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Research Paper

Changes in bone turnover markers in patients without bone metastases receiving immune checkpoint inhibitors: An exploratory analysis

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HIGHLIGHTS

- Immune checkpoint inhibitors (ICIs) are correlated with immune-related adverse events (irAEs) that may potentially affect all host tissues.
- The effects of ICIs on the skeleton are poorly investigated, thus we evaluated the changes of specific markers of bone resorption and formation.
- We found an increase of type I collagen C-terminal telopeptide (CTX-I) levels after 3 months of ICIs treatment with a concomitant reduction of *N*-terminal propeptide of type I procollagen (PINP) levels with a trend toward statistical significance.
- CTX-I increase was also associated with poor prognosis in terms of treatment response and survival.

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ABSTRACT

Immune checkpoint inhibitors (ICIs) has revolutionized the treatment of different advanced solid tumors, but most patients develop severe immune-related adverse events (irAEs). Although a bi-directional crosstalk between bone and immune systems is widely described, the effect of ICIs on the skeleton is poorly investigated. Here, we analyze the changes in plasma levels of type I collagen C-terminal telopeptide (CTX-I) and *N*-terminal propeptide of type I procollagen (PINP), reference makers of bone turnover, in patients treated with ICIs and their association with clinical outcome.

A series of 44 patients affected by advanced non-small cell lung cancer or renal cell carcinoma, without bone metastases, and treated with ICIs as monotherapy were enrolled. CTX-I and PINP plasma levels were assessed at baseline and after 3 months of ICIs treatment by ELISA kits.

A significant increase of CTX-I with a concomitant decreasing trend towards the reduction of PINP was observed after 3 months of treatment. Intriguingly, CTX-I increase was associated with poor prognosis in terms of

Abbreviations: ICIs, Immune Checkpoint Inhibitors; irAEs, Immune-Related Adverse Events; CTX-I, type I collagen C-Terminal telopeptide; PINP, *N*-terminal Propeptide of type I Procollagen; ELISA, Enzyme-Linked Immunosorbent Assay; PD-L1, Programmed cell Death Ligand 1; RANKL, nuclear factor kappa-B ligand; OPG, Osteoprotegerin; NSCLC, Non-Small Cell Lung Cancer; RCC, Renal Cell Carcinoma; OS, Overall Survival; T0, Time 0; T1, Time 1; RECIST, Response Evaluation Criteria in Solid Tumors; CT-scan, Computed Tomography Scan; TTF, Time to Treatment Failure; ECOG, Eastern Cooperative Oncology Group; IFN-γ, Interferon-γ; TNF-α, Tumor Necrosis Factor-α; IL-6, Interleukin-6; APRIL, a proliferation-inducing ligand; Th17, T helper 17.

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treatment response and survival. These data suggest a direct relationship between ICIs treatment, increased osteoclast activity and potential fracture risk.

Overall, this study reveals that ICIs may act as triggers for skeletal events, and if confirmed in larger prospective studies, it would identify a new class of skeletal-related irAEs.

1. Introduction

The introduction of immune checkpoint inhibitors (ICIs) as cancer treatment has markedly transformed the prognosis of patients in different subtypes of metastatic cancer by enhancing cytotoxic T-cells activity though the programmed cell death 1 (PD-1)–programmed cell death 1 ligand 1 (PD-L1) axis [1–4]. However, ICIs are correlated with a variety of toxicities as results of an excessively activated immune system and, therefore, considered as immune-related adverse events (irAEs). Despite these toxicities may potentially affect all host tissues [5–9], the effect on the skeleton is poorly investigated due to the few and small case series reported in literature [10,11]. These studies described skeletal adverse effects in patients treated with ICIs including serious vertebral fractures that, in some cases, affected multiple sites [10,11].

