1. Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by proliferation of malignant plasma cells in the bone marrow. Osteolysis is one of the main clinical consequences of MM and concerns 90% of the patients. Osteolytic foci in MM patients induce serious clinical manifestations such as bone pain, fractures of long bones, vertebra and/or hypercalcemia. Bone fractures occur in 60% of patients (Roodman, 2009). Moreover, bone fractures have a higher incidence in MM than in other cancer (breast, prostate and lung cancer) with a high frequency of bones metastases. Pathological fractures in MM are associated with a 20% increased risk of death (Saad et al., 2007). In the last decade, the median survival of MM patients has been improved due to the apparition of new treatments. The median survival is now approximately 5–8 years and reaches 10 years for patients with low-risk cytogenetic abnormalities (Avet-Loiseau et al., 2012; Chesi and Bergsagel, 2015). Bone resorption, due to an increase in osteoclast number, is increased in MM patients in the vicinity of plasma cell nodules (Bataille et al., 1989). Secretion of numerous cytokines by malignant plasma cells and the bone marrow microenvironment are responsible for an increased osteoclastogenesis in MM (Roodman, 2009). Several cytokines have been identified to play a key role in osteolysis: tumor necrosis factor alpha (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6), macrophage inflammatory protein-1-alpha and beta (MIP-1α and MIP-1β) and ligand for receptor activator of nuclear transcription factor-κB (RANKL) (Kato et al., 2002; Xi et al., 2016). In overt MM, a decrease in bone formation is associated, leading to an uncoupling in bone remodeling at the later stages of the disease. Inhibition of osteoblastogenesis is due to inhibitors released by plasma cells that depress the Wnt-signaling pathway: DKK1 (Dickkopf-1) and Sfrp2 (Secreted frizzled-related
protein 2) (Tian et al., 2003) or act on osteoblastic precursors: hepatocyte growth factor (HGF), interleukin-3 (IL-3) and interleukin-7 (IL-7) (Ehrlich et al., 2005; Standal et al., 2007).

Animal models can help to understand the molecular, cellular and tissue aspects of the pathophysiology of human diseases and to develop new therapeutic strategies. Several murine models of human MM have been described in the literature (see reviews in (Libouban, 2015; Paton-Hough et al., 2015)). Among them, the 5T2MM murine model was described in the C57BL/KaLwRij strain (Radl et al., 1988, 1979). It presents the most common aspects of the human disease with moderate growth, homing restricted to the bone marrow microenvironment and osteolytic lesions. A monoclonal protein (M-protein) is detected from 6 weeks (w) after injection and osteolytic lesions are radiographically visible at 8 w (Libouban et al., 2004). The 5T2MM model was extensively used to evaluate the effect of antineoplastic and antiangiogenic drugs (Deleu et al., 2009a; Deleu et al., 2009b) or inhibitors of bone resorption such as bisphosphonates (Croucher et al., 2003; N'Diaye et al., 2015; Radl et al., 1985). The model is also of the utmost importance to elucidate the pathophysiology of the disease (Buckle et al., 2012; Vanderkerken et al., 2000). It has been shown that a high bone remodeling level (as induced by ovarectomy in mice) accelerates plasma cell growth (Libouban et al., 2003). Similarly, other factors known to increase bone remodeling have also been found to burst the growth of carcinomatous cells (Hirbe et al., 2007; Ooi et al., 2010; Zheng et al., 2008, 2007, 2011). A calcium deficient diet stimulates the growth of MF-7 human breast cancer cells in the mouse by inducing a secondary hyperparathyroidism (Zheng et al., 2007). More recently, it was shown that a vitamin D deficiency promotes MDA-MB-231 breast cancer cells in the mouse by also inducing a secondary hyperparathyroidism (Ooi et al., 2010). These studies highlight the importance of calcium intake and bone remodeling on tumor growth and osteolysis. They have analyzed the pathophysiological mechanisms at the cell and tissue levels in animal models of bone metastasis; however, molecular alterations in the bone microenvironment have received little consideration. The aim of the present study was to investigate the effect of a dietary calcium deficiency on MM development at the tissue, cell and molecular levels in the 5TMM model. Microcomputed tomography (microCT), histomorphometry and gene expression profile of the microenvironment were used.

