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Bone remineralization Of Lytic Lesions in Multiple Myeloma -- The Arkansas Experience

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Abstract

Multiple myeloma (MM) patients frequently present with extensive osteolytic bone lesions. However, the impact of myeloma treatment on focal lytic lesion remineralization has not been extensively studied. In this study, the effect of anti-myeloma treatment on the extent of bone remineralization was examined and potential mediators identified. Newly diagnosed MM patients enrolled in the Total Therapy 4 and 5 (TT4; n = 231, TT5; n = 64) protocols were longitudinally evaluated for changes in radiological parameters for a median of 6.1 years. Bone remineralization

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was defined as a sclerotic CT change within the lytic lesion and quantified as a percentage of remineralization, using the initial lesion size as a reference. Such changes were correlated to clinical and biochemical parameters, and the gene expression profile of bone marrow biopsy.

Overall, remineralization occurred in 72% of patients (213/295). Of those patients that experienced remineralization, 36% (107/295) achieved at least 25% of bone remineralization. Patients with high-risk disease defined by gene expression profile signature (GEP70 ≥ 0.66) experienced significant remineralization compared to low-risk MM. Female patients were also more likely to experience bone remineralization and in a shorter median time (2.0 *vs.* 3.3 y). Factors such as serum alkaline phosphatase along with high levels of *RUNX2* and *SOX4* gene expression correlated with increasing extent of bone remineralization. This analysis demonstrated significant remineralization of lytic lesions in MM patients treated on TT clinical trials. While the underlying mechanism remains elusive these findings support the hypothesis that patient baseline bone-related factors play a fundamental role in the skeletal repair of bone lesions in MM that provide new opportunities for improving patient outcomes.

INTRODUCTION

Multiple myeloma (MM) is considered an incurable cancer of abnormal, clonal proliferation of plasma cells (1). Bone disease is a characteristic and defining feature of MM that contributes to significant morbidity and mortality due to the increased risk of skeletal-related events (2). While the driving mechanisms for MM induced lytic bone lesions remains unclear, the uncoupling of osteoblastic activity and osteoclastic activity, with the upregulation of osteoclast differentiation and bone resorption creates a feed forward loop facilitating tumor expansion, resulting in extensive bone destruction (3–5).

Despite a clear understanding of the lytic process in MM patients, little is known regarding the effects of existing treatment modalities on the lytic lesions specifically or what factors determine whether a patient will experience bone remineralization (6). We previously reported the occurrence and extent of remineralization of large pelvic lytic lesions in a subset of patient with low-risk (LR) disease (7). Here, utilizing imaging data collected in Total Therapy (TT) 4 and 5 clinical trials of bone lesions of different sizes, the extent of bone remineralization was correlated with clinical and biochemical parameters as well a patient-specific gene expression profiling (GEP) data derived from baseline bone marrow biopsy.

MATERIAL AND METHODS

Patient Eligibility and Samples

All patients provided written informed consent after UAMS Institutional Review Board approval and this retrospective review of the patients enrolled on the TT4 and TT5 protocols (8) was conducted in accordance with the Declaration of Helsinki. Three musculoskeletal radiologists reviewed a total of 469 MM patients enrolled in TT4 and TT5 protocols for eligibility and 174 patients were excluded. Reasons for exclusion were lack of initial or follow-up PET/CT, suboptimal image quality, non-visualization of the lesion due to vertebroplasty or surgery on initial or follow-up imaging studies, and focal

lesions of < 1 cm in size since measurement of lesions less than 1 cm were technically unreliable (supplement figure 11). A total of 295 patients (231 on TT4 and 64 on TT5) were included in this analysis. Stratification onto TT4 or TT5 was based on GEP70 risk-classifier strategy (9–11). TT protocols encompassed induction chemotherapy, tandem autologous stem-cell transplantation and maintenance with steroids, proteasome inhibitors, and immunomodulatory drugs as previously reported (8, 12). The TT4 enrolled low-risk MM (GEP70 <0.66) between June 2008 and January 2019, the treatment encompassed 2 cycles of upfront induction therapy with multiagent chemotherapy with novel agents bortezomib and thalidomide followed by tandem autologous stem cell transplantation with Melphalan 200 mg/m^2 with consolidation with 2 cycles of multiagent chemotherapy and novel agents and 3 years of maintenance with a triplet combination of bortezomib, revlimid and dexamethasone. In contrast TT5 protocol enrolled HR MM (GEP70 ≥0.66) and treatment included induction therapy with multiagent chemotherapy and novel agents with tandem transplant with Mel80-VRDPACE conditioning with an inter-therapy with Melphalan 5mg/m2×4 days VTDPACE (75% of dose) × 2 cycles and fixed duration maintenance therapy alternating bortezomib, revlimid dexamethasone and bortezomib, melphalan and dexamethasone for 3 years. The study schema is as shown in Supplemental Fig. 9. Monthly bisphosphonates were allowed on the study.

Identification of Bony Focal Lesion

A bony focal lesion was defined as a lytic focal area measuring ≥ 1 cm on CT. The CT study was either a standalone dedicated CT or the CT portion of the PET/CT study. The single largest lytic lesion measuring ≥ 1 cm was chosen on baseline PET/CT and followed serially in each patient. Remineralization of the lesion was defined as a sclerotic change in the previously seen lytic lesion with a decrease in the lytic component using the largest diameter compared to the baseline imaging study. Measurements were obtained on the plane showing the largest dimension of the lytic lesion. The same plane was used for the initial and final CT measurements. Most of the bone lesions were measured in the axial plane, which inherently represents the largest possible diameter. Baseline lesions measured on the CT portion of PET/CT were followed up and measured on CT portion of PET/CT only. Similarly, lesions measured on stand-alone CT were followed up on stand-alone CT only. As such, measurements were internally controlled for imaging modality precision and reproducibility. Extent of remineralization at the lytic site was calculated as a percentage, using the initial lesion size as reference. Percent remineralization was defined as (initial lesion size – final lesion size)/initial lesion size × 100 and categorized into quartiles of remineralization as follows: < 25%, 25–50%, 51–75%, and 76–100%. For data analyses, bone lesions with remineralization falling within the top 3 quartiles were then defined as positive for remineralization (≥ 25%) and those lesions with < 25% were considered negative for remineralization (supplement figure 10). The CT scans are repeated at an average of every 3 months during the first year of diagnosis and later every 3–6 months afterwards depending on the patient's disease status, subsequently continued at least once a year for patients who remain in sustained remission.

