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Bone sialoprotein as a potential key factor implicated in the pathophysiology of osteoarthritis

L. Pesesse †, C. Sanchez †, D.A. Walsh ‡, J.-P. Delcour §, C. Baudouin ||, P. Msika ||, Y. Henrotin †¶*

† *Bone and Cartilage Research Unit, University of Liege, Liege, Belgium*
‡ *Arthritis Research UK Pain Centre, Academic Rheumatology, University of Nottingham, Nottingham, United Kingdom*
§ *Bois de l'Abbaye Hospital, Seraing, Belgium*
|| *Expanscience Laboratories, IRD Direction, Epernon, France*
¶ *Princess Paola Hospital, Vivalia, Marche-en-Famenne, Belgium*

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SUMMARY

Objective: We previously identified an association between bone sialoprotein (BSP) and osteoarthritic (OA) chondrocyte hypertrophy but the precise role of BSP in ostearthritis (OA) has not been extensively studied. This study aimed to confirm the association between BSP and OA chondrocyte hypertrophy, to define its effect on molecules produced by chondrocytes and to analyse its association with cartilage degradation and vascular density at the osteochondral junction.

Method: Human OA chondrocytes were cultivated in order to increase hypertrophic differentiation. The effect of parathyroid hormone-related peptide (PTHrP), interleukin (IL)-1β or tumour necrosis factor (TNF)-α on BSP was analysed by real-time reverse transcription polymerase chain reaction (RT-PCR) and western blot. The effects of BSP on OA chondrocytes production of inflammatory response mediators (IL-6, nitric oxide), major matrix molecule (aggrecan), matrix metalloprotease-3 and angiogenic factors (vascular endothelial growth factor, basic fibroblast growth factor, IL-8, and thrombospondin-1) were investigated. BSP was detected by immunohistochemistry and was associated with cartilage lesions severity and vascular density.

Results: PTHrP significantly decreased BSP, confirming its association with chondrocyte hypertrophy. In presence of IL-1β, BSP stimulated IL-8 synthesis, a pro-angiogenic cytokine but decreased the production of TSP-1, an angiogenesis inhibitor. The presence of BSP-immunoreactive chondrocytes in cartilage was associated with the severity of histological cartilage lesions and with vascular density at the osteochondral junction.

Conclusion: This study supports the implication of BSP in the pathology of OA and suggests that it could be a key mediator of the hypertrophic chondrocytes-induced angiogenesis. To control chondrocyte hypertrophic differentiation is promising in the treatment of OA.

Introduction

Osteoarthritis (OA) is a pathology affecting every joint tissue, especially the cartilage that undergoes many structural and biochemical modifications leading to its final destruction. Many pathways are implicated in damages of cartilage, including the hypertrophic differentiation of chondrocytes. Indeed, in early and late stage OA, some differentiated chondrocytes in permanent articular cartilage are activated and acquire hypertrophy-like changes[1]. This phenotypic changes are associated with the calcification and neovascularisation of the extracellular matrix leading to an increase of cartilage stiffness (for review, see[2]). Consequently, chondrocyte hypertrophic differentiation has been hypothesized to be a key event in OA progression (for review, see[3]).

Pathological chondrocyte hypertrophy is considered as a reiteration of the endochondral ossification process normally absent in normal adult cartilage[4]. Hypertrophic differentiation of chondrocytes participates to cartilage degradation in OA because

* Address correspondence and reprint requests to: Y. Henrotin, Institute of Pathology, Level 5, CHU Sart-Tilman, 4000 Liege, Belgium. Tel: 32-4-3662516; Fax: 32-4-3664734.
E-mail addresses: l.pesesse@ulg.ac.be (L. Pesesse), christelle.sanchez@ulg.ac.be (C. Sanchez), david.walsh@nottingham.ac.uk (D.A. Walsh), jean.pierre.delcour@skynet.be (J.-P. Delcour), cbaudouin@expanscience.com (C. Baudouin), pmsika@expanscience.com (P. Msika), yhenrotin@ulg.ac.be (Y. Henrotin).

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hypertrophic chondrocytes secrete large amounts of matrix metalloproteases (MMP) responsible for cartilage degradation in OA[5]. As observed in the growth plate of growing individuals, the change in cell phenotype is accompanied by invasion of articular cartilage by blood vessels coming from the subchondral bone. Previous studies have shown that in OA, channels containing vascular structures may break the tidemark and invade the articular cartilage[6–8]. Vascular structures are usually accompanied by neural invasion[9]. Moreover, vascular invasion, a process also called angiogenesis, is associated with calcification of the extracellular matrix.

The hypertrophic phenotype is characterized by a high expression of specific markers such as type X collagen[10–12], runt-related factor 2 (runx2)[13,14] or MMP-13[15–17]. A network of positive and negative regulatory factors controls chondrocyte hypertrophy, this includes transcription factors, growth factors, hormones, extracellular matrix molecules and proteases (for review, see[18]).

We previously showed that bone sialoprotein (BSP) was highly expressed by hypertrophic chondrocytes in comparison with non-hypertrophic chondrocytes and was correlated to specific markers of chondrocyte hypertrophy[19]. BSP is a major non-collagenous extracellular matrix protein that belongs to the small integrin-binding ligand N-linked glycoproteins (SIBLING) gene family[20]. In physiological conditions, BSP is expressed by mature osteoblasts, osteoclasts and hypertrophic chondrocytes of the growth plate[21–23]. In these tissues, BSP is involved in mineralization since its interaction with collagen promotes hydroxyapatite nucleation[24,25]. As a result, BSP is firstly known as a marker of bone formation and has been considered as a marker for subchondral bone for a long time[26]. In addition to its implication in tissue mineralization, BSP also promotes cell attachment and signalling through its Arg-Gly-Asp (RGD) sequence and alternate attachment mechanisms[27,28] and has been further described as an angiogenesis enhancer. Indeed, Bellahcène *et al.* showed that BSP mediates human endothelial cell attachment and migration through the interaction of its RGD domain with endothelial cell $\alpha_v\beta_3$ integrin receptors[29].

Hence, BSP constitutes a multifunctional protein with a large clinical interest. We previously identified a preliminary association between BSP and OA chondrocyte hypertrophy[19] but the precise role and regulation of BSP in OA remained to be extensively studied. Herein, we investigated the effects of parathyroid hormone-related peptide (PTHrP), interleukin (IL)-1β and tumour necrosis factor (TNF)-α on BSP production by OA hypertrophic chondrocytes. Further, we have investigated the effect of increased dose of BSP on OA chondrocytes production of inflammatory response mediators [IL-6, nitric oxide (NO)], a major matrix molecule [aggrecan (AGG)], MMP-3 and angiogenic factors [vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), IL-8, thrombospondin (TSP)-1]. Finally, an immunohistological study performed on a set of non-OA and OA cartilage sections demonstrated the association between BSP localization, cartilage lesions severity and vascularisation.

