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Subchondral bone microdamage accumulation in distal metacarpus of Thoroughbred racehorses

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Summary

Background: Microdamage accumulation leads to subchondral bone injury and/or fracture in racehorses. An understanding of this process is essential for developing strategies for injury prevention.

Objectives: To quantify subchondral bone microdamage in the third metacarpal bone of Thoroughbred racehorses at different stages of the training cycle.

Study design: Cross-sectional.

Methods: Bone blocks from the palmar aspect of the medial condyles of third metacarpal bones from 46 racing Thoroughbred horses undergoing post mortem were examined with micro computed tomography (microCT) to detect calcified microcracks, and light microscopy to quantify bulk stained microcracks. Racing and training histories were obtained for comparison with microdamage data using regression modelling.

Results: Subchondral bone microcracks were observed in all bones with at least one method. Microdamage grade was greater in older horses, levelling off for horses five years and older (quadratic term P = 0.01), and with lower bone material density in the parasagittal groove (P = 0.02). Microcrack density was higher in older horses (P = 0.004), and with higher bone volume fraction (BV/TV) in the parasagittal groove in horses in training (interaction effect, P = 0.01), and lower in horses resting from training (P = 0.02).

Main limitations: Cross-sectional data only. Incomplete detection of microdamage due to the limits of resolution of microCT and lack of three-dimensional imaging with microscopy. Multicollinearity between variables that indicated career progression (e.g. age, number of career starts, duration of training period) was detected.

Conclusions: Fatigue damage in the distal metacarpal subchondral bone is common in Thoroughbred racehorses undergoing post mortem and appears to accumulate throughout a racing career. Reduced intensity or duration of training and racing and/or increased duration of rest periods may limit microdamage accumulation. Focal subchondral bone sclerosis indicates the presence of microdamage.

Introduction

Subchondral bone injury is common in racehorses at sites subjected to high magnitude cyclic loading such as the dorsal aspect of the carpal joints and the palmar aspect of the condyles of the third metacarpal bone in fetlock joints [1; 2]. Subchondral bone injuries occur due to the accumulation of bone fatigue which results in microcracks that develop at the calcified cartilage/hyaline cartilage interface and then propagate into the subchondral bone plate [3-5]. Damage can remain confined to the subchondral bone, propagating transversely and resulting in focal subchondral bone injury, or propagate into the trabecular bone to form fractures, some of which may be catastrophic [5; 6].

Due to the difficulty of detecting microdamage *in vivo* before significant subchondral bone injury develops, and the challenge of treatment once it occurs, minimising such damage in racehorses is highly desirable. An understanding of the factors contributing to the accumulation of subchondral bone microdamage, particularly those that might be modifiable, would inform management strategies more likely to successfully reduce its frequency. Previous studies investigating risk factors for subchondral bone injury have used gross pathology as the outcome rather than the more sensitive quantification of microdamage [2; 7]. All methods for detecting microdamage have limitations. Preparation of bone for histological examination can result in the formation of artefactual cracks in the sample. In order to differentiate cracks formed *in vivo* from those induced by sectioning, bone blocks are bulk stained with basic fuchsin before undemineralised sections are cut however this does not prevent cracking from dehydration during the staining process [8; 9]. In addition, a proportion of cracks in equine subchondral bone contain highly mineralised material which is easily observed in undemineralised blocks with backscattered electron microscopy or microCT, however this technique will not allow all cracks to be identified [9; 10]. Alternatively, examination of hydrated undemineralised blocks using bright field and differential interference contrast (DIC) microscopy avoids the induction of artefactual cracking via dehydration but not from sectioning [11].

Microdamage has been quantified in single sections from the lateral and medial condyles of the third metacarpal bone in small numbers of Thoroughbred race horses using a single method (DIC or basic fuchsin bulk staining) [11; 12]. Another study of 38 horses examined one type of microcrack in only two dimensions and found that higher densities of calcified microcracks were associated with increasing age [13]. In the study reported here we examine a new and larger cohort of metacarpi using two methods to detect microdamage in order to mitigate the limitations of each and allow more extensive examination of the parasagittal groove and condyles: microCT to quantify microdamage in three dimensions, and bulk stained sections to quantify all microcracks rather than just those that contained calcified material. Gross injury to the distal metacarpus can affect both lateral and medial aspects with subchondral bone injury more common on the medial condyle and condylar fractures more common in the lateral parasagittal groove and condyle [2; 14]. We examined medial condyles because subchondral bone injuries are more prevalent than condylar fractures [2]; we aimed to investigate the effect of previous training history on the quantity of microdamage present

and hypothesised that greater microdamage would be associated with greater racing career duration and greater duration of the current training period at the time of sampling.

Materials and methods

The sample size required to estimate the prevalence of microdamage in the population of Victorian racehorses was determined to be at least 34 horses (population size 8,828) for an estimated true prevalence of 90%, with a precision of 10%, and a confidence level of 95% [15; 16].

The medial condyle and parasagittal groove of a single third metacarpal bone were obtained from 46 racing Thoroughbred horses: 26 in training and 20 resting from training at the time of death. Resting horses were included to determine whether the increased subchondral bone remodelling rates associated with lower levels of activity were associated with microdamage levels [17]. Where the cause of death involved a fetlock injury (n = 4) the contralateral metacarpal bone was used otherwise the limb was chosen using a random number table [18]. There were 23 left and 23 right forelimbs. The palmar-distal aspect of the metacarpal bones was removed by cutting the bone at 55° to the dorsal plane (dorsodistal-palmaroproximal) through the centre of rotation of the condyles. Samples were stored in 70% ethanol until processing.

Racing and training histories were obtained from an official racing database (Racing Australia Limited) and by telephone conversation with trainers. All horses were either in a race preparation (i.e. were racing or training with the intention of racing and had been exercised within one week of sample collection) or, in the case of resting horses, had previously undergone at least one preparation up to galloping speed but had not been exercised for at least one week. Of the 26 horses in training, 15 were male (eight castrated) and 11 female; ages ranged from two to seven years (mean ± s.d., 4.42 ± 1.72) and each had raced 0 to 59 times (median; 25-75th percentile, 9.0; 1.00-16.0). The duration of the training period at the time of death in these horses was three to 26 weeks (12.0; 8.75-17.0 weeks). Of the 20 resting horses there were 13 males (nine castrated) and seven females resting between one to 14 weeks (4.0; 3.0-8.0 weeks). Ages ranged from two to seven years (3.50 ± 1.40) and each had raced 0 to 54 times (4.0; 0.0-8.0). Additional details of the histories and causes of deaths of horses are available in Supplementary Item 1.

