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Biglycan neo-epitope (BGN^{262}), a novel biomarker for screening early changes in equine osteoarthritic subchondral bone

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SUMMARY

Objective: Native biglycan (BGN), which can undergo proteolytic cleavage in pathological conditions, is well known to be involved in bone formation and mineralization. This study aimed to delineate the specific cleavage fragment, a neo-epitope for BGN (BGN^{262}), in synovial fluid (SF) from young racehorses in training, osteoarthritic (OA) joints with subchondral bone sclerosis (SCBS), and chip fracture joints.
Design: A custom-made inhibition ELISA was developed to quantify BGN^{262} in SF. Cohort 1: A longitudinal study comprising 10 racehorses undergoing long-term training. Cohort 2: A cross-sectional study comprising joints from horses ($N = 69$) with different stages of OA and radiographically classified SCBS. Cohort 3: A cross-sectional study comprising horses ($N = 9$) with chip fractures. Receiver operating characteristic (ROC) curve analysis was performed (healthy joints vs chip joints) to evaluate BGN^{262} robustness.
Results: Cohort 1: SF BGN^{262} levels from racehorses showed a statistical increase during the first 6 months of the training period. Cohort 2: BGN^{262} levels were significantly higher in the SF from severe SCBS joints. Cohort 3: SF BGN^{262} levels in chip fracture joints showed a significant increase compared to normal joints. The ROC analysis showed an AUC of 0.957 (95% C.I 0.868–1.046), indicating good separation between the groups.
Conclusions: The data presented show that BGN^{262} levels increase in SF in correlation with the initiation of training, severity of SCBS, and presence of chip fractures. This suggests that BGN^{262} is a potential predictor and a novel biomarker for early changes in subchondral bone (SCB), aiming to prevent catastrophic injuries in racehorses.

Introduction

Acute catastrophic fractures are a common cause of euthanasia in racehorses and can also cause serious injuries to the jockey[1]. Currently, there are no diagnostic tools that can aid in identifying the early stages of bone remodeling that can potentially cause acute fractures, since these subtle changes comprising asymptomatic micro fractures prior to catastrophic fractures of the bone cannot always be detected by radiography[2]. Other imaging techniques such as positron emission tomography (PET) and scintigraphy could be an option; however, these techniques are expensive and tedious to manage in a living horse[3]. Hence, there is a demand for serological markers that can aid in early screening of subchondral bone (SCB) sclerosis (SCBS) changes, development, and progression[4].

Bone sclerosis, a pathological consequence of osteoarthritic (OA) progression, starts in young racehorses and further correlates to strenuous training[5]. Fatigue-related bone pathology, a part of the OA progression, includes changes in the SCB microenvironment[6]. Fragmentation of the extracellular matrix (ECM) while undergoing remodeling and degradation (by the action of proteases: Matrixmetalloproteinases [MMPs] and A Disintegrin and

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Metalloproteinase with Thrombospondin motifs [ADAMTs] and non-proteolytic enzymes: hyaluronidases and heparanase) in OA joints is one of the most striking hallmarks of disease progression[7,8]. These circulating fragments or cleavage products are released into the bodily fluids (synovial fluid [SF], serum, urine, saliva) and can serve as potential pathological markers to assess the severity of disease progression.

Biglycan (BGN), one of the small leucine-rich proteoglycans (SLRPs) belonging to class I molecules, is found in both cartilage and bone, playing an important role in maintaining the structure and homeostasis[9]. Skeletal bone homeostasis relies on BGN as a modulator of osteoblast function since it promotes bone formation, is important in osteoclast precursors interactions, and is expressed in newly formed bone[10,11].

We previously identified dynamic changes in the cartilage secretome, regarding the release of different neo-epitopes at different time points during interleukin (IL-1)-β stimulation over 24 days[12]. Cartilage oligomeric matrix protein (COMP) fragments were observed early in the inflammatory process, with a peak at day three, followed by fragmentation of BGN. The new cleavage site of the fragmented BGN^{262} is ^{262}GLGHNQIRM with 100% homology across various species (humans, horses, cows, pigs, sheep, dogs, cats, rats, and mice), therefore the results obtained from this study can potentially be extrapolated to humans.

Early identification of bone lesions would enable timely intervention and altering of the horse's training accordingly, consequently limiting or preventing the progression of SCBS and micro fractures that can ultimately result in catastrophic intra-articular fractures.

This study aimed to develop an inhibition ELISA for the quantification of BGN^{262} in synovial fluid (SF) from young racehorses in training, normal joints and OA joints with SCBS, and chip fracture joints. We hypothesized that SF BGN^{262} concentration would increase in correlation to SCBS severity and the presence of micro fractures. Hence, we proposed BGN^{262} as a promising novel biomarker exclusively for SCB, with the aim of preventing catastrophic injuries in racehorses.

Methods

Horses and samples

The cohorts' SF was collected from the left middle carpal joint, which is prone to OA in race horses. These OA joints will represent burden of disease (B), and diagnosis (D), comprising two of the classifications within BIPED. This classification is used when conducting research into new biomarkers for joint diseases[13]. Ethical permission number for the studies performed is C62/13.