Osteoimmunology is recently emerging as a new interdisciplinary field to investigate the bi-directional interplay between bone and immune systems. Indeed, systemic activation of T cells or alterations in pro-inflammatory cytokines production stimulates osteoclastogenesis [12–17], similarly, bone cells are able to influence immune cells mainly through the receptor activator of nuclear factor kappa-B ligand (RANKL)–receptor activator of NF-κB (RANK)–osteoprotegerin (OPG) system [18]. Skeletal complications affect patients' survival and quality of life [19], thus, bone health represents a clinically relevant issue for the management of the cancer patients. Moreover, the improvement of life expectancy in patients treated with ICIs makes the attention for skeletal integrity worthy of clinical interest. All these evidence prompted us to explore the effect of immune activation, induced by ICIs, on bone remodeling. We hypothesized that ICIs treatment enhanced bone resorption through the activation of the T cells, thus increasing the risk of fractures and bone loss.

In the present study, we evaluated the changes in plasma levels of reference markers of bone turnover such as type I collagen C-terminal telopeptide (CTX-I) and *N*-terminal propeptide of type I procollagen (PINP) in patients affected by advanced non-small cell lung cancer (NSCLC) or renal cell carcinoma (RCC) treated with ICIs therapy. In addition, we correlated the levels of these bone turnover markers with treatment response and over-all survival (OS).

2. Material and methods

2.1. Study design and patient characteristics

A consecutive prospective series of 44 patients affected by advanced NSCLC and RCC treated with ICIs as monotherapy were enrolled from 2017 to 2019 and followed up until 06–2022 at Fondazione Policlinico Universitario Campus Bio Medico. Sample size estimation has been designed to achieved a power of 80 % and a level of significance 5 % (one-side) for detecting an effect size of 0.4 between CTX-I and/or PINP pairs (Time 0 (T0) vs 3 months (T1)) requiring a minimum number of 39 patients. The study was conducted in accordance with the principles of the Helsinki Declaration. All experimental protocols were approved by the Internal Review and Ethics Boards of the Campus Bio Medico University Hospital of Rome (Prot. N. 48.17OSS) and all patients provided informed consent. The inclusion criteria were patients who were at least 18 years old, with a performance status of 0–1, no signs of active autoimmune disease, without presence of bone metastases and/or previous osteoporotic fractures. We also excluded patients with conditions affecting bone, vitamin D and/or calcium metabolism (chronic liver disease, paget's disease of bone, renal failure, malabsorption, hypercortisolism); medications altering bone metabolism (e.g. denosumab, bisphosphonates, teriparatide, glucocorticoids, aromatase inhibitors, estrogen). Plasma samples were collected at the day of the first cycle of treatment be-fore the infusion and after 3 months of treatment. The patients' disease had to be measurable per Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 at baseline and had to be periodically evaluated for response to treatment by Computed Tomography Scan (CT scan). Time to Treatment Failure (TTF) was defined as the interval from initiation of ICIs to its discontinuation for any reason, including cancer progression, adverse events, patient choice, or death; Overall Survival (OS) was defined as the time from the first infusion of ICIs to death or last news.

2.2. CTX-I and PINP assessment

All samples were collected in the morning hours in the fasted state. Samples were centrifugated 1500 rpm for 15 min at room temperature and serum was isolated within two hours, aliquoted and stored at −80 °C. CTX-I and PINP were quantified by Human CTX-I ELISA Kit (Cusabio Technology LLC, Huston, TX) and Human PINP ELISA Kit (Cusabio Technology LLC, Huston, TX), respectively, according to the manufacturers' instructions.

2.3. CTX-I and PINP assessment

Descriptive statistics were reported and compared using Wilcoxon's rank to assess CTX-I and PINP changes. CTX-I and PINP values are presented as mean ± standard error (SE). Non-parametric Spearman test was used for correlation analyses. Survival curves were estimated by the Kaplan–Meier method and compared with the Log-rank test. All statistical tests were two-sided with a P value of ≤ 0.05 considered statistically significant. All statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL) and R version 3.5.0 (R Institute for Statistical Computing, Vienna, Austria).

