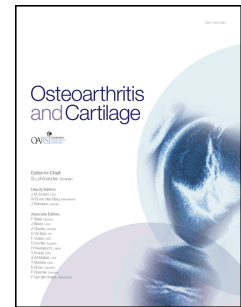


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**Subchondral involvement in osteonecrosis of the femoral head: insight on local composition,  
microstructure and vascularization**

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## 1    **Abstract (257 words)**

### 2    **Objective**

3    To determine changes of subchondral bone composition, micro-structure, bone marrow adiposity and  
4    micro-vascular perfusion in end-stage osteonecrosis of the femoral head (ONFH) compared to  
5    osteoarthritis (OA) using a combined in vivo and ex vivo approach.

### 6    **Design**

7    Male patients up to 70 years old referred for total hip replacement surgery for end-stage ONFH were  
8    included (n=14). Fifteen patients with OA were controls. Pre-operative MRI was used to assess bone  
9    perfusion (dynamic contrast-enhanced (DCE) sequences) and marrow fat content (chemical shift  
10    imaging). Three distinct zones of femoral head subchondral bone – necrotic, sclerotic, distant – were  
11    compared between groups. After surgery, plugs were sampled in these zones and Raman spectroscopy  
12    was applied to characterize bone mineral and organic components (old and newly-formed), and  
13    contrast-enhanced micro-computed tomography (CE- $\mu$ CT) to determine bone micro-structural  
14    parameters and volume of bone marrow adipocytes, using conventional 2D histology as a reference.

### 15    **Results**

16    In the necrotic zone of ONFH patients compared to OA patients: *i)* the subchondral plate did not exhibit  
17    significant changes in composition nor structure; *ii)* the volume fraction of subchondral trabecular  
18    bone was significantly lower; *iii)* type-B carbonate substitution was less pronounced, *iv)* collagen  
19    maturity was more pronounced; and *v)* bone marrow adipocytes were significantly depleted. The  
20    sclerotic zone from the ONFH group showed greater trabecular thickness, and higher DCE-MRI AUC  
21    and  $K_{trans}$ . Volume fraction of subchondral bone, trabecular number, and  $K_{ep}$  were significantly lower  
22    in the distant zone of the ONFH group.

### 23    **Conclusions**



1 This study demonstrated alterations of subchondral bone microstructure, composition, perfusion  
2 and/or adipose content in all zones of the femoral head.

3

4 **Keywords:** osteonecrosis femoral head; avascular necrosis; Raman spectroscopy, contrast-enhanced  
5 computed microtomography; dynamic-contrast enhanced MRI; bone marrow adiposity

## 1 Introduction

2 Osteonecrosis of the femoral head (ONFH) is a common disease with an increasing prevalence  
3 worldwide, more than 80% being associated with corticosteroid use and/or excessive alcohol intake<sup>1</sup>.  
4 Natural evolution of the disease leads to collapse of the femoral head in a majority of cases, often  
5 requiring total hip replacement before the age of 50. Conservative treatments such as core  
6 decompression and bone grafting produce uneven results in preserving the sphericity of the femoral  
7 head and preventing its collapse<sup>1, 2</sup>.

8 Treatment of ONFH remains a challenge mainly because of a lack of understanding of the disease's  
9 initial pathophysiology and later on why the femoral head eventually collapses<sup>2</sup>. Understanding of the  
10 disease is hindered by the absence of satisfying animal models<sup>3</sup> and human control groups in clinical  
11 studies requiring bone samples. In the latter, the choice of comparing ONFH patients to cadaver  
12 subjects was often made in previous works, with the drawbacks of an important age difference  
13 between groups (with an important impact on bone composition) and insufficient available data on  
14 disease history inherent to anatomical subjects<sup>4</sup>. Choosing patients with end-stage osteoarthritis (OA)  
15 as controls allows to have groups of comparable ages and most importantly is motivated by the fact  
16 that patients with end-stage ONFH develop OA<sup>5</sup> which becomes a confounding factor when analyzing  
17 modifications observed in ONFH patients. Initial phases of the disease lead to the constitution of a  
18 necrotic zone in the weight-bearing region, surrounded by a repairing sclerotic zone around which  
19 remains a macroscopically healthy region<sup>6</sup>. Risk factors for the eventual collapse of the femoral head  
20 include the size of the necrotic lesion, its location and individual hip morphology<sup>1, 6</sup>. While the collapse  
21 is particularly demonstrative in the trabecular region of the femoral head, doubt remains on whether  
22 the trabecular bone is indeed fragilized by the envioning medullar necrosis<sup>4</sup>, or if its collapse is induced  
23 by increased mechanical stress secondary to the defect of the subchondral shell. Indeed, whereas  
24 medullar necrosis and ischemia are extensive in the femoral head <sup>7, 8</sup>, neither the micro-structure nor  
25 the mineral composition of the trabecular bone within the necrotic zone have been found to be

significantly altered, suggesting that trabecular bone may merely be a bystander to the structural evolution of the disease<sup>4, 9</sup>. The subchondral region, composed of the subchondral plate (which includes the deep mineralized cartilage layer relying on a parallel mineralized trabeculae) and the underlying dense subchondral trabecular bone, is increasingly suspected to have a predominant role in the structural collapse of the femoral head in human and animal models<sup>10-13</sup>. Particularly, subchondral bone composition and architecture are both suspected to be altered in ONFH<sup>14</sup>, with a putative relationship with bone marrow changes both in its fat content and microvascular exchanges<sup>7</sup>. Providing further evidence of the responsibility of the subchondral bone in the collapse would reinforce the rationale of targeting specifically this bone layer with conservative therapeutic approaches, particularly hip resurfacing which has given satisfying clinical results, with similar survivorship compared to other indications of the procedure<sup>15, 16</sup>.

Magnetic resonance imaging (MRI) is the reference imaging technique for disease diagnosis and prognosis, and provides a basis for choosing between available therapeutic options<sup>1</sup>. It also offers the interesting combination of being able to measure bone marrow fat (BMF)<sup>17</sup> through sequences such as chemical shift imaging (CSI), and to assess bone micro-vascular exchanges (also known as bone perfusion) through sequences such as dynamic contrast-enhanced (DCE)-MRI, thus providing in vivo insight in known/suspected actors of ONFH<sup>7, 18-20</sup>.