Cohort 1: A longitudinal study comprising ten Standardbred trotters (STB) monitored during a long-term (24months) training program. The horses were clinically evaluated at six visits, and computed tomography (CT) and radiographic examination of the carpal joints were performed at each visit (progression of disease, P; description in supplementary). The horses were thoroughly described in a previous study[14].

Cohort 2: A cross-sectional study comprising 69 horses (STB, Thoroughbred (TB), Mixed breed, Swedish Warmblood horses (SWH), Ponies) with normal joints or different stages (mild, moderate, and severe cartilage lesions) of macroscopic OA. Macroscopic OA was defined as joints with different severity of articular cartilage fraying, erosions and loss of cartilage with denuded bone. Radiographic examination of the carpal joints was performed on post-mortem bone specimens and the severity of articular SBCS was noted (Burden of disease, B; and Diagnose, D).

Radiographic examination of cohort 2's bone specimens' postmortems

Radiographic examination of the dissected distal row of the carpal bones was performed using a Cabinet X-ray system (Model no 43855A, Hewlett and Packard, Palo Alto, CA, USA) on envelope-wrapped mammographic film (Hewlett and Packard, FUJI UM-MA HC). The bones were radiographed in a proximo-distal projection. Exposure data were 3 mA, 75 kV, times 3 min, and F.f.d. 65 cm.

Radiographs were interpreted without prior knowledge of their identity, and were characterized according to the extent of sclerotic changes. Bones showing uninterrupted cortical bone with clear delineation between cortical and trabecular bone and distinct bone trabeculae of uniform thickness were considered normal and classified as 0 = non-sclerotic, 1 = mild sclerosis, 2 = moderate sclerosis, and 3 = severe sclerosis[15]. The cohort was thoroughly described in an earlier publication[16]. Immunohistochemistry (IHC) was performed on tissue samples from 11 horses selected from this cohort, including articular cartilage and adjacent SCB from the proximal and distal joint surfaces of C3.

Cohort 3: A cross-sectional study of 9 horses with chip fractures comprising cartilage lesions down to bone, diagnosed on arthroscopy of the middle carpal joint (Burden of disease, B). This cohort was comprised of racehorses (STB, TB), as described in a previous publication[17]. The SF from normal joints with macroscopically healthy articular cartilage and no radiological SCBS from cohort 2 was used for comparison (normal joints).

Sequence of the neo-epitope, BGN^{262} and the antibody production

The selected sequence of BGN^{262}, 15 amino acids long (^{262}GLGHNQIRMIENGSC), was recombinantly produced by GenScript Biotech, The Netherlands (Lot. 5763DL290-1/PE9501, Genscript). Briefly, the BGN^{262} peptide was reconstituted in distilled water to a concentration of 1 mg/ml, aliquoted, frozen, and stored at −80ºC until use. Specific primary (rabbit polyclonal and monoclonal) antibodies against the 15 amino acid sequence were raised.

BGN^{262} immunoassay

BGN^{262} inhibitory ELISA was developed and evaluated in equine serum and SF. Nunc MaxiSorp™ Clear Flat-Bottom 96-Well Plates (Invitrogen) coated with BGN^{262} peptide (1 μg/ml, 100μl/well, GenScript Biotech, The Netherlands), diluted in 100 mM carbonate buffer (pH 9.6) and incubated overnight at 4 °C, denoted as the ELISA plate.

The calibration standard was prepared from the stock of the BGN^{262} peptide (1 mg/ml). First, the highest standard point was set at 2 μg/ml by diluting in MultiBuffer (Kementech, Denmark), thereafter using 11-steps, 1:2 serial dilution (1 ml peptide + 1 ml MultiBuffer), the calibration standard was made ranging from 0 (the 11th with no peptide) to 2 μg/ml.

The samples and standards were diluted in MultiBuffer (Kementech, Denmark). Sample dilution was determined as 1:4 (SF) and 1:20 (serum) after analyzing the sample linearity, where the primary antibody found the most peptide. The spike and recovery has been determined using three individual horse serums and two individual horse SFs. As a control, we used Equidae serum (lot.2109875, Gibco).

The primary monoclonal antibody against BGN^{262} (0.681 mg/ml, lot: U8229DL260-6, GenScript) was diluted in MultiBuffer to a concentration of 30 ng/ml. Calibration standards and samples (100μl/well, in duplicate) were added to Thermo Scientific™ Sterilin™ Clear Microtiter™ Plates (Fisher Scientific). The primary antibody (100μl/well) was added to each standard as well as samples and pre-incubated overnight in a humid chamber within a rotation incubator (39 rpm) at 37 °C.