Material and methods

Chondrocytes culture in alginate beads

Culture settings

Human chondrocytes were isolated from cartilage of OA patients undergoing total knee replacement (TKR) surgery with the approval of the Ethic Committee of Medicine department of the University of Liege (number B70720108313, reference 2010/43). Alginate beads were prepared as previously described[30] and OA chondrocytes were embedded at a density of 4.2×10^6 cells per millilitre alginate solution. OA chondrocytes in alginate beads were cultivated for 3, 4, 8, 12, 21 or 28 days in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Verviers, Belgium) either supplemented with 10 mM *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid* (HEPES), 20 μg/ml proline (Sigma–Aldrich, Bornem, Belgium), 50 μg/ml ascorbic acid (Sigma–Aldrich, Bornem, Belgium), 2 nM glutamine (Lonza, Verviers, Belgium) and 10% (v/v) foetal bovine serum (FBS) (Lonza, Verviers, Belgium) in order to induce hypertrophic differentiation of OA chondrocyte, as previously validated[19] or with 1% of a preparation of insulin, transferrin, selenous acid, bovine serum albumin (BSA), and linoleic acid (ITS+) (ICN Biomedicals, Asse-Relegem, Belgium) in order to test the effect of recombinant BSP on OA chondrocyte. Media were changed twice a week. Some chondrocytes in alginate beads were cultivated with either 20 nM PTHrP, an inhibitor of hypertrophic differentiation (Abcam, Cambridge, United Kingdom) or 170 pg/ml IL-1β (10^{-11} M) or 25 ng/ml TNFα (Roche Diagnostic, Vilvoorde, Belgium), two key cytokines activating catabolic pathways of chondrocyte metabolism. These concentrations correspond to the maximal IL-1β concentration found in the synovial fluid of patients[31,32]. These compounds were added during the complete culture period of 28 days or only for the last 7 days of the culture (+7D), when OA chondrocytes expressed a highly hypertrophic phenotype.

To investigate the effects of BSP on chondrocytes metabolism and on the production of angiogenic factors, chondrocytes in alginate beads were cultivated 12 days in medium supplemented with 1% ITS+ and increased concentrations of recombinant BSP (R&D Systems, Abingdon, United Kingdom) that is 25, 100 and 400 ng/ml. Each chondrocyte culture was realized from a pool of three to six different OA patients that displayed a grade III or IV based on the classification tree for Collins grading of OA changes at the articular surface[33]. Each culture condition was done in triplicate. Cultures were repeated three times with different pools of chondrocytes coming from different donors.

Gene expression and assays

Real-time reverse transcription polymerase chain reaction (RT-PCR) of collagen type X and BSP

RNA from approximately 2×10^6 cells was isolated using the RNeasy minikit (Qiagen, Hilden, Germany), and PCR was performed using the LightCycler SYBR Premix Ex Taq system (Takara, Brussels, Belgium) as previously described[30]. The PCR template source was either 3 ng first-strand complementary DNA (cDNA) or purified DNA standard for calibration curve. The house-keeping gene hypoxanthine phosphoribosyltransferase (HPRT) was amplified and used as an internal control to standardize messenger RNA levels. Forward and reverse primer sequences used to amplify the desired cDNA are respectively the following: *HPRT*: TGTAATGACCAGTCAACAGGG and TGCCTGACCAAGGAAAGC; collagen type X (*col10a1*): GGGAGTGCCATCATCG and AGGGTGGGGTAGAGTT; *BSP*: GTGTCACTGGAGCCAA and ACCATCATAGCCATCGT.

Western blot analysis of BSP

Protein expression of BSP by chondrocytes was analysed by western blotting with a mouse monoclonal anti-BSP antibody (1:300) (LFMb-24, sc-73634; Santa Cruz Biotechnology Inc., Germany). Protein concentrations were measured using the Micro BCA Protein assay Kit (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions. Equal amounts of protein were separated in a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and were electrically transferred to polyvinylidene fluoride (PVDF) membrane. After incubation with goat anti-mouse

horseradish peroxidase (HRP) secondary antibody (1:3000) (Dako, Heverlee, Belgium), proteins were detected using enhanced chemoluminescence reagent (ECL+) (GE Healthcare, Diegem, Belgium). Results were normalized compared to tubulin-α expression detected with mouse monoclonal antibody (1:1000) (sc-8035; Santa Cruz Biotechnology Inc., Germany). Control condition was constituted of human osteosarcoma Saos-2, known to produce BSP[34]. These cell extracts were provided by the Metastasis Research Laboratory, GIGA-Cancer at the University of Liege.

Immunoassays for TSP-1 and IL-8

TSP-1 (Duoset, R&D Systems, Abingdon, United Kingdom) and IL-8 (EASIA, Biosource Europe, Fleurus, Belgium) were measured by specific enzyme amplified sensitivity immunoassay directly in chondrocytes conditioned culture media according to the manufacturer's protocol.

Histology and immunohistochemistry study

Samples collection and processing

Cartilage biopsies were obtained from 24 patients undergoing TKR for OA aged 68.96 ± 9.388 (mean ± SD, $n = 24$) and from nine non-arthritic controls collected post-mortem aged 50.67 ± 18.81 (mean ± SD, $n = 9$). Cartilage degradation severity was evaluated macroscopically according to the photographic chondropathy score (PCS) described by Walsh *et al*[35]. Cartilage samples were fixed in neutral-buffered formalin and then wax embedded. Midcoronal sections of medial tibial plateaux were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) and 10 mM Tris buffer (pH 6.95) at room temperature (RT) prior to wax embedding. Five micrometre thick tissue sections were cut with a microtome. All histological scoring was undertaken by two different investigators, blinded to patient group, using a Zeiss Axioscop-50 microscope.