Analyzing bone quality – which directly impacts its mechanical properties – involves multi-scale assessment, from molecular composition to microscopic trabecular structure<sup>21</sup>. The available toolbox for the assessment of physicochemical parameters includes vibrational techniques among which Raman spectroscopy offers the advantage of requiring almost no sample preparation<sup>22</sup>. Raman spectroscopy provides the molecular composition of the mineral and organic matrix of bone through semi-quantitative parameters which have shown their interest for studying bone composition in physiological and pathological conditions<sup>23</sup>. At the microscopic level, X-ray microfocus computed

tomography ( $\mu$ CT) provides high-resolution and three-dimensional measurements of bone architecture with a potential for obtaining spatial resolution below 5 $\mu$ m. Assessment of bone quality additionally requires studying the medullar environment, including micro-vascularization and bone marrow adiposity <sup>24</sup>. Conventional 2D histology provides an assessment of bone marrow adiposity (BMA) <sup>25</sup>, but other techniques have the advantage of combining its analysis with three-dimensional and/or dynamic analyses of the other components of bone. Using contrast-enhancing staining agents (CESAs) such as polyoxometalates (POM), contrast-enhancing X-ray microfocus computer tomography (CE- $\mu$ CT) can not only simultaneously provide 3D measurements of bone trabecular microstructure, but it also allows to quantify the BMA content and its structural properties <sup>26</sup>.

The objective of this study was to characterize the potential involvement of subchondral bone in end-stage ONFH by determining if modifications of subchondral bone composition, micro-structure, BMA and vascular perfusion occur in ONFH compared to OA, and if so, their extent beyond the necrotic zone in the sclerotic and macroscopically healthy regions.

## Methods

### *Ethical approval and clinical trial registration*

The study was approved by the French national board (CPP Nord-Ouest 03/018/2015) and by the French drugs and health products safety agency (reference B-151581-32). The ClinicalTrials registration number is NCT02733900.

### *Patients*

Male patients up to 70 years old suffering from ONFH staged Ficat 3 or 4<sup>27</sup> and requiring total hip replacement were prospectively enrolled in the study from orthopedic and rheumatology clinics of the Lille University and Lille Catholic University hospitals from April 2016 to September 2017. ONFH was secondary to corticosteroid treatment, excessive alcohol intake ( $\geq 3$  units (30g) per day) or idiopathic.

A control group of male patients up to 70 years old suffering from hip OA and requiring total hip replacement were recruited to constitute the control group. ONFH being a very predominantly male disease and gender having an important impact on bone characteristics, females were excluded to avoid having different gender ratios between groups. The gender of birth and how the patient currently identified were required to be both male to meet eligibility to the study. Demographic data were collected at the inclusion visit, together with the disease history, alcohol intake, corticosteroid use, and any history of fracture. Patients provided written informed consent to undergo a dedicated MRI scan at a maximum 6 weeks prior to surgery and accepted to donate their femoral head for sampling and multi-technique analysis (histology, HR- $\mu$ CT and Raman spectroscopy) (**Supplementary Figure 1**).

#### *Magnetic resonance imaging*

Patients were all examined on the same 1.5-T MR scanner Optima MR 450W Gem (General Electric Healthcare, Milwaukee, IL) of the Lille Catholic University hospital in supine position. The hip was to benefit from surgery was imaged. A 3D T1-weighted sequence was used for ONFH diagnosis and to identify anatomical landmarks. It covered the pelvis with 256 slices acquired in the coronal plane. Time of repetition was 440ms and TE was 14ms. The field of view was 410 x 410mm, the matrix was 352 x 320, and the slice thickness was 1.6 mm resulting in acquired 1.2 x 1.3 x 1.6 mm voxel size. Parallel imaging (autocalibrating reconstruction for cartesian imaging) was used with an acceleration factor of 2. Acquisition time was 4 min 45. The protocols used for CSI (DIXON sequence named IDEAL IQ, General Electric brand name) and DCE sequences are described elsewhere<sup>7</sup>. The total acquisition time was about 20 minutes (including sequence calibration, shimming time and injection).

T1-weighted and DIXON images were analyzed on a PACS workstation (Carestream, Rochester, NY, USA) by two independent musculoskeletal radiologists (five and 18 years of experience in musculoskeletal radiology). The images were also post-processed on an OsiriX DICOM workstation with

DCE-Tool software (Kyung Sung, Body MRI research group, Stanford University, CA, USA) using a method previously described for DCE-MRI<sup>28, 29</sup>.

Regions of interest (ROIs) were drawn in the subchondral region in the immediate deeper layer beneath the cartilage as shown in **Supplementary Figure 2**, following a reproducible method used in previous studies<sup>7, 30</sup>. Four ROIs were drawn in the ONFH group: in the necrotic zone (bearing region); in the border between necrosis and healthy bone (sclerotic zone) (bearing region); in the distant healthy zone (not bearing region); and in the cortical bone of the femoral neck. In the OA control group, 3 ROIs were drawn in the bearing region, in the distant not-bearing region, and in the cortical of the femoral neck.

For each ROI, BMF content was measured on the “fat fraction” (BMFF) reconstruction map issued from CSI images. For DCE, we measured two semi-quantitative parameters (area under the curve, AUC, and initial slope, IS) and two pharmacokinetic parameters (transfer constant,  $K_{trans}$ , and rate constant,  $K_{ep}$ ). According to the Tofts model,  $K_{trans}$  is the volume transfer constant between blood plasma and the extravascular extra-cellular space and  $K_{ep}$  is the rate constant between the extravascular extracellular space and blood plasma<sup>31</sup>.

#### *Ex vivo Samples*

Whole femoral heads were collected during total hip replacement and immediately frozen at -80°C. Landmarks for the correct orientation of the samples were performed by the surgeon during before the femoral head was collected.

Plugs were harvested from the femoral heads using an 8mm diameter trephine. Twin plugs (2 plugs close to each other) were sampled per zone. Four zones were established in the ONFH group: the necrotic zone in the bearing region; the sclerotic zone at the border of the necrosis in the bearing region; the distant zone macroscopically not necrotic in the non-bearing inferior region; and a fourth zone in the lateral side of the femoral neck. In the OA group, twin plugs were also sampled per zone. Three zones were established: the expected bearing region (according to the orientation of the femoral

head) with the least macroscopical OA lesions (OA bearing region); the distant zone similarly to the ONFH group; and the femoral neck. Confirmation that all samples had an OARSI OA stage 2 or less was obtained from the histology study and from CE- $\mu$ CT analyses<sup>32</sup>. The samples were kept frozen at -80°C. One of the twin plugs was kept frozen until Raman spectroscopy analysis. The other twin plug from the same zone was prepared for CE- $\mu$ CT imaging by 72h fixation in 10% paraformaldehyde. The plug was then stained with hematoxylin and eosin staining, cut and prepared for conventional 2D histological analysis.