Gene Expression Profiling

Iliac crest bone marrow (BM) biopsy samples were collected at diagnosis and immediately frozen and stored in liquid nitrogen. The frozen bone biopsy samples were pulverized with a mortar in liquid nitrogen with their content of marrow intact. RNA was then extracted using an RNeasy kit (Qiagen, Germantown, MD). GEP on bone biopsies from the lesions in the 286/295 MM patients' samples were previously performed using the Affymetrix U133 Plus 2.0 gene microarray (13). Raw cel files were downloaded from the GEO website and imported into Partek Genomics Suite software. The data was subjected to a Robust Multichip Averaging (RMA), GC, quantile normalization followed by log2 transformation (14). Differential gene expression was determined by one-way ANOVA corrected for multiple testing and a gene enrichment set analysis (GSEA) was performed with 100 permutations and normalization of gene enrichment scores (NES).

Statistical Analysis

Comparisons of clinical parameters and occurrence of lytic bone lesion remineralization were performed with Student's t-test, one-way ANOVA, or Fisher's Exact test as appropriate. To correct for when multiple t-tests were applied, *P*-values were adjusted with the Benjamini and Hochberg false discovery rate (FDR) or Šídák's multiple comparisons test. These adjusted *P*-values (adj. *P*-value or FDR) are noted. All analyses were two-sided and performed using GraphPad Prism 6 software with significance considered at *P*-value, FDR, or adj. *P*-value of less than 0.05. Survival analyses were conducted with the Kaplan Meier method and significant differences were assessed by the log-rank test or Cox regression multivariate analysis using Partek Genomics Suite software.

RESULTS

Patient and Bone Lesion Characteristics

Overall, the 295 patients were followed for a median of 6.1 years with TT4 (n = 231) and TT5 (n = 64) patients having a median follow-up of 6.7 and 4.0 years, respectively. Patient baseline characteristics are shown in supplement Table 1. Bone remineralization in the form of new bone mineralization ranged widely in terms of measurable changes such as sclerosis and calcification. The first sign of remineralization was considered when estimating the time to remineralization and noted as an average of 26 months (2 – 88 months) following initial PET/CT. There were also non-measurable changes including development of thin rim of sclerosis, which was observed as early as two months after the initial PET/CT. The first evidence of remineralization was seen 4 weeks after initiation of treatment. Representative cases with remineralization and no detectable remineralization are provided in Fig. 1. Remineralization of the lytic lesion by 25–50% was documented in 42/295 (14%), 51–75% in 32/295 (11%) and 76–100% in 34/295 (12%) patients. The remaining patients (n = 187) showed less than 25% remineralization with 82 cases having no evidence of remineralization (82/295; 28%). Lytic lesions were distributed throughout the skeleton with the majority involving the pelvic bones (34.3%) (Fig. 2A). Consistent with previous surveys in MM patients (15), other lesion sites included the vertebra (19.5%), sternum (11.5%), sacrum (10.6%), ribs (8.9%), scapula (5.6%), cranial and skull (5.0%), and long bones (femur, humerus, tibia, and clavicle; 4.6%) (Fig. 2A). A similar lesion distribution was observed in

both TT protocols except lesions were less frequent in the sternum and absent in the clavicle in patients on TT5 (Supplemental Fig. 1). The average lesion size was 3.5 cm (range 0.1 cm to 16.5 cm) and the average diameter at a given site is shown in Fig. 2B. Of note, lesion size varied depending on location ($P = 0.0009$) with lesions in the ribs having the largest average diameter (4.7 ± 0.5 cm) relative to vertebra (adj. $P = 0.005$) and the cranium and skull (adj. $P = 0.005$; Fig. 2B). When bone regions were classified as either trabecular or cortical (205/295; remaining 90 were irregular) and compared for occurrence of remineralization, trabecular bones showed signs of remineralization more frequently than cortical counterparts ($P = 0.01$; Fig. 2C). However, this analysis is limited due to the low number of cortical bones affected by MM induced lesions (n = 14).

Among the patients enrolled on TT5, about one third presented with more than 100 lytic lesions at the baseline PET/CT. Since these patients presented with multiple lesions, we evaluated remineralization at a secondary site (cervical vertebra) and the index lytic lesion to assess whether there was a predisposition to remineralization at all sites. Remineralization at a secondary, cervical lesion occurred at the same frequency whether initial locations did or did not remineralize (73%, 8/11 *vs.* 75%, 5/7; $P = 1.0$, R.R. 0.95, 95% CI: 0.2 – 4.3; Supplement table A1). However, three patients with complete remineralization of the index lesion also demonstrated 100% remineralization at a secondary cervical site. Similarly, 63% (5/8) of patients with 25–75% remineralization at the primary site showed at least 25% remineralization of the cervical lesion.

Frequent and More Complete Remineralization of Lytic Bone Lesions in Patients with high-risk Disease

A significantly higher proportion of remineralization was observed in MM patients with a high-risk (HR) GEP70 risk score of ≥ 0.66 compared to those with no remineralization (32%, 34/108 *vs.* 9%, 17/187; $P < 0.0001$; R.R. 2.2, 95% C.I. 1.5 – 3.4; Table 1) indicating HR disease may be associated with robust remineralization. Remineralization was also observed in the majority of TT5 patients compared to patients on TT4 (69%, 44/64 *vs.* 28%, 64/231; adj. $P < 0.0001$; Fig. 3A). Furthermore, patients receiving TT5 were more likely to experience remineralization (H.R. 37.0, 95% C.I. 19.1 – 71.6; $P < 0.0001$; Fig. 3B) and within a shorter time frame than TT4 patients (0.8 y; range, 0.2 – 3.4 *vs.* 1.9 y (range, 0.2 – 7.3); Table 1). The more frequent bone remineralization was not the result of smaller lesion size in patients with remineralization, since these patients presented with comparable sized lesions on average to lesions with no remineralization (4.0 *vs.* 3.2 cm; adj. $P = 0.99$; Table 1 and Supplemental Fig. 2A). There was no significant difference in bone lesion size between TT4 and TT5 patients (3.6 *vs.* 3.2 cm; adj. $P = 1.00$; Table 1 and Supplemental Fig. 2B).