Palmar osteochondral disease (POD) was graded as previously described [19]. Medial and lateral condyles were separated at the sagittal ridge, then from each medial condyle, bone blocks measuring 10 mm in dorsopalmar width and 10 mm deep at the most distal point of the condyle were cut using a low speed saw (Isomet saw[a]). Each block was imaged with micro computed tomography (micro CT) (Scanco 50[b]) at 70 kVp, 200 µA and a voxel size of 20 μm^3. Further higher resolution microCT images of the subchondral bone underlying the parasagittal groove and centred at the deepest point of the groove, were also obtained at 70 kVp, 200 µA and a voxel size of 4.4 μm^3. All microCT was acquired in a sagittal plane and then reformatted to enable viewing in multiple planes, with the whole volume examined for microdamage. On microCT images, microcracks were identified as bright linear areas within the bone and are referred to as calcified microcracks in the manuscript (Fig 1). Microdamage in whole condyles was estimated using a grading system based on the number of microcracks and larger fractures observed (Table 1). Calcified microcracks in the higher resolution images of the parasagittal groove were counted. Calcified projections from the calcified cartilage surface into the hyaline cartilage and parasagittal fissures were recorded as present or absent. Bone volume fraction (BV/TV) and bone material density (density of mineralised tissue) were obtained for volumes of interest (VOI) drawn within each acquired volume using the Scanco microCT evaluation algorithms. This approach avoids edge effects and focuses on areas that typically undergo densification in response to loading [14]. The VOI for the lower resolution images was defined by selecting a region measuring 6 mm in dorsopalmar width and 6 mm in distoproximal depth at the most distal point of the condyle and then extending it transversely as far as the sagittal plane through the most proximal point of the condyle (the deepest point of the parasagittal groove; Fig 2A). The VOI defined for the high resolution microCT of the parasagittal groove was derived by selecting the central region (6 mm in dorsopalmar width and 6 mm in distoproximal depth) of each of the 600 sagittal slices centred around the deepest point of the groove (equivalent to 2.64 mm of mediolateral width of the parasagittal groove; Fig 2B).

For bulk staining, the whole bone blocks prepared for microCT were incubated in 0.2 M NaOH for 48 hours to remove the hyaline cartilage, then stained in 1% basic fuchsin in ascending grades of ethanol (80%, 90% and 100%) under vacuum (20 - 30 mm Hg) for 18 days. Bulk stained samples were embedded in methacrylate resin and oblique dorsal sections (70-90 µm) were obtained from the middle of the embedded bone blocks using a low speed saw. Bone sections were mounted onto glass slides and cover slipped using Eukitt® mounting medium[c].

High magnification images of bone sections were obtained by brightfield microscopy (Olympus BX 60[d]) and stitched using image analysis software (ImageJ[e]). Pre-existing microcracks were identified as oblique linear discontinuities extending from the calcified cartilage into the subchondral bone, and with a surrounding halo of deep basic fuchsin staining [20; 21] (Fig. 3). Microcrack density was calculated on bone sections as previously described [13]. The entire length of the calcified cartilage surface was measured, then stained microcracks that intersected with this interface were counted and expressed as the number of microcracks per millimetre (microcrack density).

Data Analysis

Five outcome variables indicating the presence and degree of microdamage were assessed – microdamage, calcified projections, parasagittal fissure, microcrack density (mm), and number of calcified microcracks. To investigate associations between outcome and explanatory variables, we fitted various regression models – an ordered logistic regression for the outcome variable microdamage (graded 0-4); logistic regression for the binary outcomes calcified projections and parasagittal fissure (graded present or absent); and linear regression for the continuous outcome variables microcrack density (mm) and number of calcified microcracks.

Explanatory variables included categorical terms for limb, sex, training status, presence of catastrophic fracture, and Australian and New Zealand classifications of race distance (whether the horse's longest race in its last five starts was short [≤1300 metres], middle [1301-1800 metres], intermediate [1801-2100 metres], long [2101m - 2700 metres], or extended [>2700 metres]). For analysis, we combined middle and intermediate ('middle') and long and extended ('staying'). Horses that had not yet raced were classified as 'unraced'. Continuous terms were also included for age, number of race starts, prize money earned, time in training since last rest period (weeks), time resting since last period of training (weeks), number of races in last 30 days, and time in days between last two starts including the start in which the horse died or was euthanased on a race day. Linear relationships between continuous outcomes and predictors, and between predictors significant in univariable analysis, were assessed by generating scatter plots. Relationships between binary and ordinal outcomes and continuous predictors were assessed using box and whisker plots. Prize money earned and time in days between last two starts were log-transformed for analysis.

Explanatory variables with unconditional associations significant at the P<0.30 level in univariable models were selected for inclusion in the multivariable models. Models were run with and without microCT variables included. Explanatory variables that were not significant (P>0.05) were removed from the model one at a time, beginning with the least significant. Excluded variables were re-entered into the final model and retained if they changed the estimated regression coefficients by more than 20%. Model diagnostics performed included the link test to identify model specification error, examinations of tolerance (<0.1) and variance inflation factor (VIF>10) to assess presence of collinearity within the models, and selection of final models based on lower Akaike information criterion (AIC) and Bayesian information criterion (BIC) compared to possible alternate models. Continuous variables were further checked for linearity in the regression models using the Box-Tidwell method. For the ordered logistic regression model for microdamage grade we assessed the proportional odds assumption and fitted a quadratic term for age in the final model. Multicollinearity within the models was detected, particularly between variables that indicated career progression. We chose not to deal with multicollinearity directly within the models, but we further assessed the relationship between age and other continuous variables that were statistically significant in univariable analysis by generating Pearson's pairwise correlation coefficients. Differences between mean age for binary variables (in training, raced) were assessed using Student's t-test. Due to model specification error in the microcrack density model, we assessed two-way interaction terms between the remaining main effects that were significant in multivariable analysis. Assumptions of normality and homogeneity of variance were checked by examining histograms of residuals from the final multivariable models and examining plots of residuals versus predicted values. Odds ratios (OR) and coefficients with their 95% confidence intervals (CIs) are presented for the logistic and linear regression models, respectively. Stata/SE 14.2 for Windows[f] was used for statistical analyses.

