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Bone lineage proteins in the entheses of the mid-foot in patients with spondyloarthritis.

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I. ABSTRACT

Introduction: Patients with juvenile-onset spondyloarthritis may develop ankylosis of the midfoot resembling the spinal changes seen in patients with ankylosing spondylitis. The study of the histopathology of the feet of patients with tarsitis could help us understand the pathogenesis of bone formation in affected structures in the spondyloarthritis. The objective of this study was to describe the histopathologic characteristics of the midfoot in patients with tarsitis associated with spondyloarthritis.

Methods: We obtained synovial sheaths, entheses, and bone samples from 20 spondyloarthritis patients with mid-foot pain/tenderness and swelling. Tissue samples underwent hematoxylin and eosin staining, immunohistochemistry for CD3, CD4, CD8, CD68, and CD20 cell identification, and immunofluorescence for bone lineage proteins, including osteocalcin, osteopontin, parathyroid hormone-related protein, bone sialoprotein, and alkaline phosphatase.

Results: Slight edema and hyalinization were found in some tendon sheaths,few inflammatory cells were detected in the entheses. In contrast, there were suggestive osteoproliferative changes, including both endochondral and intramembranous ossification, in the absence of leukocyte infiltration or any

significant increase of inflammatory cells. In entheses showing bone proliferation, we detected osteocalcin and osteopontin in cells with a fibroblast-mesenchymal phenotype, suggesting the induction of entheseal cells towards an osteoblast phenotype.

Conclusions: Osteoproliferation and abnormal expression of bone lineage proteins, but no inflammatory infiltration, characterize mid-foot involvement in patients with SpA. In this sense, tarsitis (or ankylosing tarsitis) resembles the involvement of the spine in patients with ankylosing spondylitis. Ossification may be in part explained differentiation of mesenchymal entheseal cell towards the osteoblastic lineage.

Key Indexing Terms: Ankylosing Spondylitis, Enthesitis-Related Arthritis, Juvenile, Osteocalcin, Osteopontin.

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Running footline: Bone lineage proteins in SpA enthesis.

II. INTRODUCTION

Spondyloarthritis (SpA) refers to a group of HLA-B27-positive associated rheumatic diseases that share clinical and genetic features. Signs, symptoms, and radiographic features define the diseases and conditions that constitute the group. Inflammation of the *synovium* and enthesis as well as ossification of the latter are the pathologic processes that stand behind the clinical picture of SpA, mainly ankylosing spondylitis (AS) (1,2).

In Latin American patients with SpA, the prevalence of peripheral arthritis and enthesitis is higher and occurs earlier than in Caucasians (3,4). A unique form of severe involvement of the feet, named ankylosing tarsitis (AT) has been described in Mexicans youngsters suffering SpA. AT consists of tarsal swelling, synovial inflammation, bone overgrowth, endochondral ossification, enthesophytosis, bone bridging, and ankylosis of the tarsal bones. The series of changes that occur in this form of disease resemble those found in radiographic and magnetic resonance imaging (MRI) studies of the spine in patients with AS.

The scarcity of histopathologic samples, particularly of the spine and sacroiliac joints, has hampered our understanding of the pathogenesis of SpA, especially in patients with early disease. We assume that the study of the mid foot bones of patients with SpA undergoing ossification could be a complementary approach.

Both inflammation and osteoproliferation are fundamental processes to be understood.

Histopathologic studies of the spine and sacroiliac joints of patients with AS have focused in inflammation primarily; these studies have assessed the immune cell populations and also their secreted cytokines. The predominant cell found in the infiltrates are T cells (mainly the CD4, CD8, CD45RO, and CD45RA subtypes), macrophages are also present at lower counts (5–7); these cells conform inflammatory infiltrates that locate mostly in the bone marrow. Pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) were detected in areas of inflammatory infiltration whereas the transforming growth factor beta (TGF-ß) is expressed in areas of new bone formation. Local bone marrow macrophages and dendritic cells produced interleukin (IL)-23 (8) and T helper cells 17 (Th17) produced IL-17 (9).

Ossification, on the other hand, has not been studied extensively in patients with SpA despite its relevance in patient´s prognosis and its potential as a therapeutic target. Ossification of entheseal and spinal structures in SpA involves both the endochondral or the intramembranous pathways (10,11). This ossification may result either from the proliferative response of the periosteal edges intruding on the neighboring tissues, or from the differentiation of mesenchymal cell precursors in the neighboring tissues after an osteogenic stimulus. The intimate mechanisms behind this ossification are likely diverse in order to induce ossification in a diversity of cell lineages which reside on different anatomical structures (i.e. non-entheseal zygapophyzeal cartilage vs entheseal interdiscal annulus fibrosus). In order for a tissue to become ossified, the cell precursors require osteogenic stimuli, then, the primed cells undergo stages of sequential differentiation during which,

and they express an array of stage-specific protein markers. Among those markers, some define the early stages of differentiation like bone sialoprotein (BSP) whilst the expression of others such as osteopontin (OPN) or osteocalcin (OCN) define latter stages including mature osteocytes (12,13).

The expression of these markers for bone lineage commitment has not been studied in tissues undergoing ossification the SpA. Understanding the process and the mediators for the abnormal ossification in SpA tissues could provide an additional rationale to design specific therapy against osteoproliferation.

We analyzed the histological picture of the osteoproliferative response in the mid-foot bones from patients with AT, with particular interest in the expression of bone lineage commitment proteins within the ossifying tissues.

III. METHODS

This is a cross-sectional study of adult patients with undifferentiated SpA (14) and AS (15) with active inflammation of the tarsal region (defined as the mid-foot, from the ankle to the metatarsophalangeal joints) as defined by the presence of swelling or limitation of motion accompanied by heat, pain, or tenderness for >2 weeks. Patients with a history of congenital malformations or acquired disease, trauma or surgical or immobilization procedures at any time were not included in the study. Patients who had received local foot and/or intramuscular injections and/or oral glucocorticoids in the last three months were not included in the study. The Hospital General de Mexico Review Board for human research approved the study protocol. The study was conducted according to the Declaration of Helsinki and

local. All patients signed an informed consent after being informed about the nature of the study, in particular the procedures and risks of the foot biopsy.

