3.

Table 3. The variation in seven inflammatory molecules (bDNF, MIP-1b, PDGF-bb, PIGF-1, RANTES, SDF1-a, and bNGF) resulted to be significantly correlated with the SRT in the NSCLC patient cohort. Several cytokines (24/44, 54.5%) resulted undetectable in a significant percentage of the samples and were not further analyzed.

PARAME-TERS	Wilcoxon Signed Rank Test T1/T2	Wilcoxon Signed Rank Test T2/T3	Wilcoxon Signed Rank Test T1/T3	W Randall Test (CASES)	W Randall Test (CONTROLS)
BDNF	0.594	**0.012**	0.036	**0.010**	0.078
EGF	0.953	0.123	0.123	0.135	0.127
Eotaxin	0.374	0.484	0.889	0.417	0.174
FGF-2	0.441	0.237	0.208	0.140	0.282
HGF	0.441	0.161	0.779	0.417	0.472
IFN-γ	0.767	0.889	1	0.882	0.471
IL-17A	0.859	0.161	0.327	0.135	0.420
IL-18	0.678	0.889	0.779	0.882	0.174
IL-2	0.953	0.161	0.161	0.417	0.127
MCP-1	0.575	0.263	0.674	0.542	0.452
MIP-1b	0.214	**0.035**	1	0.206	0.779
PDGF-BB	0.859	**0.012**	**0.017**	**0.010**	0.368
PIGF-1	0.779	0.093	0.123	**0.030**	0.368
RANTES	**0.028**	**0.017**	0.674	**0.010**	0.105
SCF	0.678	0.779	0.484	0.881	0.819
SDF-1a	0.110	**0.012**	**0.050**	**0.001**	0.472
TNF-a	0.678	0.176	0.327	0.206	0.109
VEGF-A	0.767	0.575	1	0.846	0.779
VEGF-D	0.953	0.674	0.674	0.884	0.368
bNGF	0.678	0.208	**0.045**	0.072	0.088

Seven of the analyzed molecules showed a statistically significant variation among different time points in NSCLC patients (figure 1): brain derived neurotrophic factor (BDNF) showed a progressive reduction among the three time point (p-value 0.012 for T2-T3 and p-value 0.010 for W Randall test T1-T2 -T3); macrophage inflammatory protein 1 beta (MIP-1b) showed an increase between T2 and T3 (p-value 0.035); platelet derived growth factor basic (PDGF-BB) showed a sharp reduction between T2 and T3 (p-value 0.012 for T2-T3, 0.017 for T1-T3 and p-value 0.010 for W Randall test T1-T2-T3); placental growth factor (PLGF-1) showed a decrease at T3 (p-value 0.030 for W Randall test T1-T2-T3);

[DOI: 10.52547/ijrr.21.2.10]

chemokine (C-C motif) ligand 5 (RANTES or CCL5) showed an immediate decrease and subsequent increase some weeks after SRT (p-value 0.028 for T1-T2 and 0.017 for T2-T3, p-value 0.010 for W Randall test T1-T2-T3); stromal cell-derived factor 1 (SDF-1a, also known as CXCL12) showed a marked increase some weeks after SRT (p-value 0.012 for T2-T3, p-value 0.001 for W Randall test T1-T2-T3); nerve growth factor beta (bNGF) showed a decrease some weeks after SRT (p-value 0.05 for T1-T3).

None of the variations among the 44 analyzed molecules at different time points had a statistical significance in the healthy controls.