Results

Evidence of gross subchondral bone injury (POD grades 1-3) on the articular surface of medial condyles was observed in 18/46 horses. In all bones examined, subchondral bone microdamage was observed with at least one method, either microCT or light microscopy. Stained microcracks were observed in 43/46 bones with light microscopy. The three bones without stained microcracks were from three 2-year-old horses, two of which had not yet started in a race and one of which had started in one race. In all three of these horses, one or

two calcified microcracks were observed on microCT. POD grade was associated with microdamage grade (P = 0.006), but not microcrack density (P = 0.9) or calcified microcracks (P = 0.6). Transverse subchondral bone fractures were observed in three horses two of which had no evidence of gross subchondral bone injury on the articular surface (Fig 4). All were experienced racehorses four to five years old, which had started in a race from five to 25 times. Parasagittal fissures were observed on microCT in 14 horses which were 2-7 years of age and had started a race 0-54 times.

Calcified microcracks were most commonly identified in the parasagittal groove on microCT whereas transverse fractures were identified at different sites across the condyle (Fig 4). Both calcified and bulk stained microcracks were predominantly oriented at 35° to 60° to the articular surface (Figs 1A and 3) as well as slightly oblique to the sagittal plane along the parasagittal groove (Figs 1B and 1C). Calcified projections extending from the calcified cartilage into the hyaline cartilage were observed in 13/46 horses (Fig 5). These structures were always continuous with a calcified microcrack and were most common in the parasagittal groove region. Multiple resorption spaces were observed in a number of the horses resting from training, some of which were immediately adjacent to microcracks (Fig 3B).

Results of univariable analysis are presented in Table 2. In multivariable analysis, microdamage grade was higher in older horses, levelling off for horses five years and older (linear term OR 27.47; 95% CI 3.57, 211.55; P = 0.001; quadratic term OR 0.75; 95% CI 0.60, 0.93; P =0.01), and in horses with lower bone material density in the parasagittal groove (OR 0.96; 95% CI 0.93, 0.99; P = 0.020). Microcrack density was higher in association with older age (Coef. 0.12; 95% CI 0.04, 0.21; P = 0.004), and in horses in training compared with those resting from training (Coef. 0.39; 95% CI 0.06, 0.72; P = 0.02). In addition, microcrack density was higher with higher BV/TV in the parasagittal groove, but only for horses in training (Fig 6; horses in training main effect, Coef. -3.38; 95% CI -6.29, -0.47; P = 0.02; BV/TV in the parasagittal groove main effect, Coef. 1.37; 95% CI -1.73, 4.47; P = 0.4; interaction term, Coef. 5.03; 95% CI 1.12, 8.94; P = 0.01). Calcified projections were associated with the number of calcified microcracks in the parasagittal groove (OR 1.31; 95% CI 1.11, 1.55; P = 0.001). Parasagittal fissures were more common in right forelimbs (OR 6.21; 95% CI 1.29, 29.83; P = 0.02) and were associated with higher microdamage grade (OR

2.24; 95% CI 1.05, 4.78; P = 0.04). Calcified microcracks in the parasagittal groove were not associated with any of the explanatory variables (data not shown).

Multicollinearity between variables that indicated career progression, such as age, number of starts, frequency of racing, duration of current training period and time resting was detected (see Supplementary Item 2). It was difficult therefore to distinguish which variables were most important biologically and worthy of inclusion in multivariable analysis.

Discussion

By detailed quantification of calcified cartilage and subchondral bone microdamage underlying the articular surface of the palmar aspect of the medial metacarpal condyle and parasagittal groove using multiple techniques, we have extended the findings of previous studies showing that microdamage is prevalent in a population of Thoroughbred race horses presenting for post mortem. We found that microdamage becomes more extensive with career progression, in those in race training compared with resting horses, and where there is greater bone volume underlying the parasagittal groove in horses in race training. In addition, gross articular surface damage is not always a good indicator of the amount of microdamage present in the subchondral bone. Other findings such as parasagittal fissures and calcified projections were less commonly observed but were associated with subchondral bone microdamage.

Microcracks were most prominent in the calcified cartilage and subchondral bone underlying the parasagittal groove extending obliquely from the calcified cartilage surface into the superficial subchondral bone. This position and orientation of microcracks is similar to previous descriptions of subchondral bone at this site, with their oblique orientation consistent with shear failure under compressive loading [5; 12].

The palmar aspect of the medial metacarpal condyle is subjected to extremely high loads during galloping exercise and is therefore prone to fatigue injury [22]. Fatigue of subchondral bone will accumulate with greater numbers of cycles of loading which equates to greater galloping distance [23]. Therefore, the microdamage resulting from bone fatigue is likely to be more extensive in older more experienced racehorses as we have observed. Three previous studies have related subchondral bone microdamage at this site with age in Thoroughbred racehorses, with one reporting a subjective increase in microdamage associated with age in

16 horses and another finding no relationship in 12 horses [11; 12]. A relationship between calcified microcracks and age observed in a previous study did not account for all microcracks [19]. Microcracks in calcified cartilage accumulate in humans with ageing, but in contrast to our findings are rare in young adults and in subchondral bone so it is unlikely that age alone is the cause in horses in training [24; 25]. Grossly visible metacarpal joint surface injury at post mortem has been shown to be associated with total lifetime starts in an older cohort of racehorses, consistent with racing history being more important than age alone [26]. Bone fatigue is also more likely to occur due to the higher loads associated with high speed exercise. It has been observed that subchondral bone microdamage fails to accumulate with exercise at speeds less than or equal to 11 m/s in 18-month-old horses [27].