Histology and grading of chondropathy

Tissue sections were stained with safranin-O to assess the severity of microscopic OA changes in articular cartilage. After nuclear staining with Weigert's haematoxylin and dedifferentiation in acid alcohol, sections were immersed in 0.02% fast green, then 0.1% safranin-O, and mounted. Microscopic histological evaluation of OA severity in articular cartilage was evaluated using the modified Mankin's score[36]. Grades for the severity of OA were the sum of four components: cartilage surface integrity [grade 0 (normal), grade 1 (surface irregularities), grade 2 (pannus and surface irregularities), grade 3 (≥2 clefts to transitional zone), grade 4 (≥2 clefts to radial zone), grade 5 (≥2 clefts to calcified zone), grade 6 (complete disorganisation)], chondrocyte appearance [grade 0 (normal), grade 1 (diffuse hypercellularity), grade 2 (cloning: a single chondron ≥5 nuclei), grade 3 (hypocellularity)], proteoglycan loss [grade 0 (no loss of safranin-O stain), grade 1 (slight reduction), grade 2 (moderate reduction), grade 3 (severe reduction), grade 4 (absence of staining)] and tidemark integrity [0 (intact) or 1 (crossed by vascular channels)]. Severity scores thus can range between 0 (no observed changes) and 14 (most severe changes).

Vascular structures immunostaining and counting

Blood vessels at the osteochondral junction were visualised using a mouse monoclonal anti-CD34 antibody (clone QBEnd 10, Dako, Cambridge, United Kingdom) directed against vascular endothelium. Sections were deparaffinized in xylene and rehydrated in distilled water. The nonspecific binding sites were blocked with horse serum (1:30) supplemented with 0.05% BSA and sections were incubated with anti-CD34 antibody (1:40) 1 h at RT. A horse anti-mouse biotinylated secondary antibody (1:100) was used prior to reaction with alkaline phosphatase-labelled ABC (both in vectastain ABC-PA kit, Vector Laboratories, Peterborough, United Kingdom). Sections were mounted with glycerol (1:10) and stored at 4°C until examination. CD34 immunoreactive structures were visualised using FastRed (Sigma–Aldrich, Dorset, United Kingdom). FastRed reaction product was detected by transmitted light microscopy and also using its fluorescent properties under UV illumination (615 nm). Osteochondral junction, calcified and non-calcified cartilage were discriminated by autofluorescence. The length of the tidemark was measured using digital electronic callipers (Mitutoyo, Kawasaki, Japan). Osteochondral vascular density was determined as the ratio between the number of vascular channels terminating in non-calcified articular cartilage and the length of the tidemark and was expressed as number of vascular structure per millimetre of tidemark.

BSP detection in cartilage

BSP was immunolocalized in cartilage using mouse monoclonal antibody (LFMb-25, sc-73630; Santa Cruz Biotechnology Inc., Germany). Briefly, cartilage sections were deparaffinized in xylene and rehydrated in distilled water. The blocking of endogenous peroxidase was performed with 0.3% H_2O_2 in methanol and the nonspecific binding sites were blocked with normal goat serum (1:30) supplemented with 0.05% BSA. Anti-BSP antibody at dilution 1:100 was applied and incubated overnight at +4°C. A negative control with a mouse IgG_1 non-immune serum (Santa Cruz Biotechnology Inc., Germany) at dilution 1:100 was included in the experiment. The tissue sections were then incubated with biotinylated horse anti-mouse antibody (1:100) (BA2001, Vector Laboratories, Peterborough, United Kingdom), followed by exposure to preformed streptavidin-biotinylated HRP complex (ABC Elite, PK6100, Vector Laboratories, Peterborough, United Kingdom). Peroxidase was revealed by the 3,3′-diaminobenzidine tetrahydrochloride reaction. Sections were finally counterstained with haematoxylin, dehydrated and mounted.

Statistical analysis

Unless otherwise specified, data were analysed by one-way analysis of variance (ANOVA) and if significant, followed by Tukey's post-test and multiple comparisons test to obtain adjusted *P*-values. The assumptions underlying the ANOVA were assessed. Data were made of independent observations and the homogeneity of variance between groups was analysed with the Bartlett's test but the number of sample in each group was too small to test the normality. However, the ANOVA was used because this statistical test is robust against violation of normality. Multiplicity adjusted *P*-values account for the number of comparisons between data groups for each experiment. The multiple comparison tests report 95% confidence interval for the difference between means of each data group. The statistical significance threshold was set to 5%, this value applies to the entire family of comparisons (experiment-wise error-rate). Tukey's test is a single-step multiple issue procedure that identifies any difference between two means that is greater than the expected standard error (SE) and corrects for experiment-wise error-rate. The same uncertainty and limitations addressed to the comparison between two groups, analysed with a Student's *t* test. Gaussian distribution of data was assessed with the D'Agostino and Pearson normality test. All data were analysed using GraphPad Prism software, version 6. Production values were reported to DNA content of the cells. The DNA content of the cultures was measured according to the fluorimetric method of Labarca and Paigen[38].

Results

In vitro investigation of BSP

The first part of this study aimed to observe BSP gene expression and protein production by OA chondrocytes incubated with or without PTHrP, a specific inhibitor of chondrocyte hypertrophy or IL-1β and TNFα, two major inflammatory cytokines. The effect of recombinant BSP on the production of IL-6, NO, AGG, MMP-3, VEGF, bFGF, IL-8 and TSP-1 by OA chondrocyte was also investigated in this part of the study.

Effect of PTHrP

Col10a1 gene expression was significantly inhibited when chondrocytes were continuously incubated with PTHrP (20 nM) for 21 days ($P = 0.0003$; −91.53), or 28 days ($P = 0.0016$; −94.38%) and when PTHrP was added in the culture medium during the last 7 days of culture (between days 21 and 28) ($P = 0.0013$; −97.12%) [Fig. 1(A)]. These results indicated that PTHrP inhibited the expression of a specific marker of chondrocyte hypertrophy. *BSP* gene was not expressed or produced during the first 3 days but well after 21 and 28 days of culture when chondrocytes expressed a hypertrophic phenotype and was almost completely inhibited when PTHrP was added in the culture medium. A significant inhibition of 93.92 ± 1.19% was observed after 21 days ($P = 0.0009$) and of 95.94 ± 1.38% after 28 days of incubation with PTHrP ($P = 0.0254$). A decrease of 92.83 ± 5.9% of *BSP* expression by chondrocytes was also observed when PTHrP was added from day 21 to 28 ($P = 0.0243$) [Fig. 1(B)]. The production of BSP by chondrocytes analysed by western blot was also reduced by PTHrP after 21 and 28 days but the decrease was very small when PTHrP was added for the last 7 days of culture [Fig. 1(A)]. A lactate dehydrogenase activity (LDH) assay was performed to ascertain that chondrocytes were not ongoing apoptosis. Whatever the culture time or condition, cell death ratio remained below 5% (data not shown).

