Leukemia (2015), 1–8

www.nature.com/leu

ORIGINAL ARTICLE

Osteolytic lesions, cytogenetic features and bone marrow levels of cytokines and chemokines in multiple myeloma patients: Role of chemokine (C-C motif) ligand 20

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The relationship between bone marrow (BM) cytokine and chemokine levels, cytogenetic profiles and skeletal involvement in multiple myeloma (MM) patients is not yet defined. This study investigated a cohort of 455 patients including monoclonal gammopathy of uncertain significance (MGUS), smoldering MM and symptomatic MM patients. Skeletal surveys, positron emission tomography (PET)/computerized tomography (CT) and magnetic resonance imaging (MRI) were used to identify myeloma bone disease. Significantly higher median BM levels of both C-C motif Ligand (CCL)3 and CCL20 were found in MM patients with radiographic evidence of osteolytic lesions as compared with those without, and in all MM patients with positive PET/CT scans. BM levels of CCL3, CCL20, Activin-A and Dickkopf-1 (DKK-1) were significantly higher in patients with high bone disease as compared with patients with low bone disease. Moreover, CCL20 BM levels were significant predictors of osteolysis on X-rays by multivariate logistic analysis. On the other hand, DKK-1 levels were related to the presence of MRI lesions independently of the osteolysis at the X-rays. Our data define the relationship between bone disease and the BM cytokine and chemokine patterns highlighting the tight relationship between CCL20 BM levels and osteolysis in MM.

Leukemia advance online publication, 20 October 2015; doi:10.1038/leu.2015.259

INTRODUCTION

Osteolytic lesions are a central feature of multiple myeloma (MM).[1] Almost all patients with MM develop bone lesions, which are frequently associated with bone pain, pathologic fractures and hypercalcemia.[1] Conventional radiography remains the standard diagnostic procedure for the detection of skeletal involvement in MM, however, the utility of this imaging technique is limited, as lytic lesions are only visible on plain films after loss of 30–50% of trabecular bone.[1–3] Magnetic resonance imaging (MRI) can be used to identify the increased bone marrow (BM) cellularity that can result from MM cell infiltration as well as focal bone lesions in the absence of osteolysis visible on X-ray.[4] Positronic emission tomography combined with computerized tomography (PET/CT) using an 18F labeled deoxyglucose has also become a more frequently used imaging technique in MM patients, and can be used to identify focal growth of MM cells before osteolysis occurs and to assess response to treatment.[2,5] Recently, the diagnostic criteria of symptomatic MM were updated including the CT and PET/CT together with X-rays in the imaging techniques to define bone lesions following CRAB criteria.[6] Moreover, the presence of more than one focal lesion at MRI has been included as a biomarker to start treatment in MM patients.[6]

Osteolytic skeletal lesions in myeloma develop as a result of abnormal bone remodeling in the MM BM microenvironment. Multiple cytokines and chemokines, including RANKL,[7,8] osteoprotegerin (OPG),[7,8] Dickkopf-1 (DKK-1),[7,9] Activin-A,[10,11] Interleukin (IL)-3,[12,13] chemokine (C-C motif) ligand (CCL)3[refs 14–16] have been identified as key regulators of the altered bone remodeling characteristic of MM bone disease. Recently, Th17[ref. 17] has been identified as a key regulator of osteoclast activation in MM, and consistent with this, high levels of CCL20, a chemokine that recruits Th17, have been observed in MM patients.[17,18]

The relationship between BM levels of these cytokines and chemokines, the cytogenetic and molecular features of MM cells, and MM bone disease has not been completely defined. Gene expression profiling studies have shown that an increased expression of DKK-1 by plasma cells correlates with the presence of focal lesions on MRI.[9] In addition, a possible relationship between the molecular profiles of MM patients and the presence of bone lesions on MRI has been suggested.[19] Serum levels of several cytokines (that is, RANKL, DKK-1, CCL3) have a potential value for assessing bone disease, but results must be further confirmed.[20–25] Finally, higher BM than peripheral cytokine levels have been observed, without a clear correlation between the two levels.[26–28] Therefore, in this study, we investigated a potential relationship between bone disease and BM levels of selected cytokines and chemokines and cytogenetic abnormalities, to better define the relationship between molecular disease features and bone disease and identify the biomarkers that best correlate with myeloma bone disease.

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Received 13 June 2015; revised 7 September 2015; accepted 10 September 2015; accepted article preview online 30 September 2015

SUBJECTS AND METHODS

Patients

A cohort of 393 MM patients including 98 with smoldering MM (SMM) and 295 with symptomatic MM were analyzed in this study. Two hundred and one MM patients were analyzed at the diagnosis and 94 at the relapse. Sixty-two patients with monoclonal gammopathy of uncertain significance (MGUS) and 27 healthy donors were also included in the study. The skeletal evaluation was performed by the X-ray survey in 209 out of 295 patients with symptomatic MM.

