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Age-related morphometric changes of the tidemark in the ovine stifle

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Age-related morphometric changes of the tidemark in the ovine stifle

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Keywords:	sheep, cartilage, knee, osteoarthritis, ageing
Abstract:	<p>Though the ovine stifle is commonly used to study osteoarthritis, there is limited information about the age-related morphometric changes of the tidemark. The objective of this study was to document the number of tidemarks in the stifle of research sheep without clinical signs of osteoarthritis and of various ages (n = 80). Articular cartilage of the medial and lateral tibial condyles and of the medial and lateral femoral condyles was assessed by histology: (1) to count the number of tidemark; and (2) to assess the OARSI (OsteoArthritis Research Society International) score for structural changes of cartilage.</p> <p>The number of tidemarks varied between anatomical regions respectively from 4.2 in the medial femoral condyle to 5.0 in the lateral tibial condyle. The axial part showed a significant higher number of tidemarks than the abaxial part, for all regions except the medial tibial condyle. While the tidemark count strongly correlated to age (Spearman Correlation coefficient=0.70; 95% confidence interval 0.67 to 0.73; P<0.0001), the OARSI score was weakly correlated to age in our cohort of sheep (Spearman Correlation coefficient=0.25; 95% confidence interval 0.19 to 0.30; P<0.0001). Interestingly, no tidemark was seen in the three animals aged 6 months.</p> <p>Our data indicate that the number of tidemarks increases with age and vary with anatomical region. The regional variation also revealed a higher number of tidemarks in the tibia than in the femur. This could be attributed to the local variation in cartilage response to strain and to the</p>

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	difference in chondrocyte biology and density.

SCHOLARONE™
Manuscripts

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3 **1 Age-related morphometric changes of the tidemark in the ovine stifle.**
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8 **3 Running title:** Tidemark in the ovine stifle
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27 **Summary**

28 Though the ovine stifle is commonly used to study osteoarthritis, there is limited information
29 about the age-related morphometric changes of the tidemark. The objective of this study was to
30 document the number of tidemarks in the stifle of research sheep without clinical signs of
31 osteoarthritis and of various ages (n = 80). Articular cartilage of the medial and lateral tibial
32 condyles and of the medial and lateral femoral condyles was assessed by histology: (1) to count
33 the number of tidemark; and (2) to assess the OARSI (OsteoArthritis Research Society
34 International) score for structural changes of cartilage.

35 The number of tidemarks varied between anatomical regions respectively from 4.2 in the medial
36 femoral condyle to 5.0 in the lateral tibial condyle. The axial part showed a significant higher
37 number of tidemarks than the abaxial part, for all regions except the medial tibial condyle.

38 While the tidemark count strongly correlated to age (Spearman Correlation coefficient=0.70;
39 95% confidence interval 0.67 to 0.73; $P<0.0001$), the OARSI score was weakly correlated to
40 age in our cohort of sheep (Spearman Correlation coefficient=0.25; 95% confidence interval
41 0.19 to 0.30; $P<0.0001$). Interestingly, no tidemark was seen in the three animals aged 6 months.
42 Our data indicate that the number of tidemarks increases with age and vary with anatomical
43 region. The regional variation also revealed a higher number of tidemarks in the tibia than in
44 the femur. This could be attributed to the local variation in cartilage response to strain and to
45 the difference in chondrocyte biology and density.

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49 **Key words:** sheep – cartilage – stifle – osteoarthritis - ageing

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53 **Number of figures in this manuscript: 4**

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53 **Introduction**

54 Osteoarthritis is a degenerative process of the diarthrodial (synovial) joint characterized by
55 progressive degeneration of the articular cartilage, combined with subchondral bone sclerosis
56 and osteophyte formation, leading to reduced joint function (Grynpas, Albert, Katz, Lieberman,
57 Pritzker, 1991; McIlwraith, 1996, p.34). Histology is considered as a gold standard technique
58 to assess normality of cartilage, disease development (Lahm, Kreuz, Oberst, Haeberstroh, Uhl
59 et al., 2006; Wucherer, Ober, Cozemius, 2012; Zamli, Adams, Tarlton, Sharif, 2013), and
60 efficacy of treatments (Huang, Simonian, Norman, Clark, 2004; Hoeman, Hurtig, Rossomacha,
61 Sun, Chevrier et al., 2005; Zscharnak, Hepp, Richter, Aigner, Schultz et al., 2010) in research
62 studies on osteoarthritis.

63 Different scoring scales have been described for microscopic assessment of cartilage, based on
64 several histological criteria such as the Mankin score, the “modified Mankin score” (Thomas,
65 Fuller, Whittles, Sharif, 2007; Piskin, Gulbahar, Tomak, Gukman, Hokelek et al., 2007; Daubs,
66 Markel, Manley, 2006), and the ICRS (International Cartilage Repair Society) -II scoring scale
67 (Mainil-Varlet, Van Damme, Nestic, Knutsen, Kandel, Roberts et al., 2010). Species-specific
68 scoring scales have been proposed by the Osteoarthritis Research Society International
69 (OARSI) histopathology initiative to ensure comparison between studies using animal models
70 of osteoarthritis, in mice (Glasson, Chambers, Van Den Berg, Little, 2010), rats (Gerwin,
71 Bendele, Glasson, Carlson, 2010), guinea pigs (Kraus, Huebner, DeGroot, Bendele, 2010),
72 rabbits (Lavery, Girard, Williams, Hunziker, Pritzker, 2010), dogs (Cook, Kuroki, Visco,
73 Pelletier, Schulz et al., 2010), horses (McIlwraith, Frisbie, Kawcak, Fuller, Hurtig et al., 2010),
74 goats and sheep (Little, Smith, Cake, Read, Murphy et al., 2010). For example in sheep, the
75 histopathological assessment includes the following parameters: cartilage structure, percentage
76 of the surface area affected by structural damage, chondrocyte density, cell cloning,
77 interterritorial Toluidine blue staining, and tidemark variations.