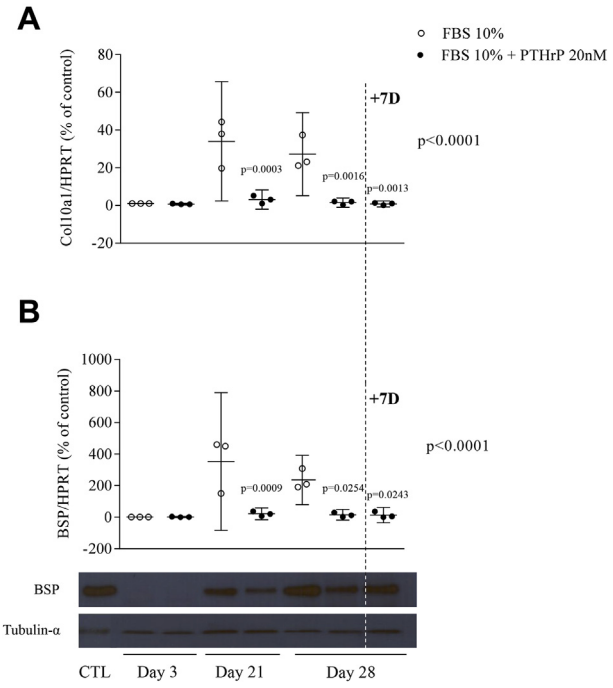

Fig. 1. Effect of PTHrP (20 nM), an inhibitor of chondrocyte hypertrophy, on collagen type 10 expression (*col10a1*) and BSP expression and production by OA non-hypertrophic (day 3) and hypertrophic (days 21 and 28) chondrocytes. (A) *Col10a1* was not expressed by non-hypertrophic chondrocytes but well by hypertrophic chondrocytes. *Col10a1* expression was repressed when chondrocytes were cultivated with PTHrP after 21 days ($P = 0.0003$) and 28 days ($P = 0.0016$) of culture and when PTHrP was added for the last 7 days of culture ($P = 0.0013$) (ANOVA: $P < 0.0001$). (B) *BSP* was not expressed or produced by non-hypertrophic chondrocytes but its expression by hypertrophic chondrocytes was repressed by PTHrP at day 21 ($P = 0.0009$) and day 28 ($P = 0.0254$) and when PTHrP was added for the last 7 days of culture ($P = 0.0243$) (ANOVA: $P < 0.0001$). Gene expression was analysed by RT-PCR and normalized by the house-keeping gene *HPRT*. BSP production (65 kDa) by hypertrophic chondrocytes, evaluated by western blot and normalized to tubulin-α (55 kDa), was clearly decreased by PTHrP after 21 and 28 days of culture and slightly decreased when PTHrP was added between days 21 and 28. Control (CTL) condition is constituted of Saos-2 cell extracts. Independent observations were analysed by a single-step multiple comparison procedure (Tukey's test) after one-way ANOVA and are presented in percentage of control with the mean ± 95% confidence interval. +7D corresponds to the addition of PTHrP for the last 7 days of culture only. Each data point is the mean of two measures performed on three different chondrocytes cultures, each made from a different pool of primary human chondrocytes coming from several different donors.

Effects of IL-1β and TNFα

After 3 days of culture, chondrocytes failed to express *BSP* and IL-1β or TNFα did not induce *BSP* gene expression or BSP protein production. At day 21 and 28, BSP was synthesized by chondrocytes. Addition of IL-1β (170 pg/ml) in the culture medium fully blocked the expression of *BSP* by hypertrophic chondrocytes when added continuously to the culture medium during 28 days ($P = 0.0115$) or when added from day 21 to 28 ($P = 0.012$). TNFα (25 ng/ml) also completely repressed *BSP* expression after 28 days ($P = 0.0139$) and when added for the last 7 days ($P = 0.0114$) (Fig. 2). BSP production was completely blocked by both cytokines as attested by the western blot analysis (Fig. 2). IL-1β and TNFα also fully repressed *col10a1* expression after 21 or 28 days ($P = 0.0476$ and $P = 0.0375$ respectively) and when added from day 21 to 28 ($P = 0.0494$ and $P = 0.0445$, respectively) (data not shown). The

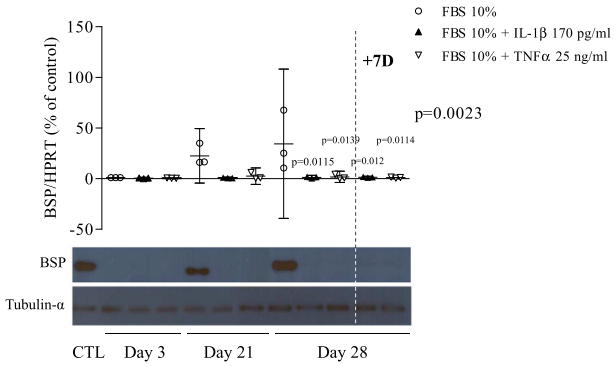

Fig. 2. Effect of IL-1β (170 pg/ml) or TNFα (25 ng/ml) on BSP expression and production by OA non-hypertrophic (day 3) and hypertrophic (days 21 and 28) chondrocytes. *BSP* was not expressed or produced by non-hypertrophic chondrocytes but its expression by hypertrophic chondrocytes was repressed by IL-1β after 28 days ($P = 0.0115$) and when IL-1β was added to the culture medium from day 21 to 28 ($P = 0.012$). TNFα also completely repressed *BSP* expression after 28 days ($P = 0.0139$) and when added for the last 7 days of culture ($P = 0.0114$) (ANOVA: $P = 0.0023$). Gene expression was analysed by RT-PCR and normalized by the house-keeping gene *HPRT*. BSP production (65 kDa), evaluated by western blot and normalized to tubulin-α (55 kDa), was completely repressed by IL-1β and TNFα after 21 and 28 days of culture and when the cytokines were added to the medium for the last 7 days of culture. Control (CTL) condition is constituted of Saos-2 cell extracts. Independent observations were analysed by a single-step multiple comparison procedure (Tukey's test) after one-way ANOVA and are presented in percentage of control with the mean ± 95% confidence interval. +7D corresponds to the addition of IL-1β or TNFα for the last 7 days of culture only. Each data point is the mean of two measures performed on triplicate conditions in three different chondrocytes cultures, each made from a different pool of primary human chondrocytes coming from several different donors.