Damaged bone can be repaired by remodelling, that is, the removal of bone by osteoclasts and its replacement with new bone by osteoblasts [28] (Fig 3B). It has previously shown that subchondral bone remodelling activity is greatest when horses are resting from training [17]. The time frame needed for significant reductions in the microdamage load in horses is unknown, but in rat bone targeted remodelling reduced microdamage by up to 40% within 10 days [28]. Although microcrack density across the condyle and parasagittal groove was lower in resting horses than in horses in race training this was not the case for microdamage grade or for calcified microcracks in the parasagittal groove. The lower resolution used to grade microdamage biased this measure towards more severe damage. Such damage might take longer to repair, especially if multicellular units are unable to cross larger disruptions in the bone and cannot access damaged areas. The accumulation of calcified microcracks within the parasagittal groove and the lack of difference in their number between horses in training and those resting suggest that bone repair in this site is less effective than in the condylar subchondral bone. Bone turnover rates showed greater differences between training and resting horses in the lateral condylar subchondral bone than the lateral parasagittal groove [17].

Previous studies have also found an association between subchondral bone damage and densification of the underlying bone (higher BV/TV) in the parasagittal groove and the condyle [12; 14; 29; 30]. This has prompted proposals that densification results in increased subchondral bone stiffness which promotes initiation of microcracks [12]. However, assuming denser bone is stiffer is flawed as there is no correlation between mechanical properties and bone density in the subchondral bone of the palmar aspect of the metacarpal

condyles down to a depth of 6 mm [31]. It is more likely that both densification and microdamage occur independently as a result of high magnitude cyclic loading, i.e. adaptation to the loading environment of the bone through modelling, and damage due to material fatigue [32]. Evidence contradicting a causal relationship between densification and microdamage includes observations that densification of trabecular bone increases its resistance to fatigue damage [33; 34]. Microdamage in human bone has been observed in areas of bone with higher material density (greater mineral content), and it has also been suggested that this is due to a greater brittleness of such bone. However, nanoindentation has shown that these areas are no stiffer than their surrounding bone further demonstrating that it is not possible to make assumptions about bone material properties from imaging [35]. Calcified projections from the calcified cartilage surface have been previously described in Thoroughbred racehorses and were associated with cartilage pathology [10; 36]. Based on our observations they appear to arise from an underlying microcrack and therefore are likely part of the pathology associated with fatigue injury along with parasagittal fissures. Similar to our findings, previous work using scanning electron microscopy found calcified projections were not associated with age or training history [36].

We used two different methods to detect microdamage to offset the limitations of each. Only calcified microcracks and larger fractures are readily identified with microCT however the ability to examine the whole condyle in three dimensions enabled extensive evaluation. Bulk staining permits all microcracks within a section to be visualised but was limited to a single section and therefore may have under-estimated the extent of damage. The mechanism for calcification of microcracks is poorly understood but as this process will take time it is reasonable to expect that calcified microcracks have been present for longer than those that have no evidence of calcification. Therefore, recent microdamage could be overlooked if only calcified microcracks are evaluated. A potential confounder was that resting horses were less likely to have suffered a catastrophic limb injury than those that were in full training at the time of death. However, we included cause of death in our analysis and no confounding effect was observed. Since many of the racing history variables examined were closely correlated and could not be differentiated in terms of individual effect on subchondral bone microdamage, age appeared to be a suitable summary of accumulated training history.

Our findings suggest that, averaged over time the rate of microdamage accumulation was greater than the rate of microdamage removal by bone repair in horses in this study. This

finding is consistent with the accumulation of subchondral bone fatigue injury over a horse's racing career. Two strategies that might limit the accumulation of microdamage are: 1. the provision of adequate periods of rest from training to accelerate microdamage removal or 2. training at a lower intensity to reduce the rate of microdamage accumulation. The length of rest period required to adequately reduce the burden of fatigued subchondral bone is unknown and will depend on bone turnover rates and the volume of damaged bone present at the commencement of the rest period. Returning horses from rest periods results in the additional risk associated with loading subchondral bone that has become more porous in response to a lower loading environment and therefore less resistant to fatigue [37; 38].

Conclusion

Fatigue damage in the distal metacarpal subchondral bone is prevalent in Thoroughbred racehorses and appears to accumulate with career progression. Independent of the age of the horse, microdamage levels are lower in resting horses which is consistent with previous work showing higher levels of subchondral bone remodelling during rest periods. Therefore, we recommend reduced intensity and duration of training and racing and/or increased duration of rest periods in order to limit the risk of fatigue injury in racehorses. Although microdamage cannot be detected in live horses with current imaging techniques, the presence of subchondral bone sclerosis, while not necessarily a problem in itself, is suggestive of localised microdamage and should prompt modification of the training regimen.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

The use of animal tissues met the requirements of the University of Melbourne Animal Ethics Committee. Owners gave informed consent for their horses' inclusion in this study.

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Authorship

R.C. Whitton and E.J. Mackie were responsible for the study concept and design, and obtaining funding. Acquisition of data was by B. Ayodele and R.C. Whitton and analysis by B. Ayodele, P.L. Hitchens and R. C. Whitton. All authors were involved in interpretation of the data, drafting the article, critical revision and final approval.

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Figure legends

Fig 1: MicroCT images of subchondral bone underlying the medial parasagittal groove of the third metacarpal bone of a 4-year-old Thoroughbred race horse. A. an oblique dorsal slice through the parasagittal groove into the subchondral bone, B. an oblique transverse slice aligned with articular surface of the parasaggital groove, C. a three-dimensional reconstruction of bone from the parasagittal groove. Numerous calcified microcracks are observed in the calcified cartilage and extending into the subchondral bone obliquely oriented to the articular surface and to the parasagittal groove (arrowheads in A). Scale bars = 1 mm.

Fig 2: MicroCT images of a third metacarpal bone illustrating VOIs for estimation of BV/TV and material density. A. The image on the left is an oblique dorsal view showing the lateromedial extent of the low resolution VOI (VOI 1); the dotted line indicates the plane of the sagittal view shown on the right, which demonstrates the dorsopalmar extent of the VOI. B. Oblique dorsal view showing the lateromedial extent of the high resolution VOI (VOI 2), which was comprised of 600 sagittal slices similar to those shown in the right image of panel A.