78

79 The tidemark is the limit between the hyaline cartilage and the calcified cartilage (Meachim &
80 Allibone, 1984; Oegema, Carpenter, Hofmeister, Thompson, 1997; Burr, 2004). At
81 microscopy, the tidemark appears as a non-cellular line of about 10 μm strongly stained with
82 hematoxylin-eosin, or toluidine blue (Lyons, Stoddart, McClure, McClure, 2005). A trilaminar
83 organization has been demonstrated by combining different histochemical staining
84 (hematoxylin and eosin, picosirius red, toluidine blue and safranin O), with a distal lamina (to
85 the side of the non-calcified cartilage), a proximal lamina (to the side of the calcified-cartilage)
86 and a central lamina. The proximal and distal laminae differ in their chemistry and, hence, in
87 their tinctorial properties. It is therefore suggested that the central lamina is actually an
88 artefactual zone due to the interpenetration of colorations of the proximal and the distal laminae
89 (Lyons et al., 2005).

90 The general consensus is that the tidemark is the result of accumulation of non-specific
91 molecules at the interface of calcified and hyaline cartilage caused by discontinuous
92 mineralization (Oegema et al., 1997). The tidemark seems to be derived from apoptotic
93 chondrocytes, and to include several molecules such as phospholipides, alkaline phosphatase,
94 type X collagen, adenosine triphosphatase, deoxyribonucleic acid, lectins, and High Mobility
95 Group Box chromosomal protein 1 (HMGB1) (Lyons et al. 2005; Simkin 2012). Chondrocytes
96 are not present in the tidemark but a few can be partially embedded in its mineralizing side
97 (Lyons et al., 2005).

98

99 Tidemark alterations, i.e. duplication, advancement and vascular invasion have been associated
100 to disease such as rheumatoid arthritis (Fassbender, Seibel, Hebert, 1992; Suber & Rosen, 2009)
101 or osteoarthritis (Oettmeier, Abendroth, Oettmeier, 1989; Bonde et al., 2005; Hulth, 1993; Suri,
102 Gill, Massena de Camin, Wilson, McWilliams et al., 2007; Bullough & Jagannath, 1983;

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3 103 Oegema et al., 1997). In the OARSI score, it is observed whether the tidemark is duplicated
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5 104 (score 1) and whether blood vessels from the subchondral bone cross the tidemark to the
6
7 105 calcified cartilage (score 2) or to the hyaline cartilage (score 3).
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11
12 107 However, multiple tidemarks can be observed in normal joints (Oegema et al., 1997; Oettmeier
13
14 108 et al., 1989). The number of tidemarks has been reported to change with ageing in humans, with
15
16 109 an average increase from 1.5 to 2.5 in femur and humerus after the age of 60 (Lane & Bullough,
17
18 110 1980). Duplicated tidemarks were visible in mature normal canine femoral articular cartilage
19
20 111 (Oegema et al., 1997). In a study on 28 cynomolgus monkeys, as many as ten tidemarks were
21
22 112 observed in normal primates over 20 years old while at least two tidemarks were present in all
23
24 113 animals (Miller, Novatt, Hamerman, Carlson, 2004). In horses, the number of tidemarks was
25
26 114 higher in non-athletic than in racehorses with articular pathology (Muir, Peterson, Sample,
27
28 115 Scollay, Markell, 2008). In non-working and working German shepherd dogs, the tidemark
29
30 116 duplication in the femur and the tibia has been suggested to be related to ageing (Francuski,
31
32 117 Radovanović, Andrić, Krstić, Bogdanović et al., 2014).

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37 118 Since tidemark duplication and advancement could be observed in diseased but also in healthy
38
39 119 animals, it is important to document how tidemark varies with age in a population of research
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41 120 animals. The sheep, in particular, is commonly used as a large animal model for osteoarthritis
42
43 121 (Little et al., 2010). In sheep, there is limited information about the variation of the number of
44
45 122 tidemarks (Appleyard, Burkhardt, Ghosh, Read, Cake et al., 2003; Frisbie, Cross, McIlwraith,
46
47 123 2006). Most of the sheep used in research are skeletally mature sheep (Huang et al., 2004;
48
49 124 Burger, Mueller, Wlodarczyk, Goost, Tolba et al., 2007; Dattena, Pilichi, Rocca, Mara, Casu et
50
51 125 al., 2009) aged between 3 and 6 years old (Hoeman et al., 2005).