In addition, the extent of remineralization was greater in HR patients with 45% (23/51) experiencing over 50% bone remineralization compared to 17% (42/244) LR patients ($P < 0.0001$; Fig. 3B). Likewise, for TT5 patients, 45% (29/64) achieved over 50% lesion remineralization whereas only 16% (36/231) of TT4 patients reached similar remineralization ($P < 0.0001$; Fig. 3C). Taken together, these data suggest that the incidence, extent, and shorter time of remineralization correlate with HR disease according to GEP70. No significant differences were observed in the proportion of patients with high ISS stage

disease within the remineralized group (65%, 122/187 *vs.* 65%, 70/108; $P = 1.00$; data not shown) despite an increased presence of stage III patients in the TT5 cohort (supplement Table 1).

Patient Sex and Serum Alkaline Phosphatase Level Affect Bone Remineralization

First, the frequency of remineralization in males versus females was assessed. The analysis detected a trend towards remineralization occurring at a slightly higher rate in females (48/110, 44% *vs.* 60/185, 32%; $P = 0.06$; Table 1 and Supplemental Fig. 3A). Female patients with lesion remineralization also experienced a shorter time to bone lesion remineralization compared to males (median time 2.0 y *vs.* 3.3 y; $P = 0.01$; Supplemental Fig. 3B). Next, serum biochemical markers associated with high bone remodeling were measured (Table 1). Consistent with a role in the mineralization of these MM patients, higher alkaline phosphatase (ALP) levels correlated with remineralization as a continuous measurement during the patient follow-up rather than the baseline level (66 IU/L *vs.* 74 IU/L; adj. $P = 0.001$ or 67 IU/L *vs.* 79 IU/L; adj. $P = 0.002$; Table 1). When serum ALP was analyzed longitudinally for a subset of patients (201/295: 74/108 with remineralization; 127/187 with no remineralization), there was a striking incidence of two additional peaks of ALP levels in patients with notable remineralization at 1.5- and 3.25-years post diagnosis. Such a phenomenon was not observed in patients without remineralization (adj. $P = 0.09$; Supplemental Fig. 4).

MM Biopsies from Patients with Remineralization of Lytic Bone Lesions Exhibit a Pro-Mitotic/Cell Cycle and Bone Forming Gene Expression Profile

Next, the GEP of BM biopsies available at the time of diagnosis from patients with (n=102) and without (n=183) bone lytic lesion remineralization were compared. There were 241 genes down-regulated and 4 genes up-regulated in the BM samples without bone remineralization relative to gene expression in BM from patients with bone remineralization (Supplemental Fig. 5, Table A2). Enrichment of particular gene sets according to a GSEA indicated most of the differentially expressed genes were of mitotic, cell cycle, and DNA/chromosome integrity related pathways with a significant down-regulation of these genes in BMs from patients without bone remineralization (Fig. 4A; Supplemental Table A4). Representative genes from these gene sets with an $FDR < 0.01$ and an average fold-change of at least 1.5 are shown in a heatmap (Fig. 4B) and several of these genes are also included in the chromosome instability signature previously identified in HR MM and other cancers (*MAD2L1*, *CCNB2*, *UBE2C*, *TRIP13*, *NEK2*, *TTK* and *KIF20A*) (16, 17). These data suggest a distinct gene expression landscape exists in remineralization *vs.* non-remineralization patients.

In addition to these gene ontology associations, two of the genes, *RUNX2* and *SOX4*, have well-known roles in bone formation (18–23). BM biopsies from patients with remineralization expressed *RUNX2* and *SOX4* mRNA at significantly higher levels compared non-remineralization patients, suggesting a potential predisposition to bone remineralization at baseline ($FDR = 0.0006$ and $FDR = 0.001$, respectively; Fig. 4B). Consistent with an association of *RUNX2* in HR MM (24), *RUNX2* mRNA was also 3-fold higher in BM samples from TT5 patients compared to TT4 patients ($FDR = 0.0002$,

Supplement Table A4). Since the majority of patients with bone remineralization were on the TT5 protocol, this finding further supports the idea that enhanced *RUNX2* mRNA is correlated with remineralization. Similarly, the GSEA from TT4 *vs.* TT5 samples mirrored the remineralization *vs.* no remineralization enrichment pattern (Fig. 4A; supplement Table A5); although, as a whole there were more up-regulated genes in BM from TT4 patients than in the BM without remineralization comparison (Supplemental Fig. 6 and Supplement Table A4). This difference is most likely due to individual samples that did not mirror the overall trend of patients that received TT5 treatment protocol experienced remineralization compared to TT4 patients. Despite remineralization of the majority of lesions from TT5 patients, there were 20 cases that did not, and these samples were then included in the no remineralization group for the GEP analysis. Conversely, there were 64 samples from TT4 patients that were considered positive for remineralization and were a part of the remineralization group for the GEP analysis. Among the significantly up-regulated genes in the TT4 samples included those involved in tumor necrosis factor (*TNFα* and *TNFRSF13C*), G-coupled protein receptor (*PRKCA, FRZB, CXCR4, MC4R*), and EGFR (*EGFR*) signaling, which have known roles in bone remodeling particularly in cancer(25–27)