Patients were considered to have bone involvement based on the presence of one or more osteolytic lesions and/or osteoporosis on the skeletal survey, according to the CRAB criteria.[6] The presence of three or more osteolytic lesions or fractures defined high bone disease (HBD). Patients with a negative skeletal survey or a positive skeletal survey with fewer than three osteolytic lesions were considered to have low bone disease (LBD). A total body PET/CT and vertebral MRI were performed in 126 and 162 MM patients, respectively. In MRI-positive patients, the BM infiltration pattern was described (normal, 'salt-pepper', focal or diffuse). PET/CT was performed 1 h after injecting a dose of 18F labeled deoxyglucose solution. In positive PET/CT scans, the number of focal bone lesions (FBLs) was assessed and the values divided in FBLs < 3 and FBLs > 3. Then, SUV (Standardized Uptake Value, a parameter used to estimate the concentration of 18F labeled deoxyglucose in the area of interest) was calculated and the lesions divided into two groups: those with values < 4.2 and those with values > 4.2.

Sample collection

BM aspirates were obtained from the iliac crest after informed consent according to the Declaration of Helsinki. Study protocol was approved by the University of Parma Institutional Review Board (Parma, Italy). Ten milliliters of BM aspirate were obtained by aspirating two separate 5-ml tubes from two different points of the iliac crest. Mononuclear cells were isolated from BM aspirates by Ficoll density sedimentation.

Fresh $CD138^+$ plasma cells were purified from isolated mononuclear cells with immunomagnetic method using anti-CD138 monoclonal antibody-coated microbeads (MACS, Miltenyi Biotec., Bergisch-Gladbach, Germany). Only samples with a purity of more than 90%, checked by flow cytometry, were considered. Plasma (5 ml) was obtained after centrifugation of the BM aspirates, and then aliquoted and stored in sterile vials at − 20 °C until analysis.

BM and peripheral blood samples were also obtained from 183 symptomatic MM patients included in the GIMEMA VMP (Bortezomib-Melphalan-Prednisone) vs VMP-T (Thalidomide) trial (median age, 72 years; range 59–87 years), previously published.[29] All patients underwent standard skeletal evaluation by X-ray.

Cytogenetic and fluorescence *in situ* hybridization analysis

Cytogenetic analysis was obtained in 210 of the 482 patients. Fluorescence *in situ* hybridization analysis was performed on fresh $CD138^+$ plasma cells, testing the presence of: del(13q) (D13 S319SO/CEP 12SG, Metasystems, Altlussheim, Germany); del(17p) (LSI ATM SG/p53SO, Metasystems); hyperdiploidy (ON9RED/15GREEN, Kreatech, Diagnostics, Durham, NC, USA); amp(1q21); del(1p32) (XL1p32SG/1q21SO, Metasystems) and chromosome 14 translocation (14 BREAK-APART, Metasystems). t(4;14) (FGFR3SO/IGHSG, Abbott Laboratories, Abbott Park, IL, USA), t(11;14) (LSI IGH/CCND1XT, Abbott Laboratories) and t(14;16) (IGH/MAF, Abbott Laboratories) were performed in $CD138^+$ cells carrying chromosome 14 translocation. Patients were divided into prognostic groups: those with either del(17p), t(4;14) or t(14;16) were considered to be high risk and others were considered to be standard risk.

Cytokine and chemokine level evaluation

The amount of Activin-A, IL-3, soluble (s)RANKL, total (t)RANKL, OPG, CCL3/MIP-1α, CCL20/MIP-3α and DKK-1, was determined in BM plasma by ELISA using commercially available kits. Results were expressed as pg per ml. In each sample, BM plasma levels of cytokines/chemokines were normalized according to the total protein concentration using the Bradford method (Bio-Rad protein assay, Bio-Rad Laboratories, Hercules, CA, USA) and expressed as pg per mg of proteins.

The assay kits for Activin-A, DKK-1, CCL3/MIP-1α and CCL20/MIP-3α were purchased from R&D Systems (Minneapolis, MN, USA); the assay kits for IL-3, RANKL and OPG were provided from Boster Biological Technology (Fremont, CA, USA), Immundiagnostik (Bensheim, Germany) and Biomedica (Wien, Austria), respectively. Cytokine and chemokine levels were tested in duplicate in the 293 samples that had an adequate plasma volume.

Statistical analysis

Quantitative variables were compared by nonparametric two-sided Kruskal–Wallis and Mann–Whitney tests as appropriate and categorical variables were analyzed by Chi-square test. Results were considered significant at $P < 0.05$.

BM cytokine and chemokine levels were calculated both in pg per ml and in pg per mg of proteins by normalizing cytokine and chemokine levels to the total content of proteins. We performed statistical analysis on both data sets. As the results were similar, data reported in this study refer to cytokine and chemokine levels in pg per ml.

A multiple logistic regression analysis was performed using the presence or absence of bone disease evaluated by the different techniques as the dependent variable and cytokine/chemokine BM levels, stage, state of disease, age, sex and cytogenetic profile as predictors.

RESULTS

To define the relationship between the presence of bone disease, cytogenetic features and the BM levels of cytokines and chemokines, we analyzed a consecutive cohort of 62 MGUS, 98 SMM and 295 symptomatic MM collected during the last years at the Hematology of Parma. The main characteristics of the patients are summarized in Table 1.