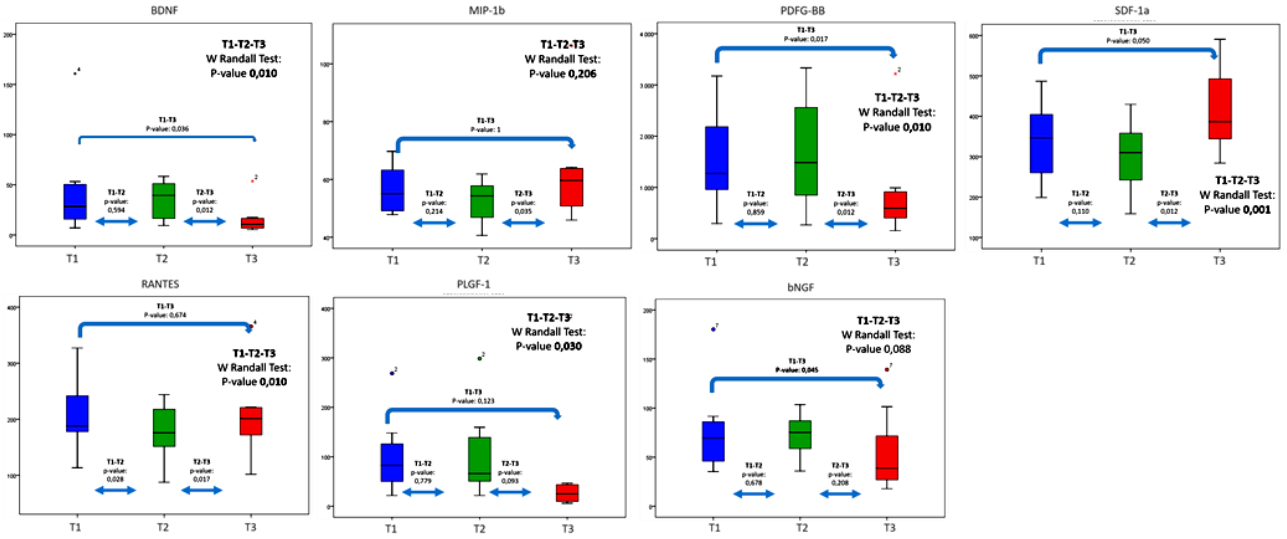

Figure 1. Seven of the analyzed molecules showed a statistically significant variation among different time points in NSCLC patients: BDNF (p-value 0.012 for T2-T3 and p-value 0.010 for W Randall test T1-T2-T3); MIP-1b(p-value 0.035, T2-T3); PDGF-BB (p-value 0.012 for T2-T3, 0.017 for T1-T3 and p-value 0.010 for W Randall test T1-T2-T3); PLGF-1 (p-value 0.030 for W Randall test T1-T2-T3); RANTES or CCL5 (p-value 0.028 for T1-T2 and 0.017 for T2-T3, p-value 0.010 for W Randall test T1-T2-T3); SDF-1a (p-value 0.012 for T2-T3, p-value 0.001 for W Randall test T1-T2-T3); bNGF (p-value 0.05 for T1-T3).

The small number of patients enrolled in this pilot study did not allow to identify correlations between the changes in plasmatic concentrations of the inflammatory molecules and clinical outcomes, including progression free survival and overall survival.

DISCUSSION

Ionizing radiations not only exert a cytotoxic effect on neoplastic cells via direct and indirect DNA damage, but also influence tumor microenvironment and the immune response. Pre-clinical reports are conflicting, as radiotherapy can induce both immunosuppressive (such as increased PD-1 expression, recruitment of T-regs and direct effector T-cells suppression) [8] and immunostimulatory effects [5,4]. In fact, radiotherapy can enhance cancer cell antigenicity by increasing tumor mutational load, with consequent accumulation of neoantigens that are released after cell killing along with damage-associated molecular pattern molecules (DAMPs). This results in the production of pro-inflammatory molecules such as the type I interferon (IFN-β) that promote the recruitment of APCs and the cross-presentation of tumor antigens to CD8+ T cells, leading to 'immunogenic cell death' [9]. Moreover, surviving irradiated cells display enhanced expression of adhesion molecules, MHC-I and co-stimulatory molecules that improve their recognition and killing by activated T cells [10]. The activation of immune response and its cascade of signal molecules might explain the anti-tumoral activity of ionizing radiations outside of the irradiated field, including the so called 'bystander effect' on nearby and loco-regional sites and systemic 'abscopal effect' on distant metastatic locations of disease. Since the first description of this phenomenon by Mole in 1953, only sporadic case reports of abscopal effect after radiotherapy alone were published in subsequent decades, questioning its relevance on clinical practice [11]. Nevertheless, several pre-clinical studies demonstrated that the combination of radiotherapy (predominantly with hypofractionated high doses) and immunotherapy can substantially increase the rate of abscopal response [12]. Immunotherapy and radiotherapy could therefore reciprocally potentiate their efficacy, triggering a virtuous circle in which ionizing radiations act as 'in situ vaccine' that increases tumor immunogenicicty and might overcome the resistance of 'cold' tumors refractory to immunotherapy, while the blockade of immunosuppressive molecules like PD-1 and PDL-1 can raise the prevalence of abscopal and bystander response [13]. It has to be noted that, as different radiation dose and delivery schedules might provoke tumor cell death through different mechanisms, the

[DOI: 10.52547/ijrr.21.2.10]

same holds true for the modulation of the immune response [14]. The large majority of pre-clinical reports of immune-stimulation and/or abscopal effect were due to multiple fractions regimens with high dose per fraction, similar to those adopted for SRT [15,16]. Considered the parallel collection of evidence of the prognostic improvement obtained in metastatic NSCLC with checkpoint inhibitors [19], the next logic step could be the combination of these treatment modalities [7]. Although several ongoing trials are evaluating this hypothesis, the optimal timing and dose are still unclear and the mechanisms underlying radiation induced immune response are far from being completely understood. Cytokines, chemokine, growth factors and other inflammatory molecules are crucial mediators of the immune system and orchestrate the complex interactions that rule anti-cancer immune response [6].