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56 126 The objectives of this study were to document the variation of the number of tidemarks of the
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58 127 stifle in a large cohort of sheep without clinical signs of osteoarthritis and of various ages.
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6 129 **Materials and methods**
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8 130 *Population*
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10 131 Eighty pairs of hindlimbs were collected, between 2012 and 2018, from crossed Texel ewes,
11
12 132 euthanatized for reasons other than hind limb lameness (mastitis, metritis), within six hours of
13
14 133 euthanasia. Animals were aged between 6 months and 3 years old (N=28), 4 to 6 years old
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16 134 (N=31) and 7 to 11 year old (N=21). Animals had no clinical signs of osteoarthritis (lameness,
17
18 135 articular swelling, and pain at manipulation). They had been used for teaching anatomy and
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20 136 were not euthanized for the purpose of the current study. The experimental protocol (KI 10/148)
21
22 137 was approved by the local ethical committee for animal welfare.
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24 138
25
26 139 *Gross anatomy*
27
28
29 140 After soft tissue dissection and joint opening, synovium and articular surfaces were assessed by
30
31 141 one investigator in a blinded manner following OARSI recommendations (Little et al., 2010).
32
33 142 Synovium was evaluated for macroscopic alterations (normal, slight, mild, moderate, marked
34
35 143 and severe): discoloration, vascularity, thickening and synovial proliferation. Macroscopic
36
37 144 scores for cartilage damages were: score 0 for intact cartilage surface; score 1 for surface
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39 145 roughening; score 2 for deeper defects (fibrillation, fissures) not involving the subchondral
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41 146 bone; score 3 for erosions down to the subchondral bone (less than 5 mm diameter); score 4 for
42
43 147 large erosions down to the subchondral bone (more than 5 mm diameter). Scoring was
44
45 148 performed in four areas of interest: the middle part of the medial tibial condyle (or plateau)
46
47 149 (MTC), of the medial femoral condyle (MFC), of the lateral tibial condyle (LTC) and of the
48
49 150 lateral femoral condyle (LFC) (Figure 1). Joint margins were observed for the presence of
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51 151 osteophytes. Joint surfaces were digitally photographed (Sony Alpha DSLR-A230 digital
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53 152 camera) with standardized lighting conditions for records (two Sony Illustar SM-300 lighting).
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154 *Histology*

155 Four mm-thick osteochondral slabs were collected from the middle part of the medial tibial
156 condyle (or plateau), medial femoral condyle, lateral tibial condyle and lateral femoral condyle
157 (Figure 1). A total of 640 samples (80 sheep x 2 limbs x 4 regions) were collected. After 48-h
158 fixation in 10% (v/v) neutral buffered formalin, samples were transferred to 70% (v/v) ethanol
159 for further processing (Little et al., 2010). They were decalcified in DC3 (non-ionic surfactants,
160 hydrochloric acid, EDTA, VWR International, Leuven, Belgium) for 2 days and embedded in
161 paraffin, and then 4- μ m sections were cut. Sections were deparaffinised with xylene and graded
162 ethanol, and then stained with Toluidine blue.

163 Each slice was examined for cartilage structure and tidemark count. Scoring of cartilage
164 structure followed the OARSI recommendations for histological evaluation of structural
165 changes in ovine articular cartilage (Little et al., 2010). Each region being divided into two
166 subregions (abaxial (Ab) and axial (Ax)), 1280 subregions were assessed (640 regions x 2).
167 Assessments were performed in duplicates by two observers to obtain a mean score. Tidemark
168 counts were obtained by one blinded observer in six equidistant locations per anatomical region.
169 Mean number was calculated and recorded. Sheep, age and limb identities were blinded to
170 histological scorers.

171

172 *Statistical analysis*

173 Statistics were performed with GraphPad Prism 7.03 (GraphPad Software, La Jolla). Statistical
174 significance was set at 0.05. Firstly, the dataset was assessed for normality, skewness and
175 kurtosis. Due to the moderate positive skewness, to kurtosis, and to non-normal distribution of
176 the data, nonparametric statistics were conducted (Pearce & Frisbie, 2010). Wilcoxon matched-

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3 177 pairs signed rank test and Friedman test were used to compare data from left and right limbs,
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5 178 and to compare data from the different (sub-)regions of each limb.
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8 179 Kruskal-Wallis test followed by a Dunn's multiple comparison test enabled to test difference
9
10 180 between age groups for tidemark count and OARSI scoring. Mean tidemark count and mean
11
12 181 OARSI scores of both limbs was considered for each sheep. Correlation between age and
13
14 182 tidemark number or OARSI scoring of the sheep was assessed using the Spearman's rank order
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16
17 183 test. Correlation was considered very weak (0.00-0.19), weak (0.20-0.39), moderate (0.40-
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19 184 0.59), strong (0.60-0.79) and very strong (0.80-1.00) depending on the absolute value of the
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21 185 coefficient.
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25 26 187 **Results**

27 28 188 *Gross anatomy*

29
30 189 Macroscopic assessment of cartilage for the 1280 anatomic areas revealed 911 zones of intact
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33 190 cartilage (71.2%), 315 score-1 lesions (24.6%), 50 score-2 lesions (3.9%) and 4 score-3 lesions
34
35 191 (0.3%). Score-2 and -3 erosions were found in 11 of the 80 sheep (13.75%). No score-4 lesion
36
37 192 was found. No signs of joint inflammation (effusion, synovitis) and no osteophyte was detected
38
39 193 at gross anatomy.
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43 44 195 *Histology*

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46 196 Thirty slides presented artifacts (folding, shredding, splitting) preventing tidemark count. Thus,
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49 197 1250 of the 1280 sub-regions were appropriately assessed.

