



## STROMAL CELLS OF GIANT CELL TUMOUR OF BONE SHOW PRIMARY CILIA

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3 **STROMAL CELLS OF GIANT CELL TUMOR OF BONE SHOW PRIMARY CILIA**  
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5 **PRIMARY CILIA IN GIANT CELL TUMOR OF BONE**  
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## ABSTRACT

Giant cell tumor of bone (GCTB) is a locally aggressive primary bone neoplasm composed by tumoral stromal cells and reactive monocytic/histiocytic cells that fuse to form osteoclast-like multinucleated cells. Recently, specific histone 3.3 mutations have been demonstrated in stromal cells of GCTB. Many of the pathways related to bone proliferation and regulation depend on the primary cilium, a microtubule-based organelle which projects outside the cell and acts as a sensorial antenna. In the present work, we aimed to study the presence and role of primary cilia in GCTB.

Ultrastructural, immunohistochemical and immunofluorescence studies were performed in order to demonstrate that the primary cilium is located in spindle-shaped stromal cells of GCTB. Moreover, we showed Hh signalling pathway activation in these cells. Hence, primary cilia may play a relevant role in GCTB tumorigenesis through Hh signalling activation in stromal cells.

**Keywords:** Giant cell tumor of bone, ultrastructural study, primary cilium, Hh signalling pathway

### Research highlights

- Transmission electron microscopy allows to describe and differentiate cellular subpopulations in Giant Cell Tumor of Bone.
- The primary cilium is present in some tumoral Stromal Cells of Giant Cell Tumor of Bone.
- Hedgehog signalling is activated in these cells.

## 1. INTRODUCTION

According to the World Health Organisation (WHO) definition, giant cell tumor of bone (GCTB) is a locally aggressive primary bone neoplasm composed of proliferative mononuclear stromal cells (SCs), numerous reactive macrophages and large osteoclast-like multinucleated giant cells (GCs) (Athanasou et al., 2013). GCTB usually appears in young adults' metaphysis-epiphysis of long tubular bones. Although malignant transformation in GCTB is uncommon, pulmonary metastasis have been occasionally described (Athanasou et al., 2013).

Two mixed mononuclear cell types are present in the GCTB: monocyte-macrophage CD14+/CD68+ cells and SCs (Wülling et al., 2003). Nowadays, it is accepted that SCs represent the proliferative tumoral component of the GCTB; meanwhile, reactive multinucleated GCs are originated from blood monocytes recruited into the tumoral tissue (Wülling et al., 2003; Kim et al., 2012;). Recently, the mutation G34W in Histone H3 has been proved as a useful diagnostic marker of SC in GCTB (Cleven et al., 2015).

Previous ultrastructural studies dealing with GCTB have been focused on characterization of mononuclear cells and their osteoblastic or osteoclastic differentiation (Anazawa et al., 2006; Aparisi et al., 1977; Garcia et al., 2013). Furthermore, ultrastructural similarities between GCTB giant cells with physiological osteoclasts and with multinucleated GCs in lesions that mimic GCTB have been also reported (Anazawa et al., 2006).

The primary cilium is a microtubular non-motile structure composed of a 9+0 axoneme originated from a modified centriole which projects to the extracellular medium (Malicki & Johnson, 2017). Its presence in skeletal cells was detected almost 50 years ago (Tonna & Lampen, 1972). The potential role that this organelle may play in bone development and homeostasis was proposed in the early 21<sup>st</sup> century (Whitfield, 2003). However, there is increasing evidence that primary cilia-dependent signalling

