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Original Article

Changes in peripheral monocyte populations 48-72 hours after subcutaneous denosumab administration in women with osteoporosis

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Abstract

Objectives: To examine the effect of denosumab administration in the peripheral blood white cell population, to further elucidate a plausible pathophysiological link between denosumab and osteonecrosis of the jaw. **Methods**: Thirty women with osteoporosis, after denosumab treatment were included. Peripheral blood samples were obtained prior to and 48-72 hours following denosumab administration. Flow cytometry gated at the monocyte population for CD14/CD23/CD123/CD16 stainings were performed. **Results**: We were able to record a number of changes in the monocyte populations between baseline and after denosumab administration. Most importantly, in the monocyte populations we were able to detect statistically significant increased populations of CD14+/CD23+ (p=0.044), CD14-/CD23+ (p=0.044), CD14+/CD123+ (p=0.011), CD14+/CD123- (p=0.011) and CD14-/CD16+ (p=0.028). In contrast, statistically significant decreased populations of CD14-/CD123+ (p=0.034), CD14+/CD16+ (p=0.037) and CD14+/CD16- (p=0.014) were detected. **Conclusions**: Our results provide evidence supporting the hypothesis that denosumab administration modifies the monocyte mediated immune response in a manner similar to that of bisphosphonates. This may partly explain the trivial immunity changes recorded with denosumab.

Keywords: Bisphosphonates, Denosumab, Macrophages, Osteoclasts, Osteonecrosis Of The Jaws

Introduction

Medication related osteonecrosis of the jaw (MRONJ) is a complication associated with the use of bone antiresorptive agents[1], mainly bisphosphonates (BPs) that are widely used for the management of osteoporosis, bone metastasis and other bone-loss related disorders[1-4]. It has been suggested that the development of MRONJ is more frequently reported with the use of high doses of IV BPs, for the treatment or prevention of skeletal related events (SREs) in patients with advanced cancer and bone metastasis compared to standard doses used for the treatment of osteoporosis[5]. Nonetheless, recent good quality of evidence suggests that MRONJ is also seen among patients receiving BPs, *per os* or intravenous for non-malignant indications[6-8]. The hallmark of MRONJ development is the finding of necrotic exposed bone in the oral cavity[1]. In the majority of cases, the precipitating event appears to be a dental extraction or other dental invasive procedures[1], and use of dentures[1,9,10]. However, 40% of MRONJ cases appear to occur spontaneously and to be unrelated to dental treatment[11,12].

Despite the increasing amount of evidence regarding the association of MRONJ with bone metabolism and anti-resorptive agents the underlying pathogenetic mechanisms remain largely unknown;[2,4,13] infection at tissue level in the

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oral cavity has also been implicated in the pathogenesis[14,15].

Denosumab (Dmab), is a fully human monoclonal antibody, which binds to receptor activator of nuclear factor kappa beta ligand (RANKL), and is a potent anti-resorptive agent used for the management of osteoporosis and the prevention of SREs in cancer patients, showing favorable results in terms of efficacy and safety[16-18]. Due to its unique pharmacokinetics Dmab exerts a maximal suppression of bone turvover during treatment, but unlike BPs that are embedded in the bone matrix, Dmab-induced suppression is reversed after treatment discontinuation[17,19].

Since osteoclasts and macrophages stem from a common progenitor cell lineage[15], it has been proposed that a plausible compromised local defense due to insufficient numbers or reduced functional capacity of macrophages, when combined with the impaired oral mucosa that has been reported in patients receiving BPs[15], could allow oral pathogens to reach the bone surface of the jaws[14]. What is more, given the more discrete RANKL pathway inhibition by Dmab, this agent might be a more appropriate target to examine RANKL inhibition effects on the immune system[15,20]. We have previously reported an increase of CD14+ peripheral blood monocyte (PBMC) populations along with a decrease of CD14- PBMC populations in breast cancer women receiving intravenous Zolendronic Acid (ZA)[21].

To shed more light in the role of anti-resorptive agents in the aetiopathogenesis of MRONJ through a possible modification of the immune system we designed this prospective study in order to examine the effect of subcutaneous administration of Dmab in postmenopausal women with osteoporosis using an immune phenotype quantified sampling profile for B-cells, T helper cells, T cytotoxic cells, Natural Killer (NK) cells, NK-like cells, Monocytes, Polymorphonuclear leukocytes (PMN) and Eosinophil granulocytes.

