Research Article

Exposure to antiresorptive therapy with bisphosphonates does not induce histological changes in human alveolar jawbone

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Aim: The identification of specific alterations in the alveolar jawbone of patients treated with nitrogen-containing bisphosphonates (NBP) but without bisphosphonate-related osteonecrosis of the jaw (BRONJ) may help to identify the early steps of BRONJ and to select patients at risk for it.
Materials and Methods: We performed a case-control study. Cases were 60 individuals treated with NBP without clinical and radiological signs of BRONJ and requiring surgical tooth extraction. Controls were 60 individuals never treated with NBP and requiring tooth extraction. Cases and controls were matched by sex (same) and age (within 5 years). 18 categorical (basophile reversal lines, osteoblasts, osteoblastic lines, osteocytes, empty osteocytic lacunae, osteoclasts, Howship's lacunae, vessel dilatation, vascular congestion, arteriolar thickening, intravascular fat globules, calcific fat necrosis, fatty bone marrow, ruptured adipocytes, granular cytoplasm of adipocytes, oil cysts, perivascular fibrosis, diffuse fibrous metaplasia) and 2 ordinal histopathological variables (inflammation and bone maturation) were investigated. Exact univariable and multivariable (correction for gender and age) logistic regression was used to test the association between NBP use and the histopathological variables. Because of multiple comparisons, the critical p-value was set to 0.0025 (0.05/20).
Results: Cases and controls did not differ for any study variable except for vascular congestion that was significantly associated with NBP use (multivariable OR = 0.24, exact 95% CI 0.10 to 0.57 for cases vs. controls, p = 0.0006).
Conclusions: Use of NBP does not produce specific histological alveolar bone alterations in the absence of overt BRONJ disease.

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Introduction

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is the first and most studied[1] of a number of bone disorders that have been linked to the use of different classes of medications, including antiresorptive drugs. These osteonecrotic processes have been recently gathered under the term of Medication-related osteonecrosis of the jaw.[2]

Although the pathogenesis of BRONJ is likely to be multifactorial,[3][4][5] prolonged therapy with antiresorptives and NBP in particular seems to influence jawbone remodeling and predispose to development of clinical signs of BRONJ.[6] Tooth extraction, oral surgery and use of corticosteroids are additional risk factors.[7][8][9]

These antiresorptive agents are worldwide administered in malignant and benign disruptive bone diseases and, because interfering with osteoclasts functions, NBP effectively reduce bone resorption rate.[10] NBP are thought to accumulate in the jaws at higher concentration than in other bones owing to the high turnover of the dento-alveolar region.[11] An increase of bone remodeling at the sites of NBP accumulation promotes osteoclasts migration and activation of NBP that, in turn, interfere with bone healing ensuing the accumulation of non-viable bone.[11][12][13] According to this hypothesis, a mucosal breakdown caused by local precipitating factors such as tooth extraction and ill-fitting prostheses would expose the “non-viable” bone and promote its superinfection. Drug-induced interference in local repair mechanisms would subsequently evolve in bone necrosis.

Alternatively, it was speculated that BRONJ initially evolves as an aseptic osteomyelitic process, in which a local inflammation promotes the development of bone necrosis.[14] In this condition, bacterial invasion through a discontinuation of the oral mucosa would subsequently lead to the exposure of infected necrotic bone.[14][15][16]

In a recent study, we found that alveolar bone histology at the time of tooth extraction can predict BRONJ occurrence in patients treated with NBP. In this study, only patients with osteomyelitis at baseline developed BRONJ within 12 months of follow-up.[17]

In addition, a number of minor inflammatory bone changes were observed in the specimens of a subset of BRONJ disease-free patients, but whose potential association with NBP use could not be adequately addressed because of the lack of a control group.[17]

It has not been previously studied whether these inflammatory bone changes are due to NBP intake and activation or, in turn, to chronic inflammatory dental/periodontal disease.

We performed a case-control study to compare the histopathological features of the alveolar jawbone of patients exposed to NBP but without clinical and radiological signs of BRONJ with those of patients never exposed to NBP.

Clearly, the identification of specific histopathological alterations of the alveolar jawbone of patients treated with NBP but without BRONJ may help to better understand the effects of NBP on jawbone and identify the early steps of disease.

Patients and methods

Study design

We performed a case-control study to test whether specific histopathological alterations are present in the alveolar jawbone of patients treated with NBP at high risk of BRONJ.

The study was conducted at the Units of Oral and Maxillofacial Surgery of the Universities of Verona and Padova (Italy) between March 2006 and August 2010. The study was approved by the local ethics committee and carried out in accordance whit the guidelines of good general practice. All subjects gave written informed consent for the treatment and bone samples drawing.

Cases were individuals with: 1) metastatic bone disease or multiple myeloma treated with high-dose intravenous NBP or non-malignant bone disease treated with oral NBP for at least 3 years; 2) dental or periodontal disease requiring tooth extraction; 3) absence of clinical and radiological signs of BRONJ in the jaw where dental extraction was required. Controls were individuals with: 1) dental or periodontal disease requiring tooth extraction; 2) no previous use of NBP.

Exposure to radiation therapy of the head and neck and benign and malignant diseases of the jaws were reasons for exclusion from the study for both cases and controls.

Controls were matched to cases in a 1:1 ratio by sex and age.

Cases and controls underwent extraction of the compromised tooth/teeth and the simultaneous harvest of an alveolar bone biopsy in the site of tooth extraction. Biopsies of the bone marrow were obtained with a bone forceps from the alveolar socket and the interradicular septa.

Alveolar bone biopsies

Biopsies were fixed in 4% formaldehyde and kept in the dark under sterile conditions for at least 24 hours. They were decalcified and embedded in paraffin blocks, which were cut into 5-μ serial sections

and stained with hematoxylin-eosin (H&E stain). Standard analysis was performed using an optical microscope (Leica DFC 280, Leica Microsystems, Wetzlar, Germany). All bone biopsies were analyzed by the same pathologist (SB), who was blinded to the patients' medical and clinical history.

At first examination bone samples were excluded from both study groups if processing or staining artifacts or insufficient amount of trabecular bone were found. Specimens with signs of osteomyelitis or osteonecrosis were also excluded (as we were interested to identify early histopathological bone alterations). The remaining specimens underwent further histopathological evaluation.

Outcome variables

We investigated 20 histopathological features as potentially associated with NBP usage. 18 categorical variables were chosen on the basis of the available evidence of potential association with BRONJ development:[18][19][20]

1 - reversal lines **(Fig. 1)**;