3. Results

3.1. Clinic-pathological findings of the patient population

From 2017 to 2019, a consecutive series of 44 patients treated with anti–PD1 therapy as monotherapy was prospectively enrolled including thirty-six patients (82 %) affected by advanced NSCLC and eight patients (18 %) suffering from advanced RCC. Twenty-three patients (52 %) received nivolumab, sixteen patients (36 %) were treated with pembrolizumab, and five patients (12 %) undergone to atezolizumab. Seventeen patients (39 %) received an anti-PD1 therapy as first-line therapy and 27 (61 %) as second or third line. The median follow up was 45 months (95 % CI 42– non-reached). The median age was 70 years (range 24–86), and female patients, all in post-menopausal state, were 19 (43 %), Patient characteristics at the study entry are summarized in Table 1.

3.2. CTX-I and PINP changes during ICIs

PINP and CTX-I were evaluated at both baseline (T0) and after 3 months (T1). We found a significant increase of CTX-I levels after 3 months of ICIs treatment (mean T0 0.388 ± 0.037 μg/L vs T1 0.455 ± 0.042 μg/L; p = 0.045), while we observed a decreasing trend towards the reduction of PINP levels (mean T0 3432 ± 123 pg/ml vs T1 3256 ±

Table 1
Clinic-pathological variables of study population.

	Overall (N = 44)
Sex	
Female	19 (43 %)
Male	25 (57 %)
Age	
≥ 70	22 (50 %)
< 70	22 (50 %)
ECOG	
0	29 (66 %)
1	15 (34 %)
Tumor type	
NSCLC	36 (82 %)
RCC	8 (18 %)
Treatment	
Nivolumab	23 (52 %)
Pembrolizumab	16 (36 %)
Atezolizumab	5 (12 %)
Treatment Line	
First line	17 (39 %)
Second or Third	27 (61 %)
Primary Tumor	
Non resected	19 (43 %)
Resected	25 (57 %)
N. metastatic sites	
1	15 (34 %)
2	17 (39 %)
>2	12 (27 %)

ECOG (Eastern Cooperative Oncology Group); NSCLC (Non-Small Cell Lung Cancer); RCC (Renal Cell Carcinoma); N. (number).

169 pg/ml; p = 0.073) (Fig. 1A and B). In particular, twenty-three patients (52 %) showed a CTX-I beyond least significant increase and seven patients (16 %) experienced a CTX-I above least significant reduction [20].

3.3. Correlation of CTX-I and PINP changes with clinical variables

Correlation with baseline CTX-I and PINP levels with clinically relevant variables (sex, age, ECOG PS, line of treatment, tumor and treatment types) showed no significant association (p > 0.05 for all).

As expected, low baseline CTX-I levels were observed in patients with the overweight range of Body Mass Index (BMI) (data not shown). Similarly, no significant correlations were observed when CTX-I and PINP changes (T0 vs T1) were associated with all clinic-pathological parameters including BMI.

Interestingly, CTX-I increase was associated with poor prognosis in terms of TTF (p = 0.029) and OS (p = 0.030) (Fig. 2). In patients with or without increased CTX-I, median TTF was 5 months (95 % CI 4.3 – 17.1) vs 12 months (95 % CI 8.0 – non-reached), respectively (Fig. 2A). Similarly, median OS was lower in patients with increased CTX-I (11 months (95 % CI 6.0 – 27.0) vs 37 months (95 % CI 13.0 – non-reached) (Fig. 2B). Moreover, ΔCTX-I (CTX-I T1 – CTX-I T0) positively correlated with tumor size changes at best response (r = − 0.36, p = 0.018) showing that a CTX-I increase was significantly associated with an increased tumor size during ICIs (Fig. 3). An additional survival analysis showed that patients with a CTX-I above "least significant increase" have a greater TTF compared to patients with a CTX-I above "least significant decrease" (Supplementary Fig. 1). No significant association was found between PINP and any clinical parameter of outcome.