#### *Raman spectroscopy*

Raman spectroscopy was performed on a LabRAM HR800 (HORIBA, Jobin-Yvon, France) with a x50 objective (NA=0.75). In brief, a monochromatic laser illuminated the sample, producing a light scatter pattern characteristic of the sample's molecular composition. The acquisition time was set to 45 sec per spectrum. Since Raman spectroscopy is sensitive to bone age<sup>33</sup>, bone areas with similar ages, known to have characteristic anatomical locations, had to be distinguished for comparisons in order to dissociate the bone age effect from the pathology effect. Under confocal microscopy, two bone ROIs – an old bone ROI and a newly formed bone ROI – were determined visually on each plug. The distinction is based on the anatomical location. In cortical bone, newly formed bone ROIs were located in the endosteal area and in the innermost layer of osteons. The old formed bone ROIs were located in the middle of the cortical bone away from osteons). In trabecular bone, recently formed bone ROIs were located in the innermost layer and old bone ROIs were located in the middle of the trabeculae (**Supplementary Figure 3**)<sup>34</sup>. Fifteen spectra were acquired in 15 different new bone ROIs of each plug, each individual spectrum corresponding to one bone ROI. The same protocol was applied for the old bone ROIs.

The physicochemical parameters characterizing bone composition and structure have been described previously<sup>35</sup>. Three mineral parameters were studied. The mineral/organic ratio = ratio of area of  $\nu_1\text{PO}_4$

(960  $\text{cm}^{-1}$ ) to the area of  $\delta(\text{CH}_2)$  (1450  $\text{cm}^{-1}$ ), which is directly related to the resistance to compressive stresses<sup>4, 23</sup>. Crystallinity which reflects the size and perfection of the crystal and tends to increase during the mineralization process<sup>36</sup> was calculated as the inverse of the full width at half maximum (FWHM) of the  $\nu_1\text{PO}_4$  band. Type-B carbonatation, which represents the substitution of phosphates ( $\text{PO}_4$ ) by carbonates ( $\text{CO}_3$ ) inducing changes in crystal conformation, was calculated as the ratio between the respective area of the type-B  $\text{CO}_3$  (1073  $\text{cm}^{-1}$ ) and the  $\nu_1\text{PO}_4$ .

Two organic parameters were studied: the hydroxyproline to proline ratio, calculated as the intensity ratio of hydroxyproline (877  $\text{cm}^{-1}$ ) and proline (853  $\text{cm}^{-1}$ ), which reflects post-translational modifications of collagen occurring during the mineralization process<sup>37, 38</sup>, and collagen maturity which reflects the structuration of bone fibrils<sup>23</sup>. Collagen maturity was calculated as the intensity ratio of two sub-component bands in the amide I band commonly described at 1660  $\text{cm}^{-1}$  for the amide I peak and 1690  $\text{cm}^{-1}$ <sup>39</sup>.

A typical Raman spectrum of bone and its mineral and organic bands of interest is shown in **Supplementary Figure 4**.

#### *Contrast-enhanced $\mu\text{CT}$*

We used CE- $\mu\text{CT}$  to simultaneously visualize the different skeletal tissues in the plugs (bone, mineralized cartilage, unmineralized cartilage and marrow adipocytes). For this, all samples were immersed for 3 weeks in a solution of 35mg of Hafnium-substituted Wells–Dawson polyoxometalate (Hf-WD POM) per ml of PBS. Subsequently, samples were scanned at 5  $\mu\text{m}$  voxel size using a NanoTom M device (GE Measurement and Control Solutions, Germany). The system's source was set to 70 kV and 300  $\mu\text{A}$ . An aluminum filter of 0.2 mm was used. The system scanning settings were: exposure time 500 ms, frame averaging 1 and skip 0, and 1600 radiographs, which allowed for a scanning time of 15 min per sample. The resulting images were analyzed with CTAn (Bruker MicroCT, Kontich, Belgium) and we selected 2 regions of interest (ROI). The first ROI was the subchondral plate which was manually selected every 50 slices with interpolation between. We applied further manual



corrections where needed. In this region two parameters were measured: plate thickness ( $\mu\text{m}$ , Plt.Th) excluding porosity; and plate porosity (% , Plt.Po). Cortical porosity was measured in the femoral neck in a ROI selected within the cortical with 500  $\mu\text{m}$  lateral and upper and lower margins. The second ROI was the subchondral trabecular bone, which was selected as a rectangle starting immediately beneath the subchondral plate with a height of 2.5mm and length of 6 mm, and automatically interpolated across slices. Two segmentation steps were performed, one to isolate trabeculae, and one to isolate the segment's bone marrow adipocytes (**Figure 1**). The following structural parameters were then obtained: bone volume per tissue volume (% , BV/TV); trabecular thickness ( $\mu\text{m}$ , Tb.Th); trabecular separation ( $\mu\text{m}$ , Tb.Sp); trabecular number (n, Tb.N); adipose volume per whole bone tissue volume (% , Ad.V/TV); and adipose volume per medullar volume (% , Ad.V/Ma.V)<sup>17</sup>.

#### *Conventional 2D histological assessment*

Once the samples were imaged with CE- $\mu\text{CT}$ , they first washed in PBS and then prepared for 2D conventional histological analysis. The samples were decalcified with a solution of formaldehyde, methanol and formic acid (Leica Decalcifier-system II®). Sections of paraffin-embedded sample blocks were stained with hematoxylin and eosin. Optical microscopic examination was performed and provided a reference for the structures observed with CE- $\mu\text{CT}$  (**Figure 2**).

#### *Statistical analyses*

Without prior available data on the expected modifications of the subchondral bone available in the literature, no sample size calculation was feasible to achieve a pre-specified power for the comparison between groups; no size effect hypotheses were defined a priori, especially since our measurements were numerous. This study being exploratory; we aimed to obtain 15 evaluable patients in each group, corresponding to the largest series found in the literature including ONFH samples.

All statistical analyses were performed using R version 3.6.1. Qualitative variables were described as numbers and frequencies of each response modality; quantitative variables were described as median [inter-quartile interval]. Due to the small sample size, ONFH and OA groups were compared using the Mann-Whitney-Wilcoxon test. In the subchondral trabecular region, comparisons between ONFH and OA groups were performed first on old bone and second on newly formed bone. The OA bearing zone served as an anatomical comparator for both the necrotic and the sclerotic zones of the ONFH group. Significance level was set at 5%. No p-values correction for multiple testing was applied as this study is exploratory.

## Results

### *Patients*

A total of 34 patients were recruited. Five of them were excluded: one patient had a Ficat stage below 3, one patient finally did not receive total hip replacement, one patient had a diagnosis of hip arthritis other than OA; also, one femoral head was lost in the operating room, and one surgery sample did not undergo the planned CE- $\mu$ CT protocol. Finally, among the 29 patients analyzed, 14 were suffering from ONFH (Ficat 4 for 8/14) and 15 from hip OA. Patients with ONFH were aged 56 [46.5 ; 59] years, and their body mass index (BMI) was 25.9 [24.8 ;29.9]. The cause of ONFH was attributed to excessive alcohol intake in 9/14 patients and corticosteroid use in 3. The remaining 2 cases were considered idiopathic. Patients with OA were aged 60 [57.5;67] years, had a BMI at 26.5 [24.3 ;29.1]. ; age differed with the ONFH group ( $p = 0.01$ ), but not BMI ( $p = 0.56$ ). 4/15 had excessive alcohol intake, while none had been treated with corticosteroids.