Fig 3: Two photomicrographs of oblique dorsal sections bulk stained with basic fuchsin: A through the parasagittal groove and condylar articular surface of the third metacarpal bone of a 4-year-old Thoroughbred racehorse in race training with multiple stained microcracks extending obliquely from the tidemark into the subchondral bone (arrow heads) , scale bar = 1 mm, and B through the parasagittal groove of the third metacarpal bone of a 5-year-old Thoroughbred racehorse that had been resting for 4 weeks. Stained microcrack (black arrows) with a resorption space forming at the tip of the microcrack (white arrows), scale bar = 100 µm.

Fig 4: Oblique dorsal microCT image of subchondral bone underlying the medial condyle and parasagittal groove of the third metacarpal bone of a 5-year-old Thoroughbred racehorse. A transverse fracture is observed extending lateromedially through the condylar subchondral bone. Scale bar = 1 mm.

Fig 5: Dorsal (A) and sagittal (B) microCT image of subchondral bone underlying the parasagittal groove of the third metacarpal bone of a 4-year-old Thoroughbred racehorse.

601 Calcified projections (arrows) are observed extending from the calcified cartilage surface into
602 the hyaline cartilage. Scale bar = 1 mm.
603
604 **Fig 6:** Relationship between microcrack density (mm) and bone volume fraction (BV/TV) of
605 the parasagittal groove for horses in training (circles) and those not in training (triangles)
606 (interaction effect, P = 0.01). Marginal effects with 95% confidence intervals are presented.
607
608

609 **Table 1:** Grading system for microCT images.
610

Finding	Grade	Description
Microdamage	0	No microcracks
	1	One or two microcracks
	2	Three to ten microcracks
	3	More than ten microcracks
	4	Fracture

611 **Table 2:** Univariable associations between subchondral bone microdamage in medial condyles of third metacarpal bones and explanatory
612 variables, from 46 Thoroughbred racehorses. Explanatory variables of P<0.30 were considered for inclusion in logistic regression models
613 reporting Odds ratios (ORs), and linear regression models reporting coefficients (Coef.) multivariable models. Calcified microcracks in the
614 parasagittal groove were not associated with any of the explanatory variables (data not shown).
615

	Microdamage grade		Microcrack density (mm)		Calcified Projections		Parasagittal fissure	
	OR (95% CI)	*P-value*	Coef. (95% CI)	*P-value*	OR (95% CI)	*P-value*	OR (95% CI)	*P-value*
Age	1.87 (1.30,2.67)	0.001	0.22 (0.12,0.32)	<0.001	1.43 (0.97,2.13)	0.07	1.11 (0.76,1.63)	0.595
Sex								
Male (entire)	Ref		Ref		Ref		Ref	
Male (gelding)	2.01 (0.52,7.75)	0.311	0.17 (-0.34,0.68)	0.514	11.25 (1.17,108.41)	0.04	0.95 (0.20,4.64)	0.954
Female	0.45 (0.11,1.77)	0.254	0.01 (-0.49,0.52)	0.957	5.00 (0.51,48.75)	0.2	0.50 (0.10,2.62)	0.412
Right vs. Left limb	1.81 (0.63,5.19)	0.269	0.21 (-0.18,0.59)	0.288	1.47 (0.43,4.98)	0.5	6.11 (1.41,26.41)	0.015
In training	1.24 (0.44,3.55)	0.684	0.56 (0.21,0.92)	0.002	0.67 (0.20,2.26)	0.5	1.04 (0.29,3.69)	0.955
Duration of training period (weeks)	1.03 (0.96,1.10)	0.377	0.05 (0.02,0.07)	<0.001	0.99 (0.91,1.07)	0.75	0.98 (0.91,1.07)	0.710
Time resting	0.92 (0.80,1.06)	0.237	-0.06 (-0.11,-0.01)	0.014	0.95 (0.80,1.14)	0.6	1.00 (0.84,1.18)	0.967
Raced	7.84 (2.11,29.19)	0.002	0.32 (-0.10,0.74)	0.140	2.17 (0.50,9.40)	0.3	1.67 (0.38,7.32)	0.499
Starts	1.06 (1.02,1.11)	0.002	0.02 (0.01,0.03)	0.002	1.02 (0.98,1.07)	0.3	1.01 (0.97,1.06)	0.520
Time between last two starts (log)	0.71 (0.28,1.82)	0.479	-0.04 (-0.31,0.22)	0.756	4.11 (1.00,16.90)	0.05	2.28 (0.79,6.59)	0.128
Races in last 30days	1.65 (1.02,2.67)	0.040	0.31 (0.14,0.48)	<0.001	0.98 (0.55,1.73)	0.9	0.88 (0.46,1.67)	0.691
Longest distance last 5 starts								
0 (unraced)	Ref		Ref		Ref		Ref	
1000-1300m	4.35 (0.92,20.63)	0.064	0.02 (-0.50,0.53)	0.946	1.43 (0.22,9.26)	0.7	1.43 (0.22,9.26)	0.708