Clinical evaluation. Demographic and clinical data were obtained in all patients. The patients included in the study underwent the following assessments: the number of painful and swollen joints, enthesis and tarsal area counts for tenderness and swelling, Schober's test, occiput-to-wall distance, and chest expansion. In addition, patients completed the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (16), Functional Index (BASFi) (17), and global (BAS-G) (18) questionnaires. We also measured C reactive protein (CRP) serum levels or the erythrocyte sedimentation rate (ESR). Radiographic studies included those of the feet as well as the sacroiliac joints; their scoring relied on the SpA-Tarsal Radiographic Index (SpA-TRI) (19) and the modified New York AS criteria for the sacroiliac joints.

Site selection and biopsy procedures. Biopsy sites were selected by two of the investigators (CP-T and AU-V). We obtained tissue samples from tendon attachments at the dorsal aspect of the tarsus, specifically the talonavicular ligament as well as the flexor tendons synovial sheaths of the toes, including those from extensor digitorum longus and brevis, extensor hallucis longus, the inferior extensor retinaculum, and the peroneus longus and brevis. Criteria for site selection included easy access to structures displaying at least one of the following characteristics: 1) spontaneous pain, 2) pain and/or tenderness elicited by digital palpation, and 3) soft tissue swelling.

Tissue samples were obtained under local, subcutaneous anesthesia (lidocaine 1% and epinephrine 0.005%) in a circular pattern around the biopsy site. Tissue samples were obtained either by open skin dissection with a scalpel or percutaneous soft tissue and bone biopsy using a Craig's needle instrument set and under fluoroscopic guidance. We obtained fragments of tendon and its synovial sheath, enthesis -ligament or tendon attachments, bone, and whenever possible, bone marrow samples. Our initial strategy targeted the tendon sheaths insertion along the tarsal bones, but based on the preliminary histologic review, after the third patient we decided to include an additional bone biopsy, therefore our analysis include 20 tendon sheaths and 18 bone biopsies.

Tissues were fixed in 4% paraformaldehyde. Bone samples were dipped and decalcified in nitric acid until suitable for sectioning. Once decalcified, all tissue samples were washed in ethanol, dehydrated in xylol, and embedded in paraffin. After sectioning, the samples were mounted on several slides for hematoxylin and eosin (H&E) staining. A pathologist (RP-T), blinded to demographic and clinical characteristics, evaluated all samples and determined the presence or absence of inflammatory infiltrates, edema, fibrosis, hyalinization, vascularization, synovitis, and bony proliferation. The pathological features of the cartilage, periosteum, and bone marrow were also described.

Immunohistochemistry of the inflammatory infiltrates was performed with specific monoclonal antibodies (DAKO) against CD3, CD4, CD8, CD20, and CD68 markers and the following protocol: after standard microwave antigen retrieval (citrate buffer at pH 6.0 for 6 min), the slides were blocked with 10% goat serum, incubated

overnight at 4ºC with the primary antibody (1:200 dilution in PBS), washed in PBS and then incubated with biotinylated secondary antibody (specie-complimentary to the primary; 1:1000 dilution in PBS) for 1 hour at room temperature. Sections were then incubated with ABC/horseradish peroxidase complex for 30 min, washed with PBS and then treated with 3,3' diamonibenzidine (Sigma D-5637) and 0.17%hydrogen peroxide in Tris buffer; counterstaining was done with hematoxylin. Immunohistochemistry for bone markers osteopontin (OPN), osteocalcin (OCN) and Parathyroid hormone-related protein (PTHrP) was also performed.

Immunofluorescence was performed with monoclonal antibodies (Santa Cruz Biotechnology) against the following markers of the osteoblast lineage cells (12,20): OPN, OCN, PTH-rP and bone sialoprotein (BSP). Following blocking with 10% goat serum in PBS, sections were incubated with primary antibody (1:200 dilution) in a humid chamber at 4°C overnight, washed in PBS and then incubated at room temperature in the dark for 1 hour with the isotype specific secondary antibody in a 1:1000 dilution: Fluorescein isothiocyanate (FITC) to OPN and OCN, Texas red (TR) to PTH-rP and Cyanine 5-labeled (Cy5) to PTHrP. Fibroblast Marker (ER-TR7) (Santa Cruz Biotechnology) was used to perform co-localizations with OPN and OCN, moreover a co-localization of OCN and OPN was carried out. Labeling was evaluated by epifluorescence microscopy (Zeiss Axioscope) and/or laser scanning confocal microsopy (LSM 700 Axio Observer, Carl Zeiss) and then analyzed with the Axiovision and/or Zen10 program. The same observer carried out immunofluorescence evaluation and interpretation and the slides were photographed under conditions optimized by the software for the exposure times.

IV. RESULTS

We included 20 patients -mostly males- with juvenile (n=11) or adult (n=9) onset AS (15) (n=13) or undifferentiated SpA (n=7). Their mean age (± standard deviation (SD) at the time of the study was 27.15 ± 8.9 years. Most patients complained of axial symptoms in combination with tarsal swelling and pain. At least 50% of the group had severe proliferative changes in the tarsus according to SpA-TRI. The mean swollen joints counts and enthesitis sites were 3.2 ± 2.75 and 8.6 ± 5.4 respectively. Twelve patients were in functional class III or IV. No patient had been previously or currently treated with biologic agents. The patients were divided based on the presence or absence of bone proliferative changes, in the group with proliferation certain tendencies were observed: they were younger at the time of the biopsy and also at disease onset, had higher swollen and tender joint and enthesitis counts, and, higher BASDAI scores, the latter was the only parameter achieving statistical significance (Table 1).

Histopathology. Most tissue samples corresponded to the peroneal and tibial tendons' sheaths and, in some cases, the dorsal aspect of the navicular bone or the naviculocunean joint. In these sheaths, slight inflammation accompanied by some interfibrillar edema and disorganization (Figure 1A) and loosening of collagen fibers (Figure 1B) was evident (Table 2). Some cases showed dilated vascular loops with some signs of vascular proliferation and redundancy (Figure 1C). In cases showing evidence of chronicity, there was more often sparse cellularity, a relative absence of fibroblasts and the development of compacted tendon sheaths, this aspect was different from that found in the extracellular matrix, which showed

collagen fiber hyalinization (Figure 1D). In one-third of the cases, we obtained synovial tissue, which showed mild inflammatory process consisting of a slight leukocyte infiltrate (Figure 1E) with no synovial cell proliferation.