2. Material and methods

2.1. The multiple myeloma cell line

We have used a 5T2MM cell line (subclone 5THL) in C57BL/KaLwRij mice as previously characterized (Libouban et al., 2004). These malignant plasma cells can be propagated into young syngeneic mice by intravenous transfer of cells from invaded bone marrow. Progression of the disease in recipient mice was assessed by measuring the serum M-protein level. Around 6 w post-injection, mice had a detectable serum M-protein and were sacrificed after 10–11 w by cervical dislocation. Femurs and tibiae were dissected, cleaned of surrounding tissues and bone marrow was flushed in Dulbecco’s modified essential medium (DMEM.mod., GIBCO, Life Technologies, France) supplemented with penicillin-streptomycin, amphotericin-fungizone and pyruvate (GIBCO). Bone marrow cells were washed once in DMEM.mod. Mononuclear cells were isolated by a Lympholyte-M centrifugation gradient (Cedarlane, Hornby, Ontario, Canada) at 450 g for 25 min. Mononuclear cells were then washed twice and counted. The recipient mice received 1.5 × 10⁶ cells in the tail vein.

2.2. Animals and study design

Seventy-six C57BL/KaLwRij female mice 6–8 w-old were used for the full study (Harlan, Gannat, France). They were acclimated for one w to the local vivarium conditions (24 °C and 12 h/12 h light dark cycle) where they were given standard laboratory food (UAR, Villemoisson sur Orge, France) and water ad libitum. The animals were housed at the animal facilities of Angers University Medical School (SCAHU). All procedures have been approved by the Institutional Animal Care and Use Committee at the University of Angers (Agreement number 49028). All procedures were done in accordance with the 2013 French animal procedure act and under the supervision of authorized investigators.

Two experimental procedures were conducted. The first one examined the modification of bone remodeling by dietary calcium deficiency in control animals and used 20 C57BL/KaLwRij mice. The second experimental procedure combined the calcium deficiency and the injection of 5T2MM cells in mice and used 56 C57BL/KaLwRij mice. The two experimental procedures were described in details below.

2.2.1. Modification of bone remodeling by dietary calcium deficiency

On day 0, twenty mice were randomized into 2 groups (10 animals per group). The first group was given standard laboratory chow with normal calcium content (0.8%); they constituted the Normal-Ca group. The second group was given a calcium deficient laboratory chow (0.05% – SAFE AUGY, Auggy, France); they constituted the Low-Ca group. At 10 w, blood samples were collected by intracardiac puncture to measure serum parathyroid hormone (PTH) level and mice were then sacrificed by cervical dislocation.

2.2.2. Combined model (dietary calcium deficiency + 5T2MM cells)

Fifty mice were injected with 5T2MM cells in the tail vein and immediately placed on the normal or low calcium diet as above. They constituted respectively the normal-Ca-MM group (n = 25) and the low-Ca-MM group (n = 25). Each group of mice was divided into 3 subgroups to perform a time-dependent analysis of osteolysis by histomorphometry and quantitative PCR (qPCR). Mice were sacrificed by cervical dislocation at 6, 8 and 10 w. Mice, which developed paraplegia before the end of each time point, were sacrificed for ethical reason. Six additional mice were sacrificed at the beginning of the study at w0 and were used as control (control-w0 group) for biochemical and histomorphometric analysis.

2.3. Bone tissue collection

Right femurs and tibias from all mice were dissected, cleaned of soft tissue, and conserved in an ethanol based fixative +4°C for further analysis. Lumbar vertebrae from L2 to L4 were also dissected for mice in experimental procedure 2, slightly cleaned of soft tissue and conserved in an ethanol based fixative for further analysis. Left femurs of mice, in the experimental procedure 2, were used for qPCR analysis.

2.4. Measurement of the M-protein level in the serum

Progression of MM was monitored by measuring the serum M-protein (lgG2αk) concentration using agar electrophoresis (Hydragel protéine, SEBIA, Issy les Moulineaux, France). The level of M-protein was expressed as the percentage of the y-peak using image analysis (NIH image for Windows) on the electrophoretic diagram. Blood was collected from the tail vein at 6 w for all 5T2MM groups to check the presence of the M-protein. For kinetic evolution of the M-protein level, blood was also collected at 8 and 10 w.
in the two groups maintained up to 10 w. Blood was collected at the time of sacrifice for mice that developed paraplegia and did not reach 10 w.