BM cytokine and chemokine levels across the groups of patients with monoclonal gammopathies

Levels of Activin-A, IL-3, sRANKL, tRANKL, OPG, CCL3, CCL20 and DKK-1 were measured in BM plasma of MM, SMM and MGUS by ELISA assays. All MM patients had higher BM levels of all cytokines and chemokines tested, including sRANKL/OPG and tRANKL/OPG ratio, as compared with healthy controls. Nonparametric analyses demonstrated that median BM levels of sRANKL ($P = 0.05$), OPG ($P = 0.003$), CCL3 ($P = 0.012$) and DKK-1 ($P < 0.001$) were significantly different. All MM patients (symptomatic and SMM) as compared with MGUS had significantly higher levels of Activin-A ($P = 0.003$), CCL20 ($P = 0.042$) and DKK-1 ($P < 0.001$). We found that BM median levels of Activin-A ($P = 0.001$), CCL20 ($P = 0.002$) and DKK-1 ($P < 0.001$) were significantly higher in MM as compared with MGUS. CCL3 BM levels were also higher in MM vs MGUS patients, although the difference between the median levels did not reach statistical significance (CCL3 median level in MM vs MGUS: 20.3 vs 11.9 pg/ml; $P = 0.072$).

BM median levels of Activin-A ($P = 0.039$), OPG ($P = 0.005$), CCL20 ($P < 0.001$) and DKK-1 ($P < 0.001$) were also significantly higher in symptomatic MM as compared with SMM (Table 2).

Prevalence of bone disease and comparison between bone imaging technologies

We initially evaluated the presence of osteolytic bone disease in MM patients by X-ray. Lytic lesions were present on 63% of skeletal surveys, while 37% were negative. Forty percent of MM patients had HBD while 60% had LBD. The overall prevalence of bone fractures, however, was 29%. Very few MM patients had evidence of osteoporosis on skeletal surveys without osteolytic lesions, therefore, further analysis comparing MM patients with osteoporosis and those with osteolysis was not carried out.

The presence of osteolytic lesions and HBD was higher in relapsed MM as compared with newly diagnosed patients ($P = 0.004$), whereas we did not find a significant relationship between bone disease and sex, age or ISS prognostic staging system ($P = $ NS).

Of the symptomatic MM patients evaluated by PET/CT scan, 69% were found to be positive and 31% negative. Sixty-nine percent of the positive scans had FBLs > 3 or SUV > 4.2. Of the total population of MM patients evaluated, 60% had positive MRIs, and 81% of the

Table 1. Main characteristics of the patients with monoclonal gammopathies analyzed in this study

Total number	455
MGUS	62
Mean age ± s.d.	64 ± 15
Sex	
M	35
F	27
SMM	98
Mean age ± s.d.	64 ± 11
Sex	
M	49
F	49
MM	295
Mean age ± s.d.	72 ± 10
Sex	
M	146
F	149
Light chain	
k	61%
l	39%
Diagnosis	68%
Relapse	32%
ISS	
I	21%
II	37%
III	42%
Bone disease (MM patients)	
Osteolysis (X-rays)	
Positive	63%
Negative	37%
High bone disease	
Positive	40%
Negative	60%
MRI	
Positive	81%
Negative	19%
PET/CT	
Positive	69%
Negative	31%
FBLs >3	
Positive	47%
Negative	53%
Cytogenetic features (MM patients)	
Hyperdiploid	47%
Del(13q)	50%
Del(17p)	15%
IgH translocations	
t(11;14)	21%
t(4;14)	9%
t(14;16)	2%
Chr 1 abnormalities	
Gain 1q21	32%
Del 1p32	25%
High risk (Del(17p), t(4;14), t(14;16))	28%

Abbreviations: F, female; FBLs, focal bone lesions; ISS, International Staging System; M, male; MGUS, monoclonal gammopathy of uncertain significance; MM, multiple myeloma; MRI, magnetic resonance imaging; PET/CT, positron emission tomography/computerized tomography; SMM, smoldering multiple myeloma.

symptomatic MM patients had MRI lesions. Fifty-three percent of MM patients had fractures evident on MRI. A comparison between X-ray, PET/CT and MRI in MM patients has also been performed, and results are reported in Supplementary Figures S1a–c.

Correlation between bone imaging features and cytogenetic profiles

No significant correlation between the primary prognostic cytogenetic abnormalities and the presence of osteolytic lesions on X-ray was observed in MM patients, aside from a statistical trend towards significance for t(11;14) (all MM patients: $P = 0.07$, symptomatic MM patients: $P = 0.056$) (Table 3). Interestingly, patients with t(11;14) had significantly higher median BM DKK-1 levels as compared with those without this cytogenetic abnormality (BM DKK-1 median levels: 10246 vs 1624 pg/ml; $P = 0.004$). Likewise, no statistically significant relationship was observed between MRI or PET/CT scan positivity and the presence of cytogenetic abnormalities (Supplementary Table 1) or the presence of hyperdiploidy, del(13q), amp(1q21), del(1p32) and BM median levels of the cytokines and chemokines tested (data not shown). Only MM patients with high-risk cytogenetics had higher levels of Activin-A ($P = 0.024$), sRANKL ($P = 0.012$), sRANKL/OPG ratio ($P = 0.015$) and CCL20 ($P = 0.05$). Interestingly, a similar correlative trend was observed between the levels of these cytokines and chemokines and the presence of del(17p) but not t(4;14) (data not shown).