50
51 198 There was no significant difference between left and right limbs for tidemark count ($P=0.5898$),
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53 199 and for OARSI scores ($P=0.2761$). The tidemark count ($P<0.0001$) showed difference upon
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55 200 (sub-)regions. The axial sub-region had a significant higher number of tidemarks than the
56
57 201 abaxial sub-region, for all regions except in the medial tibial condyle (Figure 3). The number
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59
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of tidemarks in the four regions was ranked as MFC < LFC < MTC < LTC, with an average number of 4.2, 4.5, 4.8 and 5.0, respectively; those differences were statistically significant, except between MFC and LFC.

The OARSI scores significantly differed with (sub-)regions (Figure 4), with the axial sub-regions showing higher scores than abaxial sub-regions ($P < 0.0001$). OARSI scores in the four regions were ranked as LFC < LTC < MFC < MTC, with an average score of 2.0, 2.6, 5.0 and 5.3, respectively. The differences were not significant between regions of the same bone.

The three age groups had significant different tidemark count ($P < 0.0001$) and OARSI scores ($P = 0.0197$) (Table 1), with a strong positive correlation between age and the number of tidemarks (Spearman Correlation coefficient = 0.70, 95% confidence interval 0.67 to 0.73; $P < 0.0001$). However, the OARSI score was weakly correlated to age in our cohort of sheep (Spearman Correlation coefficient = 0.25, 95% confidence interval 0.19 to 0.30; $P < 0.0001$). The correlation between OARSI scores and tidemark count was weak as well (Spearman Correlation coefficient = 0.19, 95% confidence interval 0.13 to 0.24; $P < 0.0001$). In the three young animals aged 6 months, no tidemark was visible (Figure 2).

Discussion

In this study, the number of tidemarks increased significantly with age. Interestingly, no tidemark was identified in the three sheep aged 6 months. This is in agreement with reports that calcified cartilage layer does not begin to develop until well into the first year postpartum (Martinelli, Eurell, Les, Fyhrie, Bennett, 2002). In horses, functional adaptation of articular cartilage occurs during maturation (Brama, TeKoppele, Bank, Barneveld, van Weeren, 2002). Cartilage-bone interface is a dynamic area where duplication of the tidemark and thickening of

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3 226 calcified cartilage are due to micro-trauma at the bone cartilage-interface and quick repair
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5 227 process in response to mechanical stresses over time (Burr & Schaffler, 1997).

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7 228 The effect of constraints on tidemark duplication is also illustrated by the variation of number
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9 229 of tidemarks between anatomical regions. Constraints are higher in the medial compartment
10
11 230 due to the asymmetry of load bearing and contact area in the stifle (Thomas, Resnick, Alazraki,
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13 231 Daniel, Greenfield, 1975; Baliunas Hurwitz, Ryals, Karrar, Case et al., 2002; Lee-Shee, Dickey,
14
15 232 Hurtig, 2007; Taylor, Poepplau, Konig, Ehrig, Zachow, 2011). This is associated with a higher
16
17 233 deterioration of cartilage and higher OARSI scores in those anatomical regions, as
18
19 234 demonstrated by studies in sheep (Vandeweerd, Hontoir, Kirschvink, Clegg, Nisolle et al.,
20
21 235 2013; Hontoir, Clegg, Simon, Kirschvink, Nisolle et al., 2017), and man (Arøen, Løken, Heir,
22
23 236 Alvik, Ekeland et al., 2004; Neogi, Felson, Niu, Lynch, Nevitt et al., 2009; Flanigan, Harris,
24
25 237 Trinh, Siston, Brophy, 2010). In the current study, OARSI scores were also higher in the medial
26
27 238 tibial and femoral condyles than in the lateral tibial and femoral condyles, with the axial side
28
29 239 being more affected.