Some samples showed marked vascularity (Figure 1F) and intrusion of osteoid material within the cartilage matrix (Figure 1G), evolving in a pattern that looked similar to that of the trabecular bone, suggesting endochondral ossification.

Entheses of the tibiofibular muscles in the dorsal aspects of the navicular, cuboid, and cuneiform bones showed some irregularities at the interface of the fibrous attachment to the periosteum (Figure 1H) and some indications of cortical bone proliferation and entheseal ossification (Figure 1I), sometimes away from the cortical bone (Figure 1J, 1K, 1L).

In several sections, we observed recent osteoid deposits extending directly into the enthesis. The appearance of the cortical bone aspect suggests bone apposition. Interestingly, we found no significant leukocyte infiltration in the bone marrow.

Detection of cell subpopulations by immunohistochemistry. Scarce mononuclear cell inflammatory infiltrates, mainly perivascular were found in tendon sheaths and within the entheses. In all cases the number of cells was very low to be counted (Figure 2); positive detection of isolated CD3+ cells was possible in 16% of the slides; CD4 was detected in 36%; CD8 in 20%; CD20 in 40%; and CD68 in 29% (Figure 2). This pattern of distribution replicates the findings of Benjamin in which inflammatory cells were found infiltrating the tendon or entheses but rather were associated with perivascular structures (10).

Immunofluorescence Studies. OCN, OPN, BSP and PTH-rP were found in the entheseal and osteal tissues of nine patients showing bone proliferation (Figure 3). OCN is a marker of mature post-proliferative cells; the labeling was strong and evident in bone matrix, osteocytes, and osteoblasts, but particularly intense in the entheses (77%) and periosteal tissues. OPN, which is a marker for osteoblast differentiation at early and later stages, was evident in bone matrix, in osteoblasts, and expressed in the entheses (55%). BSP staining was prominent in the bone matrix, osteoblasts, and osteocytes, but was inconsistently found in the entheses (22%). PTH-rP expression was absent from bone matrix, and seen primarily in osteoblasts, occasionally in the entheses (11%) although with a milder intensity than OCN.

We detected co-localization of OPN and OCN in several biopsies and there is co-expression in entheseal cells, although both proteins are expressed in different regions in the bone, being OCN more abundant in the periosteal and entheseal region (Figure 4C). Finally, both OCN and OPN co-localized with a fibroblast marker in the enthesis (Figure 4D and E respectively). The production of both bone lineage proteins by fibroblasts within the enthesis evidences their differentiation toward osteoid precursors.

V. DISCUSSION

Our study analyzed the histopathological findings of the entheses and joints of the mid-foot of patients with active SpA and characterized immune cell subpopulations osteoblastic lineage cells in the tissues. The rationale for selecting the feet for this study is based on the similarities found in the radiographic and MRI studies of

patients with axial SpA, particularly AS and patients with peripheral arthritis and enthesitis, specifically ankylosing tarsitis. The involvement of the foot in patients with SpA, particularly of those with juvenile onset SpA, may evolve from active inflammatory stages to partial or complete joint space narrowing and bony bridging between the tarsal bones. These changes resemble those found in the sacroiliac joints of patients with AS, including subchondral bone fusion and enthesophyte formation as consequence of intramembranous ossification of the entheses.

Interestingly, we found only scarce mononuclear-cell inflammatory infiltration, and mainly CD20+ and CD4+ cell sub-types, in the synovium of tendon sheaths and in the vascular structures adjacent to the entheses. CD4 rather than CD8 T-cell subtypes predominated in a previous study of the sacroiliac joints, which also found TNF-α and TGF-β in inflammatory and bone areas of the sacroiliac joints, respectively (21). Francois et al (7) and Appel et al (5) found CD3+ T-cells, and less frequently CD8+ cells in bone marrow inflammatory infiltrates of patients with both early and longstanding AS.

Higher cell counts in the bone marrow infiltrates have been correlated with MRI findings (6); patients with a higher activity score in MRI also exhibit higher inflammatory cell counts in sacroiliac joint biopsies.

We did not find any significant accumulation of inflammatory cells in the bone marrow of the tarsal bones. This finding could be explained by the fact that the bones where bone edema and inflammation is detectable by MRI are the large ones (calcaneus and astragalus) and the signal is weaker in cuneiforms and

navicular, nevertheless, the latter do indeed exhibit bone bridging and ankylosis. While most animal models of SpA develop important mononuclear cell infiltration including the tarsal region area before ossification of the enthesis becomes clear (22), enthesophytosis and bone ankylosis develop in the absence of bone marrow inflammation in the ankylosing enthesopathy (ANKENT) murine model of SpA.

One of the most interesting findings in our study was the identification of suggestive intramembranous ossification in the enthesis and also for subchondral osteoproliferation associated with an expression of osteoblast-associated proteins in the entheses of patients with bone proliferative changes, in the absence of extensive inflammation. Both OPN and OCN were expressed in cells with fibroblastic (mesenchymal) phenotype in the fibrous entheses whereas a more restricted-to-the-bone distribution was observed for both BSP and PTH-rP. It is worth noting that OPN is expressed in a variety of cell types and participates in remodeling of normal tissues and tumorigenesis (23). It is, however, also a marker of osteoblasts in normal and pathological calcification processes including atherosclerosis (24–27). OCN is a non-collagenous vitamin K-dependent protein expressed and secreted in the late stage of osteoblast differentiation (13). While some evidence supports a role for OCN in bone mineral maturation, its precise role in carboxylated or non-carboxylated forms in bone and other tissues remains the subject of considerable effort (28). Although OCN is not constitutively expressed on normal tendons, it is upregulated in tendons undergoing abnormal ossification. Interestingly (25,29–31), the identification of OCN in the fibrous entheses of the tarsal joints in our study suggests a role for this protein in ankylosing tarsitis. We

were unable to obtain samples from normal patients nor cadavers, and we understand that this facts limits our interpretation importantly. It is not clear at this point that the expression of bone lineage proteins in the entheses of the feet is not constitutive nor that the entheseal interface in the tarsus does not present an irregular shape. It must be mentioned however, that only half of the patients with AT had proliferation suggestive lesions, and also half had inflammatory infiltrates.