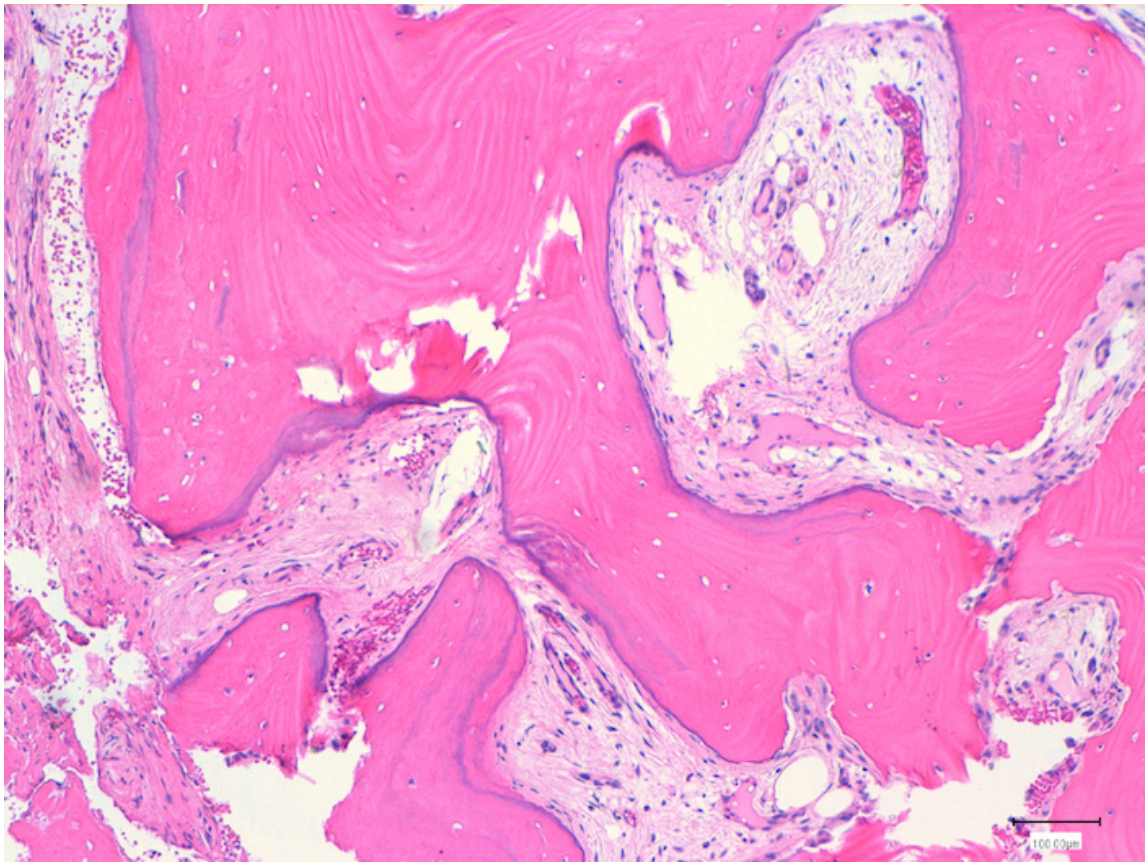

Fig. 1 - Basophilic cement lines show that the previous cellular activity at the site was resorptive. They indicate a period of several weeks called reversal phase, which separates bone-resorbing osteoclasts from bone-forming osteoblasts.

2 - osteoblasts;

3 - osteoblastic lines (rimming) **(Fig. 2)**;

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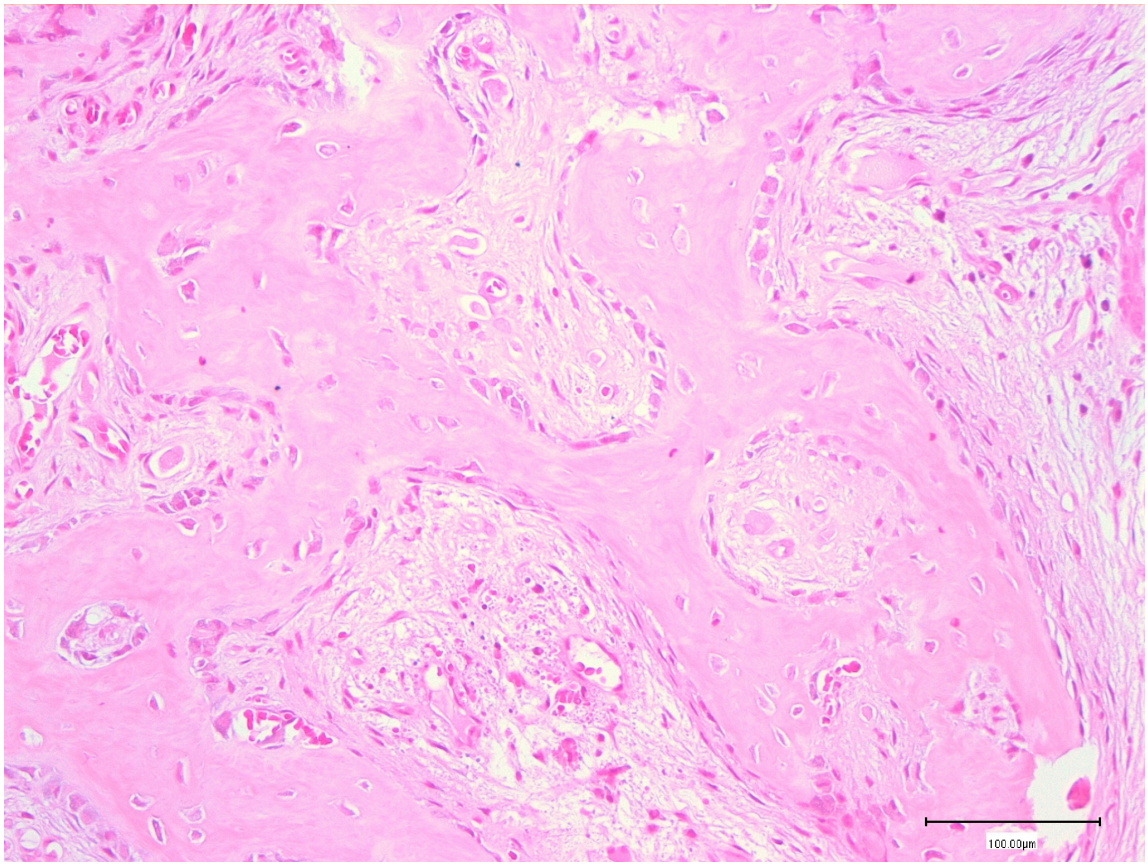

Fig. 2 - Interconnecting trabeculae of woven bone with prominent osteoblastic rimming.

4 - osteocytes;

5 - empty osteocytic lacunae **(Fig. 3)**;

