



Histomorphometric and microarchitectural analysis of bone in metastatic breast cancer patients

A. Beltran-Bless^a, M. Murshed^b, M. Zakikhani^c, I. Kuchuk^a, N. Bouganim^a, S. Robertson^e, N. Kekre^a, L. Vandermeer^a, J. Li^c, C.L. Addison^d, F. Rauch^f, M. Clemons^{a,d,*},¹, R. Kremer^{c,**},¹

^a Division of Medical Oncology, Department of Medicine, The Ottawa Hospital, The University of Ottawa, Ottawa, Canada

^b Department of Medicine, Faculty of Dentistry, Shriners Hospital for Children, McGill University, Montreal, Canada

^c Department of Medicine, Research Institute of the McGill University Health Center, Montreal, Canada

^d Centre for Cancer Therapeutics, Ottawa Hospital Research Institute, Ottawa, Canada

^e Department of Pathology, The Ottawa Hospital and the University of Ottawa, Ottawa, Canada

^f Department of Pediatric Surgery, McGill University Health Center, Montreal, Canada

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ABSTRACT

Background: Despite widespread use of repeated doses of potent bone-targeting agents (BTA) in oncology patients, relatively little is known about their in vivo effects on bone homeostasis, bone quality, and bone architecture. Traditionally bone quality has been assessed using a trans-iliac bone biopsy with a 7 mm “Bordier” core needle. We examined the feasibility of using a 2 mm “Jamshidi™” core needle as a more practical and less invasive technique.

Methods: Patients with metastatic breast cancer on BTAs were divided according to the extent of bone metastases. They were given 2 courses of tetracycline labeling and then underwent a posterior trans-iliac trephine biopsy and bone marrow aspirate. Samples were analyzed for the extent of tumor invasion and parameters of bone turnover and bone formation by histomorphometry.

Results: Twelve patients were accrued, 1 had no bone metastases, 3 had limited bone metastases (LSM) (<3 lesions) and 7 had extensive bone metastases (ESM) (>3 lesions). Most of the primary tumors were estrogen receptor (ER)/progesterone receptor (PR) positive. The procedure was well tolerated. The sample quality was sufficient to analyze bone trabecular structure and bone turnover by histomorphometry in 11 out of 12 patients. There was a good correlation between imaging data and morphometric analysis of tumor invasion. Patients with no evidence or minimal bone metastases had no evidence of tumor invasion. Most had suppressed bone turnover and no detectable bone formation when treated with BTA. In contrast, 6 out of 7 patients with extensive bone invasion by imaging and evidence of tumor cells in the marrow had intense osteoclastic activity as measured by the number of osteoclasts. Of these 7 patients with ESM, 6 were treated with BTA with 5 showing resistance to BTA as demonstrated by the high number of osteoclasts present. 3 of these 6 patients had active bone formation. Based on osteoblast activity and bone formation, 3 out of 6 patients with ESM responded to BTA compared to all 3 with LSM. Compared to untreated patients, all patients treated with BTA showed a trend towards suppression of bone formation, as measured by tetracycline labelling. There was also a trend towards a significant difference between ESM and LSM treated with BTA, highly suggestive of resistance although limited by the small sample size.

Discussion: Our results indicate that trans-iliac bone biopsy using a 2 mm trephine shows excellent correlation between imaging assessment of tumor invasion and tumor burden by morphometric analysis of bone tissues. In

Abbreviations: SREs, Skeletal related events; DEXA, Dual-energy X-ray absorptiometry; QCT, Quantitative CT; IHC, Immunohistochemistry staining; CK, Cytokeratin staining; ER, Estrogen receptor; PR, Progesterone receptor; HER2, Human Epidermal growth factor Receptor 2; HE, Haematoxylin and Eosin; VKVG, von Kossa and van Gieson; TRAP, Tartrate-resistant acid phosphatase staining; PFA/PBS, Paraformaldehyde/phosphate buffer solution; OC, Osteoclasts; OB, Osteoblasts; OS, Osteoid surface; MB, Mineralized bone; CM, Collagen material; BPs, Bisphosphonates; BTAs, Bone targeting agents; BM, Bone met; astatin AI, Aromatase inhibitors; PAM, Pamidronate; IDC, Invasive ductal carcinoma; Zol, Zoledronic acid; LSM, Limited skeletal metastases; ESM, Extensive skeletal metastases.

* Correspondence to: M. Clemons, Division of Medical Oncology, Department of Medicine, The Ottawa Hospital, The University of Ottawa, Ottawa, Canada.

** Correspondence to: R. Kremer, Department of Medicine, Research Institute of McGill University Health Center, Montreal, Canada

E-mail addresses: mclemons@toh.ca (M. Clemons), richard.kremer@mcgill.ca (R. Kremer).

¹ Contributed equally to the study.

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addition, our approach provides additional mechanistic information on therapeutic response to BTA supporting the current clinical understanding that the majority of patients with extensive bone involvement eventually fail to suppress bone turnover (Petrucci et al. 2008). This suggests that antiresorptive therapies become less effective as disease progresses.

1. Introduction

While repeated doses of highly potent BTAs, such as intravenous bisphosphonates and denosumab, are extensively used in breast cancer patients, relatively little is known about their in vivo effects on bone homeostasis, microarchitecture and quality of bone. Bone quality is a significant contributor of bone strength, i.e., bone microarchitecture, bone turnover rate, degree of calcification, and properties of bone matrix collagens (Petrucci, 2008; Weinstein, 2000; Vashishth, 2005a). In breast cancer patients assessment of bone strength is further complicated by the accumulation of naturally occurring bone aging, therapies adversely affecting bone quality and invasion of bone by tumor cells. BTAs are frequently used to prevent osteoporosis, and cancer treatment-induced bone loss (Petrucci, 2008) in this population at high risk for fractures, as well as in the adjuvant setting for reducing the risk of bone relapse (Clemons et al., 2012a; Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2015). Furthermore, in patients with bone metastases these agents are used to reduce skeletal related events (SREs), pain and to improve quality of life (Clemons et al., 2012b). Over 100 years ago Paget proposed the "seed and soil" hypothesis (Paget, 1989) where he postulated that tumor cells "seed", i.e. find a favorable terrain in the bone microenvironment (soil) where they can grow and multiply. It is also hypothesized that antiresorptive agents such as bisphosphonates and denosumab reduce SREs by suppressing bone turnover and as a result make the "soil" less favorable to skeletal metastases development (Sasaki et al., 1995a). However, a direct assessment of the efficacy of these agents on local bone turnover and invasion has not yet been reported. A greater understanding of what actually occurs in each patient could lead to more personalized therapy (Amir, 2013; Kuchuk et al., 2012a) and development of new agents (Bouganim, 2011; Kuchuk et al., 2012b; Russell, 2012). Also, both short-term side effects, such as hypocalcemia (Stopeck, 2010) and long-term toxicities such as atypical fractures, and osteonecrosis of the jaw (Whyte, 2003;

Lewiecki, 2011; Schilcher et al., 2011a; Kuchuk, 2013), could possibly be better understood. While dual-energy X-ray absorptiometry (DEXA) (Blake, 2007) and quantitative CT (QCT) are well-established non-invasive tests for bone mass assessment (MacNeil and Boyd, 2007a; Genant, 2008; Burghardt, 2010; Burghardt, 2011), and bone turnover markers have been studied extensively in osteoporosis (Bauer, 2004; Reginster, 2004; Greenspan, 2005; Delmas, 2009; Eastell, 2011) and in patients with skeletal metastasis (Ali, 2004; López-Carrizosa et al., 2010a; Zhao, 2011), these technologies cannot assess bone morphology and changes resulting from tumor bone invasion.

Currently, bone biopsy with a 7–8 mm "Bordier" trephine of transiliac bone is the gold standard for assessment of static and dynamic bone morphological parameters, and for analysis of tumor burden in bone (Helleberg-Rasmussen and Sondergaard-Petersen, 1975a; Bilezikian et al., 2008). However, the size of the needle is a concern to both patients and physicians and in clinical reality is rarely, if ever, performed.

Our group has previously used 2 mm trephine needles for tumor receptor assessment (Cawthorn et al., 2009a; Trinkaus et al., 2009a) and comparison of tumor cell yield between 2 mm iliac crest biopsies and specimens obtained by CT-guidance (Amir et al., 2008a; Hilton, 2011). We also performed a small 3-patient pilot study of morphologic bone analysis (Fralick, 2012). As previous work with necropsy specimens showed that trephines of 2 mm and 7 mm had equivalent quality (Moore, 1989), we expanded our pilot study to a more detailed analysis of 12 patients with metastatic breast cancer. Our data suggest that patients with minimally invasive metastatic disease to bone respond well to antiresorptive agents whereas patients with more advanced skeletal metastasis seem to develop resistance to these agents.

Table 1
Patients and tumor characteristics.

Patient ID	Age	Skeletal metastases	Metastatic sites	Current therapy	BTA exposure	Previous BP	Duration of BP (mo)	Current BP	BMI Kg/m ²	BMD (T-score L1–4)	Receptor status in the primary tumor
1	64	Control: No BM	Soft tissue	AI	No	Nil	N/A	Nil	34.0	−1.9	ER ⁺ /PR ⁺ /HER2 [−]
2	56	ESM	BM	AI	Yes	PAM	34	PAM	19.5	−1.3	ER ⁺ /PR ⁺ /HER2 [−]
3	59	LSM	BM	AI	Yes	PAM	24	PAM	27.9	−2.2	ER ⁺ /PR ⁺ /HER2 [−]
4	51	LSM	Lung, BM	Chemo	Yes	PAM	10	PAM	32.7	2.4	TNBC
5	57	Control ESM	BM	AI	No	Nil	N/A	Nil	23.1	1.2	ER ⁺ /PR ⁺ /HER2 [−]
6	45	ESM	BM	Chemo	Yes	PAM	27	PAM	33.5	3.7	ER ⁺ /PR ⁺ /HER2 ⁺
7	52	ESM	Liver, BM	Chemo	Yes	PAM	15	PAM	26.9	3.4	ER ⁺ /PR ⁺ /HER2 [−]
8	47	ESM	Liver, brain, BM	Tamoxifen	Yes	PAM	5	PAM	21.4	−2.9	ER ⁺ /PR ⁺ /HER2 ⁺
9	56	ESM	BM	AI	Yes	PAM	6	PAM	16.6	−0.3	ER ⁺ /PR ⁺ /HER2 [−]
10	72	LSM	BM	AI	Yes	PAM	18	PAM	26.6	1.6	ER ⁺ /PR ⁺ /HER2 ⁺
11	58	ESM	BM	HER2	Yes	PAM Zol Denosumab	20	Zol	22.3	0	ER ⁺ /PR ⁺ /HER2 ⁺
12	60	ESM	Soft tissue, BM	AI	Yes	PAM	2	PAM	23.9	3.3	ER ⁺ /PR ⁺ /HER2 [−]

LSM: limited skeletal metastases; ESM: extensive skeletal metastases; BP: bisphosphonate.