30
31 240 In the current study, the number of tidemarks was higher in the tibia than in the femur. A
32
33 241 difference in number of tidemarks has also been described in dogs (Francuski et al., 2014). In
34
35 242 femoral cartilage, tidemark multiplication was more frequently observed in working dogs than
36
37 243 in non-working dogs, whilst in the tibial cartilage it was more frequently observed in non-
38
39 244 working dogs. This particularity has not been described elsewhere. However, regional
40
41 245 differences of cartilage mechanobiology and cell biology could account for change in tidemark
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43 246 number. Mechanically, the cartilage strain is not homogeneous through the joint after exercise:
44
45 247 for example, in human, the cartilage strain (percentage of thickness change) is higher in the
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47 248 tibia (30%) compared to the femur (20%) after a 30-minutes jogging (Moscher, Smith, Collins,
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49 249 Liu, Hancy et al., 2005; Sanchez-Adams, Leddy, McNulty, O'Connor, Guilak, 2014). Moreover,
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51 250 the cartilage response to loading is different for tibial and femoral cartilage. *In vivo* assessment
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3 251 of cartilage response to load has been performed in human using compositional imaging, this
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5 252 technique revealed that tibial cartilage showed an homogeneous response for deep and
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7 253 superficial layers, whilst the femur showed an opposite response for both layers, suggesting a
8
9 254 transport of water to the deep zone of cartilage in the femur, in opposition to the general squeeze
10
11 255 of water of both tibial layers (Souza, Kumar, Calixto, Singh, Schooler et al., 2014).
12
13 256 Biologically, tibial and femoral cartilage shows different pattern, with higher
14
15 257 glycosaminoglycans and collagen content, higher chondrocyte density and proliferation rate in
16
17 258 the femur than in the tibia (Stenhamre, Slynarski, Petrén, Tallheden, Lindahl, 2008). It should
18
19 259 be reminded here that chondrocyte reaction to mechanical load varies from enhanced matrix
20
21 260 synthesis (anabolism) to catabolism, apoptosis and necrosis depending on the frequency, the
22
23 261 amplitude, or the strain-scheme for example (Sanchez-Adams et al., 2014; Bleuel, Zacke,
24
25 262 Brüggemann, Niehoff, 2015; Iijima, Ito, Nagai, Tajino, Yamaguchi et al., 2017). As the
26
27 263 tidemark originates from the chondrocytes activity (Havelka, Horn, Spohrová, Valouch, 1984)
28
29 264 and apoptosis (Simkin, 2012), the higher number of tidemarks in the tibia could be explained
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31 265 by the combination of higher strain and lower cell yield in the tibia compared to the femur.
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40 267 The correlation between the number of tidemarks and the OARSI score was weak in our sheep
41
42 268 population. In a recent research study in man, the tidemark count poorly and non-significantly
43
44 269 correlated to the human OARSI scores in the middle part of 42 lateral tibial condyles, with
45
46 270 OARSI scores ranging from 0 (normal) to 4 (superficial delamination to mid-zone erosion).
47
48 271 (Deng, Wang, Yin, Chen, Guo et al., 2016). These results support the idea, also proposed by
49
50 272 other authors (Lane & Bullough, 1980; Bonde et al., 2005; Oegema et al., 1997; Muir et al.,
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52 273 2008; Francuski et al., 2014), that tidemark multiplication is not a unique feature of
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54 274 osteoarthritis and can be found in normal animals. OARSI scores in the current study were low.
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3 275 In addition, we found no osteophytes, a feature of osteoarthritis (Little et al., 2010; Cake, Read,
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5 276 Corfield, Daniel, Burkhardt et al., 2013).

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10 278 Since there was no osteoarthritic sheep in the current research population, it is not possible to
11
12 279 infer on the association between OA and the number of tidemarks. The use of the sheep as an
13
14 280 animal model for osteoarthritis requires the surgical induction of the disease to ensure the
15
16 281 development of moderate to severe cartilage damages (Little et al., 2010). For example, in a
17
18 282 lateral meniscectomy model, average OARSI scores can reach up to 16 +/-3 for cartilage (with
19
20 283 erosion of cartilage and loss of proteoglycans to the mid/deep zone), associated to moderate
21
22 284 synovitis and osteophytes in the lateral femoral and tibial condyles (Gelse, Körber, Schöne,
23
24 285 Raum, Koch, 2017). Obviously such cases were not identified in the current population.

25
26 286 One could argue that the decalcification process is a limitation of the current study and would
27
28 287 impair assessment of the tidemark. The tidemark is basically seen as the limit between the
29
30 288 calcified cartilage and the hyaline cartilage (Meachim & Allibone, 1984; Oegema et al., 1997;
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32 289 Burr, 2004; Lyons et al., 2005). However, the tidemark is not only featured by presence of
33
34 290 calcium deposition; it contains multiple molecules (phospholipids, alkaline phosphatase,
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36 291 adenosine triphosphatase, DNA, lectins) revealed by a wide range of histologic stains
37
38 292 (Dmitrovsky, Lane and Bullough, 1978; Havelka et al., 1984; Oettmeir et al., 1989; Lyons et
39
40 293 al., 2005). Furthermore, we have purposely conducted the study according to the OARSI
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42 294 recommendation for assessment of cartilage and osteochondral junction in ovine, i.e. with a
43
44 295 decalcification step during the histological processing of osteochondral samples (Little et al.,
45
46 296 2010). Another limitation is the lack of one-year old sheep to determine the apparition of the
47
48 297 first tidemark. Those animals are not frequently available for research since they are young
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50 298 skeletally mature animal at the beginning of their reproductive career, and therefore not likely
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52 299 to be reformed.
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5 301 **Conclusion**
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Documentation of animal models is a concern in research and should be pursued to ensure accurate evaluation of the model and of the tested hypothesis. In the current study, we demonstrated that the multiplication of the tidemark is associated to ageing in the stifles of our sheep population aged between 6 months and 11 years old, without clinical signs of osteoarthritis. The tidemark count was weakly correlated to OARSI scores, confirming that tidemark count is not a feature of osteoarthritis. This might have implications in the interpretation of the OARSI histological score in sheep. Indeed, ageing seems to be more relevant to tidemark count than osteoarthritis progression in the sheep, as well as in man and dogs.