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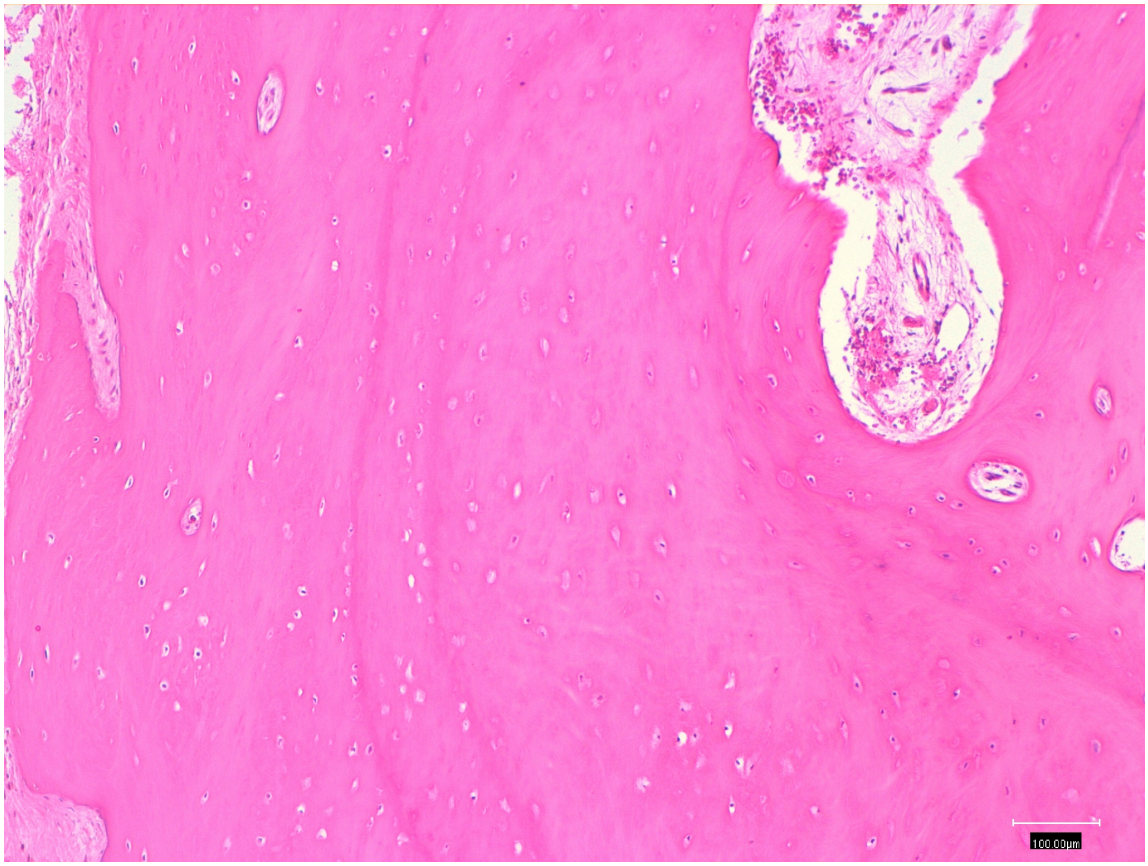

Fig. 3 - Several empty lacunae within lamellar mature bone, suggestive for osteocyte cell death (H&E stain).

6 - osteoclasts;

7 - Howship's lacunae **(Fig. 4)**;

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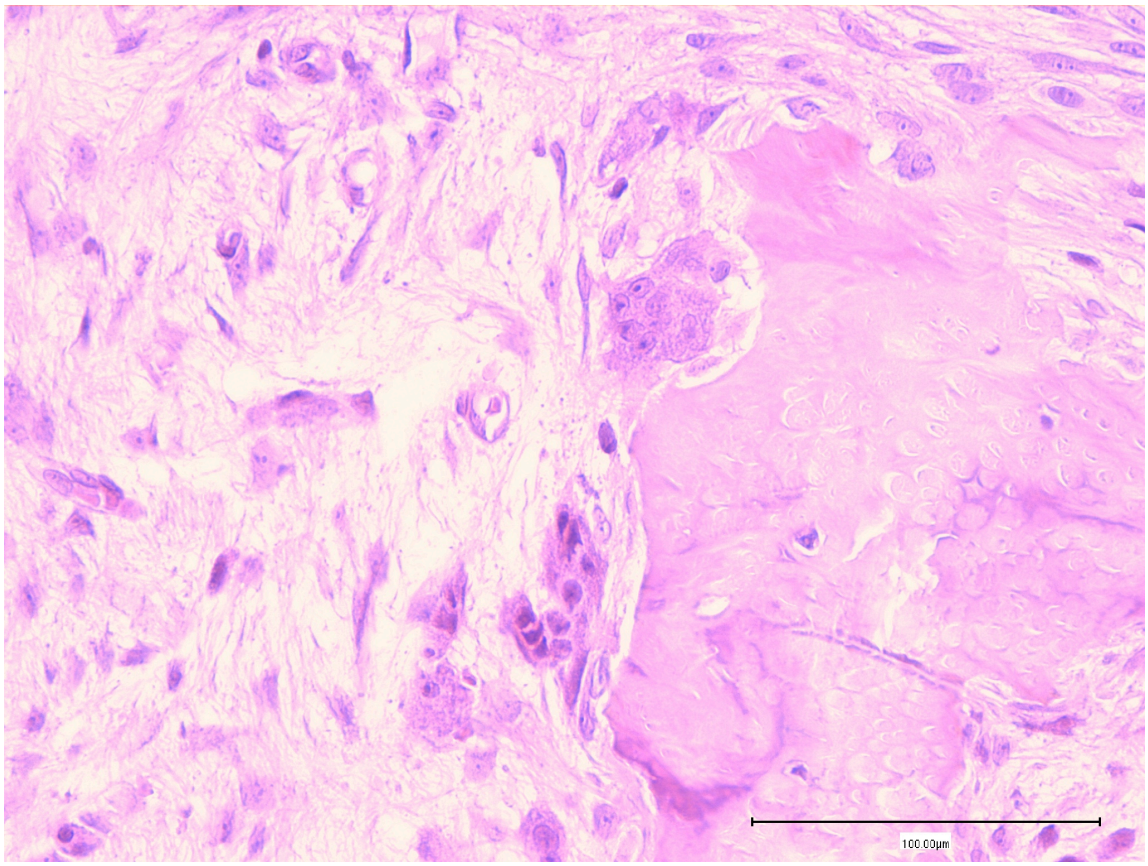

Fig. 4 - Osteoclasts-resorbing bone in Howship's lacunae, which form from the digestion of the underlying bone (H&E stain).

8 - vessel dilatation;

9 - vascular congestion **(Fig. 5a, 5b)**;

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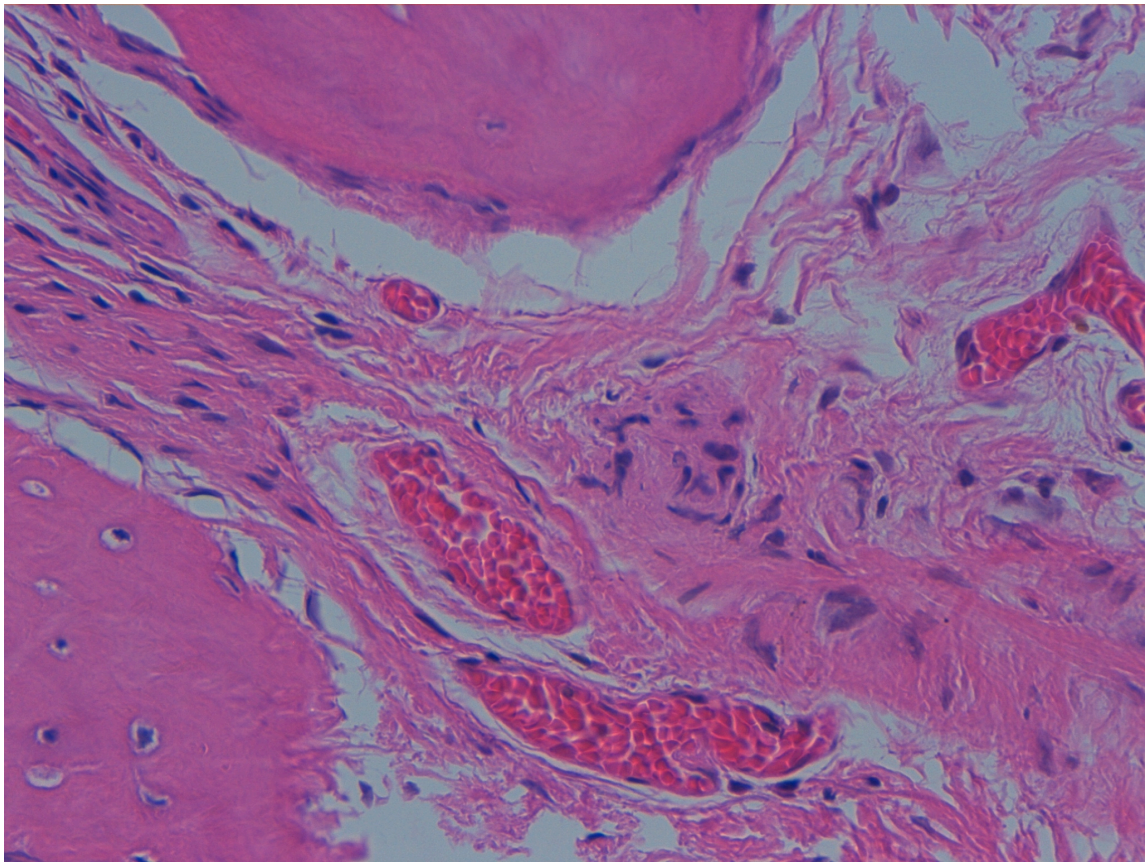

Fig. 5a - Typical vascular congestion with dilated and engorged vessels (H&E stain; 40X magnification).