AI: aromatase inhibitors; IDC: invasive ductal carcinoma; BM: bone metastases.

PAM: pamidronate; Zol: zoledronic acid; HER: herceptin (trastuzumab).

Chemo: chemotherapy; BTA: bone targeted agent; TNBC: triple negative breast cancer.

BMI: body mass index; BMD: bone mineral density; ER: estrogen receptor; PR: progesterone receptor.

HER2: human epidermal growth factor receptor 2.

N/A: not applicable.

Table 2
Static bone histomorphometry parameters.

Patient ID	Tumor characteristics	BTAs intake	N.Ob/T.Ar	N.Ob/B.Pm	N.Oc/T.Ar Per mm ²	N.Oc/B.Pm	OV/BV (%)	BV/TV	Cancer cells seen in bone biopsy	Cancer cells seen in bone marrow aspirate
1	Control (no BM)	No	142.57	14.55	2.22	0.22	0.98	24.34	No	No
2	ESM	Yes	142.57	16.98	6.68	0.8	3.02	21.27	Yes	No
3	LSM	Yes	100.25	10.91	0	0	1.66	14.41	No	No
4	LSM	Yes	73.51	5.03	8.91	0.61	1.15	19.00	No	No
5	Control (ESM)	No	100.25	11.7	20.05	2.23	10	33.14	Yes	Yes
6	ESM	Yes	118.07	8.5	26	1.92	1.58	42.24	Yes	Yes
7	ND	ND	ND	ND	ND	ND	ND	N/A	ND	ND
8	ESM	Yes	102.47	8.39	0	0	1.75	31.48	Yes	Yes
9	ESM	Yes	51.23	4.86	6.68	0.63	8.89	19.82	Yes	No
10	LSM	Yes	126.98	12.59	0	0	2.33	31.37	No	No
11	ESM	Yes	86.88	7.09	8.91	0.72	0.97	19.94	Yes	No
12	ESM	Yes	91.34	8.30	8.91	0.81	7.12	16.63	Yes	No

LSM: limited skeletal metastases; ESM: extensive skeletal metastases; BTA's: bone-targeted agent; BM: bone metastasis; N.Ob/T.Ar: number of osteoblasts/tissue area; N.Ob/B.PM: number of osteoblasts/bone perimeter; Oc/T.Ar: number of osteoclasts/tissue area; N.Oc/B.PM: number of osteoclasts/bone perimeter in different patient bone sections; OV/BV: osteoid volume/bone volume; ND: not determined.

2. Materials and methods

2.1. Patients' characteristics

Twelve patients with pathologically confirmed invasive ductal breast carcinoma, radiologic evidence of metastatic disease at any site, and ECOG performance status 0–2, provided informed consent and were deemed eligible for enrollment. Evidence of bone metastases was

diagnosed by a combination of symptoms, isotope bone scans and computed tomography scans. Magnetic resonance imaging confirmation was done when needed. Patients were enrolled without restriction in the presence or absence of bone metastases, numbers of lines of hormonal therapy, chemotherapy or use/type of BTA. As previously described in the literature, we divided patients according to whether they had less or more than three bone lesions, namely limited skeletal metastases (LSM) or extensive skeletal metastases (ESM) (Chao et al., 2005a). We used the

Patient #1

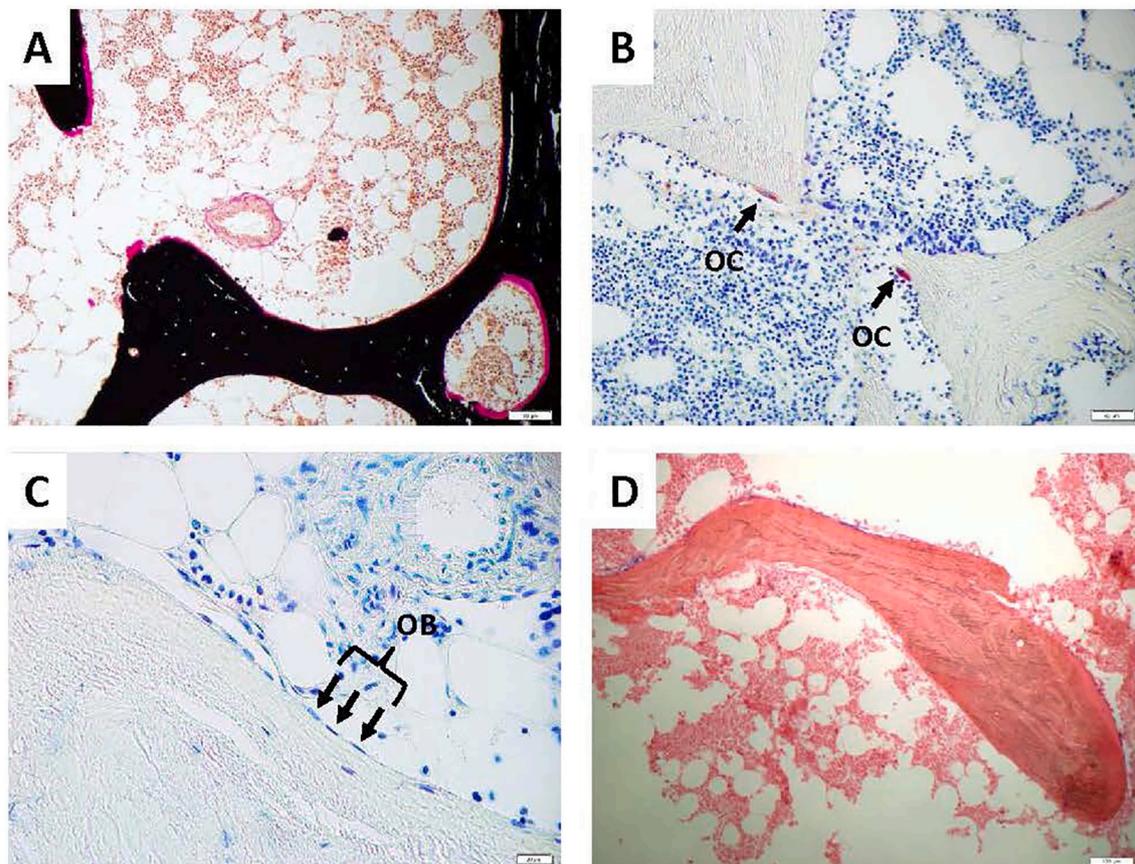


Fig. 1. (Control patient with no bone metastasis)

Patient #1 A) Normal bone structure with no evidence of metastasis. TB, OS and BM as indicated by arrows B) Arrows show the presence of OC stained by TRAP and surrounded by TB C) Arrows show OB adjacent to TB D) Blue staining indicates ALP activity (of note the trabecular bone is pink as it was counterstained with eosin, which was later omitted).

Table 3

The histomorphometry evaluation of bone dynamics by tetracycline doubles labeling technique and functional osteoblast surface.

Patient ID	Tumor characteristics	BTAs intake	Visible tetracycline labeling*	Osteoblast activity ♦
1	Control (no BM)	No	R	Positive
2	ESM	Yes	R	Positive
3	LSM	Yes	NR	Negative
4	LSM	Yes	NR	Negative
5	Control (ESM)	No	R	Positive
6	ESM	Yes	NR	Positive
7	ND	ND	ND	ND
8	ESM	Yes	NR	Negative
9	ESM	Yes	R	Positive
10	LSM	Yes	NR	Negative
11	ESM	Yes	NR	Negative
12	ESM	Yes	R	Negative

LSM = limited skeletal metastasis.

ESM = extensive skeletal metastasis.

BM = bone metastasis.

BTAs = bone-targeted agents.

ND = not determined.

R = active bone formation.

NR = no detectable bone formation.

♦ as measured by ALP-positive cell surface/bone surface.

We found positive correlation between osteoblast surface with tetracycline labeling (bone formation) in both patients with LSM or ESM, $P = 0.0364$, **Pearson Correlation Coefficient (r) = 0.6443**. There is a good correlation between tetracycline labeling and bone formation (R) and osteoblast activity as measured by ALP (Ob surface) in contrast to the absence of correlation between Ob number and tetracycline labelling and bone formation as measured by histomorphometry.

two patients who did not receive BTA (one with no bone metastases, and one with ESM) as controls. Details are shown in Table 1. All patients were post-menopausal except patient # 8. Patients with a hematological disorder causing a significant risk of bleeding associated with bone biopsies were excluded. Prior to bone biopsy all patients underwent DEXA scanning. The study was reviewed and approved by Institutional Research Ethic Board of Ottawa Hospital Cancer Center.

2.1.1. Bone specimen collection

Patients were administered two 2-day courses of tetracycline separated by a 10-day interval prior to biopsy (Hernandez et al., 2008 Nov). Trans-iliac crest bone biopsy specimens were obtained using the Jamshidi™ bone biopsy trephine (diameter 2 mm) (Cardinal Health, Dublin, Ohio, USA). Two biopsies were obtained on the same side of the posterior iliac crest of each patient. One sample was put in 70% ethanol and sent for histomorphometry analysis and the second sample was stored in 10% formalin prior to pathological evaluation. This approach is limited to the analysis of trabecular bone.