The link between inflammation and bone formation is complex. Inflammation and tissue damage constitutes the initial insult whereas healing and repairing the subsequent process perpetuating, by independent mechanisms such as mechanical factors, the reengineering of the bone. MRI studies have shown some evidence that inflammation (defined as bone edema) at the vertebral corner precedes syndesmophyte formation in anti-TNF naïve and anti-TNF treated patients despite the fact that the effect of anti-TNF agents retards the progression of the disease (32–34). In addition, it seems that the prognosis of healed lesions differs from those showing fat infiltration, but until now, the risk for syndesmophyte formation is approximately the same. Radiographic progression has been associated with high CRP levels, BASDAI, and bone inflammation on MRI (35).

The study of animal models documents the relationship between inflammation and and bone proliferation markers. Collagen I and III, BSP and OCN up regulation has been described in the proteoglycan-induced spondylitis murine model (36).. Additionally, Lories et al, have demonstrated a potential role for the bone morphogenetic proteins in the entheseal ossification and also a theoretical connection with mechano-sensing proteins of the family Wingless e Int (WNT) (37).

Aberrant WNT signaling is also been observed in the spontaneous ankylosis seen in *ank/ank* mice, which like the tissues in our present study, reveal little inflammation at site of ankylosis (38).

The decalcifying process interfered with mRNA and cytokine transcripts identification. However, the scarcity of inflammation in our samples did not warrant any significant cytokine identification. In contrast, we could identify bone lineage proteins in our tissue samples. We found no correlation between clinical findings on examination and histopathologic findings. Bone, tendon, and entheses tenderness did not correlate with histologic findings in many cases.

We report an osteoproliferative process in patients with severe involvement of the tarsal joints and entheses in patients with ankylosing tarsitis associated with SpA. Our findings suggest the differentiation of entheseal cells towards an osteoblastic lineage and the formation of bone islets embedded within the enthesis matrix. The scarcity of inflammation infiltration suggests that osteoproliferation in the tarsal region might result from a predominantly mechanical rather than an inflammatory phenomenon. Since most patients developing ankylosing tarsitis have juvenile onset disease, it is possible that bone growth factors might also play a role in this form of SpA.

VI. REFERENCES

1. Baraliakos X, Braun J. Spondyloarthritides. Best Pract Res Clin Rheumatol. 2011 Dec;25(6):825–42.

2. Ehrenfeld M. Spondyloarthropathies. Best Pract Res Clin Rheumatol. 2012 Feb;26(1):135–45.

3. Benegas M, Muñoz-Gomariz E, Font P, Burgos-Vargas R, Chaves J, Palleiro D, et al. Comparison of the clinical expression of patients with ankylosing spondylitis from Europe and Latin America. J Rheumatol. 2012 Dec;39(12):2315–20.

4. Vander Cruyssen B, Muñoz-Gomariz E, Font P, Mulero J, de Vlam K, Boonen A, et al. Hip involvement in ankylosing spondylitis: epidemiology and risk factors associated with hip replacement surgery. Rheumatol Oxf Engl. 2010 Jan;49(1):73–81.

5. Appel H, Loddenkemper C, Grozdanovic Z, Ebhardt H, Dreimann M, Hempfing A, et al. Correlation of histopathological findings and magnetic resonance imaging in the spine of patients with ankylosing spondylitis. Arthritis Res Ther. 2006;8(5):R143.

6. Bollow M, Fischer T, Reisshauer H, Backhaus M, Sieper J, Hamm B, et al. Quantitative analyses of sacroiliac biopsies in spondyloarthropathies: T cells and macrophages predominate in early and active sacroiliitis- cellularity correlates with the degree of enhancement detected by magnetic resonance imaging. Ann Rheum Dis. 2000 Feb;59(2):135–40.

7. François RJ, Neure L, Sieper J, Braun J. Immunohistological examination of open sacroiliac biopsies of patients with ankylosing spondylitis: detection of tumour necrosis factor alpha in two patients with early disease and transforming growth factor beta in three more advanced cases. Ann Rheum Dis. 2006 Jun;65(6):713–20.

8. Appel H, Maier R, Bleil J, Hempfing A, Loddenkemper C, Schlichting U, et al. In situ analysis of interleukin-23- and interleukin-12-positive cells in the spine of patients with ankylosing spondylitis. Arthritis Rheum. 2013 Jun;65(6):1522–9.

9. Appel H, Maier R, Wu P, Scheer R, Hempfing A, Kayser R, et al. Analysis of IL-17(+) cells in facet joints of patients with spondyloarthritis suggests that the innate immune pathway might be of greater relevance than the Th17-mediated adaptive immune response. Arthritis Res Ther. 2011;13(3):R95.

10. Benjamin M, Toumi H, Suzuki D, Hayashi K, McGonagle D. Evidence for a distinctive pattern of bone formation in enthesophytes. Ann Rheum Dis. 2009 Jun;68(6):1003–10.

11. Appel H, Maier R, Loddenkemper C, Kayser R, Meier O, Hempfing A, et al. Immunohistochemical analysis of osteoblasts in zygapophyseal joints of patients with ankylosing spondylitis reveal repair mechanisms similar to osteoarthritis. J Rheumatol. 2010 Apr;37(4):823–8.

12. Aubin JE. Advances in the osteoblast lineage. Biochem Cell Biol Biochim Biol Cell. 1998;76(6):899–910.

13. Liu F, Malaval L, Aubin JE. The mature osteoblast phenotype is characterized by extensive plasticity. Exp Cell Res. 1997 Apr 10;232(1):97–105.

14. Dougados M, van der Linden S, Juhlin R, Huitfeldt B, Amor B, Calin A, et al. The European Spondylarthropathy Study Group preliminary criteria for the classification of spondylarthropathy. Arthritis Rheum. 1991 Oct;34(10):1218–27.

15. Van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. Arthritis Rheum. 1984 Apr;27(4):361–8.

16. Garrett S, Jenkinson T, Kennedy LG, Whitelock H, Gaisford P, Calin A. A new approach to defining disease status in ankylosing spondylitis: the Bath Ankylosing Spondylitis Disease Activity Index. J Rheumatol. 1994 Dec;21(12):2286–91.