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3 pathways, like Hedgehog (Hh) and ~~Wnt~~, play a crucial role in regulating mammalian  
4 bone development and turnover (Day & Yang, 2008). Thus, Hh pathway activation is  
5 necessary for Runx2 expression, a transcription factor essential for correct osteoblast  
6 differentiation (Rutkovskiy et al., 2016).  
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12 Three Hh ligands have been described: Sonic (Shh), Indian (Ihh) and Desert (Dhh)  
13 (Ramsbottom & Pownall, 2016). Ihh has been mainly involved in bone formation (Yang  
14 et al., 2015). When these ligands bind the transmembrane receptor Patched (located in  
15 the ciliary membrane) it is removed from primary cilia, allowing Smo entrance in ciliary  
16 axoneme. Then, Gli proteins are activated and translocated into the cellular nucleus,  
17 where they promote target gene transcription (including Runx2) (Chahal et al., 2018).  
18 Hh activation enhances expression of genes related to proliferation, angiogenesis or  
19 epithelial-mesenchymal transition (Chahal et al., 2018). Thus, excessive Hh signalling  
20 activation leads tumor formation and maintenance of cancer stem cells (Chahal et al.,  
21 2018). Moreover, ciliary proteins, including Arl13b, have been shown to be essential in  
22 Hh signalling and in its oncogenic implications (Larkins et al., 2011; Bay et al., 2018).  
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36 Here, we show for the first time that SCs of GCTB present primary cilia. This finding  
37 might have important implications in these tumors through Hh signalling pathway  
38 activation.  
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## 46 2. METHODS

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48 Six GCTB samples biopsied from patients were included in the present study. All  
49 samples were provided by the *Hospital Clínico Lozano Blesa* from Zaragoza. All the  
50 protocols developed were approved by the Human Research Ethics Committee of  
51 Aragon (CEICA) and were in accordance with Helsinki Declaration.  
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### 59 2.1. Electron Microscopy

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3 A piece of every tumor consisting of 2.5x2.5mm was immersed in 2.5% glutaraldehyde  
4 for fixation for 4-6h. The samples were washed in PB, post-fixed with 2% osmium,  
5 rinsed, dehydrated in graded ethanol, stained with 2% uranyl-acetate, cleared in  
6 propylene oxide and embedded in araldite (Durcupan, Fluka). Serial semi-thin sections  
7 (1.5µm) were cut and lightly stained with 1% toluidine blue. Later, ultrathin (0.08µm)  
8 sections were cut with a diamond knife, collected on Formvar coated single-slot grids,  
9 counter-stained with 1% uranyl acetate and Reynold's lead citrate for 10 minutes. They  
10 were examined by a JEOL-JEM-1010 transmission electron microscope (TEM). The  
11 images were captured with Gatan Bioscan Camera with pixel size 6.45µm x 6.45µm  
12 and spatial resolution of 1024,1024, 1, 1 (x, y, z, t) and automatic gain (assigning a  
13 brightness value to each pixel depending on the clearest and the darkest intensity in  
14 every image and highlighting electron density contrast).