Patients and methods

Sample

Female patients diagnosed with postmenopausal osteoporosis and treated with denosumab for at least one year that were under regular follow up at the endocrinology outpatient clinic were candidates for enrollment. Exclusion criteria were: i) secondary osteoporosis, ii) renal and or liver insufficiency ii) medical history of cancer, iv) untreated hypo or hyperthyroidism, v) metabolic bone diseases other than osteoporosis, vi) medical history of osteonecrosis of the jaw, vii) history of previous Zolendronic acid use for the treatment of osteopororosis.

All patients gave their informed consent for participation in the study and the study was approved by the Institutional Review Board of the Faculty of Dentistry (IRB protocol 51/06-06-2019) of Aristotle University of Thessaloniki.

Anthropometric and demographic data (age, sex, place of residence, social security type, marital status) and disease status (initial diagnosis, history of treatments received, current treatment regimes) were recorded for each patient.

Study protocol

After an overnight fast, blood sample was drawn at the hospital, prior to Dmab administration. Then, a second visit was planned within 48-72 h after Dmab administration, in the hospital, for a second sample.

Flow cytometry

Immunostaining and subsequent flow cytometry were performed according to standard protocol prior to Dmab administration and 48-72 hours after, on peripheral blood samples. The antibodies used were CD45 (PerCP), CD14 (FITC), CD 23 (PE), CD 123 (PE), CD 4(FITC)/ CD 8(PE)/ CD 3(PerCP), CD 3(FITC)/ CD 16+56(PE)/ CD 45(PerCP)/ CD 19(APC)/ CD16 (PE) (BD Bioscience), as previously described[21]. Briefly, 100 μl of whole fresh blood were stained with the appropriate antibodies as instructed by the manufacturers for 30 min at RT. 2 ml of BD lysis buffer was added in order to lyse the erythrocytes and the samples were incubated for 10 min at RT. The samples were centrifuged at 500 xg and the supernatants were discarded. Pellets were washed once with serum-free PBS and centrifuged at 500 xg for 5 min. The final pellet was re-suspended in 0.5 ml serum-free PBS and the samples were immediately analyzed using FACs Calibur and Cell Quest software. 50,000 events were collected for each staining. The percentage of positive cells for each antibody was determined. The gating for each cell population has been previously described[21].

Statistical analyses

Normality explorations were performed on all variables. Non-parametric tests were used where normality assumptions were not met. Descriptives and absolute and relative frequencies for all variables were obtained. Pearson's r or Spearman's rho correlation coefficients were used, following normality explorations. Paired t-test was used for paired sample comparisons. Bootstrapping was used for internal validation. Alpha level was set at 0.05. An alpha value smaller than 0.10 was considered a trend. Statistical analyses were performed using the IBM SPSS 23.0 package (IBM SPSS Statistics for Windows, Version 23.0, Armonk, NY: IBM Corp).

Results

Patients

Thirty postmenopausal osteoporotic women under treatment with denosumab were finally enrolled in the study. The patient's anthopomorphometric, clinical and biochemical characteristics are depicted in Table 1.

Six patients (20%) had sustained at least one vertebral fracture and 4 had a history of a non-vertebral fracture (13%) (Table 1) before initiation of denosumab treatment.

No history of new or worsening vertebral fractures, hip fractures or other non-vertebral fractures were reported during treatment with denosumab.

Table 1. Anthropometric, clinical and biochemical characteristics of the enrolled patients. **: All biochemical and DXA measurements were performed before the scheduled dose of denosumab (yrs, years; VF, vertebral fractures; NVF, non-vertebral fractures; NR, normal range; BMD, bone mineral density; LS, lumbar spine; LFN, left femoral veck; LTH, left total hip).*