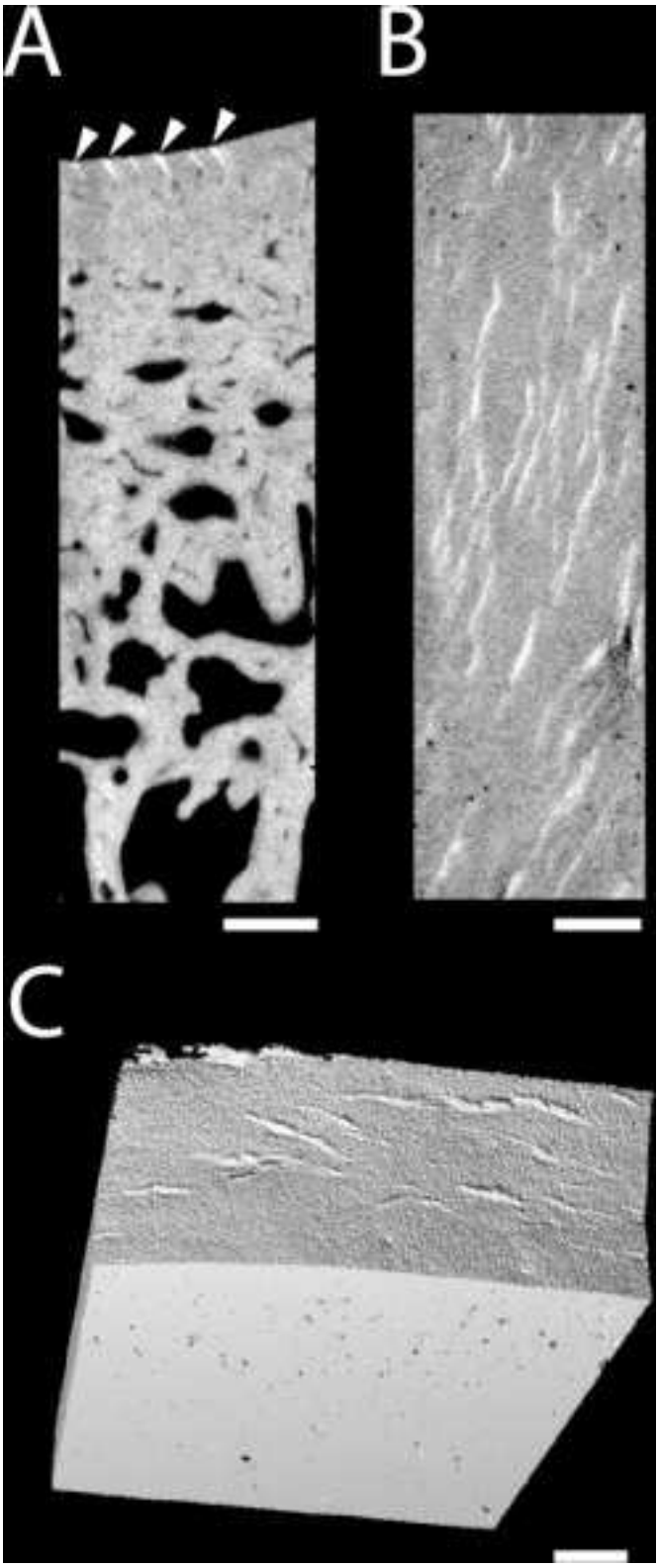
1301-2100m	11.23 (2.36,53.43)	0.002	0.27 (-0.20,0.73)	0.256	2.92 (0.57,15.05)	0.2	1.67 (0.31,8.93)	0.551
>2100m	10.63 (2.01,56.08)	0.005	0.79 (0.24,1.34)	0.005	2.00 (0.29,13.74)	0.5	2.00 (0.29,13.74)	0.481
Prizemoney (log)	1.51 (1.06, 2.13)	0.021	0.08 (-0.01,0.18)	0.097	1.08 (0.77,1.52)	0.7	1.09 (0.76,1.56)	0.628
Prizemoney								
No prizemoney (unraced)	Ref		Ref		Ref		Ref	
<100k	5.89 (1.51,22.94)	0.011	0.23 (-0.22,0.67)	0.313	1.78 (0.38,8.37)	0.5	1.46 (0.30,6.98)	0.637
≥100k	17.82 (3.27,97.03)	0.001	0.52 (-0.02,1.06)	0.058	3.33 (0.56,19.95)	0.2	2.22 (0.36,13.54)	0.386
Fracture	1.06 (0.36,3.14)	0.920	0.33 (-0.05,0.73)	0.086	0.54 (0.15,1.96)	0.3	1.18 (0.32,4.37)	0.797
BV/TV condyle	2.04 (0.00,>18k)	0.878	3.99 (0.82,7.16)	0.014	0.00 (0.00,166.89)	0.3	186.46 (0.00, >23m)	0.384
Parasagittal BV/TV	1437.9 (0.47,>4m)	0.076	5.86 (3.53,8.19)	<0.001	30.37 (0.00,>322k)	0.5	42.73 (0.00,>661k)	0.446
Parasagittal Material density	0.97 (0.95,1.00)	0.063	0.00 (-0.01,0.01)	0.966	0.98 (0.95,1.01)	0.2	0.99 (0.96,1.02)	0.574
Calcified microcracks	1.12 (1.00,1.24)	0.045	0.03 (-0.01,0.07)	0.193251	1.31 (1.11,1.55)	0.001	1.16 (1.01,1.33)	0.031
Microcrack density (mm)	3.97 (1.63,9.66)	0.002	n/a		1.21 (0.49,3.00)	0.7	2.34 (0.89,6.15)	0.086
Microdamage grade	n/a		0.25 (0.09,0.41)	0.002	1.49 (0.83,2.65)	0.2	2.18 (1.09,4.34)	0.027

Note: The final models were correctly specified according to the link test; the proportional odds assumption was satisfied for the ordered logistic regression model.

Supporting Information

Supplementary Item 1: Additional clinical and racing history.

Supplementary Item 2: Correlations between variables associated with career progression.



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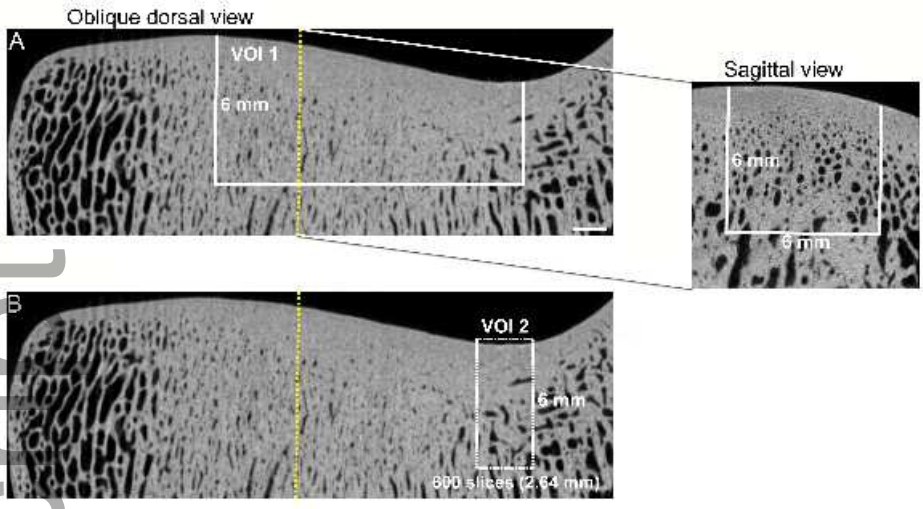