Osteolytic lesions and BM levels of cytokines and chemokines

BM cytokines and chemokine levels were compared between X-ray positive and negative patients. Higher BM levels of IL-3, OPG, CCL3, CCL20 and DKK-1 were found in MM patients with osteolytic disease as compared with those without. However, only median BM levels of CCL20 ($P = 0.006$) and CCL3 ($P = 0.026$) reached statistical significance in MM patients with at least one osteolytic lesion as compared with those without osteolysis (Table 4). Moreover, we found higher median levels of Activin-A, IL-3, sRANKL, sRANKL/OPG ratio, CCL3, CCL20 and DKK-1 in BM samples from patients with HBD as compared with those with LBD. Among these, Activin-A ($P = 0.05$), CCL3 ($P = 0.045$), CCL20 ($P = 0.004$) and DKK-1 ($P = 0.05$) reached statistical significance (Table 4).

Of the MM patients evaluated, at the diagnosis, only median BM CCL20 levels differed significantly between patients with and without osteolytic bone disease (median CCL20 BM levels: 21.87 vs 8.0 pg/ml; $P = 0.003$) (Figures 1a and c) and in HBD as compared with LBD patients (median CCL20 BM levels: 31.8 vs 11.3 pg/ml; $P = 0.004$) (Figures 1b and d).

MM patients with lesions in the spine had significantly higher median levels of Activin-A ($P = 0.016$), CCL3 ($P = 0.024$) and CCL20 ($P = 0.039$), whereas patients with femur or other long bone involvement had significantly higher median levels of CCL3 ($P = 0.005$) and CCL20 ($P = 0.002$). Significantly higher median levels of Activin-A ($P = 0.011$) and CCL20 ($P = 0.014$) characterized MM patients with bone fractures as compared with patients without fractures. In addition, evaluation of all MM patients (symptomatic and SMM) demonstrated that patients with osteolytic lesions or HBD had significantly higher BM median levels of Activin-A ($P = 0.056$ and $P = 0.003$), CCL3 ($P = 0.018$ and $P = 0.021$), CCL20 ($P < 0.001$) and DKK-1 ($P = 0.01$), respectively.

Average, standard deviation, median and range of BM cytokine and chemokine levels of osteolytic and non-osteolytic MM patients with high or LBD are summarized in Supplementary Table 2. Finally, a logistic multivariate analysis was performed by considering BM cytokine and chemokine levels and the presence or absence of osteolytic lesions identified in X-rays. Our results showed that CCL20 BM levels were the only significant predictors of osteolysis and HBD (optical density ratio: 1.016, $P = 0.025$ and 1.011, $P = 0.022$, respectively). In addition, we found a correlation between CCL20 quartile levels and the percentage of MM patients with or without osteolytic bone disease (Figure 1e).

To further validate our observations, we evaluated BM and peripheral CCL20 levels in a multicenter cohort of 183 MM patients from the GIMEMA VMP vs VMPT trial. Both mean BM and peripheral CCL20 levels were higher in patients with osteolysis as compared with those without (Supplementary Figures S2a and b), although the difference did not reach statistical significance owing to significant variation among sample collection

Table 2. BM median levels of cytokine and chemokine significantly different across the groups of patients with monoclonal gammopathies

	MGUS	*SMM*	*MM*	P-*value**	
				MM vs SMM	*MM vs MGUS*
Activin-A	365.84 pg/ml (107.98–1643.31); 4.73 pg/mg (1.35–25.27)	401.66 pg/ml (173.68–1557.42); 6.15 pg/mg (1.78–19.86)	491.61 pg/ml (173.67–2054.77); 5.88 pg/mg (1.29–31.83)	0.039	0.001
CCL20	4.26 pg/ml (0.00–634.92); 0.04 pg/mg (0.00–10.72)	4.60 pg/ml (0.00–229.80); 0.07 pg/mg (0.00–4.13)	17.69 pg/ml (0.00–1102.54); 0.22 pg/mg (0.00–13.51)	< 0.001	0.002
DKK-1	665.02 pg/ml (259.58–25669.48); 8.85 pg/mg (2.99–260.19)	1054.48 pg/ml (151.28–4630.86); 15.22 pg/mg (1.69–84.94)	2013.97 pg/ml (60.37–237452.05); 24.80 pg/mg (1.12–2611.32)	< 0.001	< 0.001
OPG	8.26 pg/ml (2.44–22.74); 2.16 pg/mg (0.57–11.34)	5.45 pg/ml (0.81–56.94); 1.65 pg/mg (0.21–20.48)	8.58 pg/ml (1.92–46.13); 2.06 pg/mg (0.36–13.34)	0.005	0.781

Abbreviations: BM, bone marrow; CCL, C-C motif ligand; MGUS, monoclonal gammopathy of uncertain significance; MM, multiple myeloma; OPG, osteoprotegerin; SMM, smoldering multiple myeloma. **P*-value calculated by nonparametric Mann–Whitney test.