Parameters	Values
Age (yrs)	67.8 ± 9
Age at menopause (yrs)	46.28 ± 4.9
Drug – naive patients (n,%)	11, 36%
History of gastroesophageal reflux disease and/or peptic ulcer (n, %)	9, 30%
Duration of previous treatment (yrs)	5.1 ± 4
Duration of treatment with denosumab	3.1 ± 1.6
Patients with a history of VF (n, %)	6, 20%
Patients with a history of NVF (n, %)	4, 13%
*Serum calcium (NR: 8.7-10.3 mg/dl)	9.5 ± 0.5
Serum phosphate (NR: 2.5-4.5 mg/dl)	3.3 ± 0.7
Serum creatinin (NR: 0.7-1.2 mg/dl)	0.7 ± 0.14
Serum PTH (NR: 11-54 pg/ml)	47.4 ± 12.8
Serum osteocalcin (NR: 9-42 ng/ml)	10.6 ± 4.8
BMD LS (gr/cm^2)	0.921 ± 0.12
T-score LS	-2.03 ± 0.96
BMD LFN (gr/cm^2)	0.743 ± 0.06
T-score LFN	-2.02 ± 0.66
BMD LTH (gr/cm^2)	0.808 ± 0.08
T-score LTH	-1.54 ± 0.71

To examine the patients' monocyte population, gating at the monocyte area with the CD14/CD123, CD14/CD23 and CD14/CD16 stainings were performed (Table 1, Figure 1). The instrument was set in order to position the cells appropriately in the dot blots by using isotype controls, voltage, and compensation tools. A dot plot of FSC versus SSC was established and the region of interest was selected (gated area), excluding any other cell type and cellular debris. Each staining was performed twice for each patient, one prior and on 48-72 hours post treatment administration (Table 2). Statistically significant increase was found in CD14+CD23+, CD14-/CD23+, CD14+/CD123+, CD14+/CD123- and CD14-/CD16+ populations. Decrease was found in CD14-/CD123+, CD14+/CD16+, CD14+/CD16- populations. No statistically significant difference was found for CD14+/CD23+, CD14+/CD23-, CD14-/CD23-, CD14-/CD123-, CD14-/CD16- monocyte populations (Table 3).

Discussion

In our sample of thirty postmenopausal osteoporotic women under treatment with denosumab we were able to record a shift towards CD23+ expression in the monocyte population, an increase in the CD14+CD123+ population while CD14-CD123+ population was decreased and a decrease in the CD14+CD16+ population.

Approximately 2-9% of the peripheral human blood leukocytes are peripheral blood monocytes (PBMC), but only 40% of the available monocytes circulate while 60% migrate[22].

CD14 (55 kDa) is a glycoprotein released by monocytes and macrophages in humans, which is located on the cellular membrane. Normal mature osteoclasts and human monocytes have been reported to express high levels of CD14[21]. In our sample, PBMC CD14+[21,23-25] populations have been found to be markedly increased following Dmab administration. In this regard, we have previously reported similar findings in PBMC of breast cancer patients treated with ZA[21]. The latter finding is in agreement with previous *ex vivo*[26] and experimental studies[27] demonstrating an increase in CD14+ expression after zolendronic acid exposure which was documented *in vitro* from human PBMC derived cultures[27] and subsequently ex-vivo from human jaw tissues[26]. Further, Dmab administration increased the population of CD14-/CD23+ monocytes, 48 hours after the infusion. CD 23 is a marker of activated macrophages associated with B-cell activation[28-30].

CD 123 antigen is present in blood dendritic cells[31,32] and it is lost when monocytes are transformed in macrophages in which CD68 and CD168 predominate[33,34]. CD123 is a molecule currently under intensive research as a potential therapeutic target for haematologic malignancies[32,35,36]. We were able to detect a subset of CD14+ that were CD123+ probably reflecting the blood dendritic cell population. Notably, the increase in this cell population following DMAB administration was similar to the increase in the original CD14+ population. In contrast, we found the CD14-CD123+ population to be decreased, a fact probably attributed to a generic decrease of CD14-PBMC following Dmab administration.

Skrzeczyńska-Moncznik J et al reported that the CD16+ subset of the CD14+ population has a potent anti-inflammatory immune action[37]. In the present study we were able to detect decrease in the CD14+/CD16+ following Dmab administration in the PBMC population of osteoporotic women under Dmab treatment. This finding is novel and may partly explain the increased infection risk that has been previously reported in patients with osteoporosis treated with Dmab[20,38]. To avoid the immune hindering effects of Dmab while maintaining its anti-resorptive efficacy, the linking of anti-RANKL with single-chain variable fragments of an antibody specific for osteonectin, a protein which is abundantly expressed in osseous tissues, has been recently proposed[39]. This approach is even more tempting if one considers that Dmab is also used for the treatment of patients with cancer related skeletal events in whom hindering macrophage mediated immunity may be a double – edged sword.