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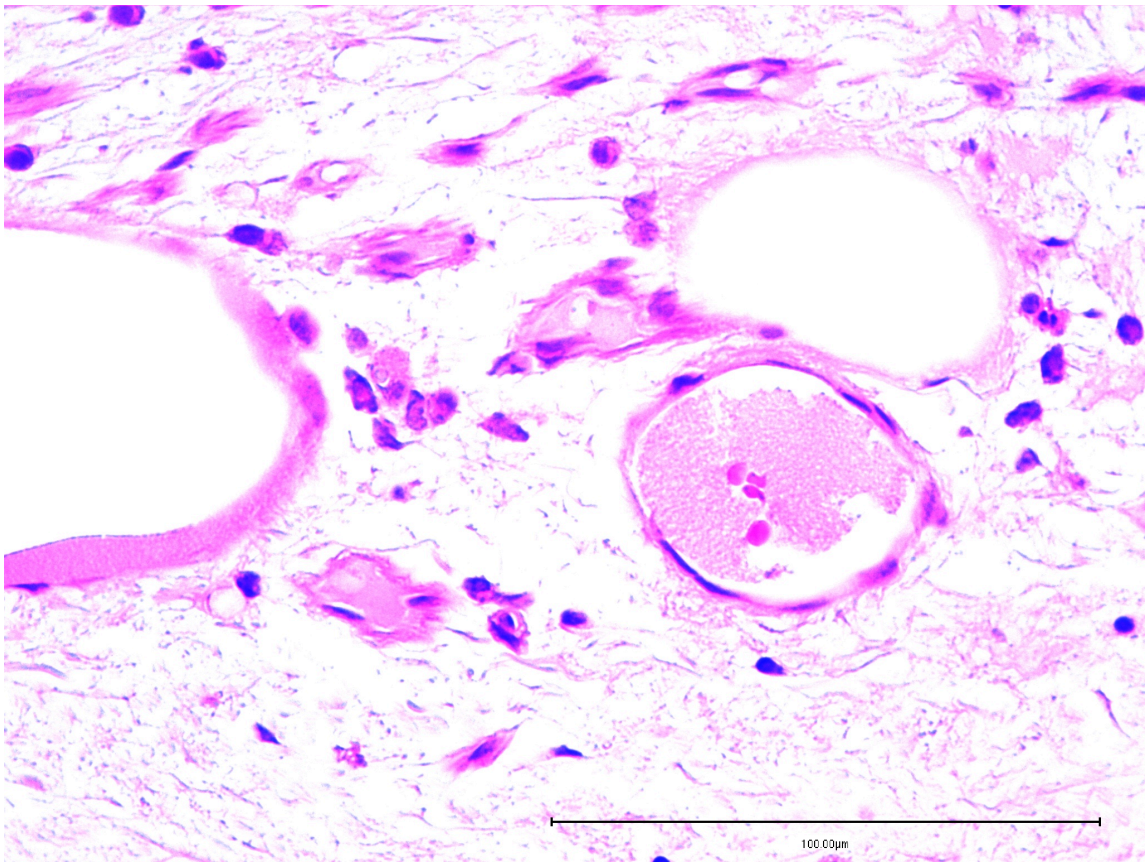

Fig. 5b - Less typical scenario of vascular congestion where blood vessels are filled with pink proteinaceous material (H&E stain).

10 - arteriolar thickening **(Fig. 6)**;

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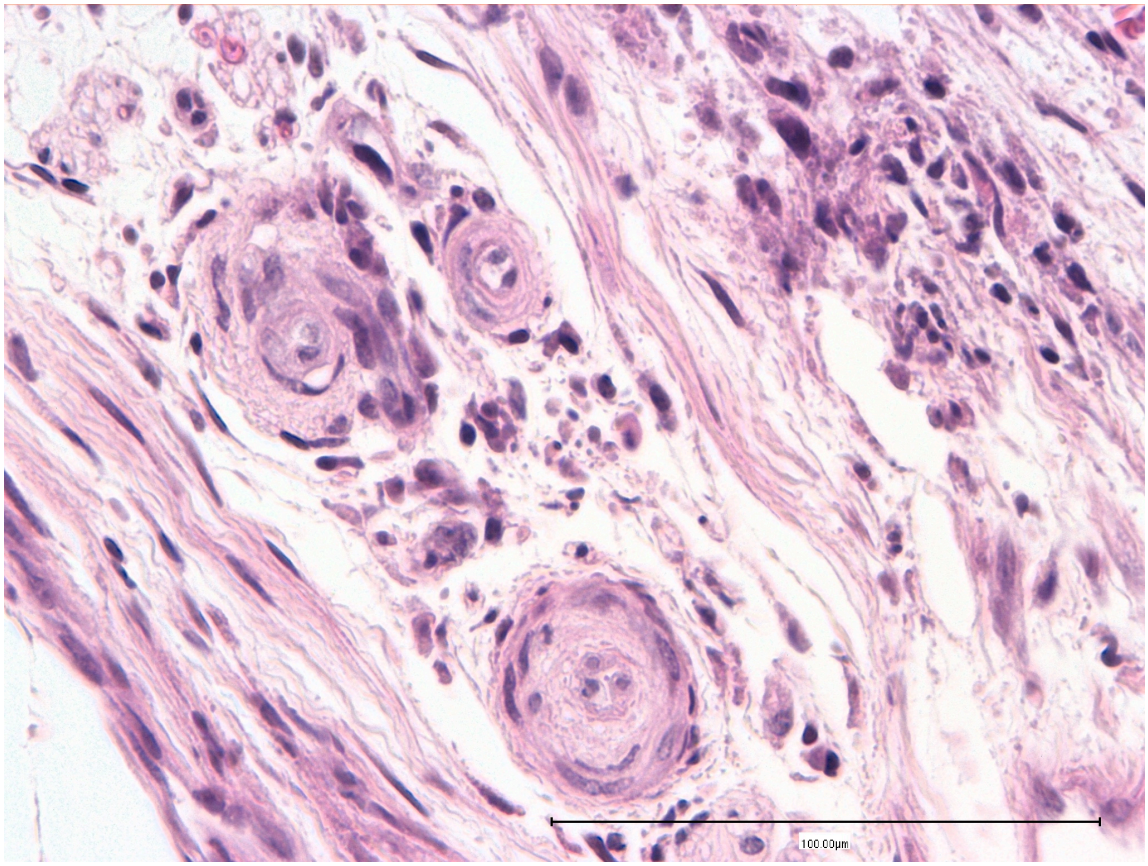

Fig. 6 - Hyaline deposition and marked thickening of the arterial wall with narrowing of the lumen (H&E stain).