Table 3. Cytogenetic abnormalities and osteolytic lesions

	Osteolysis+	*Osteolysis −*	P-*value**
Del(13q)	53%	54%	0.508
Hyperdiploid	42%	40%	0.512
Del(17p)	16%	13%	0.421
IgH translocations	50%	46%	0.408
t(11;14)	33%	19%	0.070
t(4;14)	9%	17%	0.127
t(14;16)	3%	2%	0.584
Gain 1q21	38%	34%	0.475
Del 1p32	28%	18%	0.328
High-risk cytogenetics	35%	31%	0.397

**P*-value calculated by Chi-squared test.

Table 4. BM levels of cytokines and chemokines and osteolytic lesions in symptomatic MM patients

X-ray positive vs X-ray negative	P-*value**	*High bone disease vs Low bone disease*	P-*value**
Activin-A	0.614	Activin-A	0.059
IL-3	0.378	IL-3	0.128
sRANKL	0.863	sRANKL	0.781
tRANKL	0.828	tRANKL	0.406
OPG	0.116	OPG	0.769
tRANKL/OPG	0.627	tRANKL/OPG	0.381
sRANKL/OPG	0.942	sRANKL/OPG	0.793
CCL3	0.026	CCL3	0.045
CCL20	0.006	CCL20	0.004
DKK-1	0.247	DKK-1	0.059

Abbreviations: BM, bone marrow; CCL, C-C motif ligand; IL, interleukin; MM, multiple myeloma; OPG, osteoprotegerin. **P*-value calculated by nonparametric Mann–Whitney test.

procedures across participating centers. In addition, no significant difference was found between BM and peripheral levels of CCL20 ($P = 0.82$ by Wilcoxon test). A moderate but significant correlation between BM and peripheral CCL20 levels was observed in the cohort of MM patients included in the GIMEMA trial (Supplementary Figure S2c).

PET/CT positivity and cytokine and chemokine BM levels in MM patients

Cytokine and chemokine BM levels were compared between PET/CT scan-positive and -negative MM patients. We found that only median CCL3 levels were higher in PET/CT-positive MM patients as compared with PET/CT-negative patients, with a trend towards statistical significance ($P = 0.061$) (Supplementary Table 3). Analysis of all MM patients (symptomatic and SMM) demonstrated that both BM CCL3 ($P = 0.02$) and CCL20 ($P = 0.018$) median levels were significantly higher in PET/CT-positive patients as compared with those who were PET/CT-negative (Supplementary Table 3). However, we did not find a significant correlation between the number of FBLs or the SUV and the BM levels of both chemokines (data not shown).

BM levels of cytokines and chemokines and the presence of lesions on MRI of the spine

Higher median BM levels of Activin-A, IL-3, sRANKL, tRANKL, tRANKL/OPG and sRANKL/OPG ratio, CCL3, CCL20 and DKK-1 were found in symptomatic MM patients with positive MRI as compared with those with negative MRI findings. However, only the median levels of tRANKL ($P = 0.024$) and DKK-1 ($P = 0.013$) reached statistical significance by nonparametric analysis. In our evaluation of all MM patients (symptomatic and SMM), we found significantly higher median BM levels of Activin-A ($P = 0.033$), CCL3 ($P = 0.043$), CCL20 ($P = 0.001$) and DKK-1 ($P < 0.001$) (Table 5) in MRI-positive vs MRI-negative patients. Only median DKK-1 BM levels were significantly correlated with the presence of lesions on MRI ($P = 0.045$, optical density ratio 1.000) by logistic multivariate analysis. Finally, we found that median BM DKK-1 levels were higher in patients with focal lesions as compared with those with diffuse patterns of BM infiltration on MRI (DKK-1 median levels: 3694 pg/ml vs 1520 pg/ml; $P < 0.001$). Average, median, standard deviation and range of cytokine and chemokine levels for MRI-positive and -negative patients are summarized in Supplementary Table 4.

In our analysis of MM patients based on both X-ray and MRI findings, we observed significantly higher BM levels of DKK-1 ($P < 0.001$) and Activin-A with a trend toward statistical significance ($P = 0.089$) for MRI-positive and MRI-negative patients that was independent of X-ray positivity. Significantly higher BM levels of CCL20 ($P = 0.003$) were observed in patients with both MRI and X-ray positivity as compared with those with positive MRI and negative X-rays. Comparison of both X-ray-negative and MRI-negative vs both X-ray-positive and MRI-positive patients demonstrated significantly higher median BM levels of Activin-A ($P = 0.01$), CCL20 ($P < 0.001$) and DKK-1 ($P = 0.015$) (Table 6).