2.1.2. Pathological assessment and immunohistochemistry (IHC) staining for estrogen receptor (ER) and progesterone receptor (PR) expression

Assessment of bone biopsies on paraffin embedded sections was done in decalcified sections. Since decalcification interferes with results of ER or PR staining, only surface decalcification was applied. To deal with small deposits of calcium in paraffin blocks, the block was placed face down in a weak acid decalcifier (Surgipath decalcifier I, Leica Biosystems, Buffalo Grove, IL) for 15 min, allowing the decalcifier to penetrate the block and dissolve the calcium. The block is then rinsed in water to remove residual acid and sectioned. Samples were analyzed by

a pathologist to confirm metastatic disease and to verify adequate cellularity for the ER and PR analysis. Immunostaining for ER and PR proteins were conducted using the Ventana Ultraview Detection System (UltraView Universal DAB Detection Kit, Ventana Medical Systems, Inc., Tucson AZ). The presence of positive and negative external laboratory controls was checked. Negative weak positive and strong positive breast cancer control tissues were mounted on the same slide.

2.2. Histomorphometrical analysis of bone

Primary measurements as well as structural kinetic of bone histomorphometry were used as previously described (Parfitt, 1987).

2.2.1. Static histomorphometry

For plastic sectioning, bone samples were fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffer solution (PBS), embedded in methyl methacrylate, and sectioned (5- μ m thickness). Von Kossa and van Gieson (VKVG), Toluidine blue (TB), alkaline phosphatase (ALP), or tartrate resistant acid phosphatase (TRAP) staining were applied. ALP was used to measure the activity of the osteoblasts, TB was used to count the number of osteoblasts, TRAP staining was used to enumerate osteoclasts and Von Kossa and Van Gieson staining was used to assess bone mineralization and collagen deposition. Initial sections were counterstained with eosin and therefore appear pink (patient #1, #2, #3), this was later omitted for future biopsy specimens.

Stained bone sections were analyzed for bone volume/tissue volume (BV/TV), osteoblast count and osteoclast count using the Osteomeasure software (Osteometrics, Inc., Decatur, GA). Osteoblast activity was measured by ALP-positive cell surface/bone surface. We used previously published normal values of osteoclasts in healthy post-menopausal women as references, as we did not have enough controls (osteoclast number/mm = 0.02 ± 0.03) (Arlot et al., 1990a). Images were taken at room temperature with a light microscope (DM200; Leica Biosystems, Buffalo Grove, IL) equipped with a 2.5 \times (numerical aperture of 0.07), 20 \times (numerical aperture of 0.40) or 40 \times (numerical aperture of 0.65) objectives. All histological images were captured using a camera (DP72; Olympus NDT Canada, Quebec, QC), acquired with DP2-BSW software (XV3.0; Olympus NDT Canada, Quebec, QC), and processed using Photoshop (Adobe).

2.2.2. Dynamic histomorphometry

Iliac bone sections (20 μ m thick) were analyzed at a magnification of 100 \times under fluorescent light. Tetracycline administered at a 10-day interval was incorporated into the bone tissue at sites of active bone formation and each tetracycline course generated a yellow line. This allowed measurement of the amount of bone formation that has taken place between the two tetracycline courses.

2.3. Data analysis

Given the small sample size, simple descriptive statistics were used. Patients were divided into 2 groups based on the extent of bone metastasis by imaging studies: 1) Patients with extensive (more than 3 lesions) skeletal metastases (ESM). 2) Patients with limited (less than 3 lesions) skeletal metastases (LSM). We used two patients not on BTAs as controls: one with no bone metastases and one with ESM. Two-way analysis of variance (ANOVA) were used to determine between-group differences and within-group changes over time. The bivariate Pearson Correlation was used to measure the linear association between pairs of variables (osteoblast surface over bone surface and tetracycline treat-

Patient #5

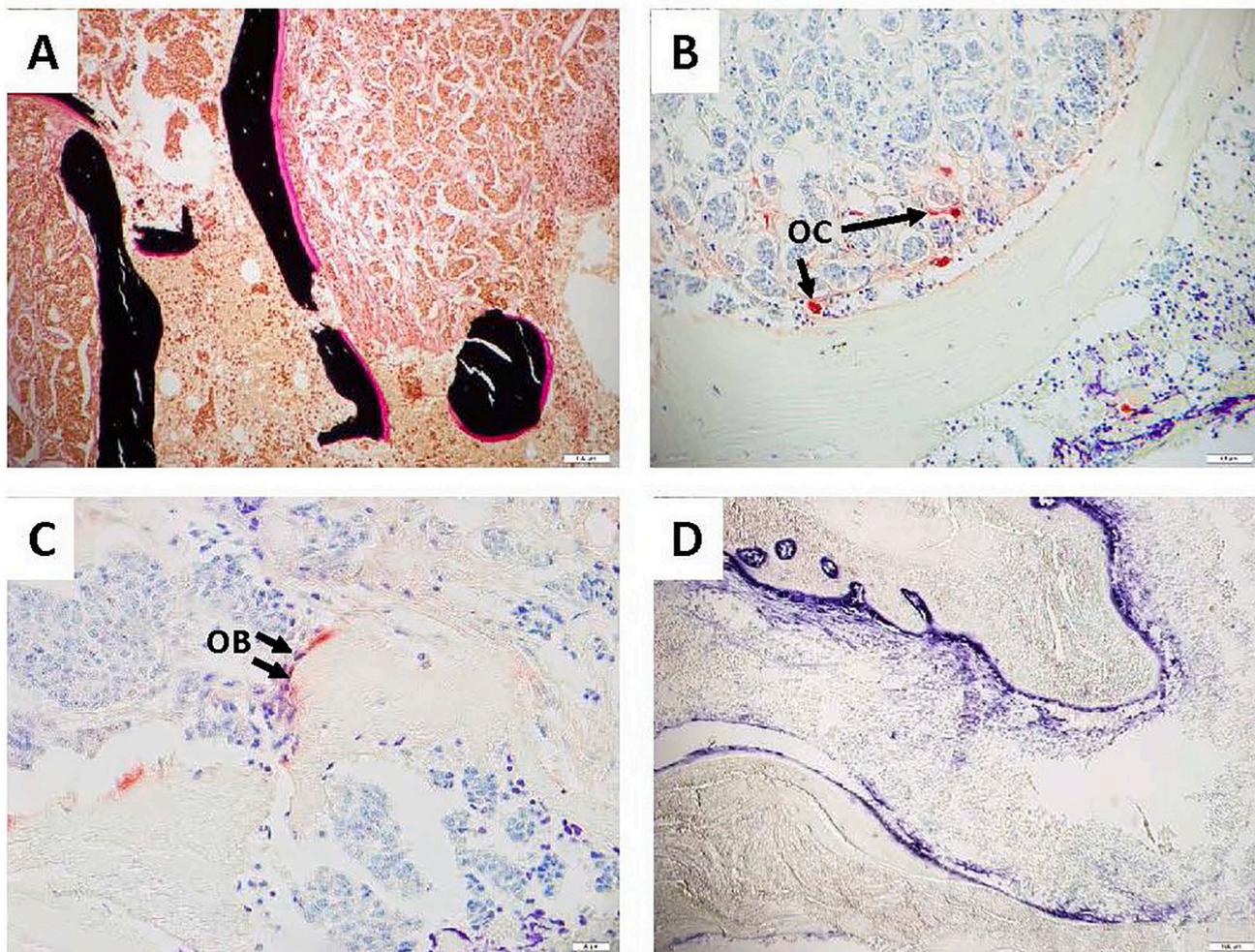


Fig. 2. (Control ESM with no BTA)

Patient #5 A) disrupted TB with clearly visible OS B) The arrows point to giant OC lining TB and one within tumor cells C) the arrows point to OB adjacent to TB D) Blue staining indicates ALP activity.

ment). Additionally, least-squares means post hoc for multiple comparisons of means (LSMEANS statement with Bonferroni correction) was applied. All statistical analyses were performed using Statistical Analysis System software, version 9.4 (SAS Institute, Cary, NC), with P values < 0.05 considered significant.

3. Results

Twelve patients consented to participate in the study and underwent bone biopsy. There were no complications due to the procedure. Demographic and tumor characteristics are presented in [Table 1](#). Most patients were postmenopausal, with hormone receptor positive breast cancer (11/12), a mean age of 51.5 (range 45–72), had bone metastases (11/12) and were receiving bisphosphonates combined with hormonal therapy (6/12) at the time of the biopsy. Bone biopsy specimens were of sufficient quality with an intact core for histomorphometry from eleven out of twelve patients (the specimen from patient #7 was of insufficient

sample quality and could not be analyzed).

3.1. Bone histomorphometry and histology

Osteoblast and osteoclast numbers per tissue area and bone perimeter are displayed in [Table 2](#).

3.1.1. Control patient (no BTA) with no bone metastases

Patient #1 ([Fig. 1](#)), was used as a control as she had no detectable bone metastasis by imaging and was not treated with BTA. The trabecular structure and osteoid tissue appeared normal ([Glorieux et al., 2000a](#)). The number of osteoclasts was similar to that previously reported in healthy post-menopausal women ([Arlot, 1990b](#)). The bone marrow had a normal appearance, with no evidence of tumor cells. Overall histomorphometry in this patient was consistent with morphologic features and bone turnover parameters seen in postmenopausal women not treated with antiresorptive agents. This patient had active