## 2.2. Immunohistochemistry

32 Samples were processed according to standard histological procedures and stained  
33 with hematoxylin and eosin (H&E). Immunohistochemical staining was performed on  
34 formalin-fixed paraffin-embedded 4-µm thick sections. All immunohistochemical  
35 products except primary antibody were obtained from DAKO (Denmark). Primary  
36 antibodies used in this study were: monoclonal rabbit anti-Histone H3.3 G34W (2.5  
37 µg/mL, Quimigen S.L., 31-1145-00, Madrid, Spain), polyclonal rabbit anti-Gli1 (1:200,  
38 ABCAM, ab49314, Cambridge, UK), polyclonal rabbit anti-Smo (1:1000, LS Bioscience,  
39 LS-B4911, Seattle, USA) and polyclonal goat anti-Patched (1:100, Santa Cruz, sc-  
40 6149, Dallas, USA). Tissue sections were deparaffinised and hydrated using routine  
41 methods and then subjected to heat-induced antigen retrieval. For Gli1 and Smo, buffer  
42 citrate pH6 was used and the samples were treated during 6 minutes in an 800-W  
43 microwave and at 360 W for 5 additional minutes. For H3.3 G34W and Patched  
44 staining, samples were treated at 96°C for 20 min in EnVision™ FLEX Target Retrieval  
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3 solution High pH. Endogenous peroxidase was blocked for 30 minutes and sections  
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5 were washed in distilled water and PBS 3 min, twice.  
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8 Afterwards, sections were incubated with primary antibodies at 4°C overnight in a  
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10 humidified chamber, washed in PBS three times and incubated with secondary  
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12 antibody Rabbit-Mouse Labelled Polymer-Dako EnVision-HRP or Polyclonal Rabbit  
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14 Anti-Goat Immunoglobulins/HRP (1:200) for 1 hour. 3,3'-diaminobenzidine was used as  
15  
16 chromogen. The samples were washed twice in distilled water, contrasted with Mayer's  
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18 hematoxylin for 7 min, washed in tap water for 15 min, dehydrated in a graded series of  
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20 ethanol, cleared in xylene and cover slipped with Eukitt.  
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23 Negative controls where based on samples where no primary antibody was added. All  
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25 antibodies were commercial and supposedly validated.  
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28 All samples were analysed and digital images from representative areas were captured  
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30 by Olympus BX53 and Olympus UIS UPLFLN Series objectives. FN: 26.5. 20x AN 0.5  
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32 and 40x AN 0.75. Images were captured with Olympus DP72 Digital Camera. Pixel size  
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34 6.45µm x 6.45µm, total number of pixel 1.5 million. Exposition mode: SFL Auto and  
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36 spatial resolution of 4140x3096x1x1 (x, y, z, t).  
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### 40 2.3. Double Immunofluorescence Microscopy

41  
42 Formalin-fixed paraffin-embedded tissues were deparaffinised and hydrated. Then,  
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44 antigen retrieval was performed at 96°C for 20 min in EnVision™ FLEX Target  
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46 Retrieval solution High pH. Sections were incubated overnight at 4°C with the following  
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48 antibodies: mouse-monoclonal anti-Acetylated tubulin (1:4000, Sigma Aldrich, T7451;  
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50 St. Louis, MO, USA), mouse monoclonal anti-Arl13b (1:50, Proteintech, 66739-1-Ig,  
51  
52 Manchester, UK) and rabbit-monoclonal anti-Histone H3.3 G34W (2.5 µg/mL, Quimigen  
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54 S.L., 31-1145-00, Madrid, Spain). They were washed in PBS and incubated with the  
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56 respective secondary antibodies: anti-Mouse AlexaFluor594 (1:1000, ThermoFisher  
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58 R37115; Waltham, MA, USA) and anti-Rabbit AlexaFluor488 (1:1000, ThermoFisher A-  
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21206; Waltham, MA, USA) for 1 hour at RT. Incubations were performed in a dark humidified chamber. After washing in PBS, DAPI (1 µg/mL, Sigma-Aldrich) was added for 1 minute for nuclei counterstaining. Sections were washed in PBS and covered with DAKO Fluorescence mounting medium.

All antibodies were commercial and supposedly validated.

Images were analysed using the following filters: U-MNUA2 for DAPI, U-MWIABA2 for Alexa Fluor 488 and U-MWIG2 for Alexa Fluor 594 in a Olympus BX53 microscope and Olympus UIS UPLFLN Series objectives. FN: 26.5. 20x AN 0.5 and 40x AN 0.75. Images were captured with Olympus DP72 Digital Camera. Pixel size 6.45µm x 6.45µm, total number of pixel 1.5 million. Exposition mode: Auto and spatial resolution of 4080x3072x1x1 (x, y, z, t). Images were analysed with DP Controller Software. Every fluorescent channel was individually photographed and channels were merged using FIJI Image\_J software, where contrast and brightness were adjusted (Schindelin et al., 2012).

Statistical analyses were performed counting ciliated cells per High Power Field (HPF = x400) and using a non parametric test (Mann-Whitney U). Hence, representative areas were analysed (total of 18 images). In each HPF, between 70 and 100 mononuclear cells were present while the number of giant cells ranges from 0 to 7.