17. Calin A, Garrett S, Whitelock H, Kennedy LG, O'Hea J, Mallorie P, et al. A new approach to defining functional ability in ankylosing spondylitis: the development of the Bath Ankylosing Spondylitis Functional Index. J Rheumatol. 1994 Dec;21(12):2281–5.

18. Jones SD, Steiner A, Garrett SL, Calin A. The Bath Ankylosing Spondylitis Patient Global Score (BAS-G). Br J Rheumatol. 1996 Jan;35(1):66–71.

19. Pacheco-Tena C, Londoño JD, Cazarín-Barrientos J, Martínez A, Vázquez-Mellado J, Moctezuma JF, et al. Development of a radiographic index to assess the tarsal involvement in patients with spondyloarthropathies. Ann Rheum Dis. 2002 Apr;61(4):330–4.

20. Aubin JE, Turksen K. Monoclonal antibodies as tools for studying the osteoblast lineage. Microsc Res Tech. 1996 Feb 1;33(2):128–40.

21. Braun J, Bollow M, Neure L, Seipelt E, Seyrekbasan F, Herbst H, et al. Use of immunohistologic and in situ hybridization techniques in the examination of sacroiliac joint biopsy specimens from patients with ankylosing spondylitis. Arthritis Rheum. 1995 Apr;38(4):499–505.

22. Van Duivenvoorde LM, Dorris ML, Satumtira N, van Tok MN, Redlich K, Tak PP, et al. Relationship between inflammation, bone destruction, and osteoproliferation in the HLA-B27/human β2 -microglobulin-transgenic rat model of spondylarthritis. Arthritis Rheum. 2012 Oct;64(10):3210–9.

23. Denhardt DT, Guo X. Osteopontin: a protein with diverse functions. FASEB J Off Publ Fed Am Soc Exp Biol. 1993 Dec;7(15):1475–82.

24. Oliva F, Barisani D, Grasso A, Maffulli N. Gene expression analysis in calcific tendinopathy of the rotator cuff. Eur Cell Mater. 2011;21:548–57.

25. Kim SY, Choi HY, Myung KB, Choi YW. The expression of molecular mediators in the idiopathic cutaneous calcification and ossification. J Cutan Pathol. 2008 Sep;35(9):826–31.

26. Vattikuti R, Towler DA. Osteogenic regulation of vascular calcification: an early perspective. Am J Physiol Endocrinol Metab. 2004 May;286(5):E686–696.

27. Mori N, Majima T, Iwasaki N, Kon S, Miyakawa K, Kimura C, et al. The role of osteopontin in tendon tissue remodeling after denervation-induced mechanical stress deprivation. Matrix Biol J Int Soc Matrix Biol. 2007 Jan;26(1):42–53.

28. Gundberg CM, Lian JB, Booth SL. Vitamin K-dependent carboxylation of osteocalcin: friend or foe? Adv Nutr Bethesda Md. 2012 Mar;3(2):149–57.

29. Rui YF, Lui PPY, Rolf CG, Wong YM, Lee YW, Chan KM. Expression of chondro-osteogenic BMPs in clinical samples of patellar tendinopathy. Knee Surg Sports Traumatol Arthrosc Off J ESSKA. 2012 Jul;20(7):1409–17.

30. Pauly S, Klatte F, Strobel C, Schmidmaier G, Greiner S, Scheibel M, et al. BMP-2 and BMP-7 affect human rotator cuff tendon cells in vitro. J Shoulder Elb Surg Am Shoulder Elb Surg Al. 2012 Apr;21(4):464–73.

31. Manjila S, Miller E, Awadallah A, Murakami S, Cohen ML, Cohen AR. Ossified choroid plexus papilloma of the fourth ventricle: elucidation of the mechanism of osteogenesis in benign brain tumors. J Neurosurg Pediatr. 2013 Jul;12(1):13–20.

32. Baraliakos X, Listing J, Rudwaleit M, Sieper J, Braun J. The relationship between inflammation and new bone formation in patients with ankylosing spondylitis. Arthritis Res Ther. 2008;10(5):R104.

33. Maksymowych WP, Chiowchanwisawakit P, Clare T, Pedersen SJ, Østergaard M, Lambert RGW. Inflammatory lesions of the spine on magnetic resonance imaging predict the development of new syndesmophytes in ankylosing spondylitis: evidence of a relationship between inflammation and new bone formation. Arthritis Rheum. 2009 Jan;60(1):93–102.

34. Haroon N, Inman RD, Learch TJ, Weisman MH, Lee M, Rahbar MH, et al. The Impact of Tumor Necrosis Factor α Inhibitors on Radiographic Progression in Ankylosing Spondylitis. Arthritis Rheum. 2013 Oct;65(10):2645–54.

35. Kiltz U, Baraliakos X, Karakostas P, Igelmann M, Kalthoff L, Klink C, et al. Do patients with non-radiographic axial spondylarthritis differ from patients with ankylosing spondylitis? Arthritis Care Res. 2012 Sep;64(9):1415–22.

36. Haynes KR, Pettit AR, Duan R, Tseng H-W, Glant TT, Brown MA, et al. Excessive bone formation in a mouse model of ankylosing spondylitis is associated with decreases in Wnt pathway inhibitors. Arthritis Res Ther. 2012 Nov 22;14(6):R253.

37. Lories RJU, Derese I, Luyten FP. Modulation of bone morphogenetic protein signaling inhibits the onset and progression of ankylosing enthesitis. J Clin Invest. 2005 Jun;115(6):1571–9.

38. Las Heras F, Pritzker KPH, So A, Tsui HW, Chiu B, Inman RD, et al. Aberrant chondrocyte hypertrophy and activation of β-catenin signaling precede joint ankylosis in ank/ank mice. J Rheumatol. 2012 Mar;39(3):583–93.