After 17 h, the coated ELISA plate was washed 4 times in the wash buffer (10 mM PBS with 0,05 % Tween, pH 7,4) using Tecan Hydro wash and thereafter blocked with synthetic blocker (Kementech, Denmark) for 30min at 37ºC. After blocking, the pre-incubated standards and samples (50µl/well) were transferred to the ELISA plate and incubated for 1 h at room temperature on an ELISA plate shaker set at 600 rpm. After incubation, the ELISA plate with primary antibody, standard, and samples was washed four times in wash buffer. The secondary polyclonal goat anti-rabbit (IgG) HRP 1 mg/ml (Abcam, ab97051) was diluted 1:50,000 in 10 mM PBS with 0, 05 % Tween and 0.1% BSA, pH 7, 4 and was added to the standard and sample wells (50µl/well) in the ELISA plate, incubated in the dark for 30 min on an ELISA shaker set at 600 rpm. Thereafter, the ELISA plate was washed eight times with wash buffer. Next the TMB (50µl/well) was added, incubated in the dark at RT, and the reactions were stopped after 20–30 min with 0.18M H_2SO_4 (50µl/well). Absorbance was evaluated at 450 nm using SPARK multifunctional plate reader with the Magellan software (Tecan). The specificity of the primary antibody against BGN^{262}, was evaluated using overlap peptide, OL, with the sequence KLYRLGLGHNQIRMIENGS as coating peptide and as antigen in the preincubation where serial dilutions were made similar to the calibration standard. The intra-assay precision was assessed using control serum (commercially purchased from Håtunalab AB, Håtunaholm, Sweden) in six replicates. The inter-assay was also examined for the control serum as six replicates in a total of three assays on different occasions.

Microscopic examination

The osteochondral samples were evaluated microscopically using the recommended assessments for OA in horses[18]. The scoring includes the articular cartilage, cartilage bone interface, SCB plate, and underlying SCB. The areas of the radial facet and the adjacent palmar part of the loaded proximal C3 were evaluated separately. Microscopic assessments of articular cartilage (total scores: 0–16) as: chondrocyte necrosis (0–4), chondrone formation (0–4), fissuring (0–4), and focal cell loss (0–4) and of bone (total scores: 0–10) as: osteochondral lesions (0–4), subchondral remodeling (0–3) and osteochondral splitting (0–3) were performed by a board-certified pathologist (SE) on haematoxylin & eosin (H&E) stained sections.

Immunohistochemistry (IHC)

The immunostaining protocol for BGN and BGN^{262} is described in detail in the supplementary methods.

IHC staining quantification

The BGN & BGN^{262} stained tissues were imaged at 200× magnification using a bright field microscope. Stained areas, including articular cartilage and underlying SCB, were quantified in photomicrographs (10–15 images per section), selecting areas of the radial facet and the adjacent palmar-loaded part of the proximal surface of C3, using the Fiji Image J program (ImageJ, National Institute of Health Bethsada, MD, USA). The data are expressed as fold change compared to the same areas with a score of 0 (control).

Statistics

Data are presented as the means with confidence intervals [CI]. The ELISA calibration standard curve fit was set as a 4-parametric with a logarithmic scale on the *y*-axis.

For cohort 1, a repeated measures mixed model was used. The BGN^{262} values were log-transformed. We included a random intercept for horses and allowed for a correlation structure using an AR^1 process for age in days. Age in days was considered a continuous variable. The residuals were evaluated to ensure that the model provided a good fit for the data.

For cohorts 2 and 3, one-way analysis of variance was used to test the difference in BGN^{262} values between the groups. The BGN^{262} values were log-transformed. Post-hoc tests were adjusted for multiple testing using Tukey's method (post-hoc *t*-tests) or Holm's method (post-hoc Wilcoxon rank-sum tests). The statistical analysis software R (https://www.r-project.org/) version 4.0.2 has been used.

Receiver operating characteristic (ROC) analysis was performed. The area under the curve was used to determine the specificity and sensitivity of the ELISA and robustness of BGN^{262} in distinguishing between samples from normal joints and chip fracture joints. Data were processed and evaluated using GraphPad Prism 9.0.1.

Statistical significance was set at $P < 0.05$. One asterisk (*) if $P < 0.05$; two (**) if $P < 0.01$; three (***) if $P < 0.001$ and four (****) if $P < 0.0001$.

Results

BGN^{262} ELISA

The primary antibody against BGN^{262} showed high specificity with clear inhibition for the BGN^{262} peptide however, when tested against OL peptide, where in the peptide is spanning over the cleavage site no inhibition has been observed. The primary antibody could not detect the OL peptide despite its high similarity with the neo-epitope (Supplementary Fig. 1). The assay was validated using equine serum (Supplementary Table 4).

The lowest and highest detection levels of BGN^{262} were 1.95 and 2000 ng/ml, respectively. The linearity test was performed using SF from three horses with dilution curves ranging from 1:2 to 1:64. The optimal dilution was 1:4. The intra-assay variation (CV %) was 13% ($n = 1$, $n = 6$), and the inter-assay variation was 14% ($n = 3$, $n = 17$). With the 50 ng/ml spiked serum from 3 different individual horses and 25 ng/ml spiked SF from 2 different individual horses resulted in a recovery between 80% and 120%.