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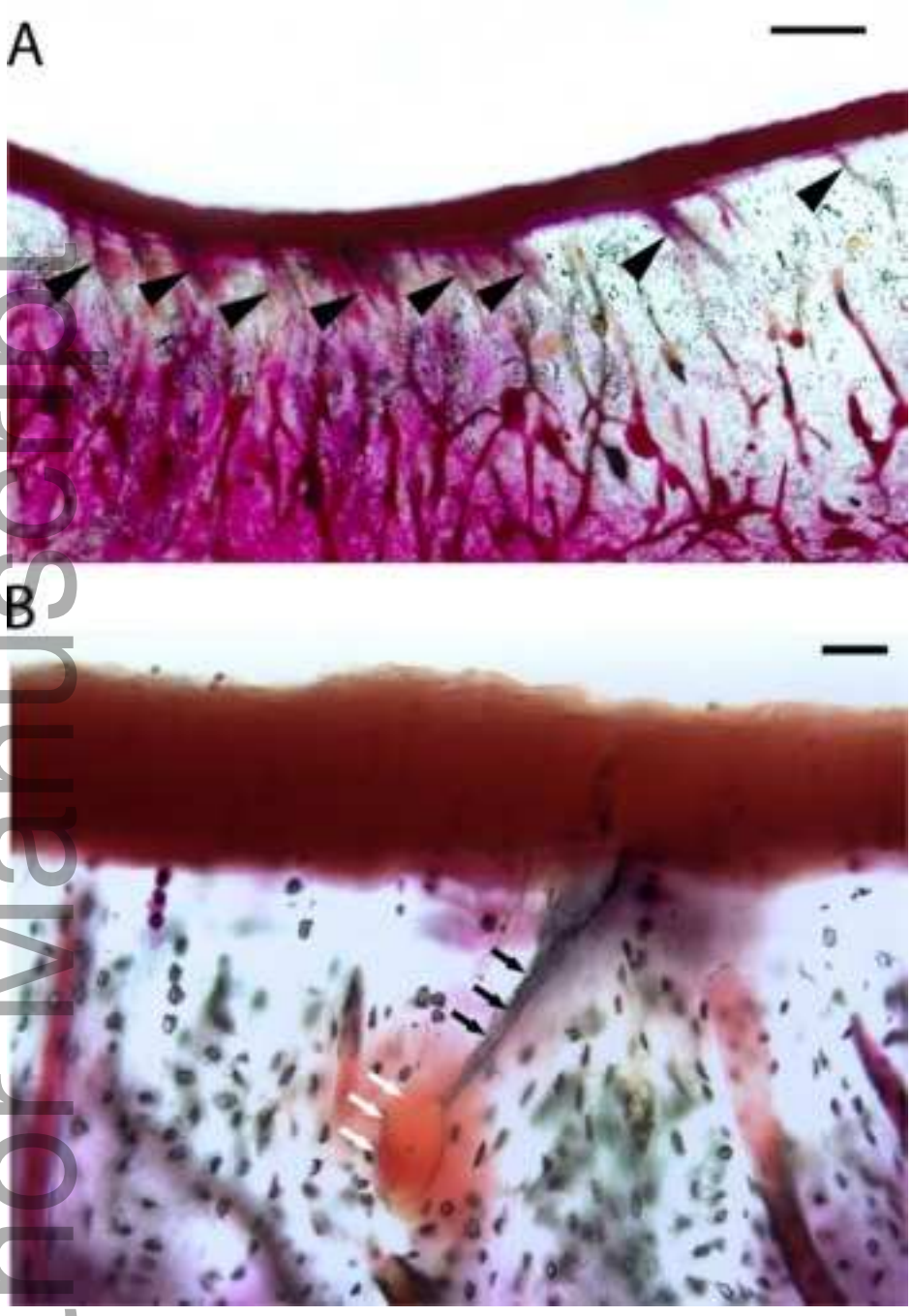

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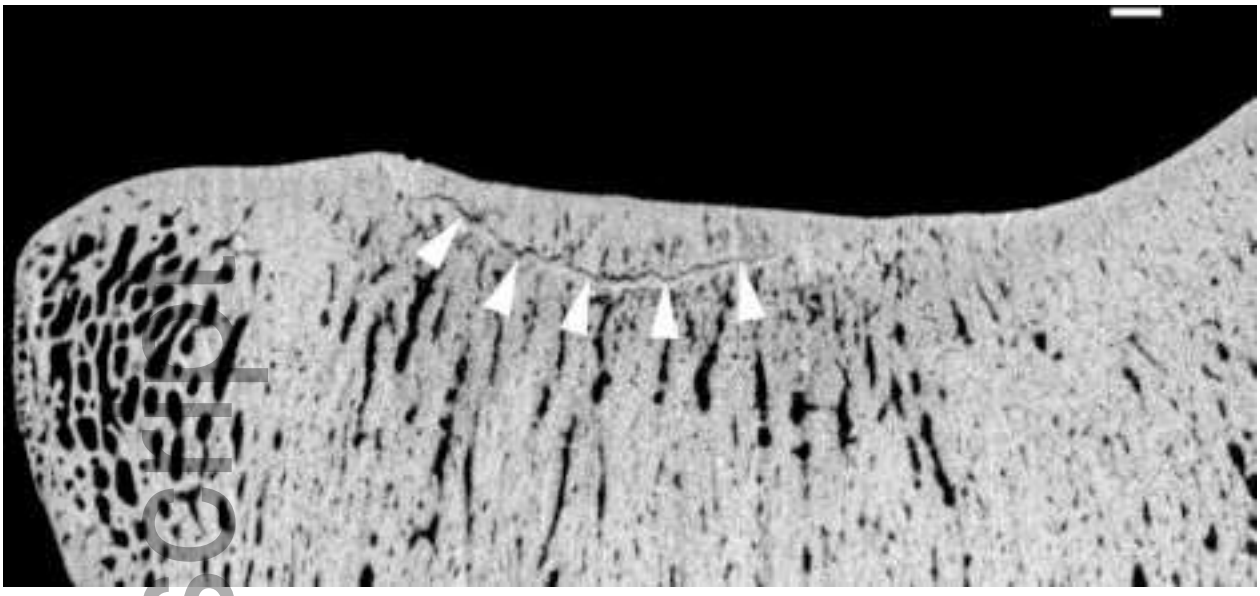