Patient #3

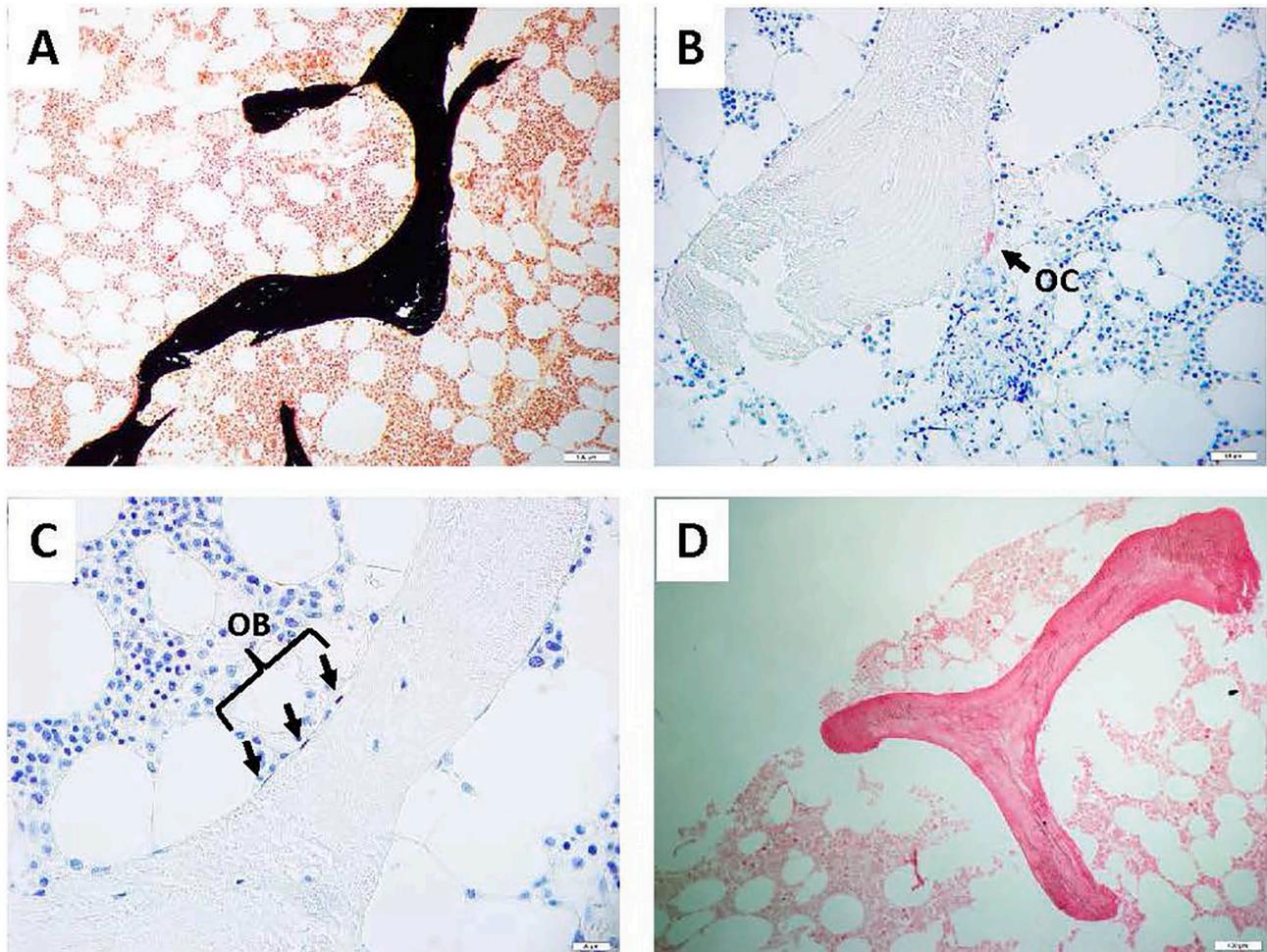


Fig. 3. Patient #3 A) The TB and BM appeared normal. No visible invasion by tumor cells in the specimen with narrow, thin OS B) No identifiable OC C) Visible OB as shown by the arrow D) Negative ALP activity (of note the trabecular bone is pink as it was counterstained with eosin, which was later omitted).

bone formation by tetracycline labeling (Table 3).

3.1.2. Control patient (no BTA) with ESM

One patient (patient #5) had ESM but did not receive BTA (Fig. 2). This patient's biopsy showed an abnormal trabecular structure with a high bone volume (BV/TV) loss of connectivity, apparent increase in non-mineralized osteoid tissue, and very high number of osteoclasts (Table 2) as compared to Patient #1 who had no evidence of skeletal metastases (Fig. 1). Patches of tumor cells were visible in the marrow. This patient had very robust bone formation by tetracycline labeling (Table 3).

3.1.3. Patients with LSM (less than 3 lesions) on BTA

Three patients had LSM and were all on BTA. Patients #3, and 10 (Figs. 3 and 4) had no detectable osteoclasts and were considered responders whereas patient # 4 (Fig. 5) was considered a non-responder, with detectable osteoclasts (Table 2). All three of these patients had no active bone formation (Table 3).

3.1.4. Patients with ESM on BTA

There were six patients (patients #2,6,8,9,11,12) [Figs. 6-11] who had ESM and were also receiving BTAs. Five of the six patients (patients #2,6,9,11,12) had extensive invasion of tumor cells throughout the marrow and were non-responders to BTA based on the presence of osteoclasts (Figs. 6,7,9-11). During intensive and sustained bisphosphonate therapy, osteoclasts number should normally be suppressed as reported earlier (Parfitt, 1987; Arlot et al., 1990a). Patient #8 (Fig. 8) had no detectable osteoclasts despite evidence of tumor cells in the same biopsy and was thus categorized as a responder (Table 2). Patients 2, 9, and 12 had high apparent osteoid volume and patients 2,6,9,11 and 12 had active bone formation by tetracycline labeling (Table 3).

3.2. Comparative analysis of ESM and LSM

Compared to untreated patients, patients treated with BTA showed a trend towards suppression of bone formation, as measured by tetracycline labelling in all patients (both LSM and ESM combined) (ANOVA, 2-

Patient #10

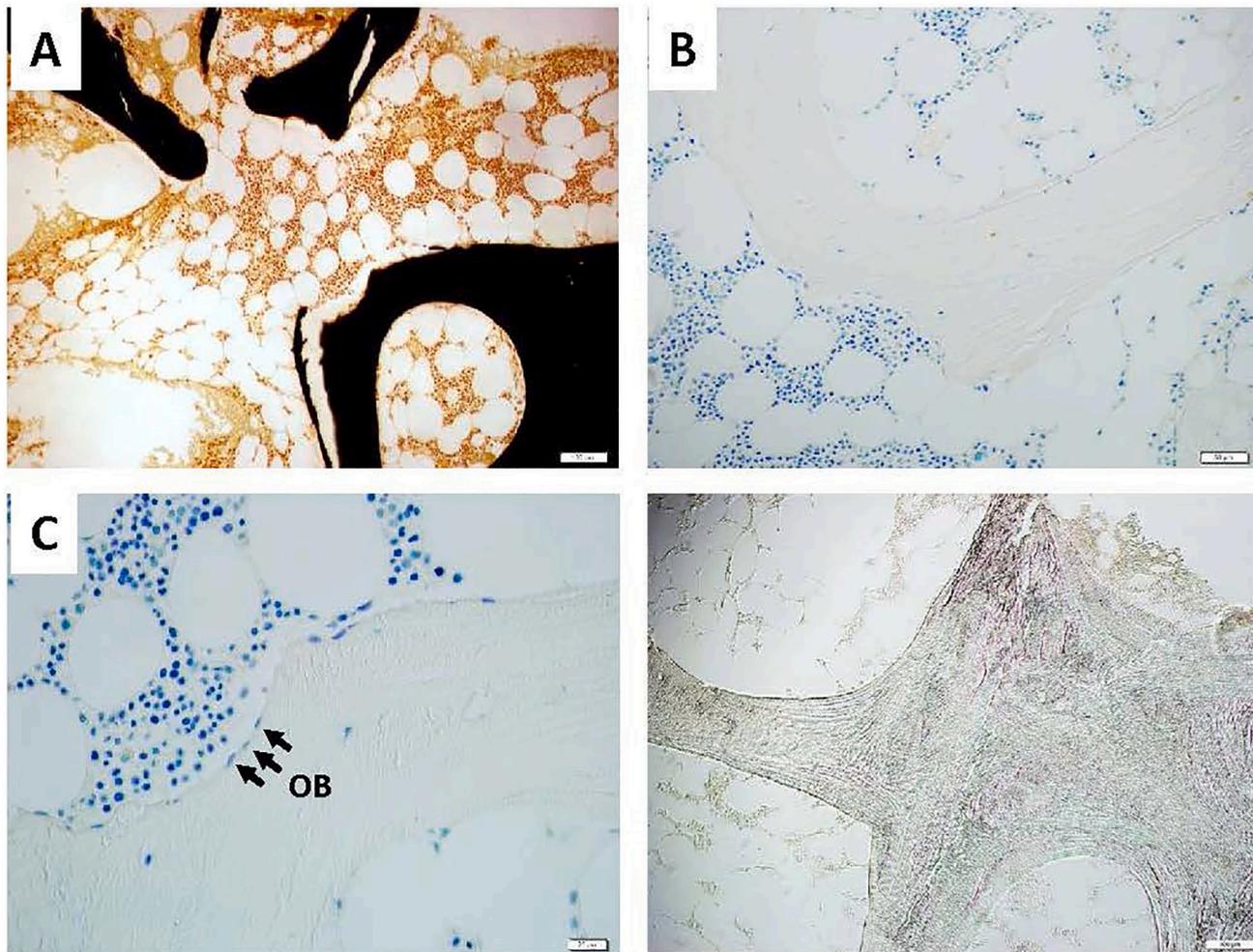


Fig. 4. (LSM with BTA) Patient #10 A) Normal looking bone structure B) No OC at the surface of trabecular bone C) Arrows point to OB at the surface of TB D) No ALP activity seen.

way, $P = 0.610$) as seen in Fig. 12.

There was also a trend towards a significant difference between ESM and LSM treated with BTA, highly suggestive of resistance (non-suppressibility of tetracycline labelling) to BTA in patients with ESM ($p = 0.814$) (Table 3).

In all groups treated with BTA, OV/TV was significantly higher in patients with ESM as compared to patients with LSM ($p = 0.0239$) (Fig. 13).

There was no significant difference in the osteoblast number between ESM and LSM treated with BTA. In contrast, the number of osteoclasts was significantly higher in patients with ESM compared to LSM treated with BTA, which may be suggestive of resistance to suppressive therapy ($p = 0.044$).

An interesting finding when we looked at the entire cohort was the positive correlation between osteoblast number and length of BTA ($p = 0.0382$). This has not been previously reported. When we divided the cohort between ESM and LSM, this correlation was not seen. We found

no correlation between osteoclast number and length of BTA treatment.

We found a good correlation between osteoblast activity and tetracycline labelling (representative of bone formation) in contrast to absence of correlation between osteoblast number and tetracycline labelling ($p = 0.0364$) (Table 3).