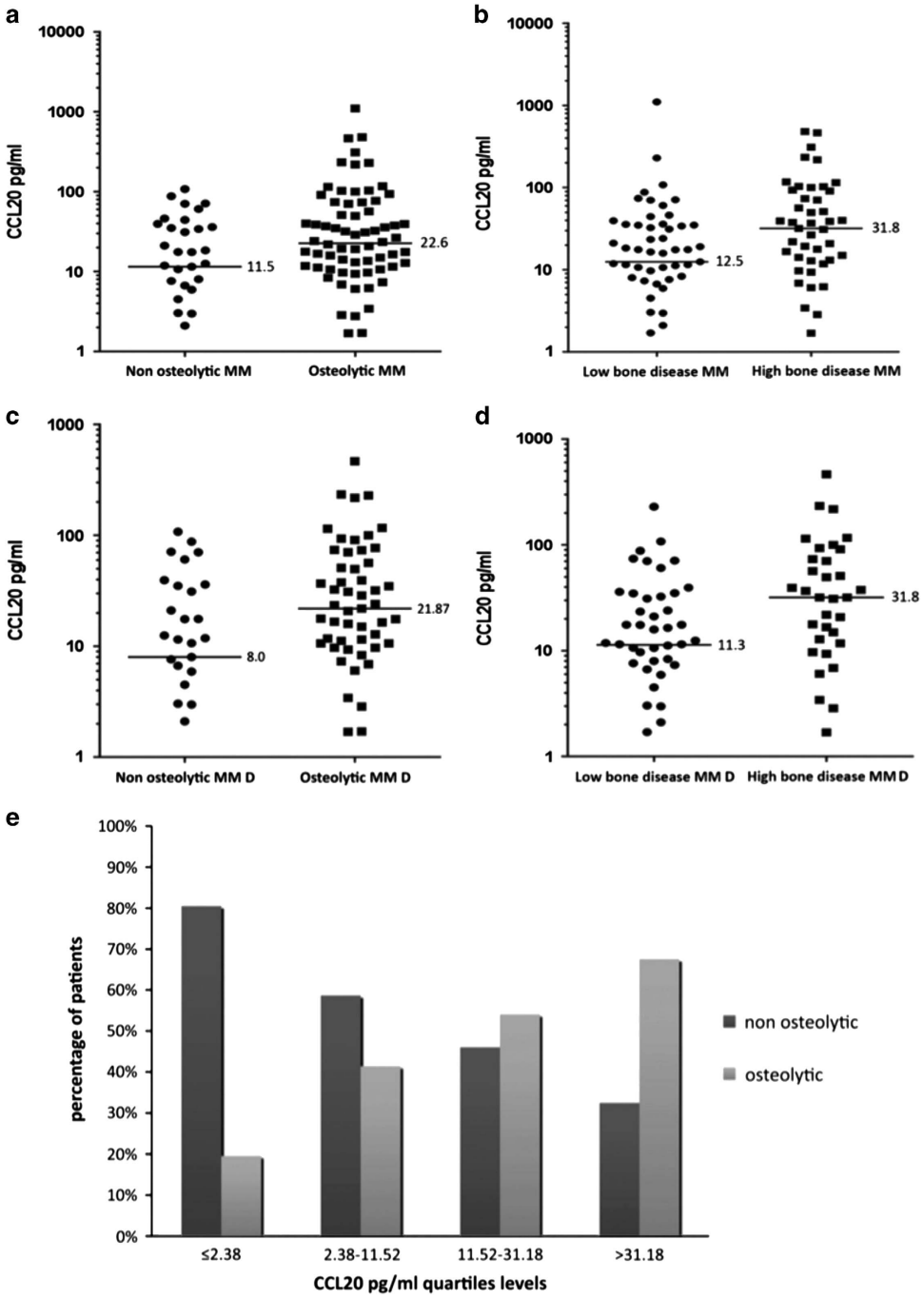

Figure 1. BM CCL20 levels in MM patients in relationship to the presence of osteolytic bone disease. BM CCL20 levels measured by ELISA assay in osteolytic or non-osteolytic patients (**a**) and in LBD or HBD patients (**b**) including all symptomatic MM patients. BM CCL20 levels in osteolytic or non-osteolytic patients (**c**) and in LBD or HBD patients (**d**) including only MM patients at the diagnosis (MM D). Plots and horizontal bars represent the single and the median CCL20 BM levels. (**e**) Relationship between the presence of osteolytic lesions and distribution of CCL20 levels. Bars represent the percentage of patients with or without osteolytic lesions in relationship with CCL20 quartile levels.

DISCUSSION

Bone disease is a hallmark of MM, however, the relationship between bone disease and BM microenvironment cytokine and chemokine levels and MM cell molecular characteristics has not been fully defined. In recent years, new technologies for evaluating skeletal disease in MM have been introduced, including MRI and PET/CT.[2,4,5,30] When compared with the standard skeletal survey, a series of X-rays, MRI has a higher sensitivity for the detection of FBLs, as it can detect early development of bone destruction.[4] PET/CT also has a higher sensitivity and specificity as compared with skeletal survey,[5] and has prognostic impact.[31,32] The potential relationships between the presence of bone lesions identified with these differing modalities and the presence of molecular BM features in MM patients are not known.

Recently, it has been reported that patients with t(11;14) and t(6;14) have more bone disease as compared with patients with t(4;14) or t(14;16).[33] Consistently, we found a higher incidence of osteolytic lesions on skeletal surveys of patients with t(11;14) as

compared with those without this translocation, with a trend of statistical significance, in our cohort of MM patients. It is likely that the higher incidence of osteolytic lesions observed in this group of patients is related to their higher BM DKK-1 levels measured as compared with those negative for t(11;14). Previously, Tian *et al.*[9] reported a lack of significant correlation between del(13q) or hyperdiploid karyotype in MM patients with lesions on MRI, while Robbiani *et al.*[19] described a statistically significant correlation between hyperdiploidy, t(4;14) or t(11;14) and at least one MRI-detected focal lesion. In our cohort of patients we did not observe a statistically significant relationship between MRI or PET/CT scan positivity and the presence of specific cytogenetic abnormalities. This demonstrates that the presence of bone disease identified by separate highly sensitive imaging procedures is not significantly correlated with any specific cytogenetic feature, with the exception of a trend towards statistical significance for patients with t(11;14) carrying more osteolytic lesions on skeletal survey.