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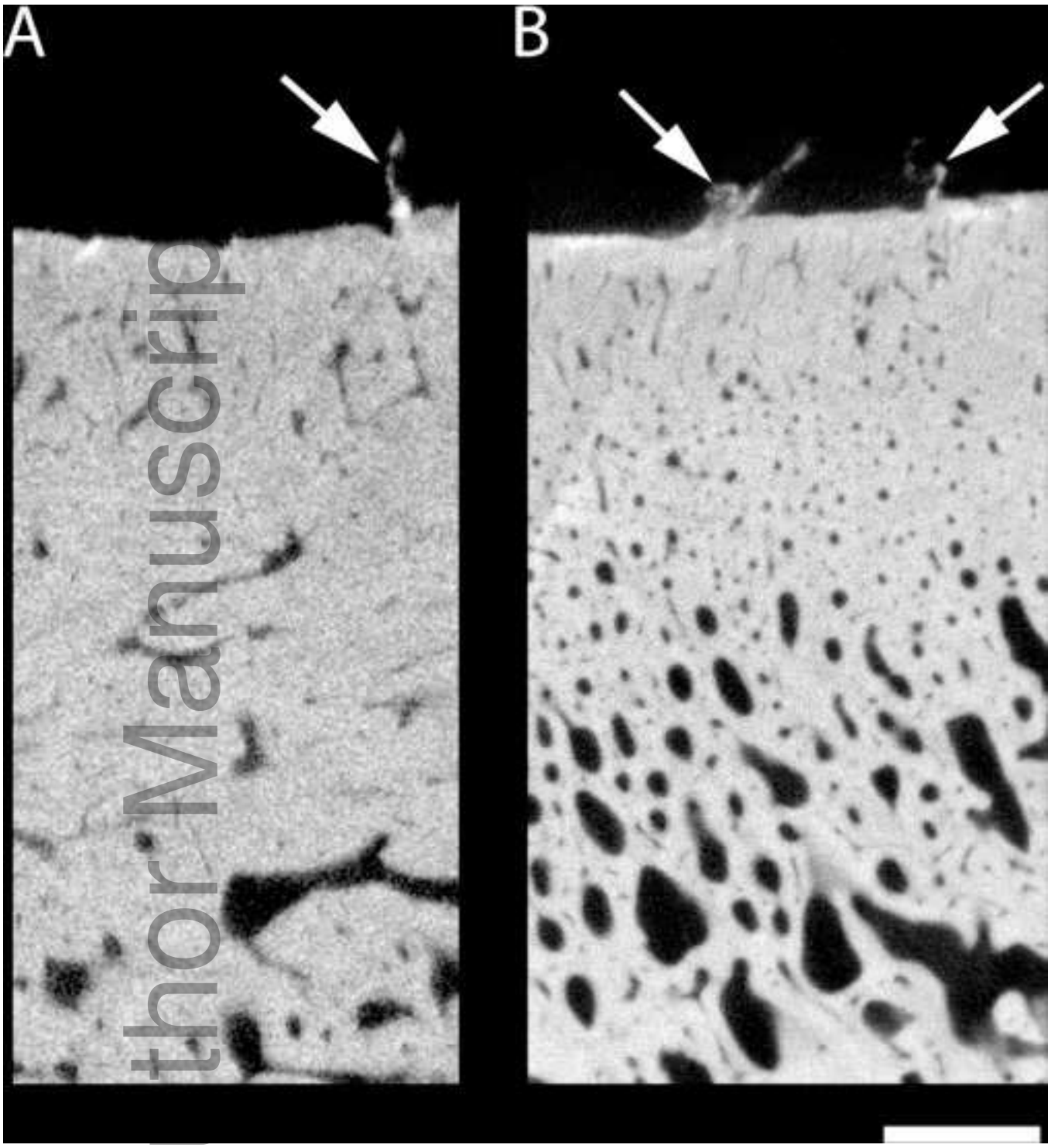

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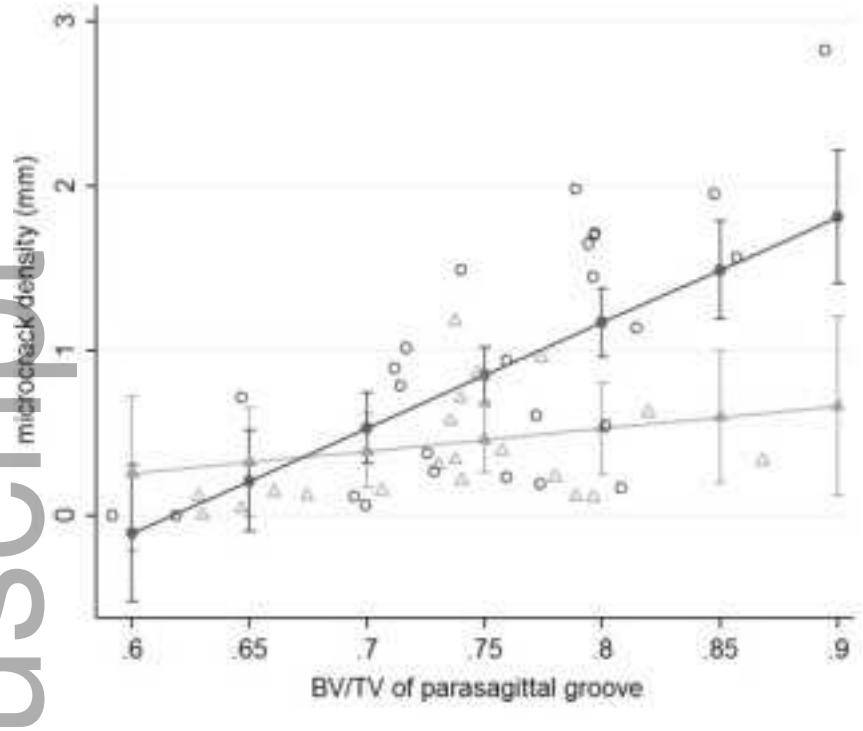

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