VII. FIGURES

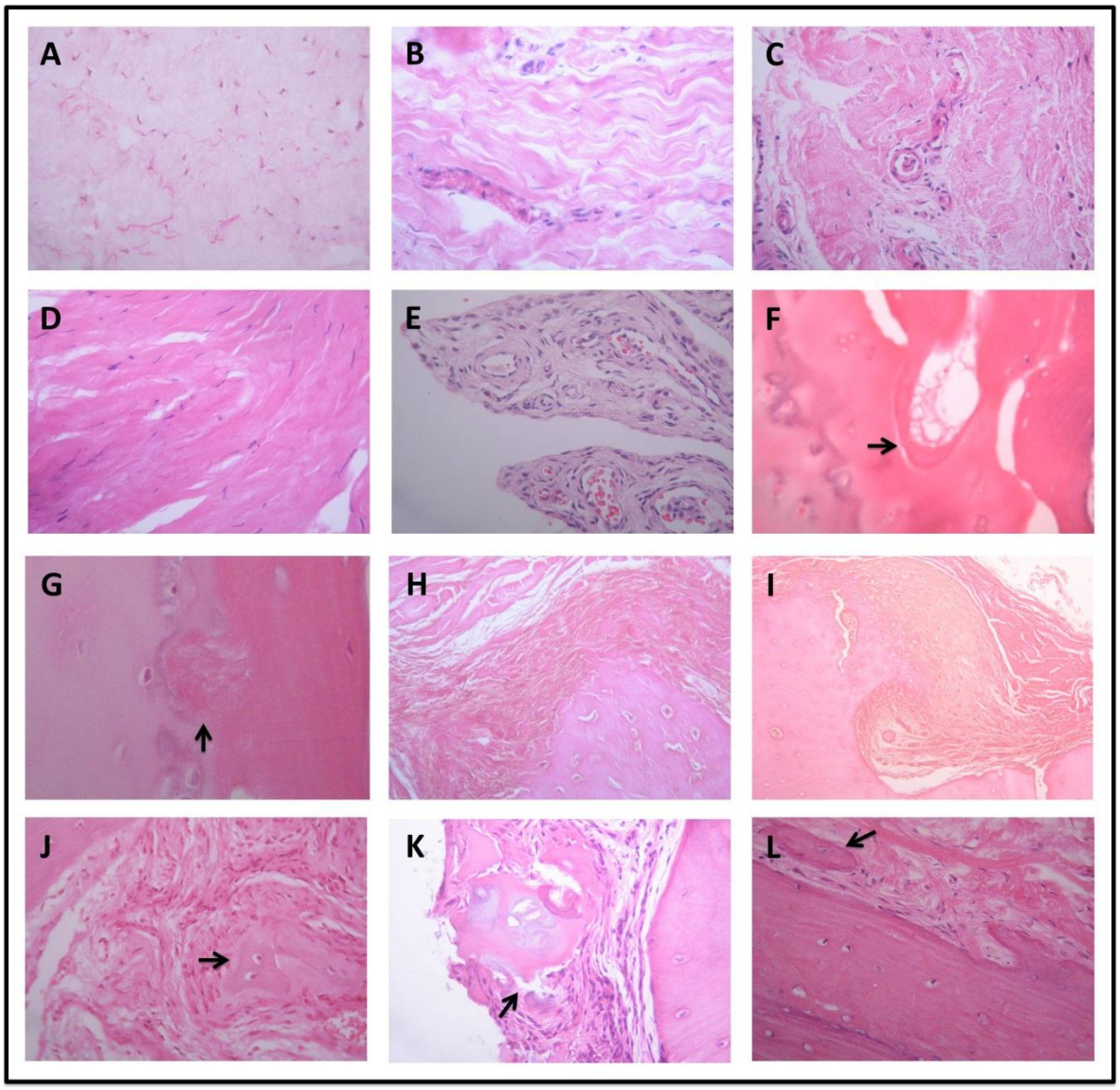

Figure 1. Histologic images of several structures in the joints and entheses of patients with Ankylosing tarsitis. Tendon sheaths presented edema with disorganization of the collagen fibers (1A,1B,1C) and vascular proliferation (1B,1C), in cases with more chronic inflammation the collagen fibers showed hyalinization (1D), sinovial membrane (1E) also showed high vascularity but no significant inflammatory infiltrates. Subchondral bone (1F, 1G) showed irregular borders with bone proliferative response and tendency to intrude in the cartilage matrix (arrows). The entheses showed blurred bone-tendon borders, disorganized fibrillar structure and proliferation of tenocytes and periostic cells (1H, 1I). Evident ossification of the entheseal tissue beyond the periosteum (1J, 1K, 1L (black arrows)) was detected suggesting a transformation of the entheseal cells toward osteoblastic lineage, no significant inflammation is detected neighbouring the ossificating tissue.