4. Discussion

To our knowledge, this is the first study evaluating the bone remodeling status during ICIs using CTX-I and PINP, useful non-invasive biomarkers for monitoring bone health and the efficacy of anti-resorptive and anabolic therapies [21–23]. In our cohort, we found a significant increase of CTX-I levels, after 3 months of treatment, and a concomitantly PINP reduction with a trend towards statistical significance. Interestingly, four patients (9 %) developed new lumbar fractures after 3 months of therapy suggesting a potential relationship between ICIs, increased osteoclast activity and fracture risk. To date, how ICIs influence bone metabolism is poorly investigated, even if the established role of activated T cells in skeletal remodeling makes plausible a direct relationship between ICIs therapy and bone health. Studies from autoimmune/inflammatory diseases associated to bone loss, such as rheumatoid arthritis and postmenopausal osteoporosis showed that the increased levels of pro-inflammatory cytokines including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels stimulated osteoclast differentiation and activity [12,24,25]. Moreover, in these inflammatory diseases, *T*-cell activation induced RANK-L production leading to a disruption of the RANK-L/OPG balance, increasing bone turnover and promoting bone loss [26–28]. Similarly, ICIs therapy induce the secretion of pro-inflammatory cytokines that are implicated in tumor destruction but, at the same time, could adversely affect bone remodeling stimulating osteoclast function, thus resulting in an increased risk of fractures. These evidences lead us to sup-pose that there is potential relationship between ICIs treatment, increased CTX-I and the development of fractures observed in our cohort.

Additional research is warranted to elucidate the significant correlation between increased CTX-I and poor prognosis, in terms of survival and treatment response. One potential explanation is that osteoclasts could create a systemic immunosuppressive microenvironment that promotes tumor growth. Evidence from multiple myeloma support the immunosuppressive role of osteoclasts that.

directly inhibit proliferation of activated CD4 + and CD8 + effector T cells. In addition, the secretion of some molecules by osteoclasts, such as galectin-9 and a proliferation-inducing ligand (APRIL), induced T cells apoptosis and enhanced tumor growth [29].

It has widely described that Th17 cells stimulate bone resorption directly through the expression of RANKL on their surface or, indirectly, by the secretion of IL-17 that enhances the expression of RANKL in osteoblasts [30,31]. A recent paper demonstrated that an increased infiltration of T helper 17 (Th17) cells caused primary resistance to ICIs in lung cancer patients [32]. Thus, another possible scenario could be that specific tumor-infiltrated *T*-cells in tumors resistant to ICIs secrete pro-osteoclastogenic cytokines. As results of an excessive osteoclast activation, we observed a CTX-I increase in primary refractory patients. Based on all these evidences, it reasonable to hypothesize a bidirectional interaction between osteoclasts and specific populations of tumor-infiltrated *T*-cell, such as Th17 cells, which creates a vicious cycle that promote cancer cell progression and bone osteolysis. Finally, we cannot exclude that the bone turnover increase in patients who experienced disease progression is associated to a general physical health deterioration that affect also bone tissue.

The results of this study are in accordance with our previous paper that demonstrated the correlation between the high levels of circulating RANKL and poor prognosis in patients with metastatic RCC treated with ICIs [33].

Our exploratory study includes a small, but adequately powered number of patients with stringent exclusion criteria that makes the data reliable and unaffected by potential biases or confounding factors. The major limitation of the study is the lack of a control group of patients who were not treated with ICIs. The adequate control group would have