2.5. Follow up of osteolytic lesions

Presence of osteolytic bone lesions was checked on digital radiographs of the pelvis and hind legs obtained with a Faxitron machine at 26 kV (Faxitron Edimex, Angers, France). Radiographs were performed at the time of sacrifice in all groups.

2.6. X-ray microtomography

MicroCT was performed on the proximal tibia and on the lumbar vertebrae (L2 to L4) with a Skyscan 1172 microtomograph (Bruker microCT, Kontich, Belgium). Bone samples were transferred to an Eppendorf tube containing the fixative and polyethylene foam was used to ensure immobilization of the sample. The tube was affixed on a brass stub with plasticine and analyzed at a resolution of 5.25 µm per pixel for the tibia and 13.38 µm for the vertebrae. After segmentation, the 3D models were constructed from the stack of 2D images with a surface-rendering program (Ant, release 2.0.5, Skyscan).

3D measurements were obtained with the CtAn software (release 2.5, Skyscan). For each tibia sample, 200 section images were obtained and corresponded to the cumulated height of the primary and secondary spongiosa (1.05 mm). Trabecular bone analysis was performed on the L2 vertebral body.

The following 3D parameters were calculated: trabecular volume (BV/TV, in%), trabecular number (Tb.N, in mm⁻¹) and trabecular separation (Tb.Sp, in µm). Cortical bone measurements were performed on 2D sections of the tibia at the metaphyseal region (1.05 mm under the growth cartilage) and at the diaphyseal shaft (3.14 mm under the growth cartilage). Mean cortical thickness (Ct.Th, in µm) was determined with the CtAn software.

2.7. Histology

Histology was performed on undecalciﬁed femurs after embedding in poly (methyl methacrylate). For each mouse, four non serial sections (50 µm apart, 7 µm in thickness) were cut dry on a heavy-duty microtome equipped with tungsten carbide knives (Leica Polycut S; Rueil-Malmaison, France, with 50° knives) and stained with Goldner’s trichrome or tartrate resistant acid phosphatase (TRACP) for the histochemical identiﬁcation of osteoclasts (Chappard, 2014). The osteoclast number was determined at the endosteal surface (on 5 mm from the growth plate) at a magniﬁcation of x200 on the TRACP-stained sections. The percentage area of the metaphysis invaded by plasma cells was measured on histological sections of the femur.

### Table 1. Biochemical analysis in the different groups.

<table>
<thead>
<tr>
<th>Serum assay</th>
<th>Control-w0</th>
<th>Control groups</th>
<th>MM groups</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Normal-Ca</td>
<td>Low-Ca</td>
</tr>
<tr>
<td>Calcium (mmol/L)</td>
<td>2.40 ± 0.02</td>
<td>2.32 ± 0.03</td>
<td>2.34 ± 0.02</td>
</tr>
<tr>
<td>w10</td>
<td>39.21 ± 6.05</td>
<td>39.21 ± 6.05</td>
<td>39.21 ± 6.05</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>29.5 ± 2.02</td>
<td>106.13 ± 23.17a</td>
<td>112.69 ± 21.4a</td>
</tr>
<tr>
<td>w6</td>
<td>46.76 ± 16.89</td>
<td>94.65 ± 27.67c</td>
<td>94.65 ± 27.67c</td>
</tr>
<tr>
<td>w8</td>
<td>19.37 ± 3.41c</td>
<td>19.37 ± 3.41c</td>
<td>19.37 ± 3.41c</td>
</tr>
<tr>
<td>w10</td>
<td>2.34 ± 0.02</td>
<td>2.34 ± 0.02</td>
<td>2.34 ± 0.02</td>
</tr>
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</table>

* vs control-w.
1 * vs w6.
2 * vs w8.
3 * vs Normal-Ca.
4 * vs Normal-Ca-MM.

2.8. PTH and calcium assays

Blood calcium was determined at 0 and at euthanasia after exsanguination in all groups on a Technicon SMA analyzer. PTH was measured at 0 and at 6, 8 and 10 w in the two control groups (normal-Ca; low-Ca) and in the two MM groups (normal-Ca-MM; low-Ca-MM). Intact PTH was dosed using the Mouse Intact PTH ELISA Kit (#60–2300, Immunotopics International, San Clemente, CA), as recommended by the supplier.