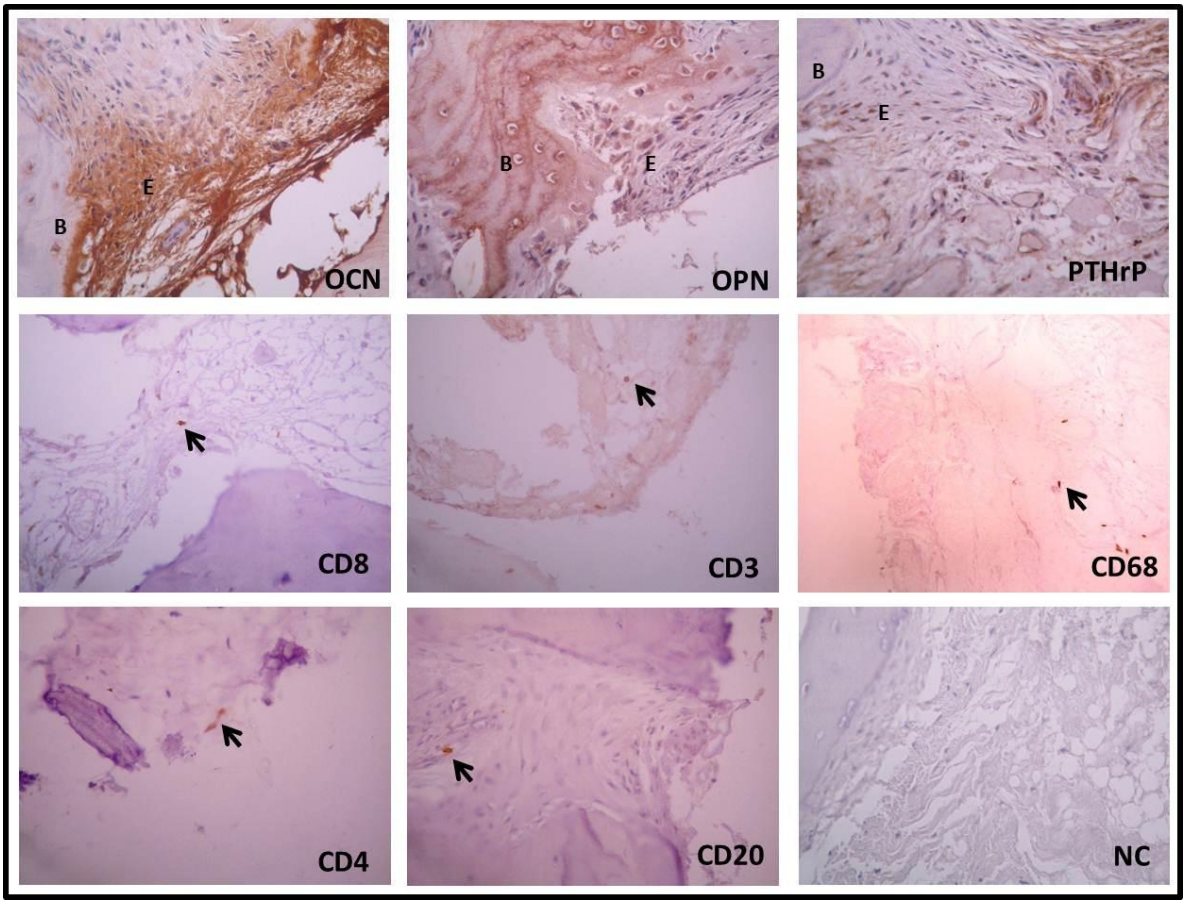

Figure 2. Immunoshistochemistry photographs of entheseal biospies in patients with ankylsing tarsitis. As can be noted, bone lineage proteins (OCN, OPN and PTH-rP) are expressed in both the bone (B) and in the entheses (E) with different patterns, being OCN by far the most abundant. Cellular subpopulation detection (CD3, CD4, CD8, CD20, CD68) showed a very isolated staining suggesting a minimal presence of immune cells. A negative control (NC) is presented as a reference.

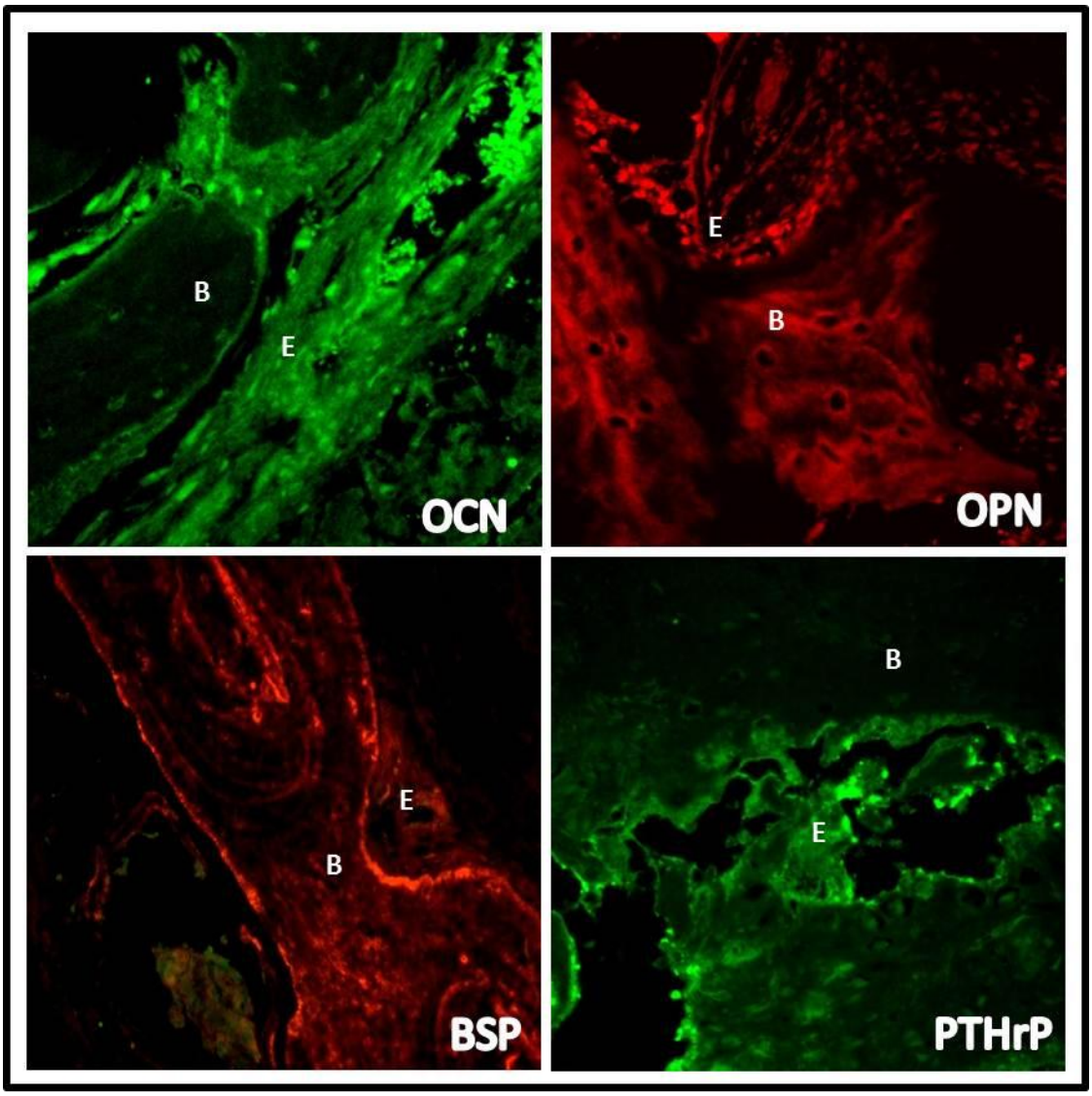

Figure 3. Immunofluorescence detection of bone lineage proteins in the bone (B) and the enthesis (E) of patients with ankylosing tarsitis. OCN is detected on osteocytes and in the enthesis even in the extracellular matrix but not in the bone matrix, OPN is detected in the bone matrix and within cells in the entheses, BSP is mostly expressed in the bone matrix and PTH-rP is basically absent from the bone matrix and osteocytes and is strongly expressed in the periostium and in the entheseal tissue.