11 - intravascular fat globules;

12 - calcific fat necrosis;

13 - fatty bone marrow;

14 - ruptured adipocytes;

15 - granular cytoplasm in adipocytes **(Fig. 7)**;

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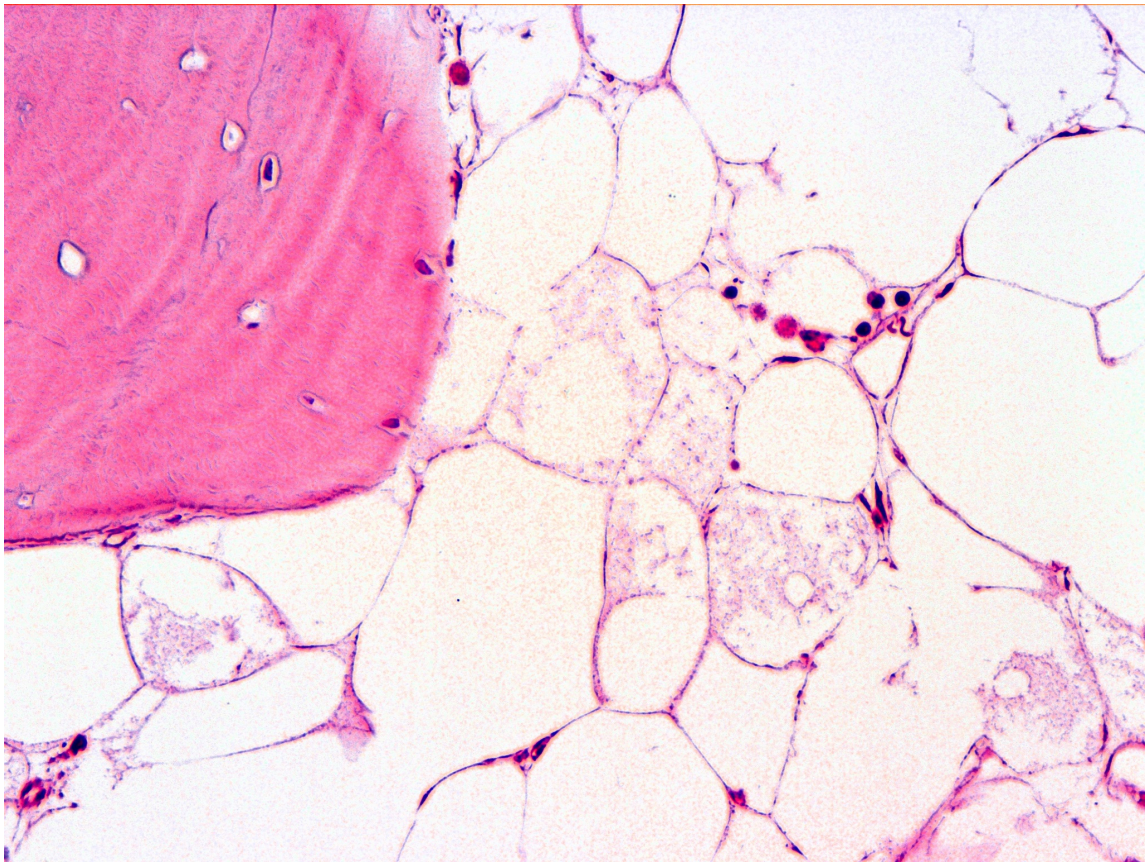

Fig. 7 - Presence of small eosinophilic granules within the cytoplasm of adipocytes (H&E stain).

16 - oil cysts;

17 - perivascular fibrosis **(Fig. 8)**;

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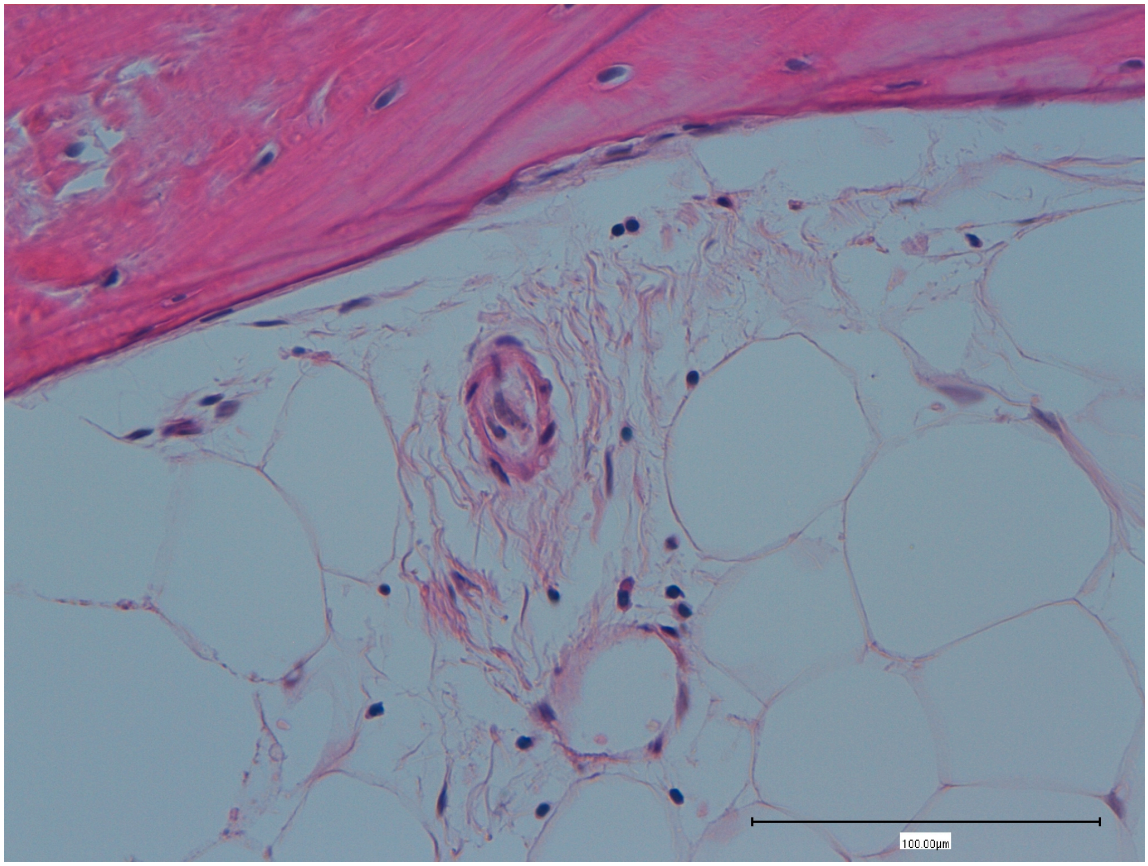

Fig. 8 - Loose perivascular fibrosis surrounding small vessels (H&E stain).

18 - diffuse fibrous metaplasia **(Fig. 9)**.