2.9. Quantitative RT-PCR analysis

The left femur of each mouse was dissected, cleaned of surrounding tissues and bone marrow was flushed in Dulbecco’s modiﬁed essential medium. Bone marrow cells were lysed and proceeded for RNA extraction using the RNeasy mini kit (Qiagen,
France) following the manufacturer procedure. After elution, total RNA was aliquoted and stored at –80 °C until use. Quality of the RNA samples was examined on a denaturing agarose gel and RNA concentrations were determined by spectrometry. One μg of total RNA sample was reverse transcribed using random hexamer primers and SuperScript II reverse transcriptase (SSII) (Invitrogen, France). Primers were designed using Primer3 software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The following primers were used: MIP-1α (forward 5′-CTCTGCTGTTCTCTGACCAC-3′ and reverse 5′-AAATGACACTTGGGTCGGG-3′); RANKL (forward 5′-TGATCTTCCAGCGCAGATG-3′ and reverse 5′-CCCCAATGTGTTGCTTCC-3′); Lrp5 (forward 5′-GTGACCTGGACGTCTCT-3′ and reverse 5′-TACGGTGTAGTTGAAAGC-3′); Lrp6 (forward 5′-TACACCTTCCGGTCG-3′ and reverse 5′-CCACACTTCCTTCTCAAC-3′). Amplification was performed in duplicates with qSYBR Supermix (Bio-Rad, France) and using a Chromo4™ real time system (Bio-Rad). Thermal cycling was initiated with 10 min incubation at 95 °C followed by 40 cycles of 15 s at 95 °C, 11 s at 55 °C and 22 s at 72 °C. Gene expression was calculated using the comparative Ct method with the normalization of the target genes to 6 housekeeping genes (G3PDH, HPRT1, B2 M, ACTB, RPS18 and HSPCB).

2.10. Statistical analysis

Statistical analysis was done with SYSTAT statistical software, release 13.00.05 (Systat, San José, CA). All data were reported as mean ± standard error of the mean (SEM). Significant differences between groups were analyzed by a non-parametric Kruskal-Wallis analysis of variance followed by the Conover-Inman test for all pairwise comparisons. The comparison between normal-Ca vs low-Ca (in control or MM groups) was analyzed by the non-parametric Mann-Whitney U test at each study time point. Differences were considered as significant when p < 0.05.

3. Results

3.1. Dietary calcium diet induced bone loss due to secondary hyperparathyroidism in control mice

Ten weeks of low calcium diet induced trabecular bone loss and thinning of the cortices as evidenced on 3D microCT models (Fig. 1A). In the low-Ca group, morphometric analysis revealed a significant decrease in BV/TV (−14.5%, p < 0.05) and cortical thickness at the metaphysis (−28.2%, p < 0.0001) and the diaphysis (−27.4%, p < 0.00001) (Fig. 1B–C). Biochemical results for the control groups appear on Table 1. Trabecular and cortical bone losses were associated with an increased level of PTH reflecting the secondary hyperparathyroidism. No significant differences were observed between the two groups after 10 w.

3.2. Effect of low calcium diet on MM progression

During the course of the disease, detection of the M-protein was evidenced at 6 w in both 5T2MM groups with no difference in the amount of the protein. In the low-Ca–MM group the kinetic became different after 8 w when an increased level was obtained more rapidly than in the normal-Ca–MM group. At 10 w, the difference between the 2 groups was significant (p < 0.05). For the low-Ca–MM group, an additional time point was added on the curve at 9 w because several mice developed paraplegia and were sacrificed for ethical considerations. Table 2 shows the incidence of paraplegia in the 2 groups of 5T2MM mice. In the two groups, no paraplegia was observed at 6 w. Fifty percent of paraplegia were observed at 8 w and 56% within 9–10 w in the low-Ca–MM group. No paraplegia was observed in the normal-Ca–MM group. X-rays performed at the time of sacrifice showed minimal “punched-out” osteolytic lesions (preferentially localized at the tibial crest) in normal-Ca–MM and low-Ca–MM groups sacrificed at 8 w (Fig. 2B: a, c). At 8 w, osteolytic lesions were observed in the low-Ca–MM group at the tibia and femur; they were associated with a marked thinning of the cortices. At 10 w, the number of osteolytic lesions was increased in the low-Ca–MM group compared to the normal-Ca–MM (Fig. 2B: b, d). Biochemical results for the MM groups appear on Table 1. Briefly, calcemia was significantly decreased in the low-Ca–MM group vs the normal-Ca–MM group after 10 w. PTH was considerably increased at 6, 8 and 10 w in the low-Ca–MM vs initial values at 0 w. In the normal-Ca–MM group, no change occurred during the time course of the study.