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540 **Table 1:** Tidemark count and OARSI score values (median and interquartile range) for the three
 541 age groups.

	6 months to 3 years old (N = 28)	4 to 6 years old (N = 31)	7 to 11 years old (N = 21)
Tidemark count			
Median	2.67	4.33	6.67
Range	(1.33 – 4.00)	(3.33 – 5.50)	(5.30 – 8.08)
OARSI Scores			
Median	1.50	2.00	3.00
Range	(1.00 – 3.00)	(1.00 – 5.00)	(1.00 – 7.00)

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 543 N= number of sheep. Mean tidemark count and OARSI scoring of both limbs were considered
 544 for each sheep.
 545 The tidemark count ($P < 0.0001$) and the OARSI scores ($P = 0.0197$) differed significantly
 546 between groups.

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3 548 **Figure legends**
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5 549 **Figure 1.** Sampling sites in the middle third of the medial tibial condyle (MTC), medial femoral
6 condyle (MFC), lateral tibial condyle (LTC) and lateral femoral condyle (LFC). Tibial slabs
7
8 550 were centered on the intercondylar eminence (black lines). Femoral slabs were obtained in the
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10 551 centre of the middle third of the circumference of the condyle (black lines and dotted black
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12 552 box). White rectangles illustrate the histological slices that were obtained, each abaxial (Ab)
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14 553 and axial (Ax) part being assessed separately at microscopy. White arrows highlight cartilage
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21 556 **Figure 2.** The osteochondral junction at histology.

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24 557 A. The white line indicates non-calcified hyaline cartilage (HC); the black line is the calcified
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26 558 cartilage (CC).

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28 559 B. White arrows indicate tidemarks.

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30 560 C. Histological slide showing the absence of tidemark in a sample of cartilage of the medial
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32 561 femoral condyle in a 6 months old sheep.
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37 563 **Figure 3:** Number of tidemarks in the different sub-regions for right and left limbs, expressed
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39 564 as median and interquartile range (bar). Asterisks means that statistical significance ($P < 0.05$) is
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41 565 reached for the difference between the axial and the abaxial part of the region.
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44 566 MFC, LFC: medial and lateral femoral condyle, respectively; MTC, LTC: medial and lateral
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46 567 femoral condyle, respectively.
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51 569 **Figure 4:** OARSI scores in the different sub-regions for right and left limbs, expressed as
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53 570 median and interquartile range (bar). Asterisks means that statistical significance ($P < 0.05$) is
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55 571 reached for the difference between the axial and the abaxial part of the region.
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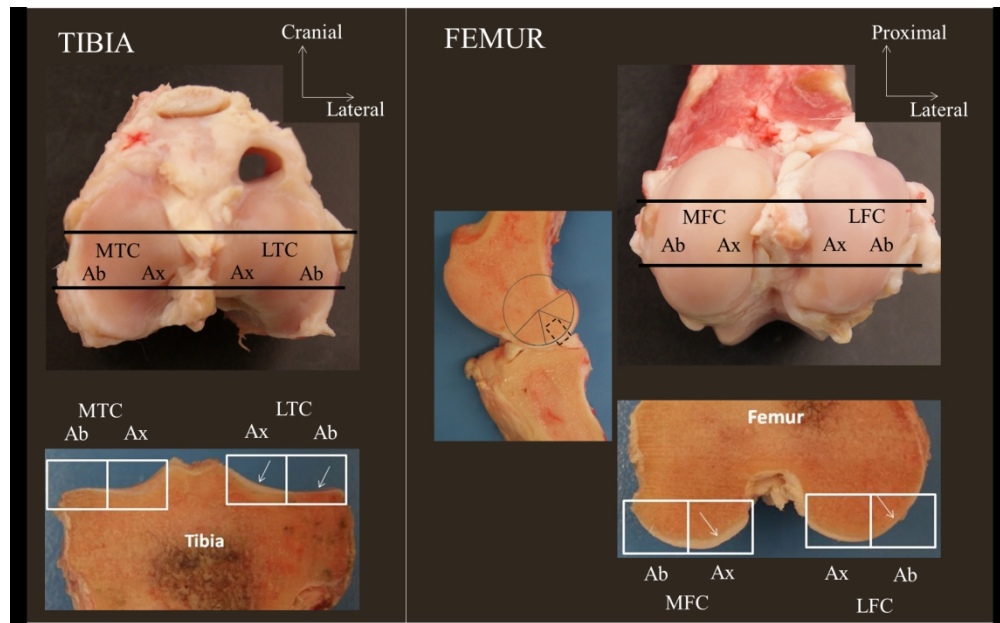


Figure 1. Sampling sites in the middle third of the medial tibial condyle (MTC), medial femoral condyle (MFC), lateral tibial condyle (LTC) and lateral femoral condyle (LFC). Tibial slabs were centered on the intercondylar eminence (black lines). Femoral slabs were obtained in the centre of the middle third of the circumference of the condyle (black lines and dotted black box). White rectangles illustrate the histological slices that were obtained, each abaxial (Ab) and axial (Ax) part being assessed separately at microscopy. White arrows highlight cartilage.