Remineralization and Progression Free Survival

Myeloma bone disease contributes to the overall performance status and comorbidity of patients, thus we next examined whether remineralization correlated with patient outcome. Overall survival (OS) of patients with remineralization of bone lesions did not differ from those with no remineralization (9.4 y *vs.* >11 y, $P = 0.41$; Fig. 5A, left panel). This lack in significantly different OS did not change when patients were further stratified according to TT protocol (TT4, >11 y *vs* >11 y, $P = 0.60$; TT5, 5.6 y *vs.* 4.9 y, $P = 0.43$; Fig. 5A, right panel). Patients with bone remineralization were noted to have a worse progression-free survival (PFS) compared to patients with no or minimal bone remineralization (5.5 y *vs.* >11 y; $P = 0.002$; Fig. 5B, left panel); however, this correlation is presumably due to the majority of these patients also having HR disease. In support of this idea, a multivariate analysis confirmed the difference in PFS based on remineralization status was not independent from TT protocol (H.R. 0.8, C.I. 0.6 – 1.2; $P = 0.33$). Accordingly, stratifying patients into TT regimen confirmed there was no difference in PFS among patients with or without bone lytic lesion remineralization (TT4, >11 y *vs* >11 y, $P = 0.54$; TT5, 2.2 y *vs.* 3.2 y, $P = 0.43$; Fig. 5B, right panel). As expected, there was an inferior OS and PFS for HR MM compared to LR MM ($P < 0.0001$; Supplemental Fig. 7). We also examined survival in male and female patients and found no significant differences in either OS (**Fig. 8A**) or PFS (**Fig. 8B**) between the two sexes regardless of TT protocol; although, males on TT5 tended to exhibit an inferior PFS with a shorter median time to progression compared to female patients (2.1 *vs.* 4.2 y, $P = 0.06$; Supplemental Fig. 8B, right panel).

DISCUSSION

MM bone disease is exceedingly common with 80% of patients having osteolytic lesions at diagnosis with ongoing risk of subsequent relapse. Apart from the significant morbidity and mortality, the impact of skeletal-related events (SRE) on health-care resource utilization

and costs for MM patients is unprecedented (28–30). In this study of both LR and HR MM we convincingly demonstrate that remineralization occurs in a significant proportion of lytic lesions of varying size and location. This study expands on previous studies demonstrating significant remineralization of large lytic myeloma bone lesions of the pelvis (7). The high incidence of pelvic lytic lesions seen in this study confirms the findings of our previous investigation that specifically examined pelvic lesions only in TT4 treated patients (7). In contrast to our prior work, we observed fewer occurrences of remineralization in TT4 patients most likely due to the current study including other sites as the index lytic lesion that may not have displayed as frequent remineralization as the pelvic lesions. Based on the GEP70 score, patients with HR MM treated on TT5 clinical trial as well as those with elevated *RUNX2* and *SOX4* mRNA levels may experience significant bone remineralization. These data provide new insight into the underlying factors that may contribute to MM bone disease remineralization.

Our work provides the first direct evidence that lytic lesions could be monitored expectedly (7). Importantly, our analysis reveals that incremental increases in serum ALP could serve as a biomarker of remineralization. Sclerotic changes were first seen early and improved during treatment. In addition, in HR disease the expression of *RUNX2* and *SOX4* may be predictive of bone remineralization. A vast majority of patients with HR MM on the TT5 protocol presented with baseline lytic bone disease and had a noticeably higher percentage of remineralization compared to patients with LR disease. This finding contrasts with previous reports of a lower prevalence of myeloma bone disease in patients with HR disease identified by the prognostic markers MAF and t (4:14) (31–33).

In the GEP analysis of BM biopsies described here, a significant enrichment of mitotic/cell cycle and osteoblast differentiation genes was observed in the BM cells from patients with remineralization. It is important to note that these BM biopsies contain a mixture of cell types and the precise percentage of MM cells within these samples is unknown. We and others have shown evidence of spatial temporal and intratumoral heterogeneity between the bony lesions and random bone marrow biopsies (34, 35), however these data point towards several potential pathways all of which are worthy of future investigation. Although, *RUNX2* expression (a master regulator of osteoblast programming) is implicated in MM disease progression (24, 36) the bone destruction initiated by HR MM most likely has additional drivers beyond merely *RUNX2*. It is conceivable that HR MM releases pro-bone formation molecules that favor bone remineralization over bone resorption. The model for how HR MM causes bone destruction that precedes or simultaneously facilitates bone remineralization will require further mechanistic studies but may well involve the activation or involvement of other bone resident cells, such as osteocytes (36). The interplay between aggressive MM and the occurrence of bone lesions may also depend on our newly discovered association with decreased *SOX4*. Similar to *RUNX2*, *SOX4* is also considered an important regulator of osteogenesis and the loss of this important transcription factor leads to compromised bone formation in mouse models (22, 23). Our findings of lower *SOX4* mRNA levels in patients that did not achieve significant bone remineralization support these previous observations regarding diminished bone formation. With such a notable difference in remineralization between patients on the TT4 and TT5 protocols, it is important to consider the effect of different MM therapies on the extent and likelihood

of bone remineralization (37–43). The study described here identifies a link between bone remineralization and MM therapy that has previously gone unnoticed. Overall, the current study provides valuable insight into the remineralization status of lytic lesions in a robust and large data set. However, the results should be viewed in the context of the limitations inherent in the retrospective nature of the study and the heterogeneous study population of HR and LR MM treated on different clinical trials. While we propose that remineralization would likely correlate with the incidence of SREs, or pain scores or quality of life measurements, these data were not available in our study, but should be considered and followed prospectively. Our ongoing studies include the biopsy of the remineralized lesions and more frequent imaging to better comprehend the actual bone composition and quality of repair. Indeed, these additional investigations are clinically pertinent to identify patients in whom routine surgical intervention of an otherwise stable myelomatous lytic lesion could be averted. Knowledge of several pathways of osteoclast activation and osteoblast inhibition including the receptor activator of nuclear factor-kB ligand/osteoprotegerin pathway, activin-A, Wnt pathway regulators such as dickkopf-1 (DKK1) and sclerostin provide new opportunities for the development of evidence-based myeloma bone disease therapies (43–47). Our data provides a strong and compelling rationale for prospective clinical trials in MM patients with comprehensive bone end points that include skeletal related events, biomarkers (alkaline phosphatase), bone marrow biopsies and more frequent imaging (PET/CT or MRI). The re-examination of bone healing in the setting of MM also supports additional studies into the use of bone anabolic agents targeting sclerostin, DKK1, and TGFβ that may enhance bone healing along with the standard anti-resorptive approach offered by standard of care bisphosphonate therapy (48–51). Nevertheless, this study has demonstrated that a significant proportion of patients treated on TT protocols incorporating novel agents exhibit significant remineralization of the lytic lesion particularity in high-risk MM and female MM patients which was previously unknown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Remineralization of bony lytic lesions was observed in majority of patients with multiple myeloma.
- Patients with high-risk multiple myeloma can present with significant lytic lesions at presentation and exhibit more complete and faster remineralization.
- Females patients with multiple myeloma demonstrate faster remineralization.
- An increased expression of *RUNX2* and *SOX4* with a pro-mitotic cell cycle and bone forming gene expression profile signature on bone marrow biopsy at diagnosis may be predictive of bone remineralization.