3.3. A low calcium diet induced a greater bone loss at the tibia

The microCT models of the tibia clearly evidenced the different progression of osteolytic lesions upon time between the normal-Ca–MM (Fig. 3A: a–c) and low-Ca–MM groups (Fig. 3A: d–f). The quantitative measurements are illustrated on Figs. 3 B–D and 2 D cortical measurements on Fig. 3E–F.

At 6 w, the tibia had a rather well preserved trabecular network in the normal-Ca–MM group without cortical perforations. BV/TV did not differ when compared with the control-w0 group; however, Tb.N decreased and Tb.Sp increased significantly, revealing an early disorganization of the trabecular microarchitecture. The trabecular network was markedly altered in the low-Ca–MM group (Fig. 3A: d); BV/TV was significantly reduced (−62%, p < 0.001) compared to normal-Ca–MM group at 6 w. The decrease of BV/TV at 6 w was associated with a significant decrease of Tb.N and a significant increase of Tb.Sp. Thinner cortices were observed in the low-Ca–MM group in both metaphyseal and diaphyseal regions as evidenced by a significant reduction in Ct.Th (Fig. 3E–F) compared to normal-Ca–MM group at 6 w. On the contrary, only significant differences were observed for Ct.Th from the metaphysis at 8 and 10 w between normal-Ca–MM and control-w0 group. At 6 w, the 3D model from the low-Ca–MM group clearly showed a non-smooth appearance of endosteal surface compared to the normal-Ca–MM group. This difference in the appearance of the endosteal surface reflected a more eroded surface in the low-Ca–MM group. The osteoclast number was significantly higher at 6 w in low-Ca–MM group compared to normal-Ca MM group (resp. 22.4 ± 4.2 vs 10.6 ± 0.8, p < 0.05). The progression of osteoclast number is presented as a 3D graph combined with evolution of the bone marrow invasion (Fig. 4). At 6 w, plasma cell invasion of the femoral diaphysis was higher in the low-Ca–MM group compared to the normal-Ca–MM group (resp. 64.5% ± 17.3 vs 43.1% ± 15.1) but this increase did not reach statistical significance.

At 8 w, BV/TV decreased significantly in the normal-Ca–MM group when compared to the control-w0 group; Tb.Sp was significantly higher and Tb.N was significantly lower compared to Tb.Sp and Tb.N measurements at 6 w. Bone destruction was evidenced in 3D models of the normal-Ca–MM group with the presence of trabecular destruction and small cortical perforations (Fig. 3A: b). Osteolytic lesions were maximized in the low-Ca–MM group

### Table 1

<table>
<thead>
<tr>
<th>Incidence of hind limb paralysis</th>
<th>6 weeks</th>
<th>8 weeks</th>
<th>10 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal-Ca-MM</td>
<td>0% (0/8)</td>
<td>0% (0/8)</td>
<td>0% (0/9)</td>
</tr>
<tr>
<td>Low-Ca-MM</td>
<td>0% (0/8)</td>
<td>50% (4/8)</td>
<td>56% (5/9)</td>
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</table>

### Table 2

<table>
<thead>
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<th>Incidence of hind limb paralysis</th>
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</tr>
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<td>Low-Ca-MM</td>
<td>0% (0/8)</td>
<td>50% (4/8)</td>
<td>56% (5/9)</td>
</tr>
</tbody>
</table>
Fig. 2. Effect of low-Calcium diet on myeloma progression. (A) Evolution of the serum M-protein level (expressed in%) during the time course of MM disease. A significant higher M-protein level was found at 10 w in the low-Ca-MM group (* p < 0.05). (B) X-ray images of the hindlimb of mice from normal-Ca-MM group at 8 w (a) and 10 w (b); from low-Ca-MM group at 8 (c) and 10 w (d). Osteolytic lacunae (→) are evidenced at 8 w in normal and low calcium diet mice. Note the thinning of the cortices (→) with low calcium diet at 8 w. Greater bone destruction is observed with low calcium diet at 10 w (d). Lacuna appeared smaller but more numerous in the tibial crest (→).