### *The subchondral plate: non-significant differences between ONFH and OA groups*

On CE- $\mu$ CT, subchondral plate thickness did not differ between ONFH and OA groups (respectively 90.8 $\mu$ m [76.6 ; 104.5] vs 101.9 $\mu$ m [77.6 ; 126.2]) (p=0.48). Plate bone porosity in the necrotic zone was 15.2% [5.8 ; 22.2] and 9.9% [5.9 ; 20.1] in the sclerotic zone, compared to 8.1% [5.1 ; 10.3] in the bearing zone of the OA group (p=0.48 and 0.28, respectively).

None of the Raman spectroscopy parameters differed significantly between the ONFH and OA groups. (Table 1).

#### *Modification of subchondral trabecular bone composition and structure inside and outside the necrotic region*

3D rendering of the CE- $\mu$ CT data of the subchondral trabecular bone structure demonstrated differences between the ONFH and OA groups. In the ONFH group, BV/TV was significantly higher in the necrotic zone (33.7 [27.5 ; 43.7] vs 25.4 [23.2 ; 26.9] p=0.04) and Tb.Th was higher in the sclerotic zone (212.8 [194.4 ; 235.2] vs 174.6 [164.9 ; 202.1], p=0.02) compared with the OA group. In the ONFH group, the distant zone exhibited lower BV/TV (15.5 [12.7 ; 20.6] vs 23.3 [18.1 ; 24.6], p=0.01) and Tb.N (0.0010 [9e-04 ; 0.0011] vs 0.0014 [0.0013 ; 0.0017], p=0.003) and higher Tb.Sp (717.3 [632.4 ; 758.6] vs 573.3 [508 ; 611.8], p=0.005) compared with the OA group (Figure 3).

On Raman spectroscopy, type-B carbonatation of subchondral trabecular old bone in the necrotic region was lower in the ONFH group (0.1365 [0.1327 ; 0.1394]) compared with type-B carbonatation in the equivalent region in the OA group (0.1436 [0.1369 ; 0.1463], p=0.02), with a similar non-significant trend observed in all zones of both newly formed and old bone. Collagen maturity of subchondral trabecular old bone in the necrotic region was higher (0.0256 [0.0254 ; 0.0259]) in the ONFH group compared to collagen maturity in the equivalent region in the OA group (0.0254 [0.0251 ; 0.026], p=0.04) (Figure 4) (Supplementary Table 1).

# 1 *Subchondral bone marrow modifications*

2 CE- $\mu$ CT data allowed to detect subchondral bone marrow modifications in the ONFH group. More  
 3 specifically, we found a much lower Ad.V/Ma.V in the necrotic zone (6.2% [1.8 ; 6.3]) compared to the  
 4 equivalent bearing region in the OA group (62.3% [60.3 ; 75.5],  $p < 0.001$ ). In the sclerotic zone,  
 5 Ad.V/Ma.V was 44.0% [26.3 ; 70.2], which did not differ significantly from the OA bearing region  
 6 ( $p = 0.18$ ). Both distant regions of the ONFH and OA groups exhibited similar Ad.V/Ma.V (respectively  
 7 61.6% [35.4 ; 81.2] and 71.3% [62.2 ; 80.7]  $p = 0.46$ )

8 On MRI BMFF was 5.4% [3.5 ; 13.1] in the necrotic zone, 8.3% [5.7 ; 15.3] in the sclerotic zone  
 9 compared to 74.0% [68.8 ; 79.1] in the bearing region of the OA group ( $p < 0.001$ ). In the distant zone  
 10 of the ONFH group, the MRI BMFF was 14% lower than in the OA group but this difference did not  
 11 reach statistical significance ( $p = 0.19$ ) (respectively 56.8% [41.6 ; 69.4] vs 71.4% [50.9 ;  
 12 85.4])(**Supplementary Figure 5**).

13 Drastic decrease of bone marrow adiposity in the necrotic zone and partially in the sclerotic zone was  
 14 observed qualitatively by conventional 2D histological analysis, showing a replacement of bone  
 15 marrow content by necrosis (**Figure 5**)

16 DCE-MRI did not detect statistically significant differences in bone perfusion parameters in the necrotic  
 17 zone owing to major in-group heterogeneity of values in the ONFH group. In the sclerotic zone, the  
 18 AUC ( $p = 0.03$ ) and the  $K_{trans}$  ( $p = 0.03$ ) were significantly higher in the ONFH group. In the distant zone,  
 19  $K_{ep}$  was significantly lower in the subchondral bone of the ONFH group ( $p < 0.001$ ) (**Figure 6**).

# 20 *Modifications of cortical and subcortical trabecular bone in the femoral neck*

21 In Raman spectroscopy, bone mineral quality did not differ between cortical bones of the femoral neck  
 22 of the ONFH and OA groups. In the trabecular bone of the femoral neck, collagen maturity was  
 23 significantly more pronounced in the newly formed trabecular bone (0.0260 [0.0256 ; 0.0266] vs

0.0252 [0.0245 ; 0.0255],  $p < 0.001$ ) and suggested non-statistically significant higher hydroxyproline-to-proline ratio ( $p = 0.16$ ) and lower carbonate B-substitution ( $p = 0.17$ ).

CE- $\mu$ CT-based comparison of the adipocyte medullar content showed a trend of higher levels in the ONFH group compared to the OA group ( $p = 0.08$ ), with a numerical difference of 18%.

## Discussion

This study showed that all subchondral trabecular components of bone – mineral trabeculae, bone marrow adiposity and perfusion – are modified inside the necrosis and beyond in end-stage ONFH when compared to zones of minor or no cartilage degradation of hip OA. None of the differences in composition and structure of the subchondral plate were statistically significant. While traces of impaired mineralization processes and bone marrow adiposity desertification could be seen in the zones of necrosis, active microvascular exchanges and bone structure thickening was observed in the sclerotic region despite end-stage disease. At the same time, subchondral bone of the apparently healthy distant zone exhibited some bone loss, a lower level of bone marrow adiposity and lower micro-vascular perfusion compared to the same zone in OA femoral heads.