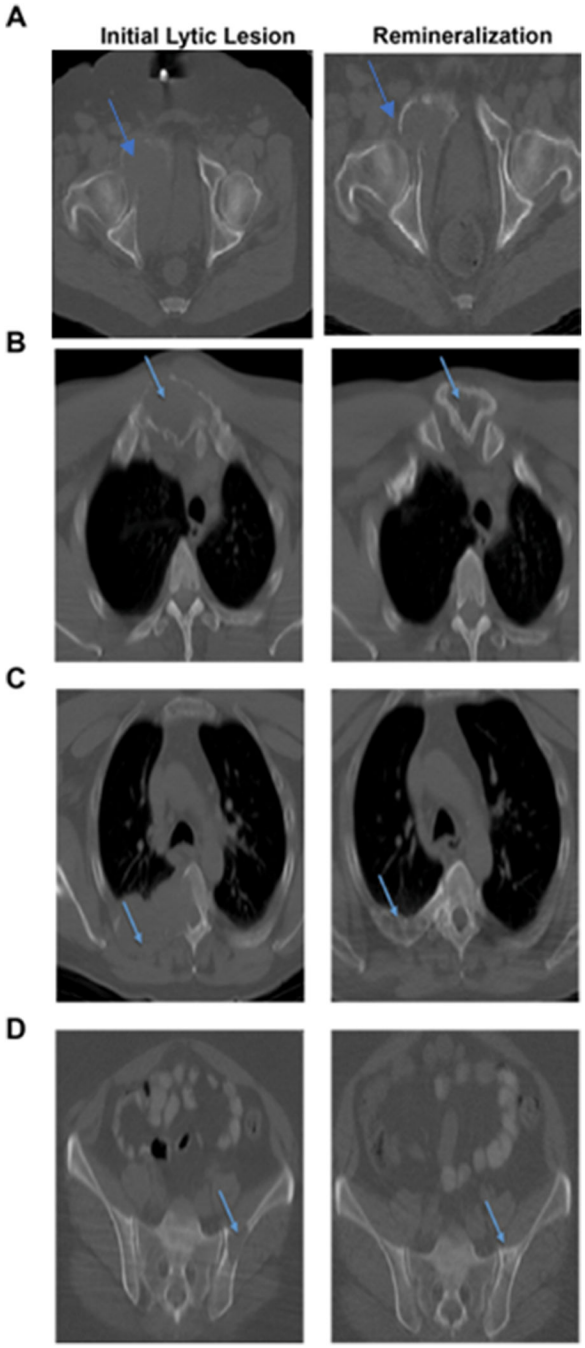

FIG. 1. Representative cases showing extent of bone remineralization.

(A) Right acetabulum showing initial lytic lesion (left; blue arrow) and remineralization (right; blue arrow). Extent of remineralization is < 25% remineralization. (B) Large Manubrial lesion showing initial lytic lesion (left; blue arrow) and remineralization (right; blue arrow). Extent of remineralization is 40% remineralization with thick sclerotic rim formation. (C): Right Rib 4th lesion showing initial lytic lesion (left; blue arrow) and remineralization (right; blue arrow). Extent of remineralization is 75% remineralization. (D)

Left IIium showing initial lytic lesion (left; blue arrow) and remineralization (right; blue arrow). Extent of remineralization is complete, 100% remineralization