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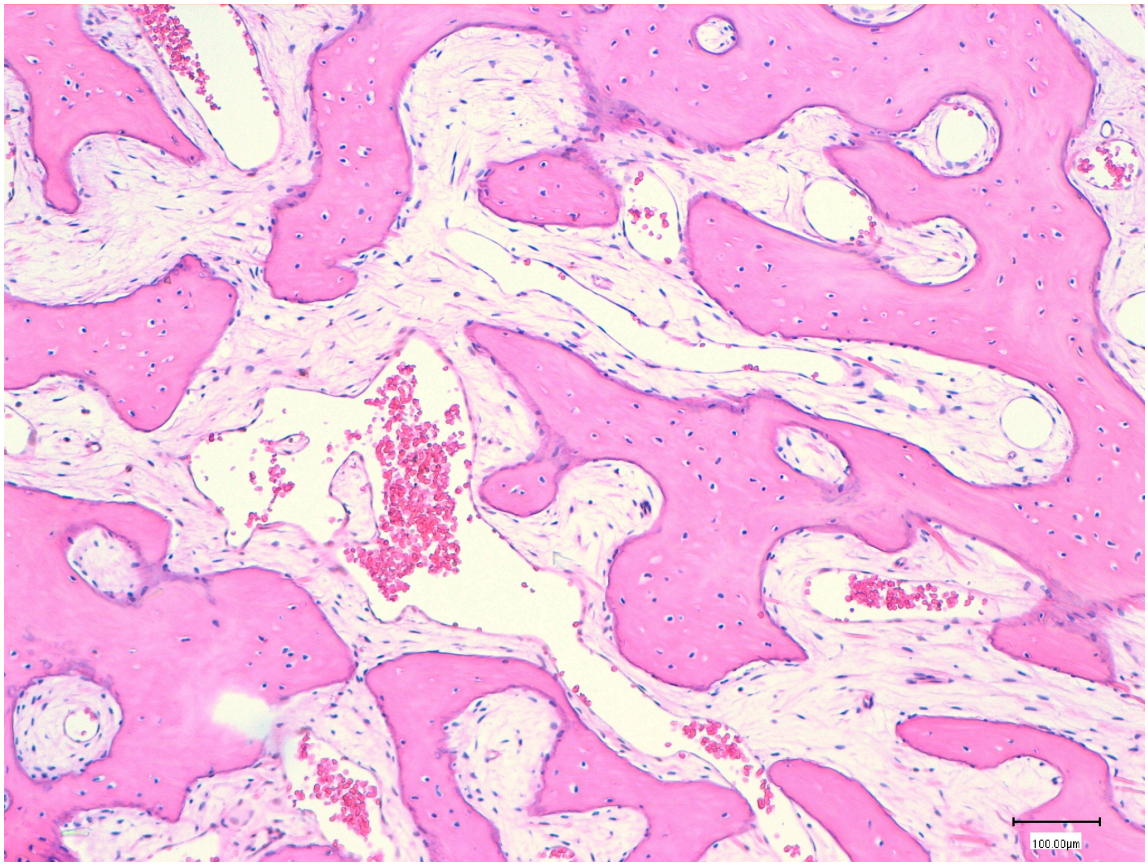

Fig. 9 - Loose fibrous tissue between fat cells remaining from original fatty marrow (myelofibrosis) (H&E stain).

Two further ordinal variables (inflammation and bone maturation) were added to the list.

Inflammation was graded as null (0), mild (1) and moderate (2), based on the amount of inflammatory cells (either neutrophil granulocytes, macrophages, plasma cells and lymphocytes) within the bone marrow. Group 1 and 2 were compared against group 0. Polarized light microscopy was used to evaluate bone maturation by comparing the amount of woven **(Fig. 10)** and lamellar bone **(Fig. 11)**.

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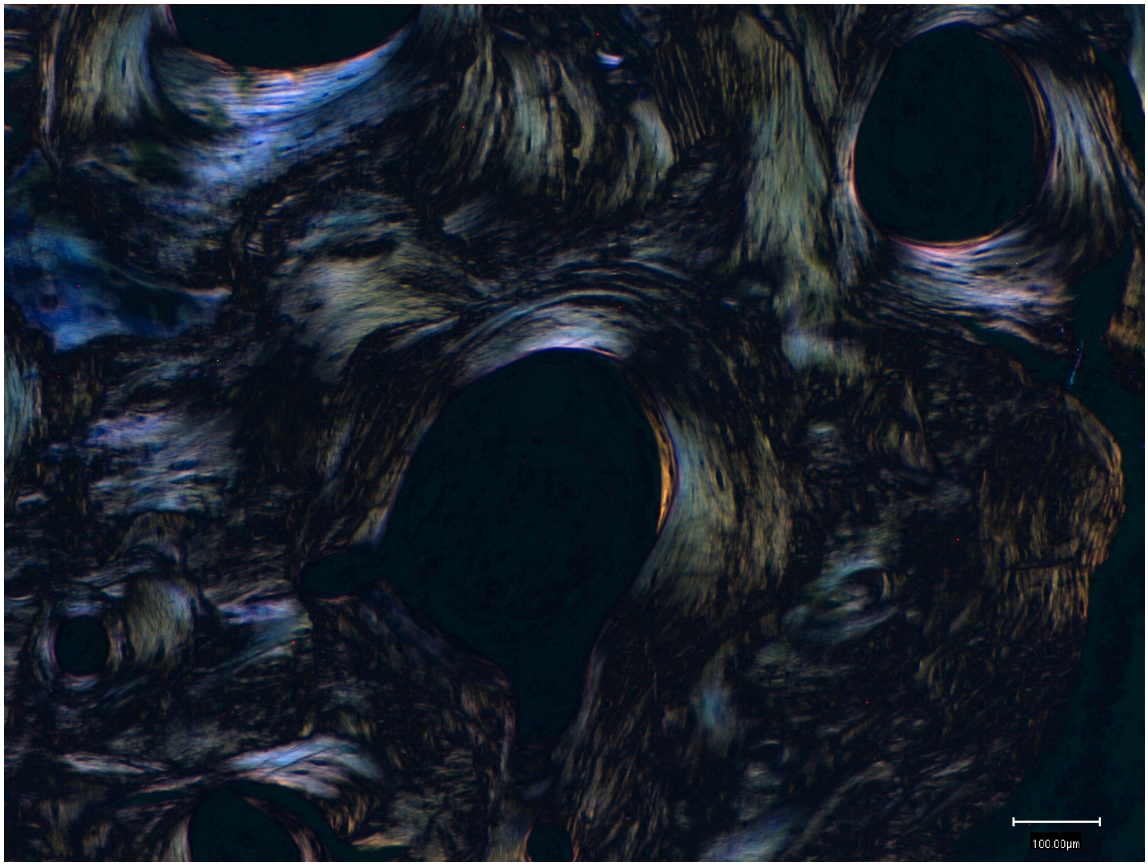

Fig. 10 - Newly formed bone, namely woven bone, with irregular osteoid trabeculae in the form of interlacing network (Polarized light microscopy).