Cytokines are as well able to induce ROS production [20], while anti-inflammatory mediators are consequently produced to balance the equilibrium [20-22], leading to continuous and often long lasting fluctuation of the concentrations of these molecules.

The efforts to define the role of each cytokine in neoplastic disease are complicated by the pleiotropy and redundancy of their activity [23] that results in a complex network of signals and interactions that can lead to opposite effect of the same molecule. The definition of patterns of inflammatory molecules linked to immunological activation or, vice versa, immunotollerance triggered by ionizing radiation and immunotherapy could clarify the processes beyond resistance to treatment and point out potential targets to enhance its effectiveness [11].

Several studies supported the hypothesis that cytokines might be considered as a biomarker to predict the clinical onset of pulmonary toxicity and clinical data confirmed a correlation between plasma levels of IL-1α, IL-6, IL-8, IL-10 and TGF-β and the risk of developing pneumonitis and lung fibrosis [24, 25]. Some papers also identified a link between plasmatic concentrations of cytokines including IL-6, IL- 10 and TGFα and worse prognosis in NSCLC patients [26-28].

Most of these analyses focused on the concentration of only one or a small number of cytokines, especially in non-metastatic and locally advanced disease treated with conventional fractionation, and the reported findings are generally considered not conclusive. Only a few published studies analyzed the modulation of the inflammatory molecules pattern in response to radiotherapy in patients affected by NSCLC.

Trovò *et al.* [29] examined the variations of the levels of 21 cytokines in early-stage NSCLC patients who underwent SRT or in locally advanced NSCLC ones who underwent radical moderately hypofractionated IMRT, often in association with chemotherapy. A significant reduction of IL-10 and IL -17 plasma levels was documented between SRT start and end, while 4 weeks after the start of IMRT several cytokines significantly decreased.

Zhang *et al.* [30] evaluated the effect of SRT in 6 patients with non-metatstatic NSCLC: peripheral CD8+ T cells significantly increased and were transformed into activated T cells, which expressed high levels of TNF-α, IFN-γ and IL-2; production of IL-2, TNF-α, and IFN-γ by CD4+ T cells was as well enhanced, while the production of TGF-β was down-regulated as well as the proportion of inhibitory T-regs.

The analysis of Ellswort *et al.* [31] assessed the variation of plasmatic levels of 30 cytokines during and after radiotherapy in 141 non metastatic NSCLC patients undergoing SRT (n=16) or radical normofractionated radiotherapy with (n=107) or without (n=18) concurrent chemotherapy. A significant variability of cytokine pattern among the different groups was observed, likely due to heterogeneity in baseline characteristics of the patients, of their disease and of the treatment. A primarily inflammatory cytokine profile was observed in all groups, but with different molecules as major determinants of plasmatic variations in each of the three groups.

The modification of 19 cytokines and 11 chemokines in 37 patients undergoing SBRT to any organ for a primary or metastatic solid tumor with various histology were evaluated by Mc Gee *et al.* [32]. Circulating levels of TNF-α and multiple chemokines (including RANTES, TNF-α, MIP-1α, IP-10 and MCP1) significantly decreased after SRT to parenchymal sites (lung and liver), but not to bone or brain.

To our knowledge, this is the first study to assess the modulation of the plasmatic inflammatory molecule pattern of NSCLC patients undergoing radiotherapy, and more specifically SRT, in a metastatic setting. Seven of the 44 analyzed molecules in our small cohort of oligometastatic or oligoprogressive patients showed a significant modification after SRT.

A gradual decrease of BDNF was observed from T1 to T3; this protein has been shown to promote different mechanisms involved in oncogenesis and metastatic spread such as migration, apoptosis inhibition and chemoresistance in preclinical studies [33]; nevertheless, its role is debated as it could also stimulate anti-tumoral immunity [34]. The chemokines MIP-1β (also called CCL4) and RANTES (or CCL5), conversely, tended to immediately decrease after radiotherapy and subsequently increase during the following weeks. These chemokines promote the recruitment of several cells of the immune system, determining an ambivalent effect as they can enhance the activity of both antitumoral Th1 response mediated by CD8+ T cells and immunosuppressive pro-tumoral M2 macrophages [35-37] and have been

[DOI: 10.52547/ijrr.21.2.10]

previously reported as central mediators of immune response to radiotherapy in clinical studies [29,31,32]. Similarly, SDF-1α (also known as CXCL12) underwent an immediate reduction followed by a marked increase some weeks after SRT. This chemokine has been reported to promote various aspects of carcinogenesis and metastatic progression such as proliferation, migration and a shift towards "cancers stemness" and chemoresistance [38]. Nevertheless, its receptor CXCR4 is also ubiquitously expressed by healthy cells and this axis mediates various physiological processes and even trafficking of immune cells that foster anti-tumor response [39].