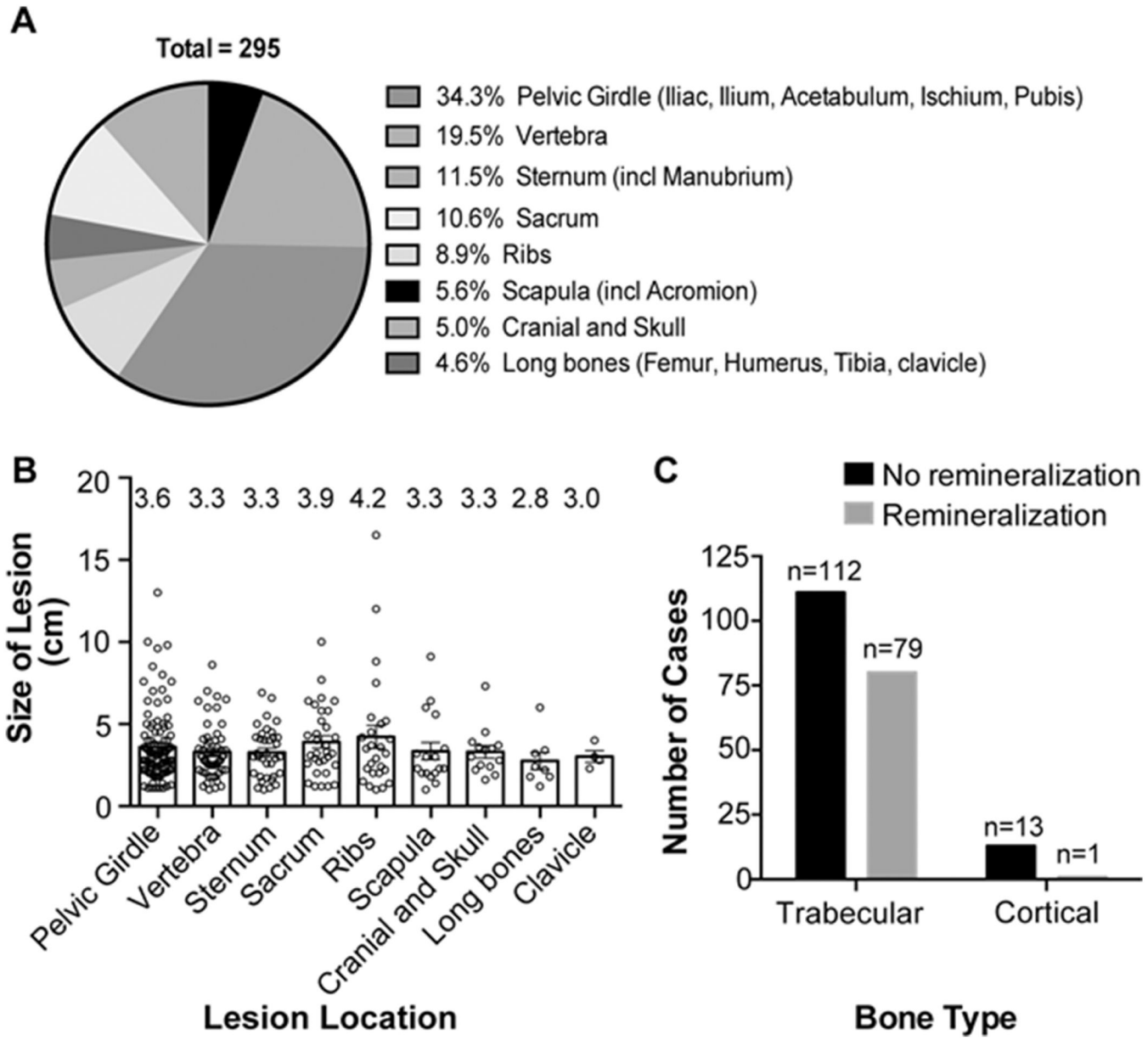

FIG. 2. Location and size of bone lytic lesions.

(A) Frequency of lytic lesions in each location for all of the MM patients. (B) Size of lesion according to lesion location. Mean size in cm is provided above each location. (C) Incidence of bone lesion remineralization according to bone regions. Black column – no remineralization; Grey column – remineralization.

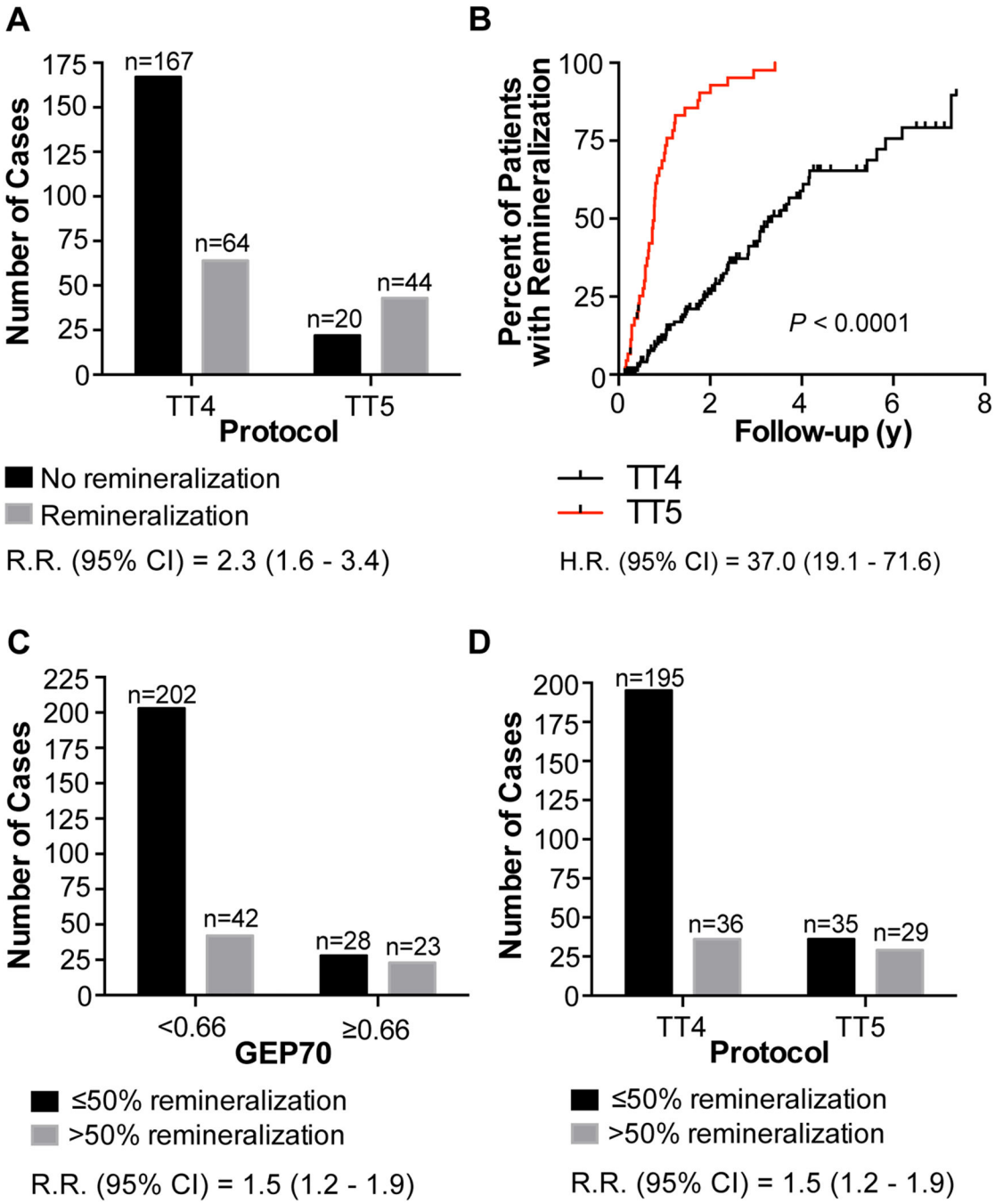

FIG. 3. Incidence of remineralization of bone lytic lesions in high-risk versus low-risk multiple myeloma patients.