3.3. Routine pathological assessment with immunohistochemistry (IHC) for receptor status in bone biopsy decalcified paraffin embedded specimens

All 12 patients had tissue available for pathology assessment. Malignant cells were identified in patients # 2,5,6,8,9,11 and 12 (Figs. 2,6,7,8,9,10,11) by either bone biopsy or by bone marrow aspirate. All of these patients had extensive bone involvement by imaging. Sufficient tumor cells for hormone receptor analysis in bone biopsies were available in 4 patients. While ER was concordant with the primary tumor in all these patients, there was loss of PR expression in three patients and one patient had only weak focal staining for PR.

Patient #4

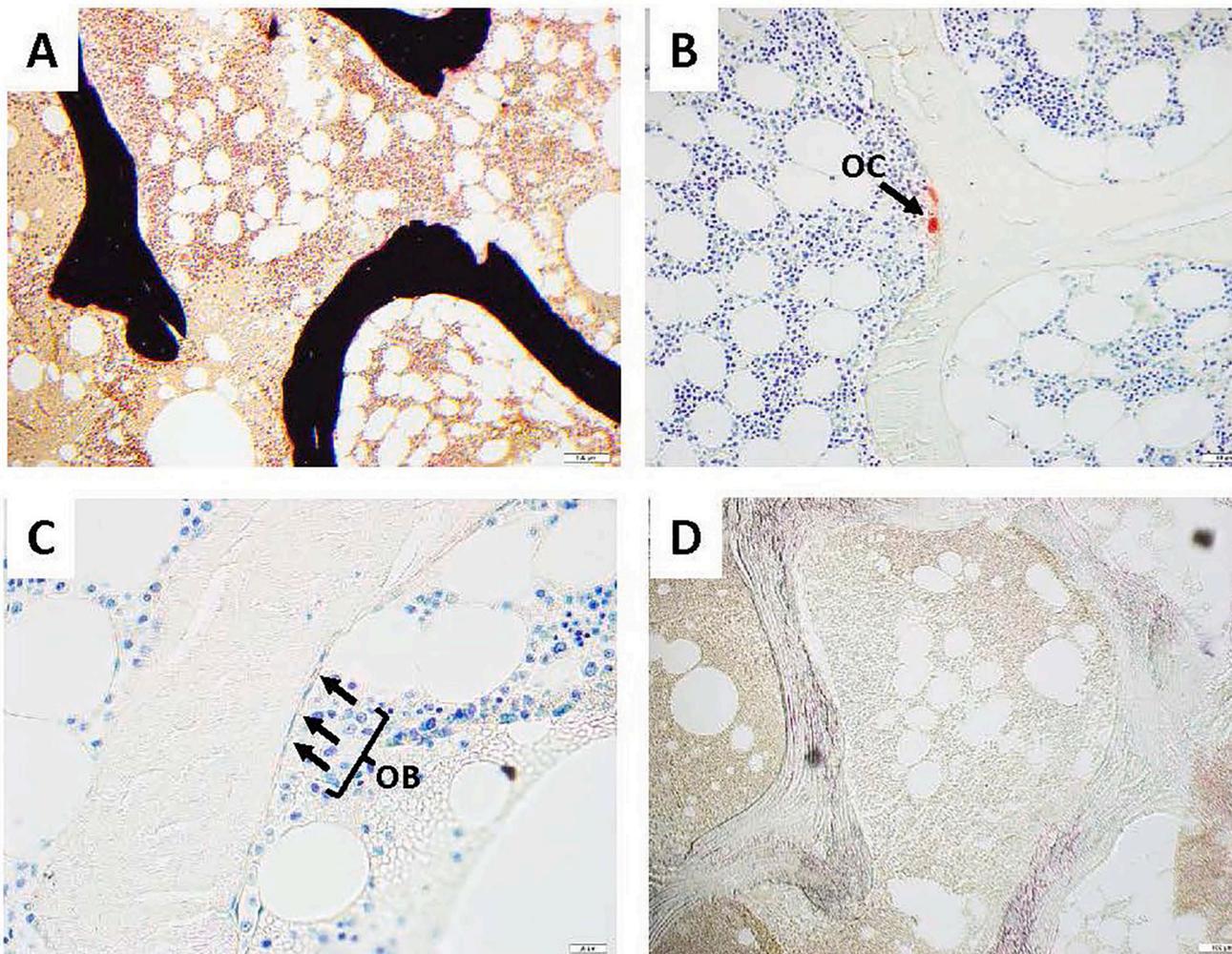


Fig. 5. Patient #4 A) Histomorphometric analysis revealed normal trabecular structure with no evidence of marrow invasion by tumor cells B) Numerous OC as indicated by the arrows C) Arrows show presence of OB at the surface of trabecular bone D) No evidence of ALP staining.

4. Discussion

Administration of antiresorptive agents (bisphosphonates and denosumab) at high cumulative dosage in relatively short time periods (Clemons et al., 2012b) (compared to their standard use in osteoporosis) in patients with both early stage and metastatic cancer is increasingly common. However, we are not aware of any studies directly investigating the effect of these therapies on tumor burden and bone turnover using bone biopsy specimens in breast cancer patients receiving active treatment. Furthermore, prolonged suppression of bone remodeling can have both beneficial and detrimental effects. Therefore, there is an urgent need for better understanding of the effects of BTAs on tumor burden, bone homeostasis, morphology and bone quality in vivo in these patients.

Several non-invasive tools including magnetic resonance imaging (MR) and MR spectroscopy, multi-detector CT, high-resolution peripheral quantitative (HR-pQ) CT and quantitative US have been developed and optimized to quantify bone architecture, metabolism, and function

in order to better predict bone strength and more sensitively monitor therapeutic interventions (Link, 2012). These imaging methodologies have shown promising results at predicting risk of fragility fractures (Boutroy et al., 2005a; Majumdar et al., 1999a; Cortet, 2000; Wehrli et al., 2001a; Khaw et al., 2004a; Szulc et al., 2011a), and in monitoring therapeutic interventions (Burghardt, 2010; Li et al., 2010) outside the context of metastatic cancer in bone. These techniques have not been used to assess the therapeutic response in patients with established skeletal metastases. Although, bone turnover biochemical markers are excellent markers of therapeutic response and might indirectly reflect bone quality status in patients with osteoporosis (Bauer, 2004; Greenspan, 2005; Delmas, 2009), these measurements reflect the effect of antiresorptive agents on the entire skeleton and have provided conflicting information as therapeutic response indicators in the context of bone metastases (Pollmann, 2007; Joerger and Huober, 2012; Brown, 2018).

Studies indicating their potential as prognostic indicators in breast cancer suggest that accelerated bone turnover may promote the

Patient #2

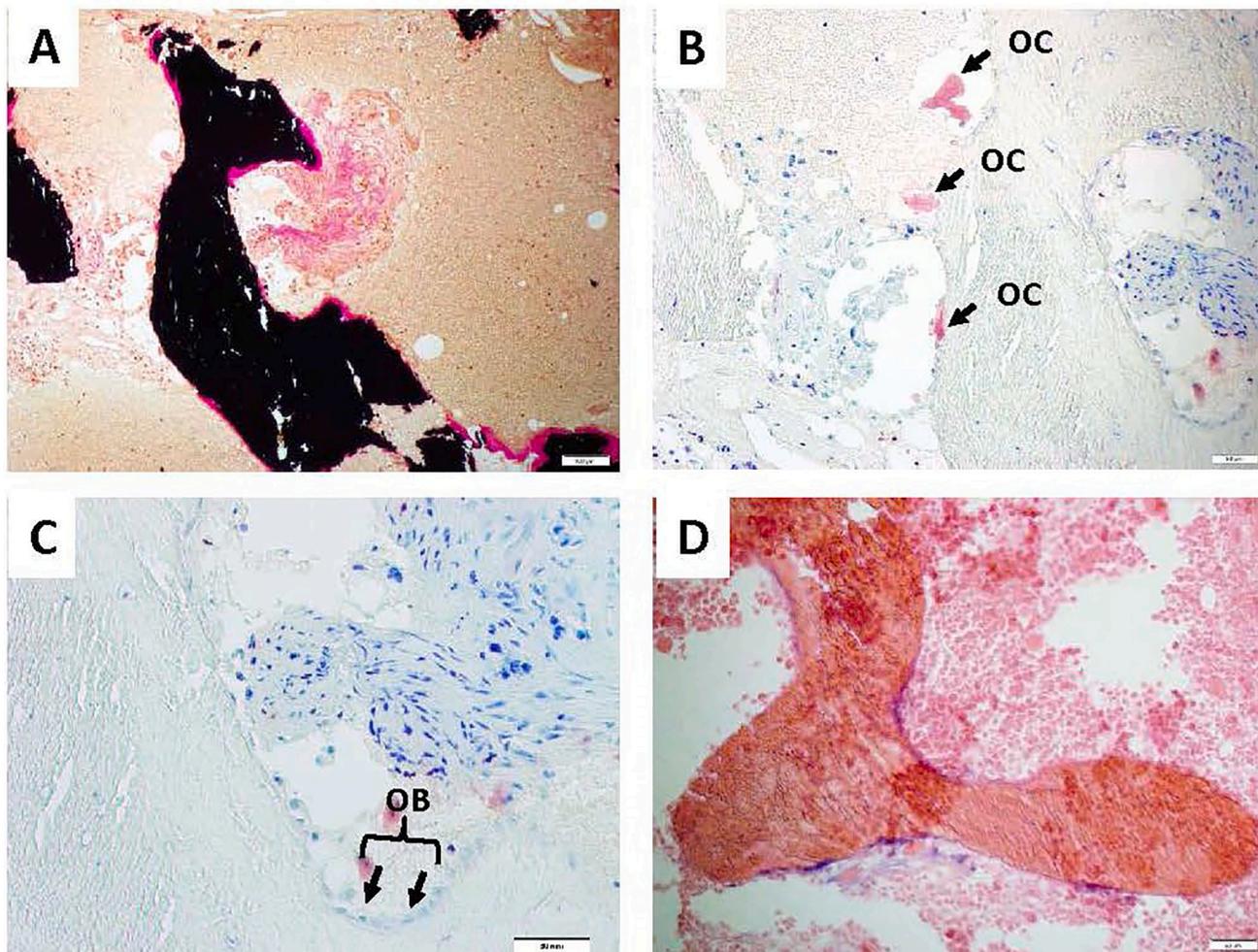


Fig. 6. Patient #2 A) On histomorphometric analysis, trabecular structure is disrupted by invasion with tumor cells with several areas of disorganized collagen-like material B) Arrows point to giant OC adjacent to bone interface and within the tumor C) Arrows point to OB lining the trabecular surface D) Strong ALP staining seen (of note the trabecular bone is pink as it was counterstained with eosin which was later omitted).

development of skeletal metastasis early on in the disease and perhaps prior to the establishment of disseminated tumor cells in the marrow (Ali, 2004).