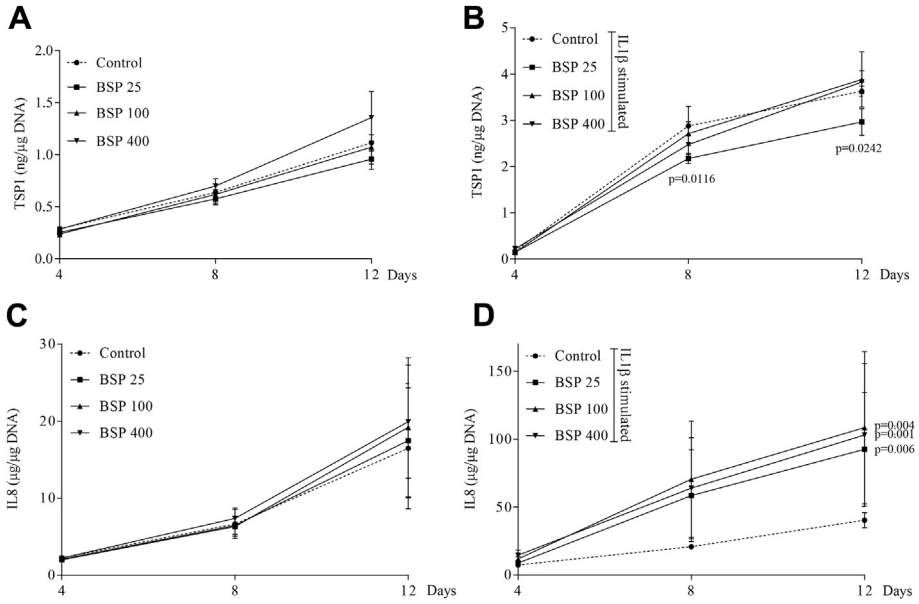

Fig. 3. Immunoassay analysis of the expression of (A and B) TSP-1 and (C and D) IL-8 by OA chondrocytes cultivated in 1% ITS+ supplemented medium (control) under the influence of recombinant BSP at 25 ng/ml (BSP 25), 100 ng/ml (BSP 100) or 400 ng/ml (BSP400) in basal (A and C) or IL-1β-stimulated (B and D) condition. IL-1β significantly stimulated TSP-1 production after 8 ($P < 0.0001$) and 12 ($P < 0.0001$) days of culture. IL-1β-stimulated TSP-1 production was significantly decreased by BSP at 25 ng/ml after 8 ($P = 0.0116$) and 12 ($P = 0.0242$) days of culture. IL-1β-stimulated IL-8 production was stimulated after 12 days of culture with recombinant BSP at 25 ng/ml ($P = 0.006$), 100 ng/ml ($P = 0.0004$) and 400 ng/ml ($P = 0.001$). Independent observations were analysed by a single-step multiple comparison procedure (Tukey's test) after one-way ANOVA and were normalized to DNA content and presented as mean ± SE cumulated in time. Each data point is the mean of three measures performed on triplicate conditions from three different chondrocytes cultures, each made from a different pool of primary human chondrocytes coming from several different donors.

LDH assay demonstrated that these cytokines did not affect cell viability (data not shown).

Effect of BSP on TSP-1 and IL-8 production by human OA chondrocytes

BSP did not significantly modify the basal production of TSP-1 and IL-8 by human OA chondrocytes whatever the dose and the period of incubation [Fig. 3(A and C)]. IL-1β significantly stimulated TSP-1 production after 8 ($P < 0.0001$) and 12 days of culture ($P < 0.0001$) and BSP 25 ng/ml decreased IL-1β-stimulated TSP-1 production after 8 and 12 days ($P = 0.0116$ and $P = 0.0242$, respectively) [Fig. 3(B)] but increased IL-1β-stimulated IL-8 production after 12 days at the concentration of 25 ng/ml ($P = 0.006$), 100 ng/ml ($P = 0.0004$) and 400 ng/ml ($P = 0.001$) compared to control condition without BSP [Fig. 3(D)].

Ex vivo investigation of BSP

The second part of this study aimed to localize BSP by immunohistochemistry in OA and non-OA human cartilage sections. Age, gender and data for PCS, Mankin score, vascular density and BSP detection of the population considered in the *ex vivo* part of the study are presented in Table I.

Analyse of cartilage degradation

PCS and Mankin score were significantly higher in OA than in non-OA cartilage ($P < 0.0001$). PCSs for OA and non-OA knee joint were 67.4 ± 15.78 ($n = 24$) and 17.83 ± 16.78 ($n = 9$) respectively (mean ± SD) and the modified Mankin scores were 6.88 ± 1.77 for OA ($n = 24$) and 2.33 ± 2.12 for non-OA samples ($n = 9$) (mean ± SD) (data not shown).

Analyse of vascular density

A significant increase in vascular density was observed in OA compared to non-OA samples ($P = 0.0029$) as previously identified[9]. Vascular density at the osteochondral junction of OA and non-OA cartilage samples was 0.31 ± 0.2 ($n = 24$) and 0.10 ± 0.13 ($n = 9$) respectively (mean ± SD) (data not shown).

Detection of BSP in cartilage

BSP-positive chondrocytes and extracellular matrix were detected in articular cartilage of the whole group of OA patients ($n = 24$) and in three samples from the non-OA group ($n = 9$). The staining was mostly localized in the superficial layer of articular cartilage and chondrocytes localized around damaged fibrillated cartilage, namely in clusters, showed a dark staining for BSP (Fig. 4).

Association of BSP with cartilage degradation and vascular density

The presence of BSP in the whole collection of OA and non-OA patients was associated with the severity of macroscopic cartilage lesions assessed with the PCS ($P < 0.0001$) [Fig. 5(A)] and with the modified Mankin score ($P < 0.0001$) [Fig. 5(B)]. A significant association was also found with the individual scoring criteria of cartilage surface integrity ($P = 0.0002$) [Fig. 6(A)], chondrocyte appearance ($P < 0.0001$) [Fig. 6(B)], proteoglycan loss ($P = 0.0081$) [Fig. 6(C)] and tidemark integrity ($P = 0.0158$) [Fig. 6(D)]. The presence of BSP in cartilage was also positively associated with vascular density in cartilage samples ($P = 0.0094$) (Fig. 7).

Discussion

Hypertrophic chondrocytes may contribute to cartilage degradation and may support extracellular matrix mineralization and neovascularization of the articular cartilage in OA. The successive modifications leading to vascularization and mineralization of the articular cartilage through phenotypic change of chondrocyte into hypertrophy are observable in physiological (growth plate) and pathological (OA) conditions. Hence, some factors involved in the induction and regulation of chondrocyte hypertrophy in the growth plate may constitute interesting targets to inhibit OA progression.