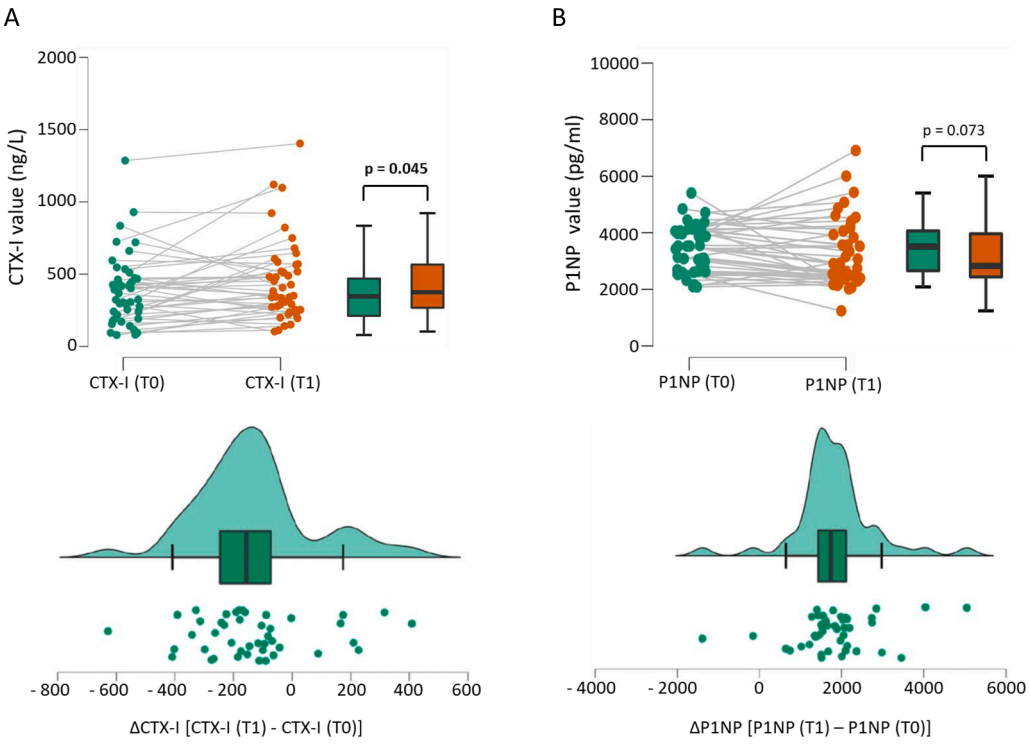
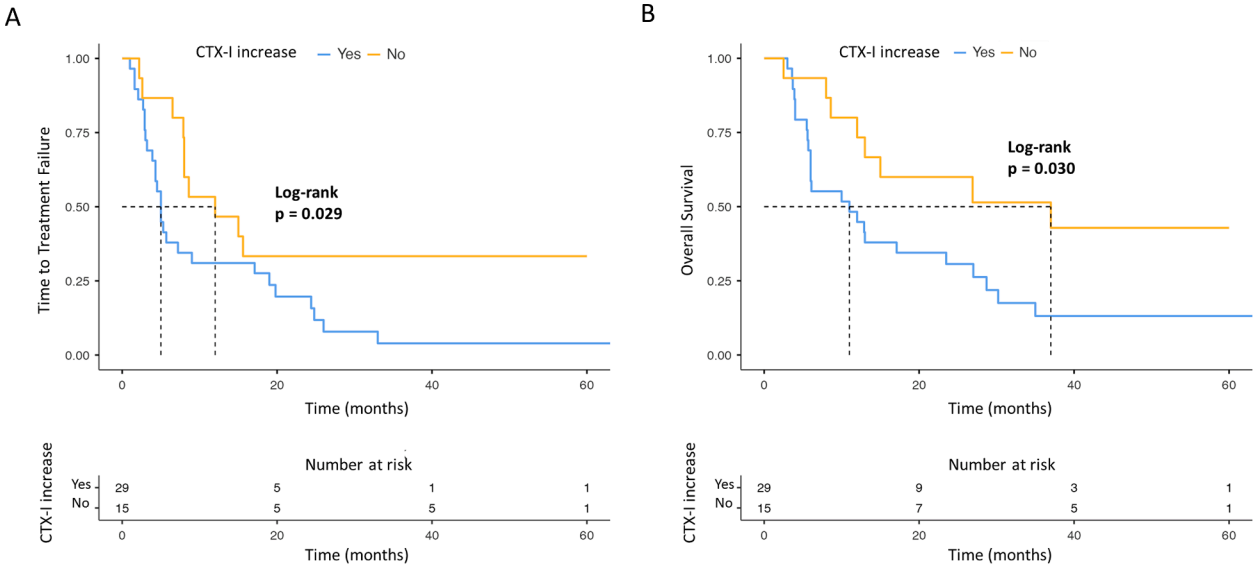