All three components of subchondral trabecular bone – mineralized bone, bone marrow micro-vascularization and bone marrow adiposity – were depressed in the distant apparently healthy region in the ONFH group as compared to the OA group. Whereas in ONFH subchondral trabecular bone there is a process favoring denser bone in the sclerotic and necrotic regions as had been observed in previous studies<sup>14</sup>, our results show that the trabecular density of bone subchondral declined in distant non-bearing regions compared to OA controls. This could suggest that the overall trabecular structure in femoral heads affected by osteonecrosis may be scarce as has been suggested by imaging studies including pre-collapse stages<sup>6, 40</sup>, and present with collagen modifications consistent with a previous study also demonstrating a decreased canalicular quality<sup>14</sup>. Bone loss in osteoporosis is usually associated with an increase in bone marrow adiposity<sup>41</sup>, a concept that would be challenged by our

1 observation of a parallel decrease in mineralized bone and bone marrow adipocytes. One likely  
2 explanation would be that in ONFH, bone marrow adiposity is not only regulated by pathophysiological  
3 processes also observed in other bone diseases like osteoporosis, but is also replaced by bone marrow  
4 necrosis. Earlier work has shown that necrotic material invades not only the necrotic and sclerotic  
5 zones of bone marrow, but apparently also the healthy distant zone<sup>4</sup>. A second explanation involving  
6 the mechanical properties of bone marrow fat could provide some insight since adipocytes may also  
7 help absorb mechanical stress<sup>42</sup>, which is inherently less pronounced in end-stage ONFH; albeit, a  
8 similar pattern should also be observed in our end-stage OA control group. How bone marrow  
9 adiposity interacts with bone turnover in the distant region of ONFH therefore remains uncertain and  
10 will need to be addressed.

11 The subchondral sclerotic zone exhibits active repair processes in end-stage ONFH. This activity was  
12 demonstrative on bone perfusion parameters as shown by the increased DCE-MRI AUC and  $K_{trans}$ . These  
13 features have also been observed in other types of osteonecrosis in other anatomical locations such  
14 as the wrist<sup>43</sup>. The mineral components of bone also displayed features of new bone formation with  
15 trends of decreased crystallinity, while trabeculae became thicker. These results are consistent with  
16 bone repair processes (sometimes in disorganized woven bone<sup>4</sup>) and they also show that the sclerotic  
17 region remains active even in end-stage ONFH.

18 Subchondral bone in the necrotic zone bears scars of impaired mineralization processes and complete  
19 replacement of bone marrow adiposity by necrosis, but sustains micro-vascular perfusion and newly  
20 formed bone is unaltered. Crystallinity reflects crystal perfection which increases with bone age, as  
21 new bone formation decreases<sup>33</sup>. Collagen maturity is also thought to increase when mineralization  
22 processes slow down, while the level of type-B carbonate substitution is a dynamic process occurring  
23 during normal bone turnover homeostasis. Although not always statistically significant, these markers  
24 indicated that 'old' subchondral trabecular bone had suffered from less than optimal bone turnover  
25 during its formation, with an expected mechanical impact on bone resistance<sup>44</sup>. Our DCE-MRI results

of subchondral bone perfusion in the necrosis are also surprising as one would have expected to find significant differences in microvascular exchanges particularly in bone marrow flooded with necrosis<sup>45, 46</sup>, but none were found. However, the distribution of perfusion parameters in the necrotic zone suggests highly heterogeneous inter-individual microvascular activity in end-stage ONFH. Surprisingly, the subchondral plate did not exhibit significant compositional nor structural significant changes despite the common fractures/disruptions of the plate observed in ONFH<sup>47, 48</sup>. This may suggest that modifications of the plate, if any, may not be as critical as subchondral trabecular alterations.

We acknowledge limitations inherent to the study design. Firstly, the downside of the main strength of the study of combining state-of-the-art in vitro and in vivo techniques was feasibility which limited sample size which induced an inherent lack of statistical power. This is why we have mentioned several visual trends even when they did not reach statistical significance. The second limitation was also inherent to the study design, which aimed to include only patients with end-stage disease from whom bone samples could be collected during total hip replacement. Results gathered in the study can therefore only be applied to end-stage ONFH and not to early disease mechanisms. Also, while patients suffering from ONFH due to purely vascular causes (traumatic/hematological diseases) were not included, we acknowledge that the diseases selected for this study may differ in how they affect bone quality independently of ONFH<sup>14</sup>. Finally, primary OA is responsible for regional bone changes of its own<sup>49</sup> which we tried to minimize by selecting only regions with no or small cartilage lesions for the control group. However, choosing fractured controls would probably have led to even more bone modifications induced by osteoporosis and would have added ethical considerations about submitting painful fractured patients to MRI scans probably unnecessary for proper management. Using cadavers as controls as we did in earlier work<sup>4, 50</sup> implies an age difference and conservation procedures, which modify many bone parameters. Bearing this limitation in mind, all results presented in this study are a comparison between zones of ONFH not to healthy zones of femoral heads, but to zones of mild cartilage lesions from OA femoral heads, having known changes, including subchondral bone thickening/increasingly mineralized.

This study showed alterations of subchondral bone mineral composition and micro-structure, bone marrow adiposity and/or perfusion across all zones of the femoral head. Such combined alterations have not been found in prior studies of trabecular bone in ONFH<sup>4, 51</sup>. These findings suggest preponderant involvement of subchondral bone in ONFH and particularly its collapse in late disease. Beyond bringing insight into the pathophysiology of ONFH, this study helps build the rationale for using conservative treatments such as hip resurfacing targeting subchondral bone, bearing in mind the presence of subchondral bone abnormalities beyond the macroscopically necrotic zone<sup>52</sup>.

#### **Competing interests**

None of the authors have any conflicts of interest relative to the content of this manuscript.

#### **Author contributions**

Tristan Pascart: Conception and design, Analysis and interpretation of the data, Drafting of the article, Critical revision of the article for important intellectual content, Final approval of the article, Provision of study materials or patients, Obtaining of funding, Collection and assembly of data

Guillaume Falgayrac : Conception and design, Analysis and interpretation of the data, Drafting of the article, Critical revision of the article for important intellectual content, Final approval of the article, Statistical expertise, Obtaining of funding, Administrative, technical, or logistic support, Collection and assembly of data.

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## **Ethics approval of research on humans**