(A) A higher proportion of patients on the TT5 protocol experienced remineralization compared to those on TT4. Black column – no remineralization defined as less than 25% remineralization; Grey column – remineralization defined as 25 – 100% remineralization. (B) Kaplan Meier curves of the incidence of remineralization in TT4 (black line) and TT5 (red line) patients. Patients on the TT5 protocol have a greater likelihood of bone remineralization compared to those on TT4. (C) A higher proportion of patients with high-risk (HR) GEP70 score (≥ 0.66) remineralize to a greater extent than those

considered low-risk. $P < 0.0001$. Black column – no remineralization defined as ≤ 50% remineralization; Grey column – remineralization defined as 51 – 100% remineralization. $P < 0.0001$. (D) A higher proportion of patients considered HR based on TT5 protocol eligibility and enrollment remineralize to a greater extent than those on the TT4 regimen. Black column – no remineralization defined as ≤ 50% remineralization; Grey column – remineralization defined as 51 – 100% remineralization. $P < 0.0001$. GEP70, 70-gene expression profile classifier for high-risk patients with short progression-free and overall survival; CI, confidence interval; TT4/TT5, total therapy 4/5; H.R., hazards ratio.

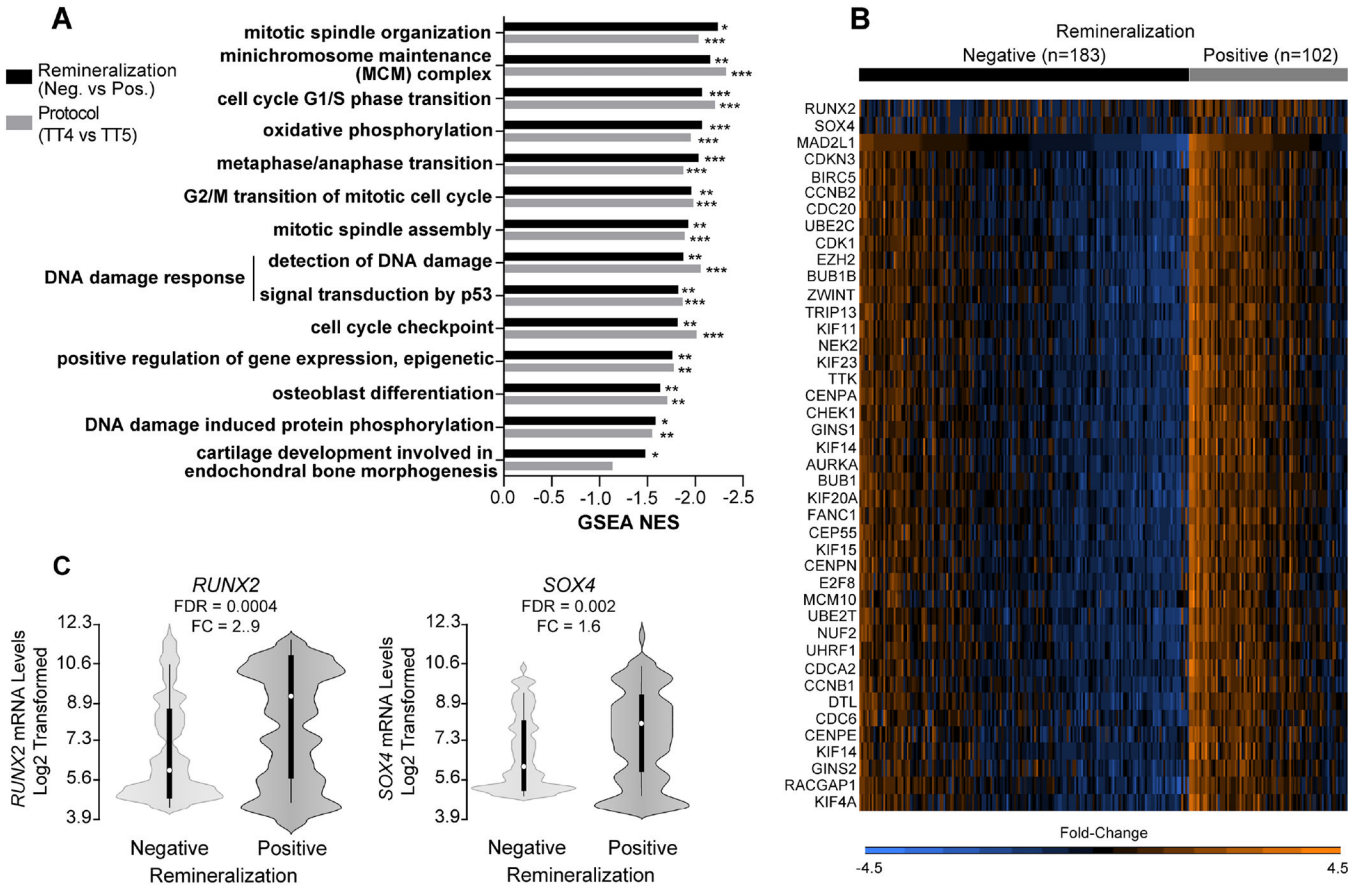

FIG. 4. Gene expression profiling of bone lesions from patients with and without remineralization.

(A) GSEA of genes differentially expressed between MM from patients with and without remineralization (black bars) and between MM from TT4 or TT5 enrolled patients (grey bars). **FDR* < 0.25, ***FDR* < 0.15, *FDR* < 0.05. (B) Heatmap of the differentially expressed genes detected in patients with (black bar) and without remineralization (grey bar). Overall, MM from patients with remineralization exhibit higher levels of the genes listed on the left. Blue – genes at low expression; Orange – genes at high expression; Black – genes with no difference in expression. (C) MM biopsies from patients with remineralization express higher levels of *RUNX2* (*FDR* = 0.0004) and *SOX4* (FDR = 0.002). GSEA, gene set enrichment analysis; NES, normalized enrichment score; FDR, false discovery rate; FC, fold change.