Cohort 1

The BGN^{262} SF concentrations increased in young STBs during the 24-month training period, with a significant increase between visits 1 and 3 (142 [55–229] and 294 [156–432]; $P = 0.0224$). Thereafter, BGN^{262} continued to remain high for the rest of the training period (Fig. 1). There was no significant association between SF BGN^{262} levels, and radiographic examination of the joint (supplementray data table 2), and scintigraphic scores (data not shown). The BGN^{262} SF concentrations did not differ with the horses' age.

Cohort 2

The concentration of BGN^{262} in SF was significantly higher in moderate radiographic SCBS (score 2, 198 [126–271]) vs SCBS score 0, 119 [90–145]; $P = 0.0392$) (Table I; Fig. 2). However, there was no significant association between SF BGN^{262} levels in joints with different macroscopic cartilage defects[14]. The BGN^{262} SF concentrations did not differ with the horses' sex, breed, or age (supplementary data table 3).

Cohort 3

The SF BGN^{262} levels were significantly increased in chip fracture joints (548.2 [302–794]) compared to normal joints (120

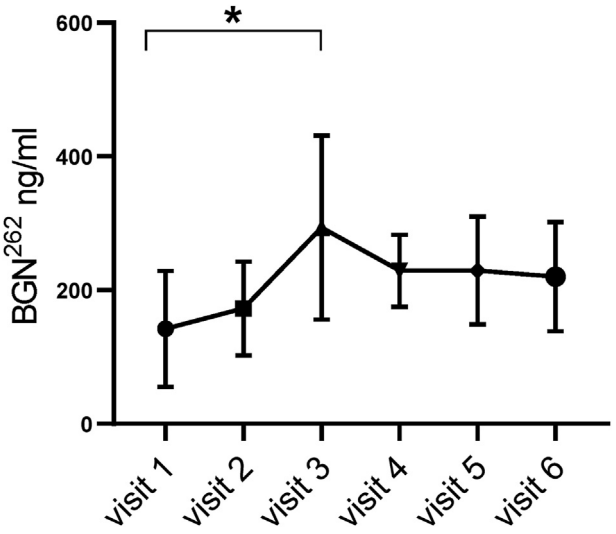

Fig. 1 Osteoarthritis and Cartilage

(Cohort 1) The data were shown as mean [C.I]. The concentration of the BGN^{262} increased in SF of the young Standardbred trotters during the 24-month training period with a significant increase between visit 1 and 3 ($P = 0.0224$) and thereafter the increase continues to remain high for the rest of the training period.

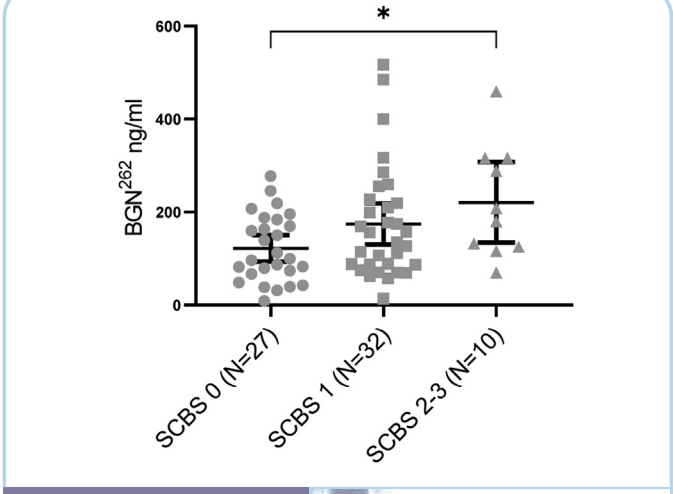

Fig. 2 Osteoarthritis and Cartilage

(Cohort 2) the data were shown as mean [C.I]. The Concentration of BGN^{262} in SF showed a significant increase in horses with radiographic (sub-chondral bone sclerosis) SCBS (2–3) vs SCBS (0) ($P = 0.0392$).

ID	rSCBS	OL	SR	OS	Total
10A	0	0/0	0/0	0/0	0/0
53B	1	0/0	0/0	0/0	0/0
51A	1	0/0	0/0	0/0	0/0
29	0	0/0	0/0	0/0	0/0
32	1	0/0	0/0	1/0	1/0
55A	2	0/0	0/0	1/0	1/0
66B	3	0/0	1/0	1/1	2/1
16B	1	1/0	1/0	3/0	5/0
19B	2	2/0	2/0	2/0	6/0
71A	1	3/0	1/0	3/0	7/0

Scoring (0/0) = radial facet/adjacent palmar area.
OL, Osteochondral lesions; SR, Subchondral remodeling; OS, osteochondral splitting; rSCBS, radiographic subchondral bone sclerosis.
Histological grading of explants. Microscopic assessments of bone: osteochondral area (0–10) as osteochondral lesions (0–4), subchondral remodeling (0–3) and osteochondral splitting (0–3) were performed as previously described by McIlwraith *et al.* 2010[11] using hematoxylin & eosin (H&E) staining.

Table I Osteoarthritis and Cartilage

Grading of the proximal subchondral bone of the third carpal bone in horses

[77–163]; $P < 0.00002$) (Table I; Fig. 4(a)). The BGN^{262} SF concentrations did not differ with the horses' sex, breed, or age.