With regard to the reversal of the hindered macrophage immune function to cure MRONJ, Ogata et al[40] used an experimental design to demonstrate that serum-free conditioned isolates from mesenchymal stem cells

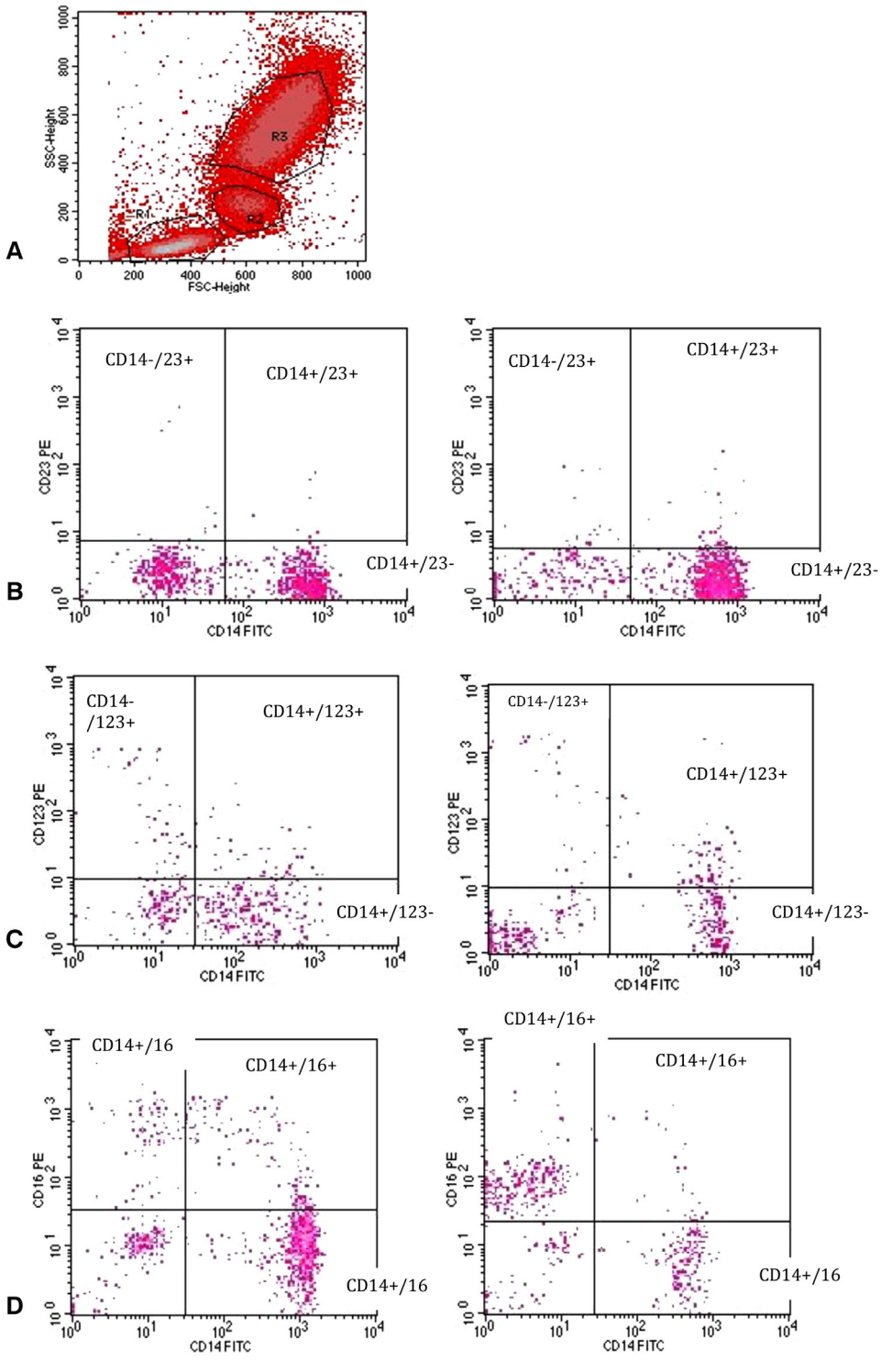

Figure 1. Representative flow cytometry analysis of a patient prior (left Column) and 48-hours following (right Column) denosumab administration. FACS plot of Forward scatter (FSC) vs side scatter (SSC) is presented, indicative of the experiments. The dot blots represent the percentages of single or double positive cells for the indicated markers (CD14/CD23, CD14/CD123 and CD14/CD16) from gated monocyte population. ***(A:*** *Gating all populations; R1: Lymphocytes, R2: Monocytes, R3: Granulocytes.* ***B:*** *Increased CD14-/CD23+ - CD14-/CD23+ and increased CD14+/CD23+ - CD14+/CD23+. Left: Before; Right: After Denosumab administration. CD14+/CD23- - CD14+/CD23- increased and CD14-/CD23- - CD14-/CD23- decreased in case image but not statistically significant in total sample of patients.* ***C:*** *Increased CD14+/CD123+ - CD14+/CD123+, CD14+/CD123- - CD14+/CD123 and decreased CD14-/CD123+ - CD14-/CD123+. Left: Before; Right: After Denosumab administration. CD14-/CD123- - CD14-/CD123- decreased in case image but not statistically significant in total sample of patients.* ***D:*** *Decreased CD14+/CD16+ - CD14+/CD16+ and decreased CD14+/CD16- - CD14+/CD16, along with increased CD14-/CD16+ - CD14-/CD16+. Left: Before; Right: After Denosumab administration. CD14-/CD16- - CD14-/CD16- decreased in case image but not statistically significant in total sample of patients).*

Table 2. Descriptives of antigen expression prior and 48-72 hours following subcutaneous denosumab administration. CD14/C23/CD123/CD16 stainings. Thirty postmenopausal osteoporotic women under treatment with denosumab.

	1st measuremet (Baseline)		2nd measurment	
Staining	Mean	Std. Deviation	Median	IQR