Fig. 3. Effect of low-Calcium diet on bone loss at the tibia. (A) X-ray microCT reconstructions of the proximal tibia from normal-Ca-MM group (a-c) and low-Ca-MM group (d-f) resp. at 6, 8 and 10 w. (B-D) 3D microCT measurements of resp. BV/TV, Tb.Sp and Tb.N on proximal tibia. (E-F) 2D microCT measurements of cortical thickness in resp. the metaphyseal region and the diaphyseal shaft of the tibia.
Numerous TRACP positive osteoclasts were observed in both groups. The osteoclast number from both groups was significantly increased at 8 w compared to 6 w. Comparison of both groups at 8 w did not show significant difference between low-Ca and normal-Ca MM groups (resp. 33.1 ± 2.8 vs 39.2 ± 5.2) (Fig. 4).

At 10 w, severe trabecular and cortical bone destruction were evidenced in both MM groups with an increase in cortical perforations. However, lesions were more pronounced in the low-Ca MM group (Fig. 3A: d, f). The osteoclast number of low-Ca and normal-Ca MM groups was not significantly different (resp. 38.4 ± 5.1 vs 39.9 ± 2.1) and was similar to the number of osteoclasts at 8 w (Fig. 4). At 10 w, the bone marrow was entirely invaded by plasma cells in the low-Ca-MM group (100%); in the normal-Ca-MM, the percentage of bone marrow invasion was 82.5 ± 17.5.

3.4. A low calcium diet induced severe vertebral bone destruction

3D models of lumbar vertebra showed progressive disappearance of trabecular bone from 6 to 10 w in the low-Ca-MM group (Fig. 5A: d–f). Lumbar vertebra in the two groups showed two anatomic holes at the posterior side of the lumbar vertebral body; these holes are not due to the tumor as they are observed in control mice (data not shown) and correspond to vascular sinuses. Cortical perforations were observed from 8 w in the low-Ca-MM group inducing an enlargement of the anatomical holes (Fig. 5A: e–f). Similar cortical perforations could be observed in the normal-Ca-MM group but only at the terminal stage of the disease (Fig. 5A: c).

Morphometric analysis showed no significant difference in BV/TV, Tb.N and Tb.Sp in the normal-Ca group compared to control-w0 at 6, 8 and 10 w (Fig. 5B–D). In the low-Ca-MM group, BV/TV decreased significantly from 6 w (w6: −33.65%, p < 0.01; w10: −57.61%, p < 0.01) compared to the normal-Ca-MM group. Reduction of BV/TV was associated with a significant disappearance of trabeculae as from 6 w (reduced Tb.N and increased Tb.Sp).

3.5. A low calcium diet modified gene expression profile

An increase in MIP-1α expression was found in the bone marrow of mice from the low-Ca-MM groups at 6 and 8 w with a ratio respectively of 1.6 and 3.9 compared to the expression in the normal-Ca–MM group (Fig. 6A). On the opposite, expression of MIP-1α was significantly lower at 10 w in the low-Ca group compared to the normal-Ca group. There was an insignificant reduction in the expression of RANKL (Fig. 6B) at 6 and 8 w Significant differences were obtained in the expression of some genes of the Wnt pathway. The Lrp5 co-receptor was significantly less expressed at 6 w in the low-Ca-MM group compared to the normal-Ca-MM group with a ratio of −2.6 (Fig. 6C). Expression of Dkk1 was significantly reduced at 6 and 8 w in the low-Ca-MM group with ratio of respectively −2.5 and −4.5 (Fig. 6D). No significant differences of Dkk1 expression were observed between the two groups at 10 w.