The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

## References

1. Mont MA, Salem HS, Piuze NS, Goodman SB, Jones LC. Nontraumatic Osteonecrosis of the Femoral Head: Where Do We Stand Today?: A 5-Year Update. *J Bone Joint Surg Am* 2020; 102: 1084-1099.
2. Chang C, Greenspan A, Gershwin ME. The pathogenesis, diagnosis and clinical manifestations of steroid-induced osteonecrosis. *J Autoimmun* 2020; 110: 102460.
3. Xu J, Gong H, Lu S, Deasey MJ, Cui Q. Animal models of steroid-induced osteonecrosis of the femoral head-a comprehensive research review up to 2018. *Int Orthop* 2018; 42: 1729-1737.
4. Pascart T, Falgayrac G, Migaud H, Quinchon JF, Norberciak L, Budzik JF, et al. Region specific Raman spectroscopy analysis of the femoral head reveals that trabecular bone is unlikely to contribute to non-traumatic osteonecrosis. *Sci Rep* 2017; 7: 97.
5. Larson E, Jones LC, Goodman SB, Koo KH, Cui Q. Early-stage osteonecrosis of the femoral head: where are we and where are we going in year 2018? *Int Orthop* 2018; 42: 1723-1728.
6. Huang Z, Tan B, Ye H, Fu F, Wang R, Chen W. Dynamic evolution of osseous structure in osteonecrosis of the femoral head and dynamic collapse risks: a preliminary CT image study. *J Orthop Surg Res* 2020; 15: 539.
7. Cultot A, Norberciak L, Coursier R, Putman S, Cortet B, Paccou J, et al. Bone Perfusion and Adiposity Beyond the Necrotic Zone in Femoral Head Osteonecrosis: A Quantitative Mri Study. *Eur J Radiol* 2020; 131: 109206.
8. Calder JD, Pearse MF, Revell PA. The extent of osteocyte death in the proximal femur of patients with osteonecrosis of the femoral head. *J Bone Joint Surg Br* 2001; 83: 419-422.
9. Pascart T, Paccou J, Colard T, Norberciak L, Girard J, Delattre J, et al. T1-weighted MRI images accurately represent the volume and surface of architectural mineral damage of osteonecrosis of the femoral head: Comparison with high-resolution computed tomography. *Bone* 2020; 130: 115099.
10. Li W, Sakai T, Nishii T, Nakamura N, Takao M, Yoshikawa H, et al. Distribution of TRAP-positive cells and expression of HIF-1alpha, VEGF, and FGF-2 in the reparative reaction in patients with osteonecrosis of the femoral head. *J Orthop Res* 2009; 27: 694-700.
11. Kolb AR, Patsch JM, Vogl WD, Benca E, Stelzeneder D, Windhager R, et al. The role of the subchondral layer in osteonecrosis of the femoral head: analysis based on HR-QCT in comparison to MRI findings. *Acta Radiol* 2018: 284185118786070.
12. Zheng LZ, Liu Z, Lei M, Peng J, He YX, Xie XH, et al. Steroid-associated hip joint collapse in bipedal emus. *PLoS One* 2013; 8: e76797.
13. Hofstaetter JG, Wang J, Yan J, Glimcher MJ. Changes in bone microarchitecture and bone mineral density following experimental osteonecrosis of the hip in rabbits. *Cells Tissues Organs* 2006; 184: 138-147.
14. Fowler TW, Acevedo C, Mazur CM, Hall-Glenn F, Fields AJ, Bale HA, et al. Glucocorticoid suppression of osteocyte perilacunar remodeling is associated with subchondral bone degeneration in osteonecrosis. *Sci Rep* 2017; 7: 44618.

- 1 15. Pyda M, Koczy B, Widuchowski W, Widuchowska M, Stoltny T, Mielnik M, et al. Hip  
2 resurfacing arthroplasty in treatment of avascular necrosis of the femoral head. *Med Sci*  
3 *Monit* 2015; 21: 304-309.
- 4 16. Amstutz HC, Le Duff MJ. Hip resurfacing results for osteonecrosis are as good as for other  
5 etiologies at 2 to 12 years. *Clin Orthop Relat Res* 2010; 468: 375-381.
- 6 17. Bravenboer N, Bredella MA, Chauveau C, Corsi A, Douni E, Ferris WF, et al. Standardised  
7 Nomenclature, Abbreviations, and Units for the Study of Bone Marrow Adiposity: Report of  
8 the Nomenclature Working Group of the International Bone Marrow Adiposity Society. *Front*  
9 *Endocrinol (Lausanne)* 2019; 10: 923.
- 10 18. Bainbridge A, Bray TJP, Sengupta R, Hall-Craggs MA. Practical Approaches to Bone Marrow  
11 Fat Fraction Quantification Across Magnetic Resonance Imaging Platforms. *J Magn Reson*  
12 *Imaging* 2020; 52: 298-306.
- 13 19. Budzik JF, Lefebvre G, Behal H, Verclytte S, Hardouin P, Teixeira P, et al. Assessment of the  
14 zonal variation of perfusion parameters in the femoral head: a 3-T dynamic contrast-  
15 enhanced MRI pilot study. *Skeletal Radiol* 2018; 47: 261-270.
- 16 20. Badr S, Legroux-Gerot I, Vignau J, Chauveau C, Ruschke S, Karampinos DC, et al. Comparison  
17 of regional bone marrow adiposity characteristics at the hip of underweight and weight-  
18 recovered women with anorexia nervosa using magnetic resonance spectroscopy. *Bone*  
19 2019; 127: 135-145.
- 20 21. Donnelly E. Methods for assessing bone quality: a review. *Clin Orthop Relat Res* 2011; 469:  
21 2128-2138.
- 22 22. Penel G, Delfosse C, Descamps M, Leroy G. Composition of bone and apatitic biomaterials as  
23 revealed by intravital Raman microspectroscopy. *Bone* 2005; 36: 893-901.
- 24 23. Gamsjaeger S, Mendelsohn R, Boskey AL, Gourion-Arsiquaud S, Klaushofer K, Paschalis EP.  
25 Vibrational spectroscopic imaging for the evaluation of matrix and mineral chemistry. *Curr*  
26 *Osteoporos Rep* 2014; 12: 454-464.
- 27 24. Zapata-Linares N, Eymard F, Berenbaum F, Houard X. Role of adipose tissues in osteoarthritis.  
28 *Curr Opin Rheumatol* 2021; 33: 84-93.
- 29 25. Lucas S, Rosen CJ, Bravenboer N. Editorial: Bone Marrow Adiposity: Establishing Harmonized,  
30 Mechanistic, and Multidisciplinary Approaches to Reach Clinical Translation. *Front Endocrinol*  
31 *(Lausanne)* 2020; 11: 604110.
- 32 26. Kerckhofs G, Stegen S, van Gastel N, Sap A, Falgayrac G, Penel G, et al. Simultaneous three-  
33 dimensional visualization of mineralized and soft skeletal tissues by a novel microCT contrast  
34 agent with polyoxometalate structure. *Biomaterials* 2018; 159: 1-12.
- 35 27. Ficat RP. Idiopathic bone necrosis of the femoral head. Early diagnosis and treatment. *J Bone*  
36 *Joint Surg Br* 1985; 67: 3-9.
- 37 28. Budzik JF, Lefebvre G, Behal H, Verclytte S, Hardouin P, Teixeira P, et al. Bone marrow  
38 perfusion measured with dynamic contrast enhanced magnetic resonance imaging is  
39 correlated to body mass index in adults. *Bone* 2017; 99: 47-52.
- 40 29. Budzik JF, Ding J, Norberciak L, Pascart T, Toumi H, Verclytte S, et al. Perfusion of subchondral  
41 bone marrow in knee osteoarthritis: A dynamic contrast-enhanced magnetic resonance  
42 imaging preliminary study. *Eur J Radiol* 2017; 88: 129-134.
- 43 30. Budzik JF, Lefebvre G, Forzy G, El Rafei M, Chechin D, Cotten A. Study of proximal femoral  
44 bone perfusion with 3D T1 dynamic contrast-enhanced MRI: a feasibility study. *Eur Radiol*  
45 2014; 24: 3217-3223.
- 46 31. Tofts PS, Brix G, Buckley DL, Evelhoch JL, Henderson E, Knopp MV, et al. Estimating kinetic  
47 parameters from dynamic contrast-enhanced T(1)-weighted MRI of a diffusable tracer:  
48 standardized quantities and symbols. *J Magn Reson Imaging* 1999; 10: 223-232.
- 49 32. Pritzker KP, Gay S, Jimenez SA, Ostergaard K, Pelletier JP, Revell PA, et al. Osteoarthritis  
50 cartilage histopathology: grading and staging. *Osteoarthritis Cartilage* 2006; 14: 13-29.