CD14+/CD23+	2,6546	3,81430	9,9927	20,78235
CD14+/CD23-	59,0086	30,06067	69,8865	23,04598
CD14-/CD23+	,9821	1,00949	1,6569	1,42718
CD14-/CD23-	25,9381	22,39671	23,2454	19,53608
CD14+/CD123+	9,0567	11,86702	17,3707	17,27212
CD14+/CD123-	62,2593	19,98991	73,0904	20,50811
CD14-/CD123+	6,5161	4,14880	3,9878	4,56302
CD14-/CD123-	14,0196	11,19632	13,6048	12,44166
CD14+/CD16+	15,6682	18,63325	8,2926	4,60213
CD14+/CD16-	65,1189	22,28524	56,0948	24,47873
CD14-/CD16+	10,0671	6,55533	15,4348	13,83929
CD14-/CD16-	9,1775	10,43060	6,4259	6,26783

2nd measurement, 48-72 hours following subcutaneous denosumab administration.

Table 3. Mean differences of antigen expression prior and 48-72 hours following subcutaneous denosumab administration. CD14/C23/CD123/CD16 stainings. Thirty postmenopausal osteoporotic women under treatment with denosumab.

Staining	Paired Differences				
	Mean	Std. Deviation	95% Confidence Interval of the Difference		p-value
			Lower	Upper	
CD14+/CD23+	7,193	17,25673	0,223	14,163	0,044
CD14+/CD23-	13,358	37,36448	-1,733	28,450	,080
CD14-/CD23+	,605	1,45269	,018	1,191	,044
CD14-/CD23-	-1,511	26,79658	-9,312	12,334	,776
CD14+/CD123+	8,461	16,00697	2,128	14,793	,011
CD14+/CD123-	10,161	19,03547	2,631	17,691	,010
CD14-/CD123+	-2,273	5,28709	-4,364	-,181	,034
CD14-/CD123-	-,699	15,71201	-6,914	5,516	,819
CD14+/CD16+	-7,502	17,76660	-14,531	-,474	,037
CD14+/CD16-	-8,773	17,32542	-15,627	-1,919	,014
CD14-/CD16+ - CD14-/CD16+	5,394	12,06589	,620	10,167	,028
CD14-/CD16- - CD14-/CD16-	-2,902	10,83524	-7,188	1,384	,176

Statistical significance typed in bold. Increase (positive difference) typed in green. Decrease (negative difference) typed in red.

conditioned media (MSC-CM) which contained various cytokines (to facilitate the recruitment of cells during osteogenesis, angiogenesis and cell proliferation), showed function maintenance in osteoclasts despite the presence of RANKL inhibitors[40].