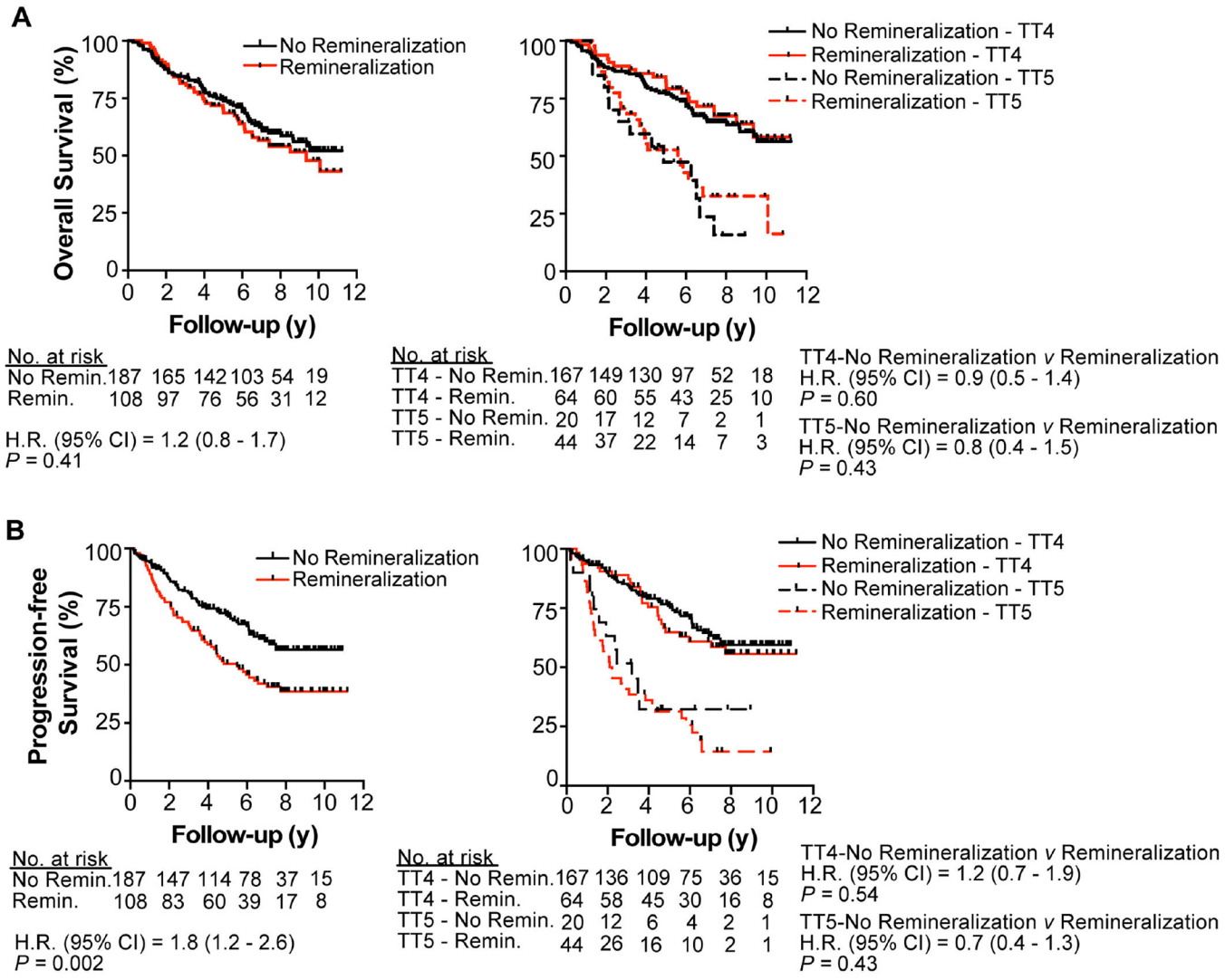

FIG. 5. Overall and progression-free survival of multiple myeloma patients with and without bone lesion remineralization.

(A,B) Kaplan Meier curves for the overall survival (A) and progression-free survival (B) of MM patients with (red lines) or without (black lines) remineralization of bone lytic lesions. Left panels display patients on both TT4 and TT5 protocols and right panels display patients stratified according to TT4 (solid lines) or TT5 protocols (dashed lines). Tables indicate the remaining patients at the given time point shown above on the x-axis (Follow-up (y)). CI, confidence interval; TT4/TT5, total therapy 4/5; H.R., hazards ratio.

Table 1.

Patient comparisons according to remineralization and Total Therapy protocol.

Characteristic		Remineralization*			Protocol		
		< 25%	25 – 100%	*P*-value	TT4	TT5	*P*-value
	n	187	108		231	64	
Median age, years (range)		61 (36 – 75)	60 (30 – 74)		58 (30 – 75)	59 (33 – 74)	
Sex:							
	Male	125 (68)	60 (32)	*0.06*	148 (64)	38 (58)	*0.47*
	Female	62 (56)	48 (44)		83 (36)	27 (42)	
Mean diameter of lytic lesion, cm (range)		3.2 (1.0 – 12.0)	4.0 (0.1 – 16.5)	*0.99*	3.6 (1.0 – 16.5)	3.2 (1.0 – 9.0)	*1.00*
Median time to remineralization, years (range)		-	1.9 (0.2 – 7.3)		1.9 (0.2 – 7.3)	0.8 (0.2 – 3.4)	*<0.0001*
Mean Alkaline Phosphatase, IU/L (range):							
	At diagnosis	66 (22 – 167)	69 (33 – 155)		66 (22 – 167)	72 (33 – 155)	
	Continuous	66 (28 – 173)	74 (33 – 225)	*0.72* *0.002*	67 (28 – 225)	78 (33 – 196)	*0.43* *0.02*
B2M ≥ 3.5 mg/L		104 (55)	60 (56)	*0.90*	119 (52)	45 (69)	*0.02*
GEP70 ≥ 0.66		17 (9)	34 (32)	*<0.0001*	2 (0.9)	49 (75)	*<0.0001*

Note: Data are presented as No. (%) and at time of diagnosis unless otherwise noted

P-values determined by Fisher's Exact Test or Student's t-test and adjusted with Šídák's multiple comparisons test.

*Remineralization defined as ≥25% remineralization of lytic lesion as determined by CT scan.

TT4/5, total therapy; B2M, beta-2 microglobulin; GEP70, 70-gene expression profile classifier for high-risk patients with short progression-free and overall survival; ND, not determined as data was not collected.