Table I
Characteristics of the immunohistochemistry study population. The PCS was obtained based on the method described by Walsh *et al*[35]. The Mankin score was calculated as described in the Material and methods section. The vascular density corresponds to the ratio between the number of vascular structures and length of the tidemark (in millimetre). For BSP, "+" stands for presence of BSP and "−" stands for absence of BSP. Data are presented in percentage (gender and BSP) or as mean ± SD. NS: not specified

	Age	Gender	PCS	Mankin score				Vascular density	BSP
				Surface integrity	Chondrocytes appearance	Proteoglycan loss	Tidemark integrity		
Non-OA samples	46	M	4.2	0	0	0	1	0.28	−
	41	F	9.8	1	0	2	0	0	−
	26	M	7	0	1	0	0	0	−
	69	F	17	0	0	0	0	0	−
	70	M	NS	2	2	1	1	0.28	+
	36	F	17	0	0	0	0	0	+
	29	M	7	0	1	1	0	0	−
	71	M	24.9	0	1	1	1	0.10	−
	68	M	55.7	1	2	1	1	0.27	+
Mean ± SD	50.67 ± 18.81	M/F (%): 66.7/33.3	17.83 ± 5.93	2.33 ± 2.12				0.10 ± 0.13	+/− (%): 33.3/66.7
				0.44 ± 0.73	0.78 ± 0.83	0.67 ± 0.71	0.44 ± 0.53		
OA samples	81	M	91.25	2	2	2	1	0.27	+
	52	F	38.05	2	2	1	1	0.3	+
	81	M	86	2	3	2	0	0	+
	74	M	39.7	3	2	2	1	0.55	+
	57	M	53.4	1	2	2	1	0.44	+
	64	F	58.8	1	2	0	1	0.42	+
	63	M	44.85	2	2	2	1	0.32	+
	70	F	64.85	4	2	2	0	0	+
	84	F	79	3	1	2	1	0.7	+
	65	M	69.7	2	2	2	1	0.37	+
	76	M	79	4	1	2	1	0.34	+
	63	F	58.8	2	1	1	1	0.46	+
	73	F	61.9	2	1	1	0	0	+
	64	M	72.8	2	2	3	1	0.43	+
	78	M	69.3	3	2	2	1	0.12	+
	62	M	77.25	2	2	1	1	0.13	+
	60	M	66	3	2	2	0	0	+
	61	F	89.5	3	2	2	1	0.27	+
	78	F	46.4	2	2	2	1	0.23	+
	75	F	52.6	2	2	1	1	0.59	+
	61	F	73.75	1	1	2	1	0.16	+
	79	NS	76.3	2	2	2	1	0.36	+
	55	F	89.5	6	3	3	1	0.49	+
	79	M	79	2	2	1	1	0.38	+
Mean ± SD	68.96 ± 9.39	M/F (%): 50/45.8	67.4 ± 3.22	6.88 ± 1.77				0.31 ± 0.2	+/− (%): 100/0
				2.42 ± 1.1	1.88 ± 0.54	1.75 ± 0.68	0.83 ± 0.38		

Previously, we have characterized a culture model for studying chondrocyte hypertrophy. In this model, chondrocytes were cultured in alginate beads for 28 days in serum-supplemented medium in order to induce chondrocyte hypertrophic differentiation[19]. Using this model, we have investigated the expression of angiogenic factors by hypertrophic or non-hypertrophic cells. By this way, we have identified BSP as the most upregulated pro-angiogenic factor by OA hypertrophic chondrocytes in culture. BSP was well correlated with the most relevant hypertrophic markers like type X collagen, runx2 or MMP-13 suggesting that BSP could be a key mediator of the hypertrophic chondrocyte mediated cartilage vascularization. The present study confirms the previous one and brings new information on the role played by BSP in cartilage degradation.

PTHrP is described as an inhibitor of chondrocyte hypertrophy[39–41]. Our results showed that PTHrP inhibited BSP expression and production by OA hypertrophic chondrocytes confirming that BSP was related to the hypertrophic status of chondrocytes. As a control of the inhibitory effect of PTHrP on chondrocyte hypertrophic differentiation, type X collagen expression was analysed. Type X collagen was chosen as representative of the hypertrophic phenotype of chondrocytes because it is the most widely accepted marker of chondrocyte hypertrophy[10–12]. In regard of the review written by R. Dreier (Germany) who stated in conclusion that the number of signalling molecules involved in chondrocyte proliferation and differentiation during endochondral ossification also plays a regulative role in articular cartilage during OA[18], our results confirm that PTHrP regulates OA chondrocytes hypertrophy.

More surprising were the effects of IL-1β and TNFα on BSP and type X collagen gene expression and protein production by hypertrophic chondrocytes. These results suggest that pro-inflammatory cytokines may repress the hypertrophic differentiation of chondrocytes, consistent with evidence that they may also globally inhibit chondrogenesis[42,43]. However, these results are not in accordance with the rest of the work and can't be convincingly explained. One explanation would be that OA is the result of a combination of mechanical, inflammatory and ageing related factors that is not mimicked by adding one single cytokine in the culture medium of *in vitro* experiments.

To study the effect of BSP on the production of matrix molecules, catabolic or inflammatory mediators or other angiogenic factors, concentrations of recombinant BSP added in the culture medium were chosen according to data found in the literature and corresponded to pathologic condition[44–46]. Production of NO, AGG, MMP-3, IL-6, VEGF and bFGF was not modulated by BSP (data not shown). BSP decreased chondrocyte production of TSP-1, an anti-