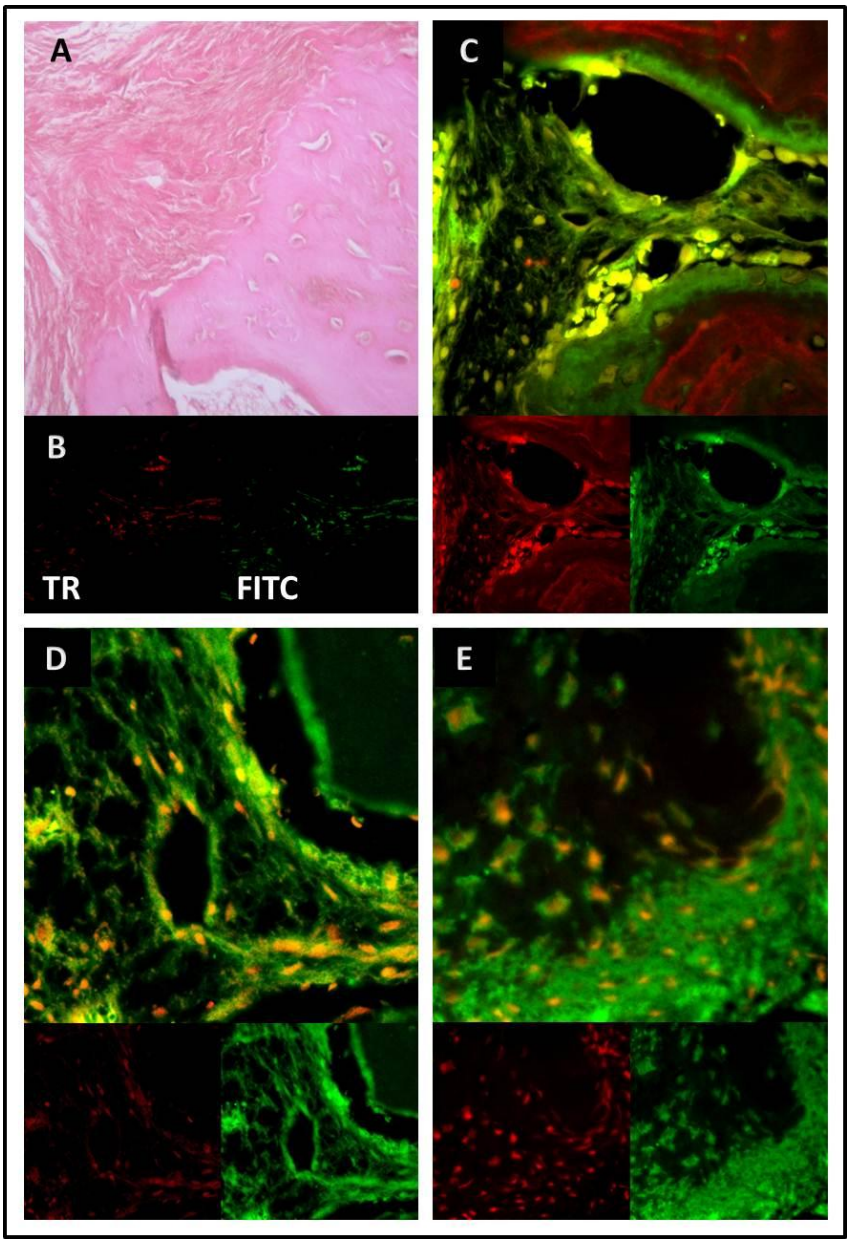

Figure 4. Expression of OCN and OPN by entheseal fibroblast. A) Hematoxylin and eosin reference micrograph (40x) of an enthesis. B) Confocal micrographs of negative control staining sampling both wavelengths. C) Co-localization confocal micrographs of OPN (red) and OCN (green) in the enthesis showing a stronger expression of OPN in the bone matrix and of OCN in the periosteal bone, both co-localize in the entheseal cells confirming their commitment toward bone lineage. D) Co-localization confocal micrographs of OCN (green) and fibroblast marker (red) confirming the expression of OCN by entheseal fibroblasts. E) Co-localization confocal micrographs of OPN (green) and fibroblast marker (red) showing the expression of OPN by entheseal fibroblasts.

VIII. TABLES

Table 1. Comparison of patients group defined by the presence or absence of proliferation in bone biopsies

Patients (n=18)	Patients with no proliferation (n=9)	Patients with proliferation (n=9)	p value
Sex (M/F)	7/2	6/3	0.45
Age (yrs)	30.78 ± 8.27	23.78 ± 2.68	0.08
Age at onset	24.78±8.85	19.78±8.30	0.23
Adult /Juvenile onset	5/4	3/6	0.63
Disease duration	4.30±1.43	2.39±0.79	0.24
Swollen joint count (0-66)	2.56±2.02	3.67±2.95	0.37
Enthesitis index	7.78±4.96	10.78 ± 5.44	0.24
SpATRI (≤2/≥3)	6/3	3/6	0.17
BASDAI	3.66±1.43	6.20±1.21	**0.004**
BASFi	3.94±3.18	6.54±2.79	0.155
BASG	1.77±0.85	2.5±0.87	0.141

Table 2. Findings in tendon sheaths biopsies and bony tissues

Structure	Findings	N (%)
Tendon sheaths (n=20)	Edema	7 (35)
	Vascular proliferation	9 (45)
	Fiber hyalinization	7 (35)
	Synovial lining cells	7 (35)
	Inflammation	4 (20)
Bone (n=18)	Proliferation	9 (50)
	Inflammation	8(44.4)
	Combined inflammation proliferation	4 (22.2)
	Bone marrow observed	6 (33.3)
	Vascular proliferation	15 (83.3)
	Cartilage (hypertrophic)	7 (38.9)