Direct assessment of tumor burden and bone turnover in metastatic patients using bone biopsy for histomorphometric evaluation should be the gold standard as it is currently in non-cancer patients. However, the standard procedure using the Bordier trephine is an invasive procedure associated with pain, inconvenience for the patients and is rarely, if ever, used in routine clinical practice. In contrast, bone biopsy using the 2 mm Jamshidi™ is a minimally invasive procedure used routinely for assessment of marrow involvement in hematologic disorders. We recently showed that it can provide sufficient material for analysis of bone quality by both microCT and histomorphometry (Fralick, 2012). It has, therefore, the potential of becoming a useful tool to assess the impact of a variety of cancer-related therapies (denosumab, tamoxifen, aromatase inhibitors, and bisphosphonates) on bone turnover and

quality in the context of bone metastatic disease. It can also directly assess the extent of tumor burden within bone. Understanding changes in bone homeostasis is especially important given the extensive use of these agents not only in palliative care but also in the adjuvant setting and potentially in primary breast cancer prevention (Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2015; Liu, 2019).

We performed immuno-histochemistry staining on paraffin-embedded bone specimens and examined ER/PR status in patients with extensive bone metastases. This additional pathological information has clinical importance because previous studies had shown that ER/PR status seen in the primary tumor may differ from metastatic sites (Amir, 2008b). Consequently, ER/PR and Her2 status in cancer cells in bone may provide additional important information for the management of bone metastatic disease, given the reported significant proportion of discordance of their expression between primary tumor and metastatic sites (Hilton, 2011). In the current study ER expression was concordant

Patient #6

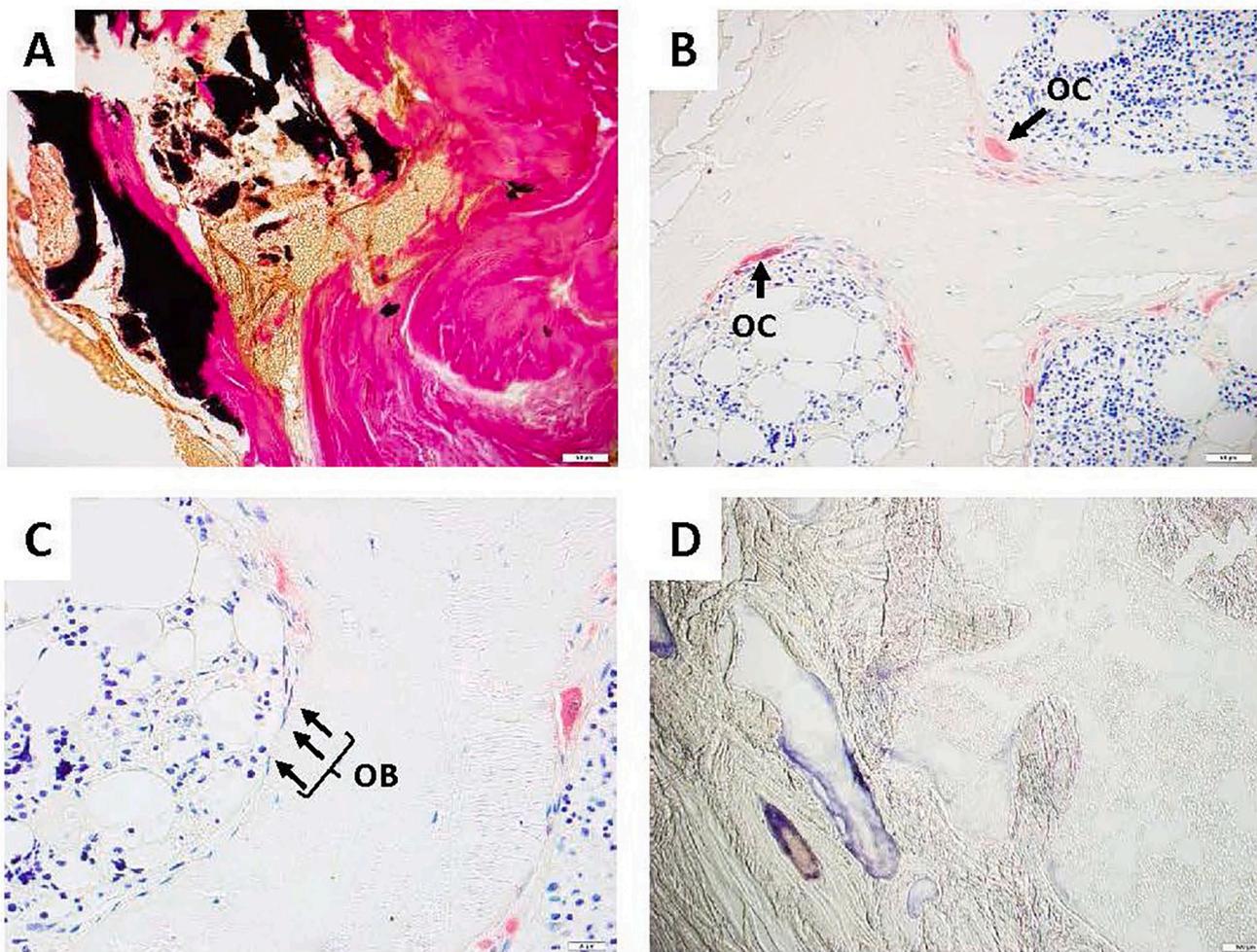


Fig. 7. Patient #6 A) Trabecular structure appeared disrupted by invasion of tumor cells with disorganized thick enlarged OS and large areas of non-mineralized collagen-like structure B) Arrows point to giant OC lining TB C) Arrows point to OB lining TB D) Positive ALP staining.

in all patients; however, loss of PR was seen in most samples possibly resulting in decreased sensitivity to endocrine therapy.

A limitation of our study was the limited sample size. This study was planned as a pilot study to explore the feasibility of using the minimally invasive 2 mm Jamshidi™ bone biopsy and not for further analysis of BTA response in this population. We saw that this procedure was very well tolerated with no complications. Despite the small sample size, we did make a number of very interesting observations. In one patient (patient #1) who did not have evidence of bone metastases but who was treated with aromatase inhibitors and did not receive bisphosphonates, bone turnover was similar to post-menopausal osteoporotic women as cited in previous studies (Arlot, 1990b). This would be expected in a severely estrogen deficient state. However, it was much lower than another patient with ESM who was also on aromatase inhibitor and not treated with bisphosphonates (patient #5). Furthermore, the majority of patients with ESM had high levels of bone osteoclastic activity even when treated bisphosphonate therapy and irrespective of the type of

anticancer therapy (AIs or chemotherapy). Patients with LSM treated with bisphosphonates showed expected suppression of bone turnover even in the presence AI. We also observed high osteoclastic activity at the interface between trabecular bone and tumor (refer to Figs. 3,4,6,7). The osteoclasts in these regions appeared enlarged resembling giant osteoclast (see Fig. 3). These giant osteoclasts are compatible with prior data that found non-resorbing, apoptotic osteoclasts associated with bisphosphonate treatment (Mac-Way et al., 2014a). Furthermore, we noticed on many samples' osteoclasts embedded within tumor, the significance of which remains to be established (Fig. 3). There was a significant difference between the number of osteoclasts in patients with ESM compared to LSM treated with BTA, which may be suggestive of resistance to suppressive therapy ($p = 0.044$). We did not see a significant change in the number of osteoblasts between LSM and ESM in patients treated with BTA.

Only 50% (3 out of 6) of patients with ESM responded to BTA as compared to 100% (3 out of 3) of patients with LSM based on osteoblast