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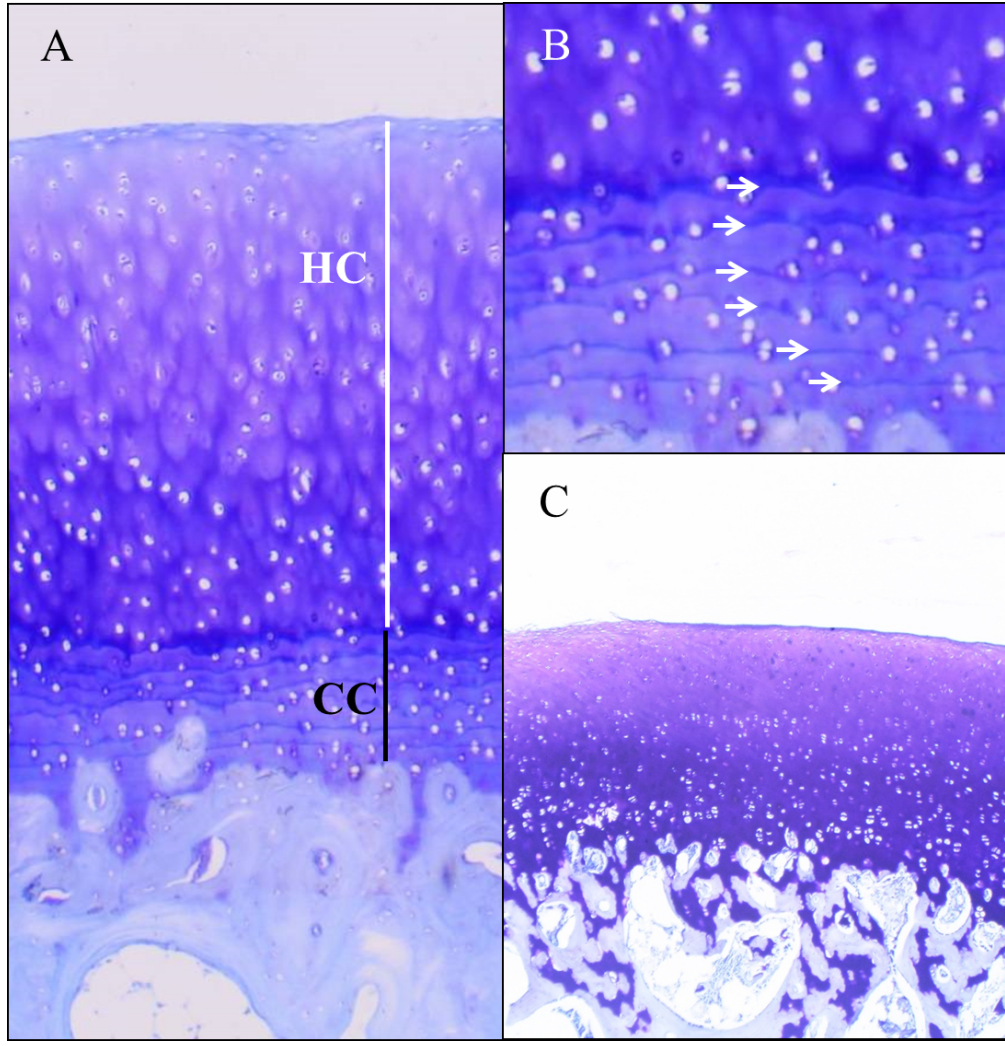


Figure 2. The osteochondral junction at histology.
 A. The white line indicates non-calcified hyaline cartilage (HC); the black line is the calcified cartilage (CC).
 B. White arrows indicate tidemarks.
 C. Histological slide showing the absence of tidemark in a sample of cartilage of the medial femoral condyle in a 6 months old sheep.