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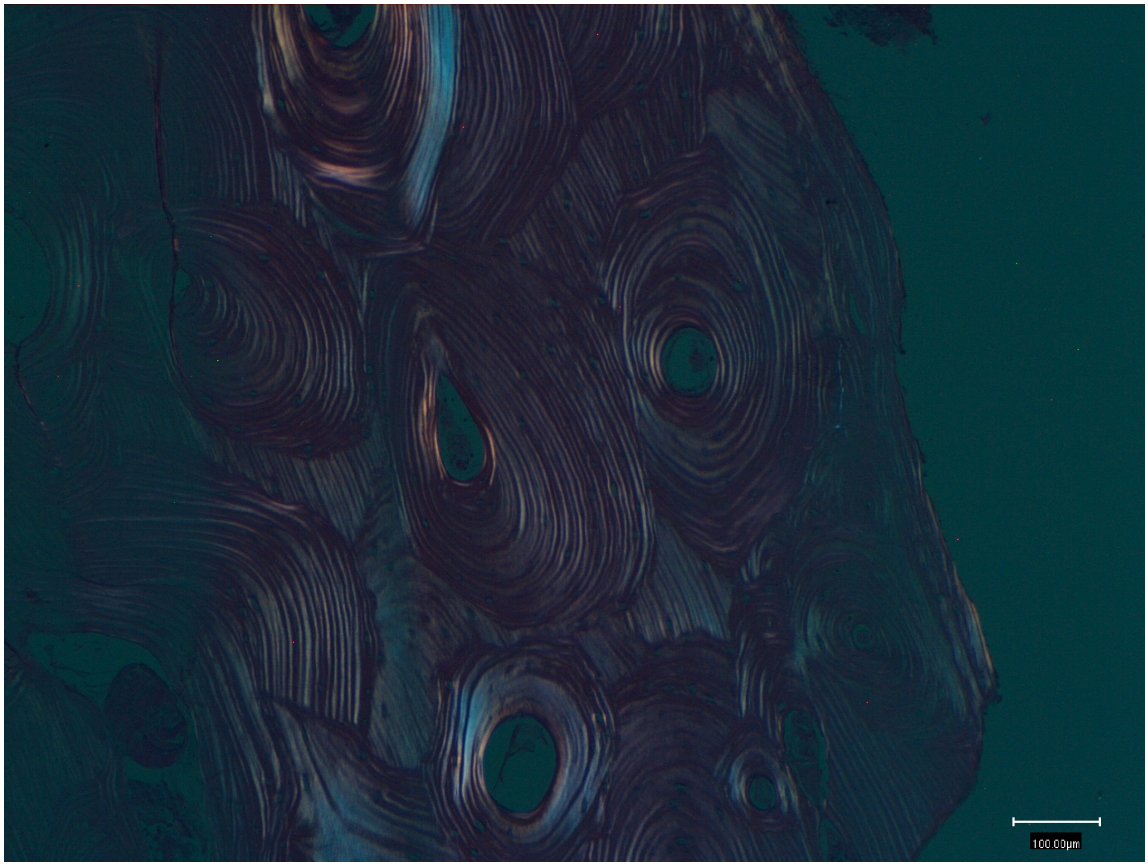

Fig. 11 - Mature bone, namely lamellar bone, formed by the regular distribution and parallel alignment of mineralized bone lamellae (Polarized light microscopy).

Woven bone was graded as it follows: absent (0), where only lamellar bone was present; scanty (1), where the amount of newly formed bone was up to 1/3 of the entire surface of the specimen; equal (2), when the amount of woven bone and lamellar bone were comparable; prevalent (3), when the woven bone largely exceed lamellar bone. The first group was identified as the reference group for the estimation of the p-value.

All samples were photographed and recorded (Leica Application Suite 2.8.0, Leica Microsystems, Switzerland).

Statistical analysis

Continuous variables are given as median and interquartile range (IQR) because of skewed distributions. IQR is reported as the difference between the 25th and 75th percentiles. Categorical variables are given as the number or percentage of subjects with the characteristic of interest.

Between-group comparisons were performed using the Wilcoxon-Mann-Whitney test. Exact logistic regression was used to evaluate the association between the outcome [1 = NBP use (case) vs. 0 = no NBP use (control matched for gender and age)] and the 20 histopathological lesions (see above). Exact odd-ratios and 95% confidence intervals are reported for a univariable model and for a multivariable model taking into account gender (1 = male; 0 = female) and age (continuous and modeled as years/10).[21] [22] Because of the 20 multiple comparisons, the critical p-value used to define an association as statistically significant was set to 0.0025 (0.05/20) using Bonferroni's correction. Statistical analysis was performed using STATA 11 (Stata Corp, College Station, TX, US) and LogXact 9 (Cytel Inc., Cambridge, MA, USA).

Results

During the study period (from March 2006 to August 2010) we prospectively collected 122 alveolar bone specimens of patients potentially suitable as cases (see above). 21 of these specimens were not considered for further analysis because of the following reasons: 1) staining artifacts (n = 7), 2) insufficient amount of trabecular bone (n = 10) and, 3) acute or chronic osteomyelitis (n = 4). During the same period, we prospectively collected 100 bone specimens in patients potentially suitable as controls (see above). 40 of these specimens were not considered for further analysis because of the following reasons: 1) staining artifacts (n = 18), 2) insufficient amount of trabecular bone (n = 21) and, 3) acute osteomyelitis (n = 1). Thus, 101 specimens from potential cases and 60 from potential controls were left for further analysis.

After performing the preplanned 1:1 matching by sex and age, 60 alveolar bone biopsies of cases were matched to 60 alveolar bone biopsies of controls. The biopsies had been performed in 14 males (23%) and 46 females in each group. The median (IQR) age of cases and controls was 66 (16) and 66 (14) years, respectively (p = 0.9435).

Among the 20 histopathological variables investigated as potential risk factors for NBP use, only vascular congestion was associated with the outcome after Bonferroni's correction (multivariable OR = 0.24, exact 95% CI 0.10 to 0.57 for cases vs. controls, p = 0.0006) **(Table 1)**. It should be noted that univariable and multivariable OR were nearly identical for all histopathological lesions, showing no confounding from sex and age.

Table 1 - Frequency of histopathological bone alterations in cases and controls. Abbreviations: OR = odds ratio; CI = confidence interval (see text for details).

*** = correction for sex and age; ** = vs. reference group**

	Cases		**Controls**		Logistic regression	
	n	%	n	%	Univariable OR	Multivariable* OR
(exact 95% CI)					(exact 95% CI)	
exact p to value					exact p to value	
1. Basophile reversal lines	57	95	60	100	0.25 (0.00 to 2.40)	0.25 (0.00 to 2.40)
					p = 0.2437	p = 0.2423
2. Osteoblasts	50	83	58	97	0.17 (0.02 to 0.88)	0.17 (0.02 to 0.87)
					p = 0.0295	p = 0.0288
3. Osteoblastic lines	34	57	33	55	1.07 (0.49 to 2.34)	1.07 (0.49 to 2.33)
					p = 1.0000	p = 1.0000
4. Osteocytes	60	100	60	100	NA	NA
5. Empty osteocytic lacunae	13	22	16	27	0.76 (0.30 to 1.91)	0.76 (0.30 to 1.91)
					p = 0.6702	p = 0.6704
6. Osteoclasts	32	53	18	30	2.64 (1.18 to 6.06)	2.60 (1.17 to 5.91)
					p = 0.0157	p = 0.0166
7. Howship's lacunae	22	37	12	20	2.30 (0.95 to 5.80)	2.28 (0.94 to 5.50)
					p = 0.0674	p = 0.0674
8. Vessel dilatation	18	30	11	18	1.90 (0.75 to 5.00)	1.88 (0.75 to 4.90)
					p = 0.2003	p = 0.2045
9. Vascular congestion	12	20	31	52	0.24 (0.09 to 0.56)	0.24 (0.10 to 0.57)
					p = 0.0005	p = 0.0006
10. Arteriolar thickening	6	10	9	15	0.63 (0.17 to 2.15)	0.63 (0.17 to 2.16)