PDGF-BB and PlGF-1 are growth factors involved in several cellular processes that support cancer progression, including cell proliferation, migration, invasion, and angiogenesis [40, 41]; the expression of PDGF-BB in neoplastic samples from NSCLC patients seems also to be correlated with increased risk of lymphatic metastasis [40]. Both these molecules decreased at T3 compared to T1 and have been already proposed as biomarkers of response to ionizing radiations in previous studies [42, 43]. Finally, beta-NGF, a growth factor not only involved in the development of the nervous system, but also in proliferation, migration and invasion of multiple cancer cell lines [44], gradually decreased from T1 to T3.

Altogether, our analysis showed a post-treatment profile of inflammatory molecules with a predominance of mediators that trigger the immune response and a decrease of some growth factors involved in tumor progression and metastasis. While certain molecules that were already reported as possible markers of the response to ionizing radiations (like MIP-1β, RANTES/CCL5, PDGF-BB and PlGF-1) were confirmed as relevant promoters of immune response to radiotherapy in this study, surprisingly we did not observe significant variations of other cytokines with an established role in neoplastic disease and anti-cancer response (including IL-1, IL-2, IL-6, IL-8, IL-10, IL-12, VEGF, IFN-γ and TGF-β).

We must as well recognize the limitations of our study. Firstly, the small sample size of our cohort of patients reduces the possibility to draw conclusive results with statistical significance. Moreover, a substantial proportion of the analyzed molecules resulted under the minimum detectable concentration. It should be noted that this problem was previously described in a similar analysis performed by MC Gee *et al.* that reported undetectable values in more than 75% of patients for 15 of 30 considered molecules [32].

The measurement of circulating cytokines and chemokines is cumbersome due to several issues, especially when multiple molecules are analyzed at the same time [45]. Plasmatic levels of these molecules are often very low or undetectable under physiological conditions and are impacted not only by neoplastic and inflammatory diseases, but also by circadian rhythm and physical exercise [46]. Proper sample handling is also crucial, as cytokines are extremely prone to fast degradation, and the necessity of sample dilution might also further reduce the assay sensitivity [46]. Many circulating proteins, including heterophilic antibodies, lectins, soluble receptors and complement system might also interfere with immunoassays [46]. Other obstacles to the adequate dosing of cytokines are represented by epitope loss due to denaturation, degradation and micro-heterogeneity of the studied molecules within the sample [47]. This could partly explain the under-detection of molecules previously associated with response to radiotherapy.

The aim of this pilot study, on the other hand, was to perform a preliminary analysis to identify cytokines, chemokines and growth factor with a significant role in the immune response to SRT, In order to design a prospective study on a larger population aimed to identify possible biomarkers within the panel of selected molecules. Moreover, clinical presentation of radiation-induced, immunotherapy-induced and also infective pneumonitis largely overlap [48], suggesting a shared immunologic mechanism that is also confirmed by preliminary clinical experiences [49]. Future larger analysis of circulating inflammatory molecules should thus take into account eventual ongoing immunotherapy and/or infective processes to avoid confounding factors that could modify circulating cytokine's pattern and distort study results.

CONCLUSIONS

Seven of the analyzed molecules showed a significant variation after SRT including both proteins previously reported in clinical studies as involved with response to radiotherapy (such as MIP-1β and RANTES) and molecules that were not formerly described as linked to this process (like βNGF, CXCL12 BDNF). Nonetheless, the results of this study will contribute to define a selection of cytokines and chemokines that will be analyzed in a prospective trial with a larger sample of patients.

ACKNOWLEDGEMENTS

None.

***Funding*:** The study was funded by AIRO (Associazione Italiana di Radioterapia ed Oncologia Clinica).

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

***Ethical consideration*:** This study was approved by Spedali Civili of Brescia Ethic Board (Registration

[DOI: 10.52547/ijrr.21.2.10]

number: NP 3553, Registration date: 16 dec 2019). The study was conducted in agreement with the Declaration of Helsinky and all patients provided written informed consent before enrollment.

***Author contribution*:** All the authors have equally contributed in conceptualization, analysis, evaluation, investigation, data curation and in writing this research paper.

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[DOI: 10.52547/ijrr.21.2.10]

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[DOI: 10.52547/ijrr.21.2.10]