33. Gourion-Arsiquaud S, Burket JC, Havill LM, DiCarlo E, Doty SB, Mendelsohn R, et al. Spatial variation in osteonal bone properties relative to tissue and animal age. *J Bone Miner Res* 2009; 24: 1271-1281.
34. Falgayrac G, Farlay D, Poncon C, Behal H, Gardegaront M, Ammann P, et al. Bone matrix quality in paired iliac bone biopsies from postmenopausal women treated for 12 months with strontium ranelate or alendronate. *Bone* 2021; 153: 116107.
35. Morris MD, Mandair GS. Raman assessment of bone quality. *Clin Orthop Relat Res* 2011; 469: 2160-2169.
36. Olejnik C, Falgayrac G, During A, Cortet B, Penel G. Doses effects of zoledronic acid on mineral apatite and collagen quality of newly-formed bone in the rat's calvaria defect. *Bone* 2016; 89: 32-39.
37. Aparicio S, Doty SB, Camacho NP, Paschalis EP, Spevak L, Mendelsohn R, et al. Optimal methods for processing mineralized tissues for Fourier transform infrared microspectroscopy. *Calcif Tissue Int* 2002; 70: 422-429.
38. Karampas IA, Orkoulas MG, Kontoyannis CG. A quantitative bioapatite/collagen calibration method using Raman spectroscopy of bone. *J Biophotonics* 2013; 6: 573-586.
39. Gamsjaeger S, Robins SP, Tatakis DN, Klaushofer K, Paschalis EP. Identification of Pyridinoline Trivalent Collagen Cross-Links by Raman Microspectroscopy. *Calcif Tissue Int* 2017; 100: 565-574.
40. Baba S, Motomura G, Ikemura S, Yamaguchi R, Utsunomiya T, Hatanaka H, et al. Is bone mineral density lower in the necrotic lesion in pre-collapse osteonecrosis of the femoral head? *J Orthop Res* 2020; 38: 2434-2442.
41. Woods GN, Ewing SK, Sigurdsson S, Kado DM, Eiriksdottir G, Gudnason V, et al. Greater Bone Marrow Adiposity Predicts Bone Loss in Older Women. *J Bone Miner Res* 2020; 35: 326-332.
42. Simkin PA. Marrow fat may distribute the energy of impact loading throughout subchondral bone. *Rheumatology (Oxford)* 2018; 57: 414-418.
43. Muller G, Mansson S, Muller MF, Johansson M, Bjorkman A. Increased perfusion in dynamic gadolinium-enhanced MRI correlates with areas of bone repair and of bone necrosis in patients with Kienbock's disease. *J Magn Reson Imaging* 2019; 50: 481-489.
44. Mandair GS, Morris MD. Contributions of Raman spectroscopy to the understanding of bone strength. *Bonekey Rep* 2015; 4: 620.
45. Chan WP, Liu YJ, Huang GS, Lin MF, Huang S, Chang YC, et al. Relationship of idiopathic osteonecrosis of the femoral head to perfusion changes in the proximal femur by dynamic contrast-enhanced MRI. *AJR Am J Roentgenol* 2011; 196: 637-643.
46. Hayashi S, Fujioka M, Ikoma K, Saito M, Ueshima K, Ishida M, et al. Evaluation of femoral perfusion in a rabbit model of steroid-induced osteonecrosis by dynamic contrast-enhanced MRI with a high magnetic field MRI system. *J Magn Reson Imaging* 2015; 41: 935-940.
47. Lafforgue P. Pathophysiology and natural history of avascular necrosis of bone. *Joint Bone Spine* 2006; 73: 500-507.
48. Brown TD, Baker KJ, Brand RA. Structural consequences of subchondral bone involvement in segmental osteonecrosis of the femoral head. *J Orthop Res* 1992; 10: 79-87.
49. Goldring SR, Goldring MB. Changes in the osteochondral unit during osteoarthritis: structure, function and cartilage-bone crosstalk. *Nat Rev Rheumatol* 2016; 12: 632-644.
50. Pascart T, Cortet B, Olejnik C, Paccou J, Migaud H, Cotten A, et al. Bone Samples Extracted from Embalmed Subjects Are Not Appropriate for the Assessment of Bone Quality at the Molecular Level Using Raman Spectroscopy. *Anal Chem* 2016; 88: 2777-2783.
51. Wang C, Wang X, Xu XL, Yuan XL, Gou WL, Wang AY, et al. Bone microstructure and regional distribution of osteoblast and osteoclast activity in the osteonecrotic femoral head. *PLoS One* 2014; 9: e96361.
52. Park CW, Lim SJ, Kim JH, Park YS. Hip resurfacing arthroplasty for osteonecrosis of the femoral head: Implant-specific outcomes and risk factors for failure. *J Orthop Translat* 2020; 21: 41-48.

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## **TABLES and FIGURES**

**Table 1.** Subchondral plate micro-structural and compositional measures in end-stage osteonecrosis of the femoral head (ONFH) and hip osteoarthritis (OA) groups. CE- $\mu$ CT: Contrast-enhanced micro-computed tomography. Values expressed in medians and [inter-quartile range]. p-value provided by the Mann-Whitney Wilcoxon comparison of medians, considered significant if  $<0.05$ .

**Figure 1.** Differential binarization mineral bone and bone marrow in contrast-enhanced biopsies scanned with HR- $\mu$ CT. A. Native image of an 8mm deep from the cartilage surface biopsy from a distant (non-bearing) zone of the femoral head of a control patient with the delineation of the subchondral plate (green) and subchondral trabecular bone (orange) regions of interest, B. super-imposition of the subchondral plate (top) and subchondral trabecular region (bottom) binarized to isolate mineralized bone and C. super-imposition of the subchondral trabecular region binarized to isolate bone marrow adipocytes. C: cartilage; Pl: subchondral plate, Tb: subchondral trabeculae, Md: Medullar space.