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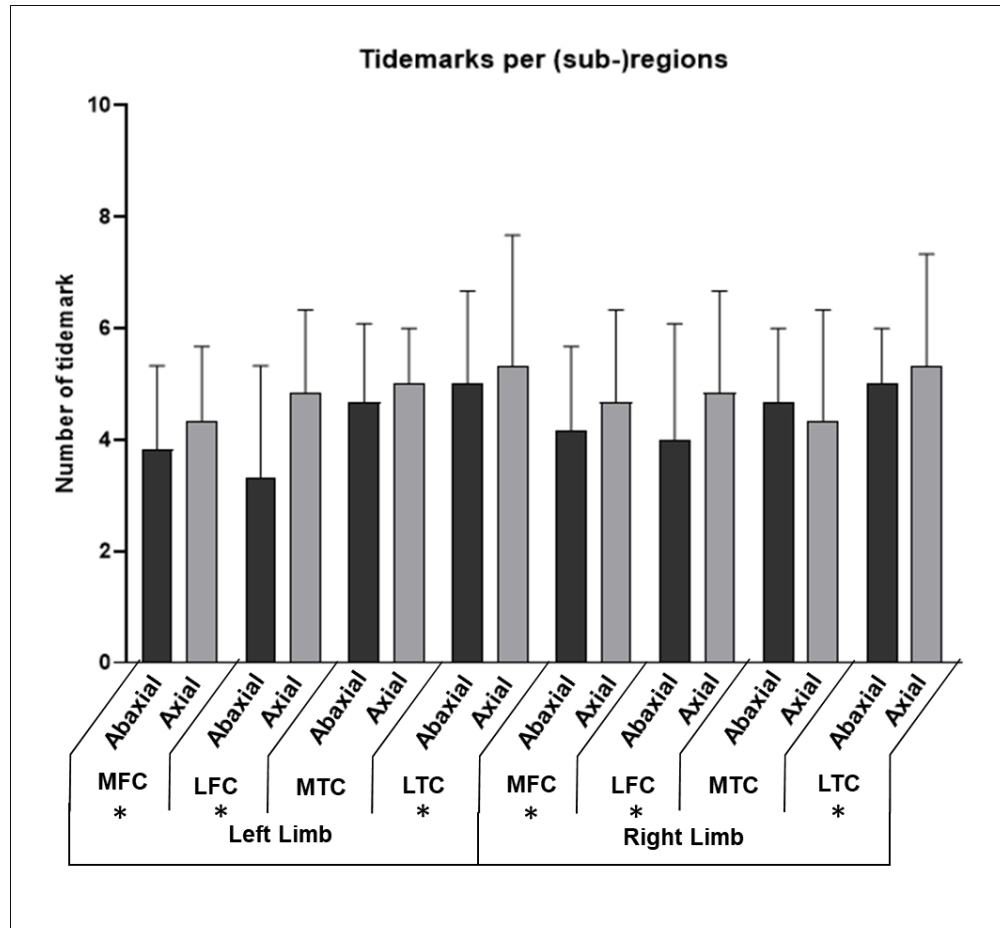


Figure 3: Number of tidemarks in the different sub-regions for right and left limbs, expressed as median and interquartile range (bar). Asterisks means that statistical significance ($P < 0.05$) is reached for the difference between the axial and the abaxial part of the region.

MFC, LFC: medial and lateral femoral condyle, respectively; MTC, LTC: medial and lateral femoral condyle, respectively.

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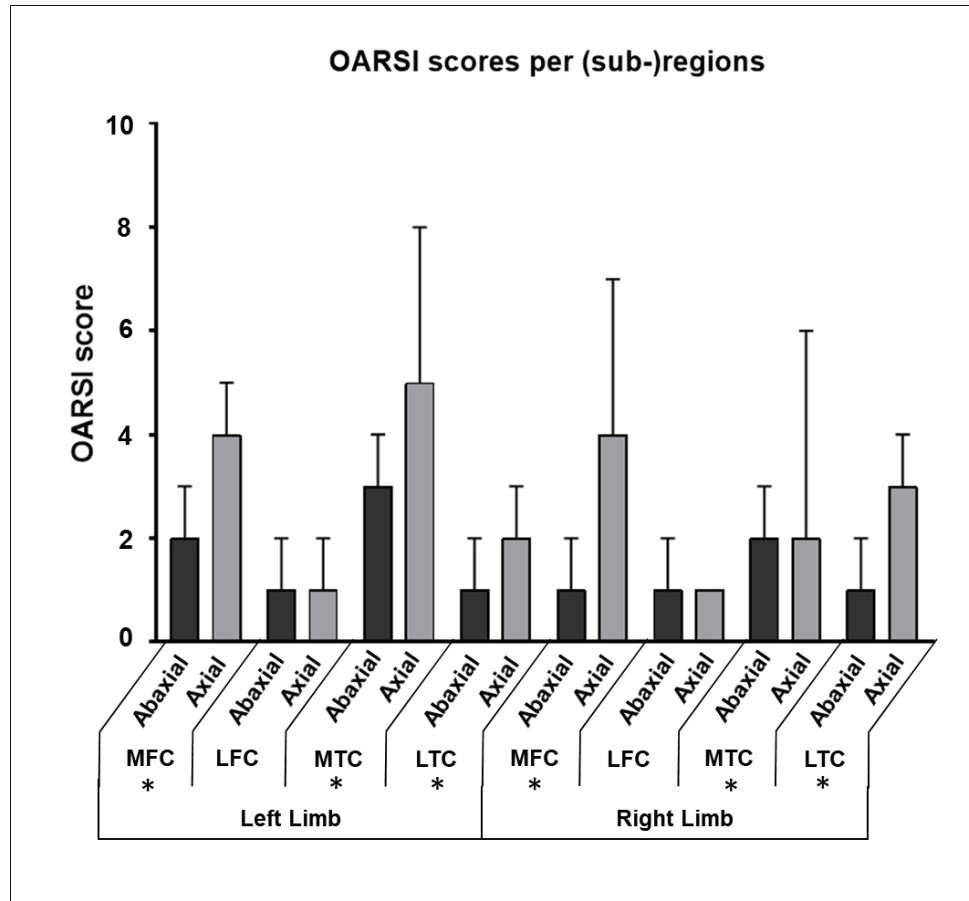


Figure 4: OARSI (OsteoArthritis Research Society International) scores in the different sub-regions for right and left limbs, expressed as median and interquartile range (bar). Asterisks means that statistical significance ($P < 0.05$) is reached for the difference between the axial and the abaxial part of the region. MFC, LFC: medial and lateral femoral condyle, respectively; MTC, LTC: medial and lateral femoral condyle, respectively.

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