					p = 0.5822	p = 0.5870
11. Intravascular fat globules	8	13	19	32	0.34 (0.11 to 0.90)	0.34 (0.12 to 0.91)
					p = 0.0276	p = 0.0292
12. Calcific fat necrosis	0	0	0	0	NA	NA
13. Fatty bone marrow	45	75	39	65	1.61 (0.68 to 3.86)	1.60 (0.68 to 3.80)
					p = 0.3193	p = 0.3236
14. Ruptured adipocytes	34	57	33	55	1.07 (0.49 to 2.34)	1.07 (0.49 to 2.33)
					p = 1.0000	p = 1.0000
15. Granular cytoplasm of adipocytes	3	5	2	3	1.52 (0.17 to 18.80)	1.53 (0.17 to 18.80)
					p = 1.0000	p = 0.9927
16. Oil cysts	12	20	14	23	0.82 (0.31 to 2.15)	0.82 (0.31 to 2.14)
					p = 0.8250	p = 0.8257
17. Perivascular fibrosis	45	75	56	93	0.22 (0.05 to 0.74)	0.22 (0.05 to 0.75)
					p = 0.0109	p = 0.0113
18. Diffuse fibrous metaplasia	6	10	4	7	1.55 (0.35 to 7.89)	1.54 (0.35 to 7.76)
					p = 0.7430	p = 0.7451
19. Inflammation						
Absent	41	68	46	77	Reference	Reference
Mild**	18	30	12	20	1.68 (0.67 to 4.31)	1.66 (0.67 to 4.24)
					p = 0.3152	p = 0.3222
Moderate**	1	2	2	3	0.56 (0.01 to 11.22)	0.56 (0.01 to 11.27)
					p = 1.000	p = 1.000
20. Woven bone						
Absent	8	13	2	3	Reference	Reference

Scanty	15	25	20	33	0.19 (0.02-1.17)	0.19 (0.02-1.17)
					p = 0.0830	p = 0.0818
Equal**	16	27	18	30	0.23 (0.02-1.139)	0.23 (0.02-1.138)
					p = 0.1353	p = 0.1337
Prevalent**	21	35	20	33	0.27 (0.02-1.58)	0.27 (0.02-1.57)
					p = 0.1928	p = 0.1900

In addition, the level of inflammation was equally distributed between NBP users and non-users. In particular, the majority of the specimens from both groups did not show any inflammatory infiltrate.
The degree of bone maturation did not disclose significantly between the experimental and the control groups. Absence of woven bone was found in few specimens only, while specimens with scanty, equal or prevalent newly formed bone were equally represented in each group and between groups.

Discussion

This is the first study to test whether there are specific histopathological alterations in the alveolar jawbone of patients treated with NBP and at high risk of BRONJ. We evaluated 18 histopathological alterations of the lamellar and trabecular bone on the basis of a previous study and we considered also inflammation and bone maturation. Similar histological alterations were also described by other researchers in patients with non-ulcerated variants of chronic ischemic jawbone disease such as neuralgia-inducing cavitational osteonecrosis.[23] In a recent letter, it was suggested that bisphosphonate-associated jawbone disease is another in the long list of osteonecrotic disorders, but no evidence of underlying bone marrow disease has been presented so far, which is an essential feature of ischemic osteonecrosis.[20]

The histologic bone changes presented here can be related for the first time to initial stages of ischemic bone disease also in patients taking NBP. Nevertheless, our data show that these ischemic changes are not induced by NBP accumulation within the jaws.
Bone vascularization is compromised since the early stages of ischemic osteonecrosis, with typical signs of vessel dilatation (capillaries, veins and sinusoids), congestion, thickening of arterioles, and

presence of intravascular fat globules. In the present study, only vascular congestion differed between cases and controls, being significantly less frequent in the former (multivariable OR = 0.24, exact 95% CI 0.10 to 0.57). Several researchers have suggested that the initiating event of BRONJ may be an alteration of blood supply secondary to NBP use.[24] The vascular lesions induced by NBP in experimental and animal studies have however not been confirmed in humans. Our present findings suggest that NBP at standard doses may not have the anti-angiogenic properties observed in vitro.

Another potential mechanism of BRONJ is the suppression of bone turnover induced by a high local level of NBP.[4][11] High doses of NBP have been associated with suppressed bone turnover in experimental and animal studies.[25] [26] NBP are known to interfere with bone remodeling mostly by induction of osteoclast apoptosis and inhibition of osteoclast maturation.[27]

NBP have cytotoxic effects on osteoblasts,[28] even if the reduction of osteoblast activity is explained mainly by diminished bone remodeling.[29] An apoptotic effect of NBP has been hypothesized also for osteocytes but it remains controversial.[30][31][32]

A detailed quantitative assessment of bone cell population was beyond the scope of the present study. It is to be noted, however, that bone cellularity and activity did not differ between cases and controls. NBP accumulate in the jawbones, but their release and activation is dependent on the rate of bone remodeling.[33] On the basis of the finding of no association between the study variables and NBP use in the present study, we hypothesize that NBP accumulation cannot suppress bone remodeling without the local increase in bone turnover. This hypothesis is corroborated by the finding that the jaws retain their ability to produce new bone in the presence of NBP. Pending confirmation by further studies, our findings tend to reject the hypothesis that NBP alone reduce bone turnover to the extent that they cause adynamic bone.

NBP have proinflammatory effects in experimental and animal studies[34][35] and an association between bone marrow inflammation and BRONJ has been recently reported in a small clinical series. [36] In the present study, however, an inflammatory infiltrate was equally absent or mild in cases (98%) and controls (97%) and there was no association between NBP use and the degree of inflammation. Thus, our findings do not support the hypothesis that NBP use is linked with early bone marrow inflammation.

Even if this is the first study to systematically test the hypothesis that there are specific histopathological alterations in the alveolar bone of patients treated with NBP and at high risk of

BRONJ, it is not without limitations. First, even if the case-control design is sometimes excellent at detecting cause-effect relationships, it is by no means the best way to search for causal associations. However, it is a great design for generating hypotheses that can be further tested by cohort studies. Second, we studied a high number (20) of histopathological bone alterations. To take into account the possibility of spurious associations between the outcome and such lesions, we employed a corrected p-value of 0.0025. It appears, however that the frequency of some lesions is very low (e.g. granular cytoplasm of adipocytes, 5% in cases vs. 3% in controls) or high (e.g. basophile reversal lines, 95% in cases vs. 100% in controls) and that much higher sample sizes than those employed in this study are needed to test these differences with an acceptable power. It is however debatable if characteristics which are so rare or common in both groups will ever be useful for prognostic applications. Third, 40% of bone specimens of potential controls were of low-quality (vs. 17% of potential cases). This was not completely unexpected as most of the potential controls underwent flapless tooth extraction so that the amount of bone accessible for biopsy was limited. The amount of trabecular bone was the most critical factor for the evaluation of bone specimens in both cases and controls. Biopsies taken from the buccal and lingual cortices of the alveolar sockets had to be often discarded because of the predominance of cortical bone. On the contrary, bone biopsies taken from the interdental septum provided adequate quantities of cortical and trabecular bone and have to be regarded as ideal specimens for future studies.

In conclusion, use of NBP is not associated with specific bone lesions in the alveolar jawbone of patients treated with NBP but without BRONJ. Further histopathological studies are needed to confirm and expand our findings.

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