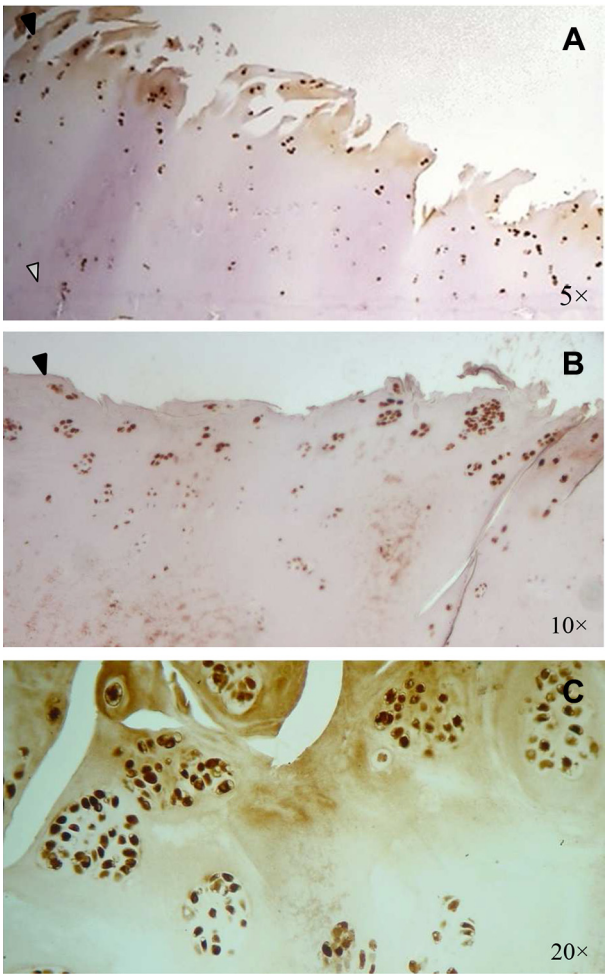

Fig. 4. BSP is detected on sections of OA cartilage by immunohistochemistry with a monoclonal antibody. Representative pictures of three different sections of OA cartilage. (A) BSP-positive chondrocytes and extracellular matrix are localized in the superficial damaged layer of OA cartilage. (B) BSP is detected in clones of chondrocytes located around damaged and fibrillated cartilage. (C) Greater magnification of BSP-positive clones of chondrocytes. Dark arrows indicate the surface of cartilage. Clear arrow indicates the tidemark (magnification 5×, 10× and 20×).

angiogenic factor[47] while it increased the production of IL-8, a pro-angiogenic factor[48–50]. This could create an imbalance between pro- and anti-angiogenic factors favourable to angiogenesis of cartilage. These data are in accordance with those of Bellahcène *et al.* demonstrating that BSP promotes angiogenesis[29]. These results also suggest that IL-8 is a key mediator of the BSP-induced angiogenesis. Indeed, BSP had no effect on VEGF and bFGF, two other well-known pro-angiogenic factors. Taken together, these data support the hypothesis that BSP could be a key mediator of cartilage neovascularisation mediated by hypertrophic chondrocytes.

The *ex vivo* part of this study was essential to place our findings in the context of human OA articular cartilage. BSP-positive chondrocytes were detected in OA cartilage tissue biopsies. As expected, BSP was also detected in subchondral bone. The extent of BSP-positive structures in bone seemed to increase with the severity of the disease (data not shown). This observation is in accordance with the results obtained by de Bri *et al.* on the guinea-pig model of OA which suggested that altered BSP abundance could be a potential bone marker for late stage OA[51]. However, this issue was not particularly dealt with in this article since it focuses on cartilage. The presence of BSP in growing cartilage and OA bone–cartilage junction has been described for several years by D. Heingard *et al.* (Sweden) in two different animal models[51,52]. In 1995, Shen *et al.* published results of a study in which they detected BSP mRNA in hypertrophic chondrocytes of the femoral heads of growing rats by *in situ* hybridization[52]. Later, de Bri *et al.* investigated the distribution of BSP at the osteocartilaginous interface in the guinea pig at different stages of primary OA by ultrastructural immunolocalization. Their results showed that BSP immunolabelling was concentrated at the bone–cartilage interface of the medial condyle in all age groups and that the presence of BSP was increased in the OA medial condyle compared to non-OA lateral condyle and was correlated to cartilage fibrillation in advanced OA. Both studies on animal models of differentiating articular cartilage and of OA notably concluded on an implication of BSP in the process of mineralization. Compared to these studies, the novelty of our work is that we detected BSP in human OA cartilage samples in order to associate its presence with cartilage degradation and vascular density at the osteochondral junction. Data showed a highly significant correlation between the presence of BSP and cartilage degradation of OA human samples based on both macroscopic and microscopic observations. Indeed, in OA samples, the presence of BSP was associated with high severity of cartilage degradation. Moreover, in non-OA samples, BSP was most of time undetectable or only a light staining of chondrocytes in the superficial layer of cartilage was detected. Detailed association between BSP and components of Mankin score revealed that cartilage surface integrity, proteoglycan loss, chondrocyte appearance and tidemark integrity were individually associated with the presence of BSP-immunoreactive chondrocytes. The vascular density at the osteochondral junction was associated with the Mankin score ($r = 0.67$; $P < 0.0001$) and with the PCS ($r = 0.54$; $P = 0.0015$) (data not shown) confirming that vascular density is associated with cartilage degradation as previously described[8]. Our results demonstrated an association between the presence of BSP and vascular density. These findings suggest that, as previously described in other diseases, BSP could in part constitute a pro-angiogenic factor in OA. This hypothesis is supported by the initial basic description of BSP, characterized by the presence of a cell attachment recognition RGD tripeptide sequence associated with the integrin-like cell-binding receptor[53].

In conclusion, results from the *in vitro* study confirmed the association of BSP with hypertrophic differentiation of chondrocytes and suggested that BSP could be a promoter of cartilage angiogenesis. The *ex vivo* part of the study allowed to detect an association between the presence of BSP in cartilage and the severity of this tissue degradation. In a previous work recently published[19], we showed that BSP was highly expressed, produced and released in the culture medium of hypertrophic OA chondrocytes and that its expression was correlated with markers of chondrocyte hypertrophy. Moreover, we showed that conditioned media from hypertrophic OA chondrocytes stimulated migration, invasion and adhesion of endothelial cells, three essential steps of the angiogenic process. We also demonstrated that an RGD domain protein was implicated in the adhesion of endothelial cells mediated by hypertrophic OA chondrocyte conditioned media. Consequently, results from our previous and present studies attested the potential role of BSP in OA physiopathology and particularly in cartilage vascularization. Indeed, in addition to its well described role in mineralization of bone, its upregulation in osteotropic cancers and its pro-angiogenic effect on endothelial cells, BSP may now be associated with cartilage degradation, chondrocyte hypertrophy and neovascularization of the articular cartilage in the pathology of OA. However, these conclusions should be tempered by the limitations of this study: (1) we lack of information regarding the hypertrophic differentiation and angiogenic activity of normal