ROC analysis was carried out to evaluate the discriminative capacity of BGN^{262} between the normal and chip fracture joints. The area under the curve was 0.957 [0.868–1]; $P < 0.0001$, indicating a good separation between the groups [Fig. 4(b)].

Microscopic evaluation of the articular cartilage of C3

The articular cartilage was graded histologically and assigned different grades, as shown in Supplementary Table 1. The articular lesions were clearly focal and found in the radial facet area (Fig. 3(a) H&E). Only 1 horse (51A) had equal cartilage grading in the radial facet and the adjacent palmar area.

The osteochondral samples were assessed separately for bone changes and assigned to different grades (Table I). SCB lesions were focal and present in the radial facet area. A mild osteochondral splitting could be identified in the adjacent palmar area of only 1 horse (55A). The histological grading of the radial facet area revealed four groups with increasing bone involvement: four joints with grade 0, three joints with grades 1–2, two joints with grades 5–6 and one joint graded as 7. All horses with grade 5–7 had osteochondral lesions, subchondral remodeling, and osteochondral splitting (Table I).

Immunolocalization of BGN^{262} in the cartilage and the subchondral bone

The immunostaining results of the radial facet and the adjacent palmar areas are described below.

Normal: BGN staining can be seen in the osteoid outlining some of the bone trabeculae of the intertrabecular areas of the SCB, but not in the superficial bone plate close to the calcified cartilage. BGN^{262} is found in a few osteocytes of the bone trabeculae, bone lining cells, and endothelial cells within the intertrabecular areas of the SCB and is both nuclear and cytoplasmic. The ECM of the superficial and middle layers of the non-calcified articular cartilage stain for BGN in the pericellular, territorial, and interterritorial matrix. Only cytoplasmic immunostaining for BGN^{262} was observed