Fig. 1. CTX-I and PINP changes. *Upper panel.* CTX-I (A) and PINP (B) values at baseline and after 3 months of ICIs treatment. *Lower panel.* Rain-cloud difference plot showing ΔCTX-I (A) and ΔPINP (B) calculated for each patients.



	95% Confidence Interval				
	Records	Events	Median	Lower	Upper
Yes	29	27	5.0	4.3	17.1
No	15	10	12.0	8.0	NaN

	95% Confidence Interval				
	Records	Events	Median	Lower	Upper
Yes	29	24	11.0	6.0	27.1
No	15	8	37.0	13.0	NaN

Fig. 2. Survival analysis. Kaplan-Meier curves reporting the TTF (A) and OS (B) of patients who showed an increased CTX-I after 3 months of ICIs treatment.

been patients mainly treated with chemotherapy, but it is known that anti-neoplastic agents and steroids used to prevent emesis both affect bone homeostasis creating potentially confounding elements in the analyses. Although we can not rule out that CTX-I increase is independent from ICIs therapy, the association between CTX-I changes and ICIs response make less plausible this second hypothesis. The study is limited also by immortal time biases since patients who died before 3 months of ICIs treatment were not included. Furthermore, bone turnover markers were evaluated only at baseline and after 3 months without a longitudinal assessment of their dynamics over time in long-responder patients.

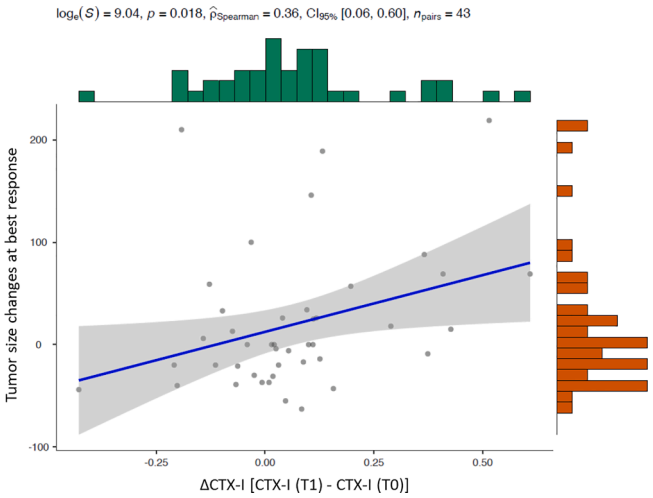


Fig. 3. CTX-I and tumor size changes. Scatter Plot representing the correlation between ΔCTX-I and tumor size change at best response.

Overall, the key message of this study is that ICIs may act as triggers for skeletal events, but larger prospective studies are needed to confirm if this treatment really affects the bone. In this regard, laboratory and imaging studies should be performed in patients who are candidates to ICIs treatment for risk stratification and early detection of porotic fractures. In addition, the presence of risk factors for impairments of bone metabolism/fracture risk factors such as pre-existing osteoporosis, fragility fractures, concomitant inflammatory arthritis or other autoimmune disease, genetic or environmental factors should be taken in consideration before starting treatment. As future clinical perspective, the use of anti-osteoclastic drugs such as bisphosphonate and denosumab could represent a novel combination strategy with ICIs not only to prevent skeletal lesions but also to improve patients' clinical outcome. In this regards, recent studies evaluating the combination efficacy of anti-osteoclastic agents and ICIs have already shown promising results [34–37].

Ethics approval and consent to participate.

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of the Fondazione Policlinico Universitario Campus Bio Medico (Prot. N. 48.17OSS).

Declaration of Competing Interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbo.2022.100459.

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