4. Discussion

The use of a dietary calcium diet to increase bone remodeling was based on previous data in both animal and clinical studies which showed an increase in bone resorption due to low calcium intake (Eklou-Kalonji et al., 1999; Iwamoto et al., 2004; Lips, 2001; Seto et al., 1999). In the present study, a long-term calcium diet deficiency induced a sustained secondary hyperparathyroidism which, in turn, caused a major bone loss at the trabecular and cortical level. Secondary hyperparathyroidism is due to extrinsic changes causing a drastic decrease in plasma ionized calcium thus inducing PTH secretion to correct plasma calcium. If low calcium availability is not corrected, patients are characterized by a long term high level of parathyroid hormone (Fraser, 2009). This is in accordance with data obtained from the present study and others where PTH level is maintained high even until 10 w of low calcium diet (Iwamoto et al., 2004). The increase in PTH due to a low calcium diet is rapid and observed as soon as 12 h after diet initiation in rats (Seto et al., 1999). Short-term study of a low calcium diet in rats, showed that the rapid increase in PTH induced rapid effects on bone remodeling; the number of osteoclasts and osteoblasts along the trabecular bone surfaces were increased after 1 and 2 days, respectively (Seto et al., 1999). Such a high bone resorption level is maintained during the entire period of the low calcium intake (Eklou-Kalonji et al., 1999; Iwamoto et al., 2004). Moreover, an increase in osteoclast number was shown in both cancellous and endosteal surfaces, in accordance with the bone loss at these two bone envelopes noted in the present study (Xiong et al., 2014). When considering bone formation, increases in osteoblast number are maintained throughout the entire time of the study in animals with a low calcium diet but bone formation and mineralization rates are decreased (Eklou-Kalonji et al., 1999; Iwamoto et al., 2004; Seto et al., 1999). Biochemical markers of bone formation (alkaline phosphatase, c-propeptides) are also increased during a low calcium diet in animals and humans (Eklou-Kalonji et al., 1999; Harris et al., 2001; Meunier et al., 2005). Secondary hyperparathyroidism due to low calcium intake is common in middle-age and elderly population. Several studies have shown that nutritional supplementation of calcium in food or drinking water reduces serum PTH level and bone markers (Bonjour et al., 2013; Meunier et al., 2005).

When MM cells injection was associated with a low calcium regimen, tumor growth was accelerated from 8 w. It was associated with a high incidence of paraplegia (which were absent in normal-Ca–MM mice); this corresponds to a more severe form of MM. Mice showed an increased osteolysis at the end stage of the disease due to an earlier development of osteolytic lesions at the tibia and lumbar vertebrae. Histomorphometric measurements confirmed that secondary hyperparathyroidism in MM mice has led to an increased bone destruction. Moreover, MM mice with a normal calcium diet did not show trabecular destruction in the lumbar vertebral body, although lumbar vertebrae had cortical perforations at the end stage of the disease. The dramatic osteolysis at lumbar vertebrae...
may explain the incidence of paralysis in the deficient group. Histologic data showed that cortical perforations in the vertebral body allowed extension of plasma cells in the extradural space and compression of the spinal cord (Libouban, 2015). Our results based on X-ray analysis, microCT and M-protein data are in accordance with our previous study on a combined model associating MM and ovariectomy (Libouban et al., 2003). They are also in accordance with data reported in murine model with bone metastasis in which a low calcium diet promoted tumor growth (Zheng et al., 2008; Zheng et al., 2007). The experimental designs of these two studies slightly differed from our experiment because the low calcium diet was initiated at the same day than injection of MM cells. Excepted this experimental difference, data are similar in term of increase in the tumor growth and osteolytic lesions (Seto et al., 1999).

It now exists strong evidences that a preexisting high bone remodeling promotes tumor growth (MM or metastasis) and would represent a risk of converting a smoldering form into an overt one. We and others have hypothesized that a high bone remodeling alters the bone microenvironment by enriching it with growth factors and cytokines, thus providing a more “fertile soil” on which tumor cells growth is enhanced (Guise, 2000). Moreover, treatment with the RANK-L inhibitor osteoprotegerin prevented the development of osteolytic lesions and reduced the tumor growth despite a calcium diet (Zheng et al., 2007). Authors emphasized the fact that the increase of tumor growth and the extend of bone lesions are directly due to high bone resorption induced by low calcium independently of direct effect of PTH. (Zheng et al., 2007). In addition, the intermittent injection of PTH (1–34) has been tested in murine models of myeloma and the effect of PTH delayed MM progression (Pennisi et al., 2010). In our model, PTH levels are continuously elevated and thus the effects on bone remodeling and tumor growth appear opposite to the above results. In addition,
we did not include neither a group with normal calcium diet and continuous PTH infusion nor tested a neutralizing antibody to verify whether the continuous increased in PTH is responsible for the observed effects.