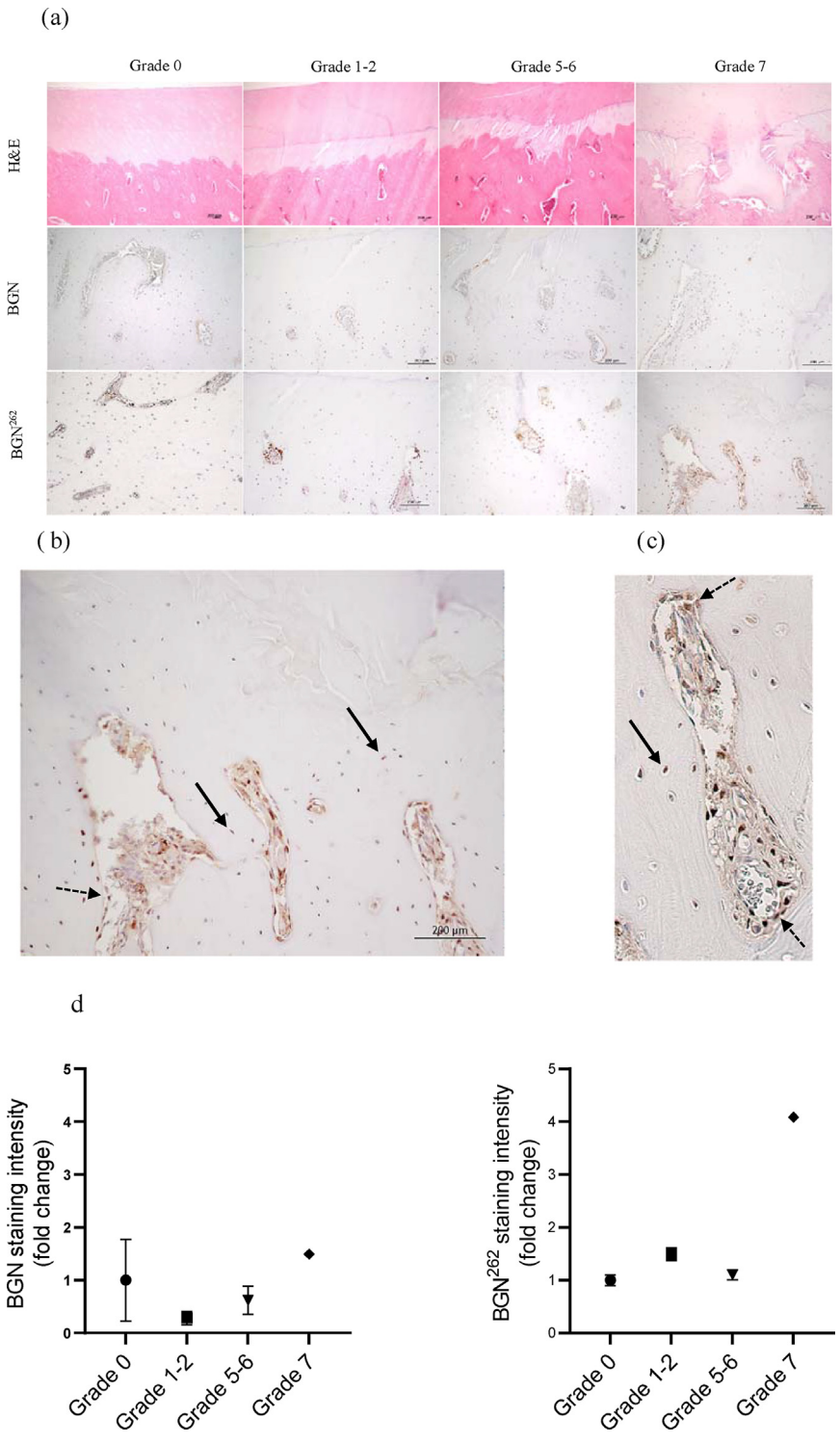

Fig. 3

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3a: Upper panel shows H&E staining of radial facet area with various grades of cartilage and bone changes. Middle panel BGN staining (IHC) of subchondral bone radial facet. Lower panel BGN^{262} staining (IHC) of subchondral bone radial facet. 3b & 3c: BGN^{262} staining of subchondral bone radial facet (grade 7) with IHC (20×) image. Arrow showing cytoplasmic and intra-nuclear stainig of osteoblasts (solid arrow) and bone lining cells (dashed arrow). 3d (cohort 2) BGN and BGN^{262} IHC quantification of subchondral bone of radial facet and adjacent palmer area of the third carpal bone (C3).

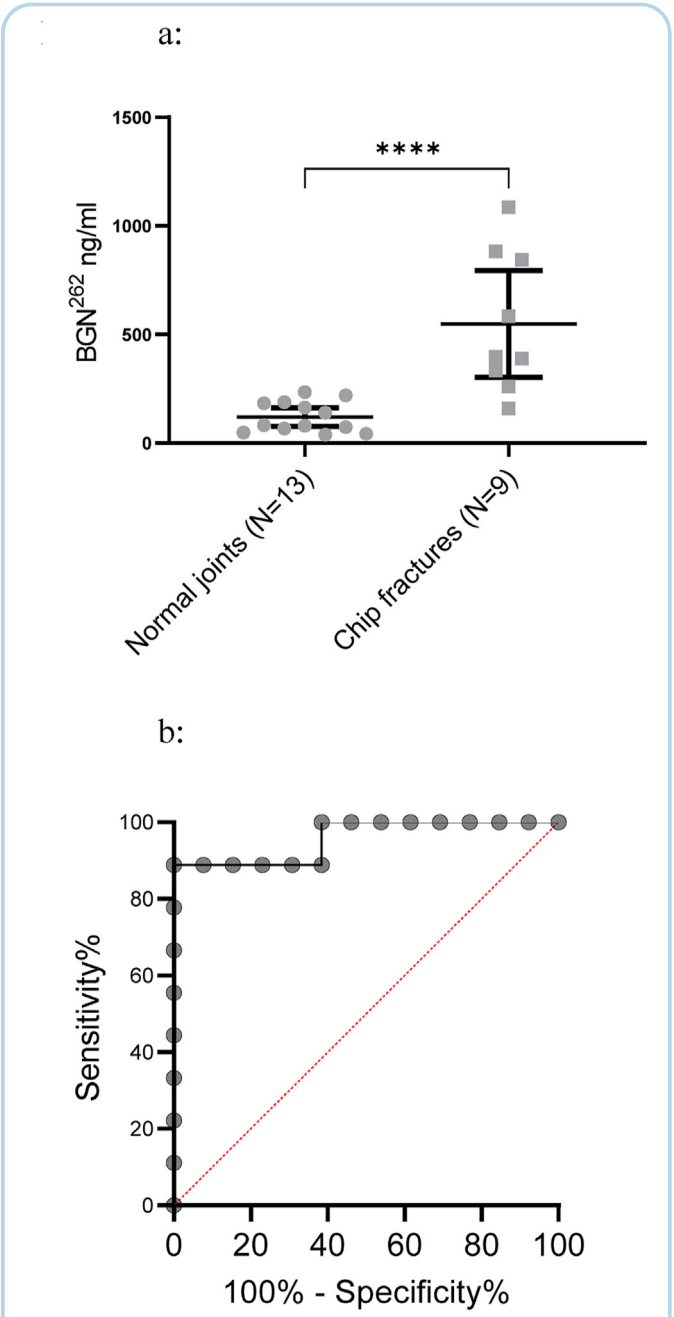

Fig. 4 Osteoarthritis and Cartilage

4a (cohort 3) the data were shown as mean [C.I]. The Concentration of BGN^{262} in SF showed a significant increase in joints with chip fractures compared to normal joints ($P < 0.0001$). 4b (cohort 3) the receiver operating characteristics curve (ROC) analysis was performed. The area under the curve was used to determine the specificity and sensitivity of the ELISA and how well the assay can distinguish between samples from normal joints and joints with chip fractures. The area under the ROC curve (AUC) was 0.957 (95% confidence interval 0.868 to 1.000; $P = 0.0004$), indicating there was a good separation of BGN^{262} concentration in synovial fluids between the two groups.

in the chondrocytes of the superficial layers of the non-calcified articular cartilage (data not shown).