**Figure 2.** Biopsy plug of a sclerotic zone: (A) Conventional 2D histological examination with hematoxylin and eosin staining, and (B) corresponding cross-sectional contrast-enhanced  $\mu$ CT image. Ad: bone marrow adipocytes, C: unmineralized cartilage, N: necrosis, P: subchondral plate, T: trabeculae.

**Figure 3.** Scatter plots of subchondral trabecular (trab) bone contrast-enhanced  $\mu$ CT (CE- $\mu$ CT) micro-structural parameters (with medians and interquartile ranges): (A) bone volume to tissue volume ratio (BV/TV), (B) trabecular thickness (Tb.Th), (C) trabecular number (Tb.N) and (D) trabecular separation (Tb.Sp). CE- $\mu$ CT slices of subchondral trabecular bone of distant zones of (E) a patient of the osteoarthritis (OA) group and (F) a patient of the osteonecrosis of the femoral head (ONFH) group. \*  $p<0.05$  ; \*\*  $p<0.01$

**Figure 4.** Scatter plots of subchondral trabecular (trab) bone compositional measures in end-stage osteonecrosis of the femoral head (ONFH) and hip osteoarthritis (OA) groups, in old and newly formed bone. Raman spectroscopy measurements of mineral parameters (A) mineral-to-matrix ratio, (B) type-B carbonate substitution, (C) bone crystallinity, and organic parameters (D) collagen maturity and (E) hydroxyproline-to-proline ratio (medians and interquartiles ranges). (F) Raman spectra of a necrotic zone of a patient with ONFH and of a bearing zone from a patient with OA. \*  $p<0.05$

**Figure 5.** Assessment of biopsy plugs of (A) necrotic, (B) sclerotic and (C) distant zones using 2D conventional histology with hematoxylin and eosin staining. C: cartilage, T: trabeculae, Ad: adipocytes, N: necrotic material.

**Figure 6.** Subchondral bone marrow adipose content and micro-vascular exchanges in end-stage osteonecrosis of the femoral head (ONFH) and hip osteoarthritis (OA) groups. (A) Adipocyte volume

to bone marrow volume ratio measured with high-resolution micro-computed tomography (HR- $\mu$ CT) and (B) fat content measured with MRI Dixon-sequence. DCE-MRI measurements of (C) the area under the curve (AUC), (D) the initial slope (IS), (E)  $K_{trans}$  and (F)  $K_{ep}$ . \*  $p < 0.05$ ; \*\*  $p < 0.01$  – Medians and interquartile ranges.

**Supplementary Table 1.** Contrast-enhanced microCT (CE- $\mu$ CT), Raman spectroscopy and magnetic resonance imaging (MRI) parameter values of subchondral trabecular bone of the osteonecrosis of the femoral head (ONFH) and osteoarthritis (OA) zones (medians and interquartile ranges).

**Supplementary Figure 1.** Study design (n=29 patients). Each patient had a 1.5T MRI scan before surgery. The femoral head was stored at  $-80^{\circ}\text{C}$  just after surgery. Four localizations were selected in the ONFH group, and 3 localizations in the OA group. A pair of biopsies were harvested per localization. For each pair, one biopsy was stained and analyzed by HR- $\mu$ CT, the other was analyzed by Raman microspectroscopy. Nec.: necrotic zone; Scl.: sclerotic zone; Dt.: distant zone; F.N.: femoral neck; ONFH: osteonecrosis of the femoral neck; OA: osteoarthritis.

**Supplementary Figure 2.** Localization of regions of interest (ROIs) on magnetic resonance imaging T1 sequences of a left osteonecrosis of the femoral head. The ROIs are represented by circles of the in the necrotic (red), sclerotic (bearing – orange) and distant (green) subchondral zones

**Supplementary Figure 3.** Confocal microscopic images of cortical and trabecular bone with localizations of old and newly formed bone.

**Supplementary Figure 4.** Typical Raman spectrum of with bands of interest of mineral (blue) and organic (pink) components.

**Supplementary Figure 5.** Representative images from DIXON sequences (IDEAL IQ, General Electrics) from a 58 years old patient presenting with right osteonecrosis of the femoral head (yellow arrow) without contralateral osteonecrosis.



**Table 1.** Subchondral plate micro-structural and compositional measures in end-stage osteonecrosis of the femoral head (ONFH) and hip osteoarthritis (OA) groups. CE- $\mu$ CT: Contrast-enhanced micro-computed tomography. Values expressed in medians and [inter-quartile range].

|                                         | ONFH group (n=14)           |                             |                             | OA group (n=15)             |                             |
|-----------------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                                         | Distant zone                | Sclerotic zone              | Necrotic zone               | Distant zone                | Bearing zone                |
| CE- $\mu$ CT plate thickness ( $\mu$ m) | 67.2<br>[66.6 ; 86.6]       | 82.3<br>[61.6 ; 108.3]      | 90.8<br>[76.6 ; 104.5]      | 76.1<br>[66.4 ; 89]         | 101.9<br>[77.6 ; 126.2]     |
| CE- $\mu$ CT plate porosity (%)         | 5.3<br>[2.8 ; 6.5]          | 9.9<br>[5.9 ; 20.1]         | 15.2<br>[5.8 ; 22.2]        | 5.9<br>[5.1 ; 9.3]          | 8.1<br>[5.1 ; 10.3]         |
| Plate mineral-to-matrix ratio           | 9.22<br>[8.85 ; 10.38]      | 9.1 [7.9 ; 9.99]            | 9.06<br>[8.08 ; 9.49]       | 9.69<br>[9.48 ; 10.66]      | 9.79<br>[8.91 ; 10.35]      |
| Plate type-B carbonate substitution     | 0.1332<br>[0.1321 ; 0.1385] | 0.1317<br>[0.1226 ; 0.1363] | 0.1327<br>[0.1315 ; 0.14]   | 0.1361<br>[0.1313 ; 0.1437] | 0.1391<br>[0.1332 ; 0.144]  |
| Plate crystallinity                     | 0.0596<br>[0.0586 ; 0.0604] | 0.0588<br>[0.0578 ; 0.0607] | 0.0607<br>[0.0604 ; 0.0611] | 0.0604<br>[0.06 ; 0.0611]   | 0.0601<br>[0.0595 ; 0.0608] |
| Plate collagen maturity                 | 0.0258<br>[0.0255 ; 0.027]  | 0.0259<br>[0.0254 ; 0.0262] | 0.0255<br>[0.025 ; 0.0259]  | 0.026<br>[0.0259 ; 0.0262]  | 0.0252<br>[0.0251 ; 0.0258] |
| Plate hydroxyproline-to-proline ratio   | 0.642<br>[0.621 ; 0.716]    | 0.617<br>[0.602 ; 0.678]    | 0.647<br>[0.608 ; 0.685]    | 0.613<br>[0.597 ; 0.663]    | 0.615<br>[0.592 ; 0.647]    |