Several studies have investigated peripheral serum levels of cytokines and chemokines in MM patients, mainly in comparison with healthy donors or MGUS subjects,[20–23,26,34–36] demonstrating that peripheral CCL3, DKK-1, sRANKL and RANKL/OPG ratio are increased in MM patients as compared with controls.[9,20,21,23,26,37] However, the relationships between these peripheral cytokine levels and patient bone disease are not consistent. In addition, it has been reported that peripheral and BM cytokine levels of many cytokines do not correlate with each other.[26–28] Moreover, most of these studies analyzed peripheral levels of cytokines and chemokines without evaluating the more biologically relevant BM levels. The majority of these cytokines and chemokines are soluble factors and they have local actions in the BM in areas of MM cell infiltration.

Peripheral sRANKL and tRANKL levels and sRANKL/OPG ratio were reported to be higher in MM patients as compared with healthy donors by several groups,[8,20–23,28,36,38] showing a significant impact as a prognostic index.[21,39] However, OPG levels were reported to be either increased or decreased in MM patients as compared with healthy donors.[8,21,22,35,40] In addition, high levels of sRANKL and high sRANKL/OPG ratio have been reported only in MM patients with advanced bone disease.[8,21] In our cohort of patients, BM sRANKL and OPG levels and sRANKL/OPG ratio were increased in MM patients as compared with healthy donors. However, sRANKL levels and sRANKL/OPG ratio did not differ significantly between MM and MGUS or between MM and SMM patients. Consistent with this, it has been reported that sRANKL levels were also higher in MGUS as compared with MM patients.[34] We found that sRANKL levels and sRANKL/OPG ratio were not significantly higher in osteolytic vs non-osteolytic MM patients or in PET/CT-positive vs PET/CT-negative patients, although their levels were higher in MM patients with HBD as reported by others.[21] BM RANKL levels were higher in MM patients with an MRI-positive scan at the spine as compared with those with a negative MRI scan. This observation is in line with the prognostic role of peripheral RANKL levels reported in other studies.[21,39] Overall, our evidence and that of the literature indicate that RANKL levels do not correlate strongly with the presence of osteolytic lesions in MM patients.

DKK-1 is a well-known Wnt signaling cytokine that is associated with MM bone disease.[7,9,37] Elevated serum DKK-1 levels are correlated with the presence of FBLs on MRI.[9] Moreover, a correlation has been reported between DKK-1 levels and the number of osteolytic lesions detected by X-ray.[41,42] In this study, we show that DKK-1 levels are tightly related to the presence of focal MRI lesions, as DKK-1 levels are higher in MRI-positive patients as compared with MRI-negative patients, independent of the presence of osteolysis. This supports the role of DKK-1 as a marker of FBLs on the MRI as opposed to osteolysis on X-ray.

A similar BM level profile was observed for Activin-A, a recently identified cytokine that promotes osteolysis in MM[10] mediating the pro-osteoclastogenic effect of IL-3.[11] Activin-A levels were higher in MM patients with HBD as compared with patients with LBD and we demonstrate a correlation between the presence of lesions on MRI and Activin-A levels.

Several chemokines are involved in osteoclast and osteoblast function.[26,43] Our data indicate that both CCL3 and CCL20 levels are associated with the presence of osteolytic bone disease in MM patients identified on both X-rays and PET/CT. Importantly, we used a logistic multiparametric analysis to demonstrate that median BM CCL20 levels are the only significant predictor for the presence of osteolytic lesions on X-ray. BM CCL20 levels were also related to the presence of bone fractures. Interestingly, subgroup analysis of MM patients based on both X-rays and MRI findings demonstrated significantly higher BM levels of CCL20 in patients with both MRI and X-ray positivity as compared with those patients that were MRI positive but X-ray-negative, suggesting that CCL20 is tightly related to the presence of osteolysis, rather than MRI lesions, an opposite profile as that seen with DKK-1.

CCL20 is a chemokine involved in Th17 recruitment through CCR6.[44] Recently, we have demonstrated the pathophysiologic role of CCL20 in MM-induced bone disease, showing that CCL20

Table 5. Comparison between BM levels of cytokines and chemokines and MRI positivity

MRI positive vs MRI negative	P-*value**
Activin-A	0.033
IL-3	0.584
sRANKL	0.893
tRANKL	0.111
OPG	0.072
tRANKL/OPG	0.277
sRANKL/OPG	0.680
CCL3	0.043
CCL20	0.001
DKK-1	< 0.001

Abbreviations: BM, bone marrow; CCL, C-C motif ligand; IL, interleukin; MRI, magnetic resonance imaging; OPG, osteoprotegerin. **P*-value calculated by nonparametric Mann–Whitney test.

Table 6. median levels of cytokines and chemokines in patients according to the X-ray and MRI

Cytokine/chemokine	*X-ray − /MRI − (A)*	*X-ray − /MRI+ (B)*	*X-ray+/MRI+ (C)*	P-*value**		
				A vs B	*B vs C*	*A vs C*
Activin-A	375.06 pg/ml (173.67–942.23)	501.52 pg/ml (344.40–921.43)	514.27 pg/ml (311.23–1347)	0.089	0.635	0.01
CCL20	4.26 pg/ml (0.00–141.22)	4.51 pg/ml (0.00–36.00)	27.22 pg/ml (1.69–464.58)	0.911	0.003	< 0.001
DKK-1	1022.32 pg/ml (272.08–4602.99)	3539.58 pg/ml (1259.40–68855.74)	2232.20 pg/ml (478.91–43521.10)	0.001	0.238	0.015

Abbreviations: CCL, C-C motif ligand; MRI, magnetic resonance imaging. **P*-value calculated by nonparametric Mann–Whitney test.