Grade 1–2: BGN staining is found in the osteoid outlining the bone trabeculae, mostly in the SCB, but focally in the osteoid of the SCB plate close to the overlying calcified cartilage. BGN^{262} is seen in a few osteocytes close to the intertrabecular spaces. It is also present in bone lining cells, superficial osteoid, and endothelial cells of some intertrabecular areas, similar to the normal sections. The staining of the articular cartilage was similar to that of the normal cartilage/bone sections.

Grade 5–6: Staining with BGN is concentrated to the osteoid outlining the intertrabecular spaces in the bone throughout the section. Staining with BGN^{262} is found in osteocytes, bone lining cells, and osteoblasts (larger cells outlining the bone trabeculae) of the SCB and focally in the SCB plate, mainly adjacent to the OA cartilage of the radial facet. The immunostaining of the cartilage was similar to that in the normal section, except for nuclear staining of a few chondrocytes close to the OA lesion.

Grade 7: Clear staining of BGN is found in the osteoid outlining the bone trabeculae in the entire bone and in the underlying bone of the OA cartilage. The staining of BGN^{262} is present in many osteocytes (nuclear and cytoplasmic), bone-lining cells, osteoblasts, osteoid, and endothelial cells in the bone underlying the OA lesion [Fig. 3(a)].

The BGN antibodies stained the ECM of non-calcified articular cartilage. There is also clear nuclear and cytoplasmic staining of BGN^{262} in chondrocytes within the OA lesion.

Quantification of staining intensity for BGN and BGN^{262} in the non-calcified articular cartilage did not show any correlation with OA grading of the cartilage (data not shown). However, there was an increasing trend of intensity in the BGN^{262} staining associated with higher scores of the osteochondral lesions in both the radial facet and adjacent palmar areas [Fig. 2(b) and (c)].

Discussion

Physiological bone sclerosis develops in racehorses during training, which can progress to pathology with the presence of micro fractures, eventually leading to acute chip fractures[19]. Fatigue-related bone pathology is part of the OA process, with changes in the SCB microenvironment[5]. Currently, it is not possible to diagnose the early stages of bone remodeling that lead to micro fractures associated with pain and subsequent lameness. Thus, subtle sclerotic changes comprising micro-fractures prior to catastrophic fractures of the bone cannot be detected in a live horse with currently available imaging techniques[20].

The ongoing structural and molecular changes in the bone are of crucial importance in understanding the initiation and pathogenesis of OA[5,21]. Therefore, there is an urgent need for diagnostic biomarkers for early screening of SCB changes. The results from our study in young racehorses (cohort 1) showed an initial increase in SF BGN^{262} levels during the first 6 months of training, and this increase remained steady for the rest of the training period. There was no association between BGN^{262} levels and radiography score throughout the training period (Table II). Interestingly, by the end of the training period (visit 6), the mean radiographic score was 1.1. This agrees with data from cohort 2, where there was no association between BGN^{262} levels and the mild SCBS group (radiography score of 1); Of note there was a significant correlation with the severe SCBS group with a score of 2–3. This highlights the potency of BGN^{262} as a more sensitive indicator than radiographic changes of early ongoing SCB changes in horses undergoing training. Thus, our study presents a potential biomarker for predicting early bone

Cohort	Age	Breed STB, SWH, TB, CB	Sex (S (G) M)	SCBS Radiography score	BGN^{262} (ng/ml)
1	Visit 1-6	10,0,0,0	5(0)5	Visit 1-6	Visit 1-6
$n = 10$	607[587–626]			0.3	142[55–229]
	689[662–715]			0.3	173[102–243]
	773[740–807]			0.5	294[156–432]
	901[877–926]			0.6	229[175–283]
	1,012[987–1,037]			0.8	229[149–310]
	1,299[1,240–1,358]			1.1	220[138–131]
2					
$n = 69$					
	5.2[3.2–71]	15,9,1,2	11(0)6	0 ($n = 27$)	122 [93–150]
	5.8[4.5–7.2]	22,7,1,2	20(9)3	1 ($n = 32$)	174 [130–218]
	5.9[3.1–8.6]	7,1,3,0	8(1)1	2 ($n = 10$)	221 [134–308]
3					
$n = 21$					
Normal joints ($n = 13$)	2.9[1.9–3.8]	6,6, 1,0	5 (7) 1	0 ($n = 13$)	120 [77–163]
Chip fracture ($n = 9$)	4[3.3–4.7]	6,0, 3,0	5 (1) 3	NA	548.2 [302–794]

*cohort 1-age in days; cohort 2 & 3 – age in years.
Age, sex and breed distribution of the different cohorts. Values for age are given as mean [C.I]. Values for sex equal the number of horses.
STB, Standard bred trotters; SWH, Swedish Warmblood horses; TB, Thoroughbred horses; CB=Crossbreed, S, stallion; G, gelding; M, mares; n, number of horses: SCBS, subchondral bone sclerosis. Radiography score expressed as mean.