Patient #8

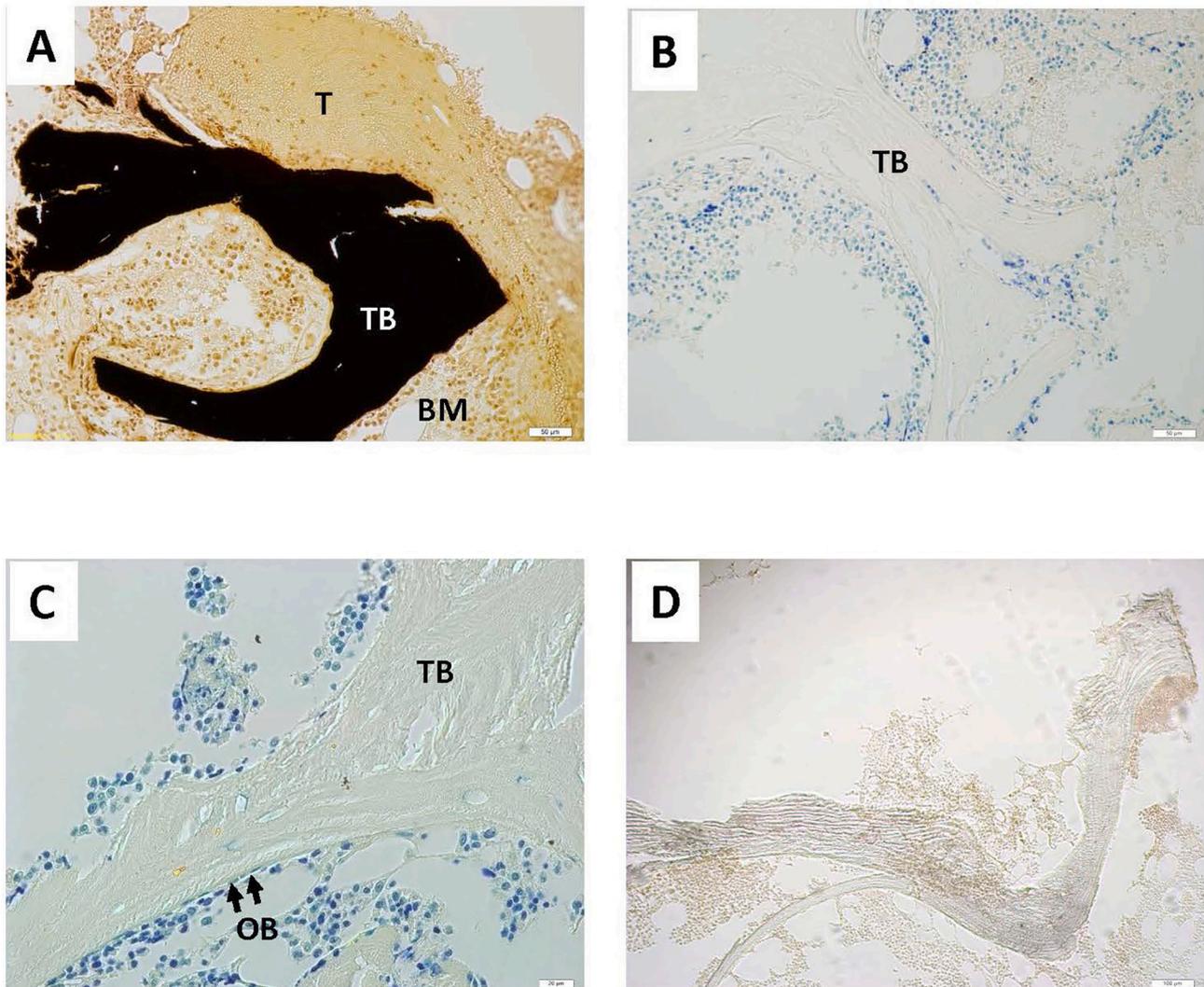


Fig. 8. Patient #8 A) the trabecular bone is disrupted with evidence of tumor cells B) No OC was seen C) Arrow points to OB D) No ALP staining.

activity (as measured by alkaline phosphatase) and bone formation (as measured by tetracycline labeling). Although suggestive of resistance to BTA in the ESM group, the size of the groups was not sufficient to show significance (p value = 0.814).

An interesting finding was the fact that all patients that classify as ESM on imaging, had presence of tumor cells on their bone biopsies. In contrast, all three patients with LSM on imaging had no bone metastases on bone biopsy and marrow aspirates. This likely reflects the fact that iliac crest biopsies do not represent sites of metastases in the LSM group. Therefore, in patients with ESM, our analysis showed that our biopsy samples reflected disease status with a high degree of correlation from skeletal survey assessed by X-rays and bone scan. However, only 3 out of 7 patients with ESM, had positive tumor cells by bone marrow aspirates whereas 7 out of 7 showed tumor cells by bone biopsy demonstrating the

superiority of bone biopsy in detecting tumor cells in patients with ESM.

5. Conclusion

In conclusion, our results suggest that the minimally invasive 2 mm Jamshidi™ bone biopsy procedure may be used as a practical clinical tool for providing important additional information on tumor burden within bone, bone microarchitectural parameters and response to BTA (as assessed by osteoclast number and dynamic histomorphometry) in patients with ESM. In patients with LSM, it can also provide additional information on the adequate response to BTA's. Further research with larger samples size in a variety of patients treated with the current panoply of anti-resorptive agents in combination with cancer targeted therapies is needed to determine their impact on bone health.

Patient #9

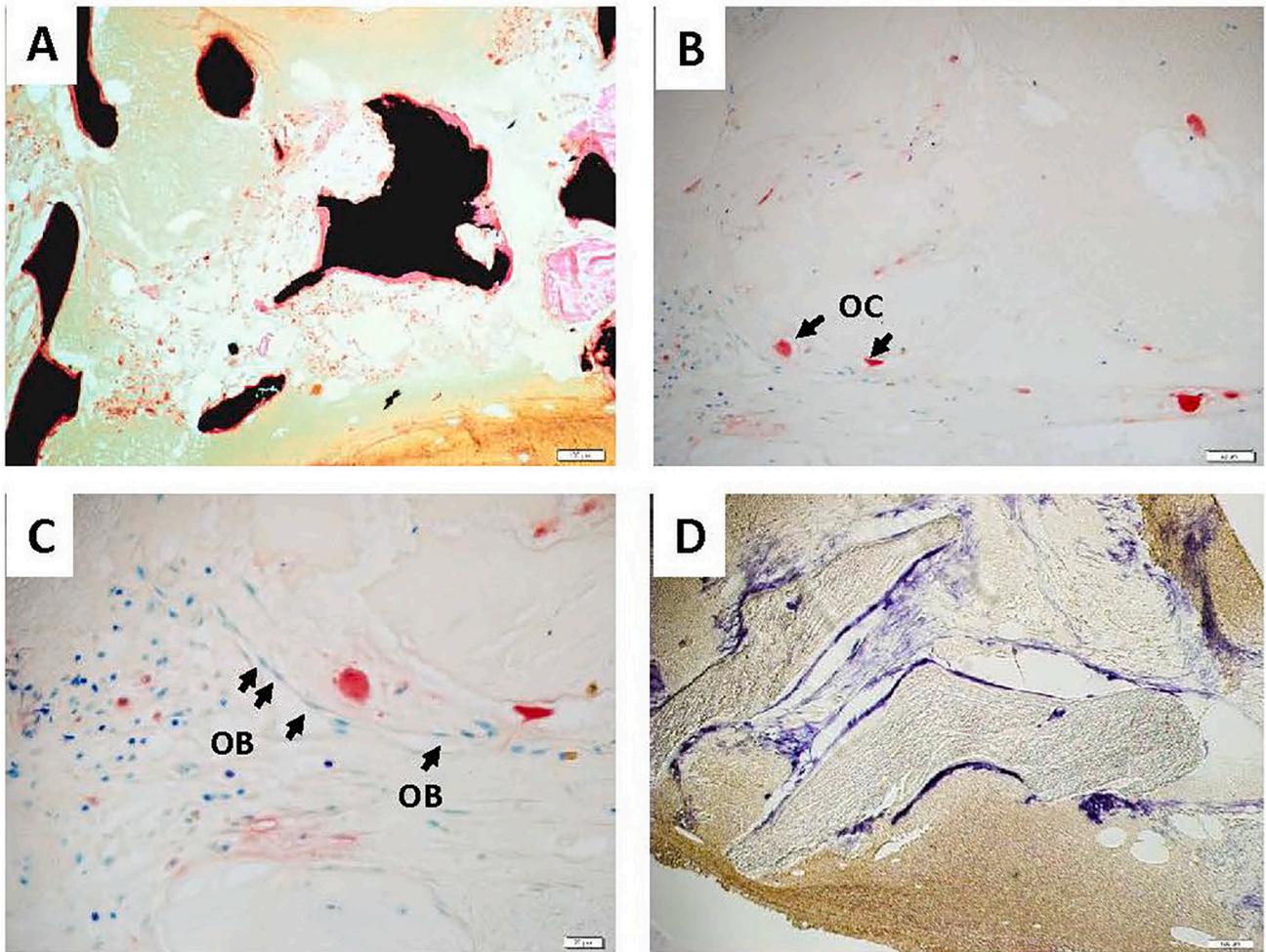


Fig. 9. Patient #9 A) the trabecular structure appears disrupted and disorganized adjacent to tumor cells Arrows point to OS B) Arrows point to giant OC seen lining TB and embedded within tumor C) Arrows point to OB lining TB D) Intense staining indicating high ALP activity.

Patient #11

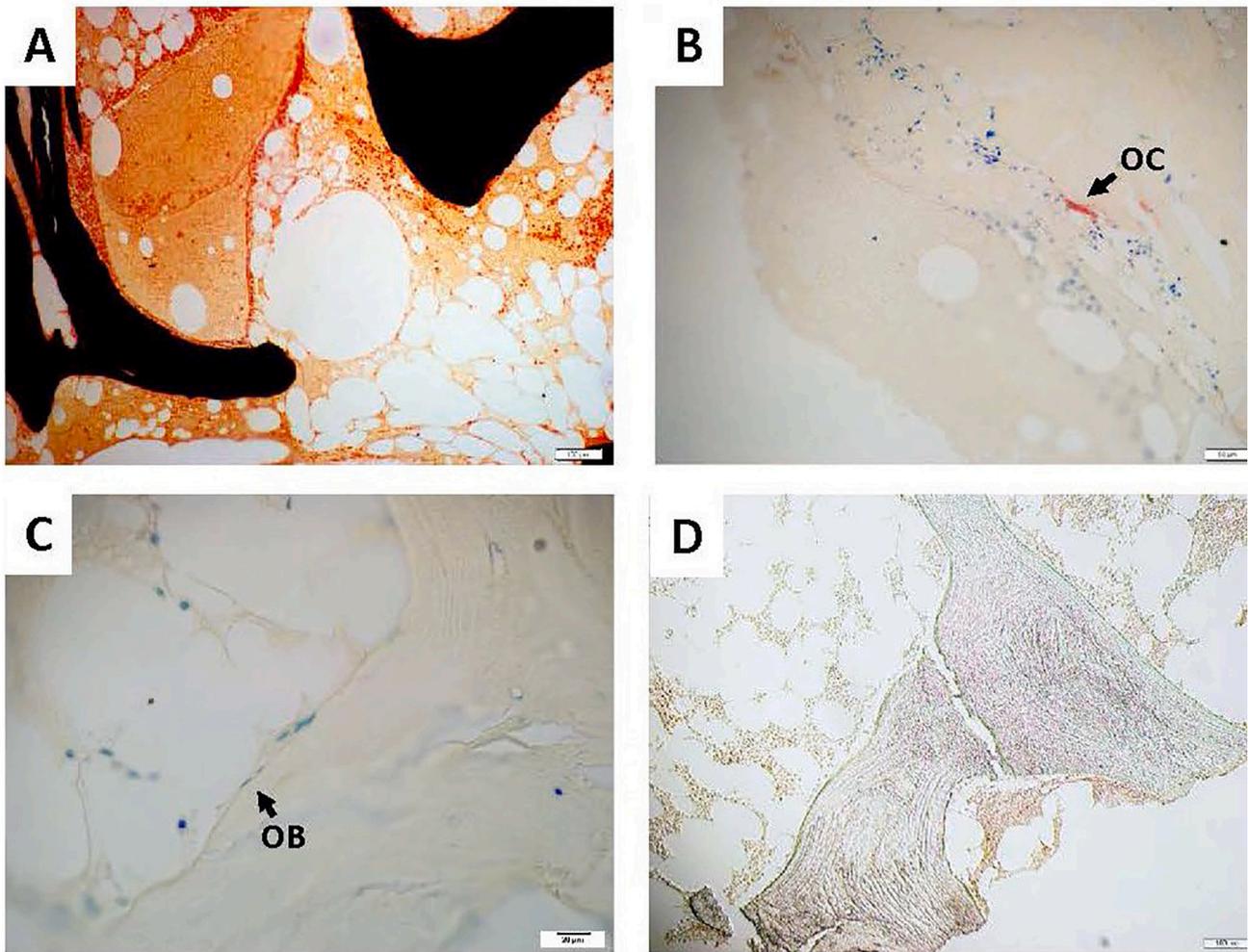


Fig. 10. Patient #11 A) TB appears disrupted with invaded tumor cells and thin layer of OS B) Arrows point to OC C) Arrow pointing to OB at the surface of trabecular bone D) Negative ALP staining.