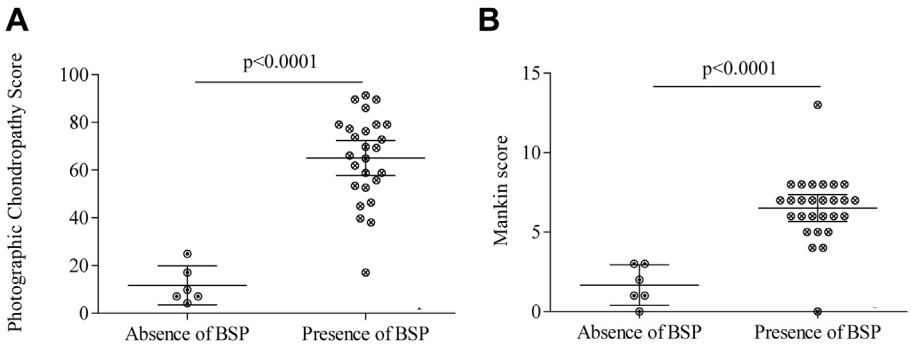

Fig. 5. Comparison of cartilage degradation scores between samples in which BSP was absent and those in which BSP was present. Cartilage samples come from biopsies from OA ($n = 24$) and non-OA ($n = 9$) patients. The photographic chondropathy score (PCS) was obtained based on the method described by Walsh *et al*[35]. The Mankin score was calculated as described in the Material and methods section. (A) PCS and (B) the Mankin score were higher in cartilage in which BSP was present ($P < 0.0001$). Data were analysed by Student's *t* test and are presented as mean ± 95% confidence interval.

chondrocytes; (2) we don't know the effect of BSP on healthy chondrocytes; (3) we can't convincingly explain why IL-1β and TNFα decrease BSP and finally (4) we failed to present a direct demonstration that BSP is the pro-angiogenic factor responsible for the angiogenic effect of hypertrophic chondrocytes. Regarding this last issue, one solution would be to block BSP activity by neutralizing antibodies in order to perform functional experiments.

Authors contributions

Study conception and design: L. Pesesse, C. Sanchez, D. Walsh, C. Baudouin, P. Msika, Y. Henrotin.

Provision of study materials and patients: J.-P. Delcour, D. Walsh.

Acquisition, collection and assembly of data: L. Pesesse.

Statistical analysis: L. Pesesse.

Analysis and interpretation of the data: L. Pesesse, C. Sanchez, D. Walsh, Y. Henrotin.

Critical revision of the article: all authors.

Final approval of the version to be submitted: all authors.

Conflict of interests

Caroline Baudouin is in charge of Research and Development in Cellular Biology and Scientific Communication at Expanscience and Philippe Msika is the Director of Innovation, Research and Development of Expanscience. This work was supported by an educational grant of Laboratoires Expanscience. The authors declare no other conflict of interest.

Role of the funding sources

This study was founded by Expanscience and the Fonds Leon Fredericq. Caroline Baudouin and Philippe Msika, from

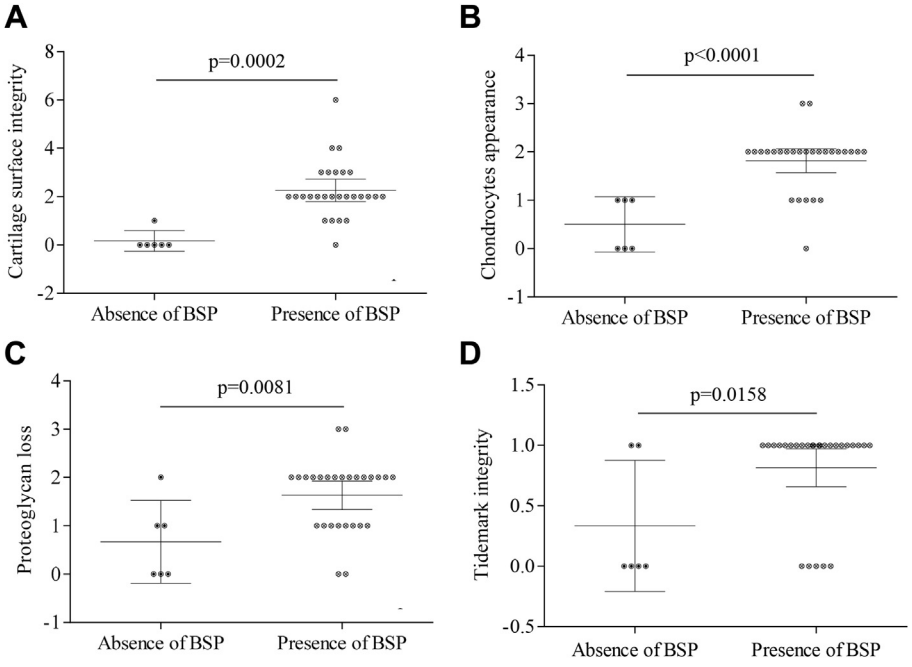

Fig. 6. Comparison of each component of the Mankin score between samples in which BSP was absent and those in which BSP was present. Cartilage samples come from biopsies from OA ($n = 24$) and non-OA ($n = 9$) patients. (A) Cartilage surface integrity (B) chondrocytes appearance (C) proteoglycan loss and (D) tidemark integrity were higher when BSP was present in cartilage samples ($P = 0.0002$, $P < 0.0001$, $P = 0.0081$, $P = 0.0158$, respectively). Data were analysed by Student's *t* test and are presented as mean ± 95% confidence interval.

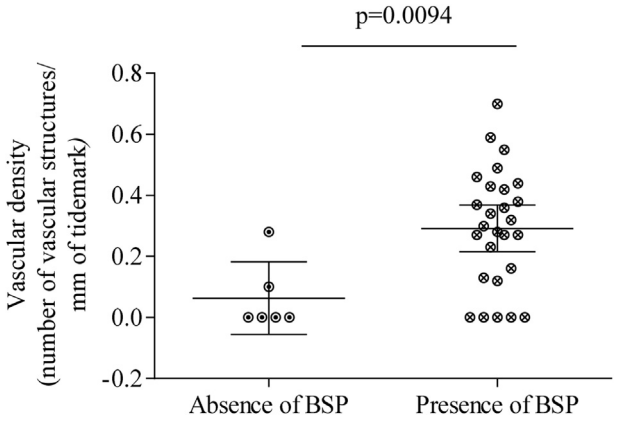

Fig. 7. Comparison of the vascular density between samples in which BSP was absent and those in which BSP was present. Cartilage samples come from biopsies from OA ($n = 24$) and non-OA ($n = 9$) patients. Vascular density was higher when BSP was present in cartilage samples ($P = 0.0094$). Vascular density corresponds to the ratio between the number of vascular structures and length of the tidemark (in millimetre). Data were analysed by Student's t test and are presented as mean ± 95% confidence interval.

Expanscience, contributed to the study conception and design, the critical revision of the article and final approval of the article. The Fonds Leon Fredericq was only a funding source and didn't contribute to this study content.

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