Table II

Demographics of the cohorts

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changes *in vivo*. This knowledge can enable us to prevent or stop OA development and progression at an early stage. There are similar studies describing neo-epitopes in several pathologies derived from degradation of ECM components such as COMP, collagen type I, collagen type II, collagen type X, collagen type VI, and BGN both *in vivo* and *in vitro*[21–27]. These neo-epitopes can be of great diagnostic value as they can be disease specific, and more precisely stage-specific, unlike their parent counterparts.

There are studies suggesting SCB has a possible role in post-traumatic OA[28]. The SCB osteoblasts can induce phenotypic changes in the chondrocytes of the OA articular cartilage[29,30]. This effect is a result of pathological bone/cartilage crosstalk, which eventually orchestrates OA progression[31]. Our data shows that BGN^{262} is present in OA articular cartilage, which is in line with the fragmentation of BGN in cartilage reported in advanced OA and RA.[32,33]

A recent study of post-traumatic OA in mice found that the small proteoglycans – decorin and BGN have different activity – with decorin playing a role in cartilage degeneration and BGN in regulating SCB tissue, respectively[34]. Our results are in line with this. The severity of SCB changes was associated with increased BGN^{262} bone staining and high SF concentrations with increased radiographic SCBS score, indicating that soluble BGN^{262} is being released predominantly from bone. Moreover, no association between BGN^{262} and the progression of articular cartilage damage was found (cohort 2).

In this study, we examined the middle carpal joints. SCB sclerosis of the third carpal bone (C3) is seen in racehorses, often in combination with clinical lameness, with a higher grade of sclerosis more likely to be present in lame horses[35,36]. Furthermore, the OA lesions of the carpal bones can lead to micro-fractures, with subsequent intra-articular fractures, which can result in catastrophic injuries[5,37–39]. Hence, there is an urgent need for biomarkers that can aid in screening racehorses in training that are at increased risk of sustaining such fractures. We found a significant increase in SF BGN^{262} levels in chip fracture joints compared to normal joints, with the ROC analysis indicating a good separation between the groups (cohort 3). It was also evident that the SF concentration did not change with the horse's age, sex, and breed. This substantiates BGN^{262} as a promising biomarker, which can aid in screening horses undergoing training and hopefully prevent catastrophic injuries. There are risks of infections associated with SF collection but in terms of preventing catastrophic injuries benefits might outweigh the risk. The future studies are focused on validation of BGN^{262} in serum and saliva which are relatively easy to collect and more feasible for screening of the horse.

Our IHC results show both nuclear and cytoplasmic expression of BGN^{262} in chondrocytes, osteocytes, and bone lining cells including osteoblasts within osteochondral lesions, unlike uncleaved BGN, which is extracellularly distributed. This is in accordance with our previous study on COMP fragmentation in articular cartilage from equine OA joints and atherosclerosis in humans, which indicated that the COMP neo-peptide is localized intracellularly, whereas the native COMP is present in the ECM[23,40]. This indicates an internalization of the neo-epitopes post-fragmentation.

BGN^{262} could therefore be a matrikine with different biological functions from the native molecule. Matrikines are small bioactive peptide fragments of ECM proteins that are cleaved off during the inflammatory process, which can be internalized or bind to specific receptors to regulate multiple cellular activities[41]. BGN functions as a signaling molecule by binding ligands such as TGFβ, BMP2/4, LRP6, and FGF2, and plays a crucial role in inflammation by binding to TLR4/TLR2, thereby modulating various cellular functions in health and disease.[42–45]

The race horses undergo slow but progressive changes pertaining to OA at a very young age as a consequence of training which can be similar to human athletes. In conclusion, the horse could be a relevant model for human OA, since post-traumatic OA is common in horses like it is in humans; additionally, there is extensive data available from OA clinical trials that can help understand and decipher the cause and progression of OA at an early

stage, which would not be possible in human subjects[46–49,13,23,24]. Longitudinal monitoring of BGN^{262} in racehorses may identify horses with increasing bone sclerosis/osteochondral lesions at an early stage. Currently, the available diagnostic tools are expensive and can identify the damage caused only upon some serious clinical/physical indications, which eventually are irreversible. Altering the training regimens in racehorses with high concentrations of BGN^{262} in the SF may help prevent catastrophic injuries. To make this biomarker a more viable, easy to use option, serum levels must also be established in racehorses. Further mechanistic and functional studies are needed to establish the biological activity of BGN^{262}. Since the BGN^{262} cleavage site is conserved in all species, including humans, it could possibly be of high clinical significance to validate this for human use by screening large OA cohorts with varying degrees of SCBS pathology.

Author contributions

ES, SE, AL and SA were in charge of the overall direction and planning. UJ and ES set up, validated the inhibition ELISA, and UJ ran all the samples. SA performed all IHC and WB experiments. SE evaluated the histology, including IHC. JL performed the statistical analysis. All authors contributed to and approved the final manuscript.

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Conflcit of interest

SE, AL, and ES are stakeholders of SGPTH Life Science holding the patent covering the BGN ^{262} neo-epitope. The other co-authors have no conflicts of interest to declare.

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Supplementary data

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