Table 7. Chemokines and cytokines whose levels are significantly increased in relationship with the presence of bone disease

Osteolysis (X-rays)	*High bone disease (X-rays)*	*MRI*	*PET/CT*
MM	*MM*	*MM+SMM*	*MM+SMM*
CCL3 ↑	Activin-A ↑	Activin-A ↑	CCL3 ↑
CCL20 ↑	CCL3 ↑	CCL3 ↑	CCL20 ↑
	CCL20 ↑	CCL20 ↑	
	DKK-1 ↑	DKK-1 ↑	

Abbreviations: CCL, C-C motif ligand; MM, multiple myeloma; MRI, magnetic resonance imaging; OPG, osteoprotegerin; PET/CT, positron emission tomography/computerized tomography; SMM, smoldering multiple myeloma.

and its receptor CCR6 are upregulated in the bone microenvironment by MM cells and contribute to osteoclast formation and osteolytic bone lesions in MM patients.[18] In addition, we reported higher CCL20 levels in MM patients with osteolytic bone lesions as compared with patients without osteolytic bone lesions.[18] In this study, we expand our previous findings, identifying BM CCL20 levels as the major biomarker of osteolysis in MM patients. The role of CCL20 as a biomarker of bone disease was also confirmed by testing CCL20 levels in MM patients enrolled in a multicenter Italian trial.[29] Consistent with our findings, it has been reported that MM patients with bone disease have larger populations of Th17 cells as compared with those without skeletal involvement.[17,45] Interestingly, CCL20 overexpression has been reported in Langerhans cell histiocytosis,[46] a disease characterized by typical coin lytic lesions[47] similar to those observed in MM patients. The tight relationship between CCL20 BM levels and the presence of osteolytic lesions supports the hypothesis that MM is characterized by an inflammatory microenvironment[48] or the role of T cells in the development of bone lesions in MM patients.[17,49] Indeed, previous data demonstrate that the MM microenvironment has a pro-inflammatory cytokine pattern, and that T lymphocytes can produce both pro-osteoclastogenic and anti-osteoblastogenic cytokines.[49,50]

Among the chemokines involved in MM bone disease, bone microenvironment cells produce CCL20, while MM cells mainly produce CCL3, which can attract osteoclasts and stimulate bone destruction.[7,14–16] CCL3 is also involved in MM-induced bone disease through the reduction of osteoblast function.[51] Many studies have identified peripheral serum levels of CCL3 as a possible marker of bone disease, as it is higher in patients with osteolytic lesions.[15,16,25,52] Moreover, in a recent study, it was reported that CCL3 and DKK-1 levels are significantly higher in MGUS patients as compared with healthy donors.[53] In our cohort of MM patients, BM CCL3 levels were related to the presence of osteolytic lesions and HBD on X-rays, as well as the presence of lesions on PET/CT. All of these evidences suggest a relationship between BM CCL3 levels and osteoclast activation that is consistent in all patients with monoclonal gammopathies, including MGUS, SMM and MM.[54]

The main clinically relevant result of the present study demonstrates a significant relationship between CCL20 quartile levels and osteolytic bone disease in a population of MM patients. These findings provide the opportunity to stratify MM patients on the basis of BM CCL20 levels, to identify MM patients with osteolytic disease. These findings could allow clinicians to limit the use of X-rays as a screening tool for identification of patients with osteolysis, but does not alter the utility of MRI and PET/CT in the evaluation of MM patients as both of these technologies have high sensitivities and prognostic impact in MM patients.

The main results of the manuscript are summarized in Table 7.

In conclusion, we demonstrated that BM levels of CCL3 and CCL20 are related to the presence of osteolysis on skeletal surveys, and identified BM CCL20 levels as the main biologic marker associated with the presence of osteolytic lesions. In addition, we define BM DKK-1 levels as the primary factor associated with the presence of focal lesions on MRI in MM patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Rebecca Silbermann, Simona Colla, Paola Storti and Dirce Gennari for the technical support. This work was supported in part by a grant from the Associazione Italiana per la Ricerca sul Cancro (AIRC) IG2014 n°15531 (NG). Collaborators: Antonio Palumbo, Fortunato Morabito, Francesco di Raimondo, Massimo Offidani, Francesca Patriarca, Maria Teresa Petrucci, Davide Rossi, Vittorio Montefusco, Tommaso Caravita, Chiara Nozzoli, Magda Marcatti, Tommasina Guglielmelli, Luca De Rosa, Manuela Rizzo, Annibali Ombretta, Mario Boccadoro and Michele Cavo for the GIMEMA (Gruppo Italiano Malattie Ematologiche dell'Adulto) Italian Myeloma Network.

AUTHOR CONTRIBUTIONS

NG designed the study, analyzed the data and wrote the manuscript. MP performed all statistical analyses. MDF performed the X-ray survey. LR performed the total body PET/CT and the vertebral MRI. BDP, FA, LC and NG provide clinical data and patients. MB and FC collect plasma from BM biopsies. PO and RR provided plasma samples. GS performed cytogenetic and FISH analysis. DG performed ELISA assays. NG had full access to the data in the study and take responsibility for accuracy of the data analysis. BDP and NG were involved in interpretation of the results. FA read, provided comments and approved the final version of the manuscript.

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