Kambayashi et al reported augmented matrix metalloproteases expression and tumor associated proliferation following RANKL treatment in CD14+ cells isolated from PBMCs of healthy donors[41]. Thus the RANK/RANKL pathway may further contribute to the development and maintenance of the immunosuppressive tumor microenvironment and denosumab may even be a promising adjuvant therapy for targeting tumor associated macrophages (TAMs) in other cancers[41]. Dmab has already shown favorable results for the treatment of Giant Cell Tumor of Bone[42], however, neoplastic cells with certain mutations survive denosumab treatment and undergo dramatic histological changes in response to this agent[43]. Still, because high RANKL mRNA expression has been reported in patients with aneurysmal bone cyst, fibrous dysplasia, osteosarcoma, chondrosarcoma and enchondroma[44], primary bone tumors present new therapeutic targets for denosumab, particularly those tumors expressing RANKL and those involving bone resorption by osteoclasts[44].

From an epidemiological perspective, MRONJ presents only in a very small percentage of osteoporotic patients receiving Dmab. Furthermore, 40% of MRONJ cases appear spontaneously with no previous documented mucosal injury. It has been reported that the initiating event in MRONJ is likely the infection, instead of the low bone turnover[14]. In this regard, sterile inflammation alone in the soft tissues surrounding the jaw was not found to be enough to induce ONJ[14]. Thus the presence of bacterial populations is also a requisite for MRONJ[45]. The pathogenesis of MRONJ could be a series of events initiating from infection, followed by inflammation which might also be augmented by the use or bone antiresorptive agents[46]. It has been reported that the presence and function of macrophages and monocytes could be crucial in the development of local infection[14]. MRONJ has been reported to be associated with various bacterial pathogens populations, the numbers of whom do not decrease despite antimicrobial chemotherapy[47,48]. These might be the reasons for the differential response to monocyte impairment in patients receiving antiresorptive agents. Differences in the populations of macrophages but also differences in the oral flora might explain the occurrence of MRONJ only in some patients, of whom some even develop MRONJ without mucosal injury.

A second significant side effect of antiresorptives is the occurrence of atypical femoral fractures[16]. We have previously reported that altered microdamage repair and microfractures accumulation, "fatigue" could be implicated in the pathogenesis of ONJ[49,50]. In this regard an experimental study showed that treatment with granulocyte colony-stimulating factor (G-CSF) result in increased bone healing along with upregulation of monocytes, granulocytes and macrophages[51], Other experimental studies suggested that CD34+ and CD31+ cells isolated from peripheral blood might be potential therapeutic autologous treatments to augment fracture healing[52,53]. Interestingly, monocytes appear to express both CD34 and CD31[54,55]. A study of the potential changes in those monocyte subpopulations would be required to explore the possible aetiopathogenetic link between Dmab and altered microdamage repair.

Through this study we were able to document changes in the peripheral blood monocyte population, 48-72 hours following subcutaneous Dmab administration. Denosumab has long been identified as a cause for MRONJ[19] and this is – to the best of our knowledge – the first clinical report to suggest that it is associated with PBMC changes in a similar pattern to ZA. Our finding when placed in the context of currently existing evidence regarding the trivial, yet existent deterioration in immunity following anti-resorptive drug administration, warrants further studies targeting the peripheral blood and the tissues of patients under bisphosphonates or Dmab treatment to explore how the changes in the peripheral blood monocytes are reflected in the tissues of those patients. As the passage of monocytes to tissues is a complex process[21,22,56], newly designed studies should be able to overcome the obscuring effects of the latter complexity.

To conclude, in the present study we were able to document an increase in the peripheral blood monocyte CD14+ population and a decrease in the CD14- PBMC population in female patients with osteoporosis, following Dmab administration. This finding is in accordance with currently existing evidence and creates further research queries that need to be addressed by future studies.

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