Patient #12

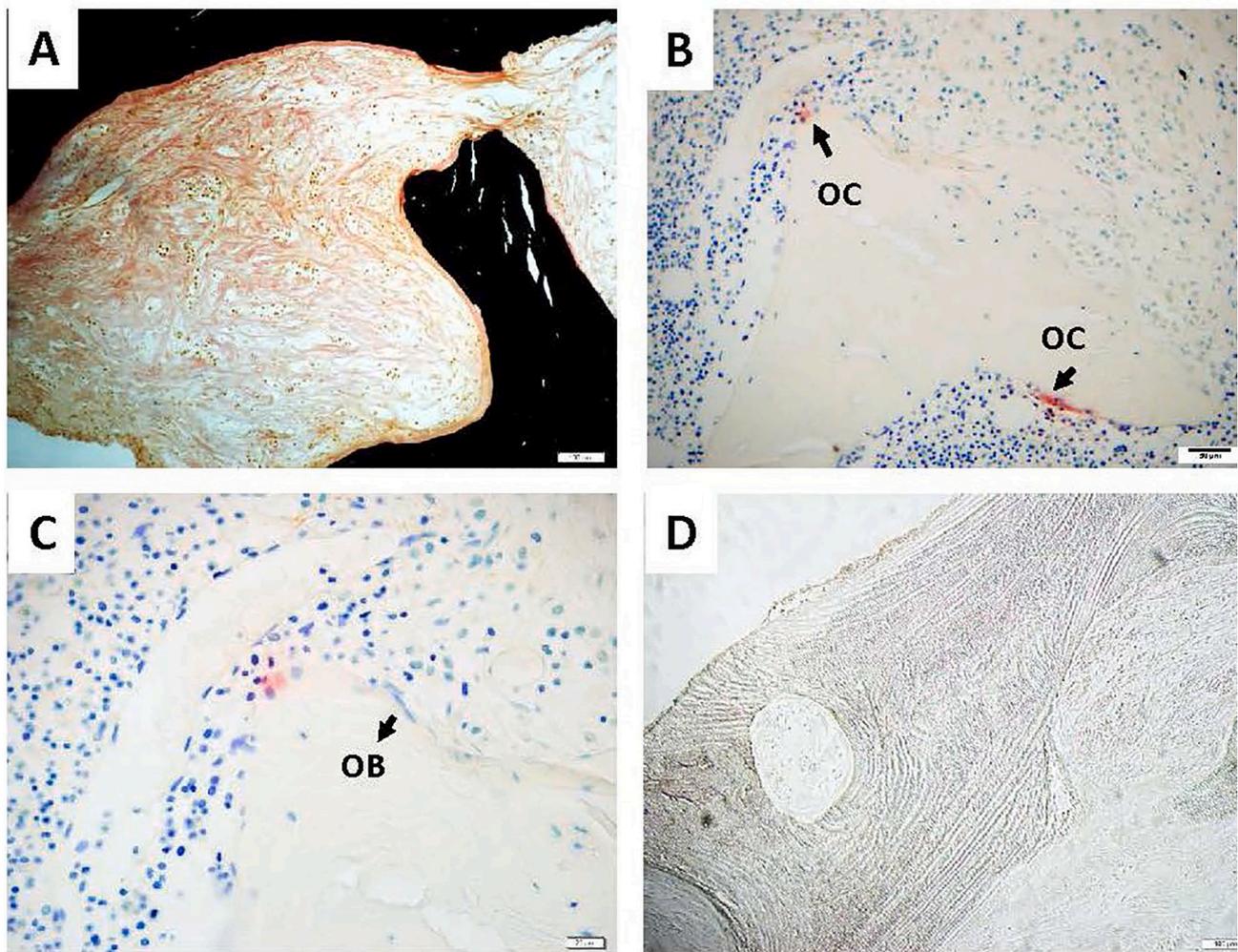


Fig. 11. Patient #12 A) TB is disrupted with large areas of tumor cells and visible OS B) Arrows point to large number of OC C) Arrow points to OB D) Negative ALP staining.

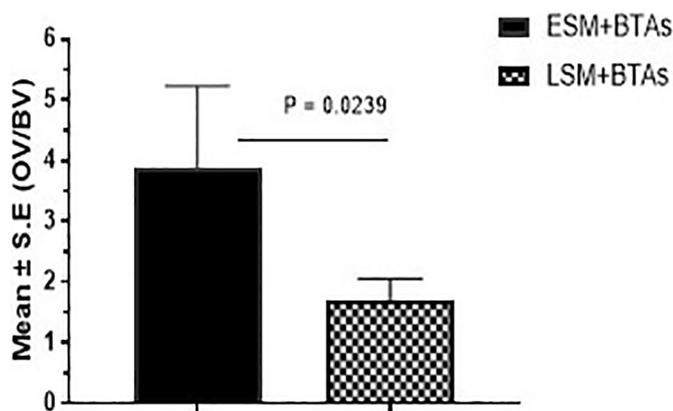


Fig. 12. OV/BV measurement in patients: The comparative analysis of the bone parameters turnover markers measurement and bone formation between patients with LSM and ESM, confirms an augmentation of Osteoid volume density over bone volume density (OB/BV) in patient treated with BTA, regardless of skeletal metastases intensity.

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CRediT authorship contribution statement

A. Beltran-Bless: Writing – reviewing and editing, Investigation, Formal analysis **M. Murshed:** Methodology, Writing – reviewing and editing, Supervision, Resources, Investigation, Formal Analysis **I. Kuchuk:** Investigation **M. Zakikhani:** Data curation, Formal analysis, Writing – review and editing, Investigation **N. Bouganim:** Investigation **S. Robertson:** Investigation.

N. Kekre: Investigation **L. Vandermeer:** Data curation, Investigation **J. Li:** Investigation, Formal analysis **C.L. Addison:** Investigation **F. Rauch:** Methodology, Resources, Investigation **M. Clemons:** Conceptualization, Methodology, Writing – original draft preparation, Writing – review and editing, Supervision, Funding acquisition, Resources, Investigation **R. Kremer:** Conceptualization, Methodology, Writing – original draft preparation, Writing – review and editing, Supervision, Funding acquisition, Resources, Investigation.

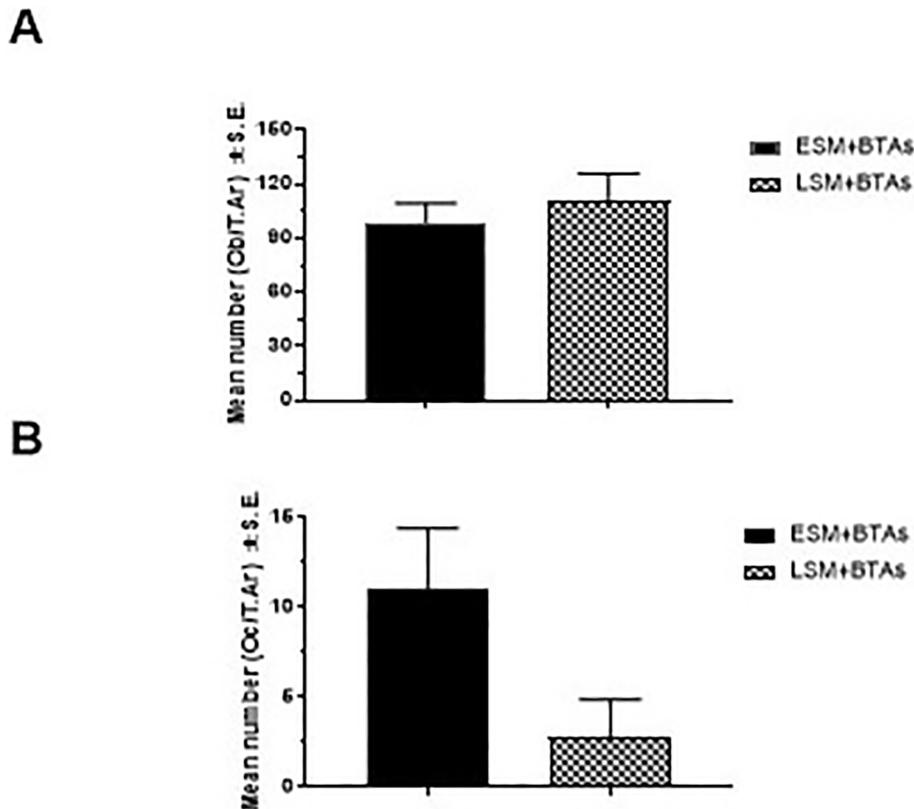


Fig. 13. (A) The comparative analysis of the number of osteoblast (Ob) and (B) Osteoclast (Oc) over tissue area (T.ar) or bone perimeter (B.PM), $P = NS$, $P = 0.044$, respectively.

Declaration of competing interest

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

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