Our results clearly showed that a preexisting high bone turnover had a strong impact on MM development at an early time, 6w following MM injection. At 6w, the M-protein level was similar between the different groups and the bone marrow invasion was higher in the low-Ca-MM groups although the difference did not reached statistical significance. However, when plotting together the% of marrow invasion and N.Oc in a 3D graph, differences between the two groups was more clearly evidenced due to psychological perception (Systat, 2009). In addition, the 3D presentation of data clearly showed that the low calcium diet accelerates the mechanisms of MM progression at the early phase of MM development that induce then a more severe bone destruction.

Until now, no study was performed to characterize the molecular enrichment of the microenvironment. In this study, we evaluated the expression of genes strongly involved in MM growth. In MM, considerable attention are being paid on cellular and molecular mechanisms responsible for a high bone resorption. RANK/RANKL and the chemokine MIP-1α constitutes the key “osteoclast activating factors” in MM (Choi et al., 2000; Pearse et al., 2001). At low calcium diet, MIP-1α was highly expressed in the earlier phase of MM development. This result is in accordance with histological data of TRACP positive osteoclasts at the endosteal surfaces; osteoclasts were increased as early as 6w in the low-Ca-MM group, confirming the higher resorption in this group. MIP-1α promotes osteoclastogenesis independently of RANKL and enhances interaction between MM cells and stromal cells (Oba et al., 2005). Surprisingly, at 10w the level of MIP-1α was much lower compared to the level at 6 and 8w and also compared to normal-Ca-MM group at the same time. However, as the marrow invasion is at a level of 100% in the low-Ca-MM group and because MIP-1α is mainly secreted by medullary cells, it is not so surprising that the level expression of MIP-1α was low. More recently, it has been shown a role for MIP-1α in inhibition of osteoblast formation via osteocalcin down regulation (Vallet et al., 2011). In our study MIP-1α was a strong indicator of MM aggressiveness; surprisingly, no significant difference in the expression of RANKL was observed. RANKL gene expression is increased in a mouse model of secondary hyperparathyroidism after 7 days (O’Brien et al., 2005). This would be in favor of an early hyper-expression of RANKL in the low-Ca-MM group occurring before 6w (the onset of our kinetic study).

The Wnt pathway is involved in the decline of bone formation in MM (Roodman, 2010). Studies on bone formation in MM have focused on DKK1 expression that is upregulated in malignant plasma cells (Roodman, 2010; Tian et al., 2003). At an early stage of MM, we observed a decrease in the co-receptor of the Wnt pathway LRP5 (which is a key molecule to enhance bone formation). The lack of LRP5 in mice induces an inhibition of osteoblast function associated with a decrease in osteoblast number (Kato et al., 2002). The low LRP5 expression in mice with the low calcium diet can reflect the decrease in bone formation. We did not observe a high level of DKK1 expression in the low-Ca-MM group. There is no data on DKK1 expression in secondary hyperparathyroidism which could explain our result. However, in a previous study of gene expression in a mouse model of disuse, DKK1 appeared as a late inhibitor of bone formation (Marchand-Libouban et al., 2013). This observation could be in accordance with our data at 10w when a non-significant increase of DKK1 expression was observed in the low-Ca-group (compared to the normal-Ca-group).

5. Conclusions

The present study by using X-ray microtomography and quantitative PCR analysis clearly showed that altered bone remodeling due to calcium deficiency increase the severity of MM bone disease with a higher osteolysis and a higher alteration of bone genes expression. Little attention has been placed on investigating the mechanisms involved in humans for the sudden progression from indolent myeloma (revealed by a monoclonal gammapathy of undetermined significance – MGUS) into an aggressive MM. However, evolution to an overt MM, is related to both intrinsic changes of malignant plasma cells and modification of bone microenvironment (Bataille, 2015; Tageja et al., 2014). So, calcium deficiency would represent a real cause for progression of smoldering MM to overt MM by inducing secondary hyperparathyroidism. MM is a disease frequently observed in the elderly where a secondary hyperparathyroidism (increasing bone remodeling) is also frequently observed.

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