### 3. RESULTS

#### 3.1. Preliminary histological study.

Histopathological techniques routinely confirmed the diagnosis of GCTB (Fig.1). Thus, H&E revealed the presence of multinuclear GCs scattered among mononuclear cells (Fig.1a). CD68 antibody labelled mononuclear histiocytic cells and multinucleated GCs (Fig.1b) while Histone H3.3 mutation G34W antibody labelled tumoral SCs (Fig.1c). Ki67 was exclusively expressed by mononuclear cells (Fig.1d).



### 3.2. GCTB ultrastructural findings

Electron microscopy examination evidenced two well-defined groups: mononuclear and multinucleated giant cells. In turn, mononuclear cells included two different phenotypes based on cytoplasm electrondensity and nuclear chromatin condensation. Thus, histiocytic cells showed lesser electrondensity and homogenous chromatin, while SCs cytoplasm were more electrondense and their nuclei showed condensed chromatin constituting marginal clusters in contact with nuclear envelope (Fig.2a).

SC morphology varied from fusiform-spindle to oval. They showed fine membrane prolongations contacting neighbour cells (Fig.2b). They presented cytoplasmic electron-light no-membranous inclusions conferring a characteristic vacuolated appearance (Fig.2b). Some mitochondria, scarce granular endoplasmic reticulum and abundant free ribosomes were observed. Centrioles, located near nuclei, showed subdistal appendages and they were related to intermediate filaments and numerous Golgi-derived vesicles (Fig.2c-d). All these findings are suggestive of centriolar activation.

Primary cilia were specifically observed in SCs (Fig.2e-f). Primary ciliogenesis sequentially ordered in Figure 3. Firstly, the ciliary vesicle (constituted by fusion of Golgi-derived vesicles) localizes on the distal pole of the mother centriole, which forms cilia basal body (Fig.3a). Then, the 9+0 axoneme grew from the basal body (Fig. 3b). Occasionally, abnormal duplication of centrioles was evidenced by detection of three centrioles, two of them originating cilia (Fig.3c). Figures 3d and 3e show ultrastructural features corresponding to a primary cilium in its maximum length. Basal body presented subdistal appendages and was anchored to cell membrane by transition fibres. The axoneme was originated from the basal body and it was located in a cell membrane invagination called ciliary pocket, where coated vesicles are concentrated as a sign of important molecular exchange (Fig.3e). In some ciliated cells, nuclei showed characteristic nuclear envelope prolongations (Fig. 3d-e). These nuclear structures have been previously called envelope-limited chromatin sheets (ELCS).

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3 Primary cilia in these cells are originated from non-displaced centrioles and, therefore,  
4 goes through the cytoplasm towards the extracellular medium.

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7 The mononuclear histiocytic cells showed a less electron-dense cytoplasm (Fig.4a),  
8 with abundant mitochondria, granular endoplasmic reticulum and prominent Golgi  
9 apparatus. Although their nuclei showed a diverse morphology, peripheral nucleoli  
10 were always found. They presented many lysosomes and fine phagocytic  
11 prolongations (Fig.4b). These cells contact each other through cytoplasmic  
12 prolongations (Fig.4b). A reduced number of these histiocytic cells showed scarce  
13 organelles, abundant ribosomes and multivesicular bodies, all features suggestive of  
14 an undifferentiated phenotype (Fig. 4c).

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24 Multinucleated GCs included 10 to 30 nuclei. They showed oval or irregular  
25 morphology and an electron light appearance. They presented a fine marginal  
26 chromatin contacting nuclear membrane and prominent nucleoli (Fig.4d). Their  
27 cytoplasm presented a homogenous distribution of organelles, abundant mitochondria  
28 and lipid inclusions (Fig. 4d). Surrounding multinucleated GCs, both, mononuclear  
29 histiocytic and stromal cells were located. Although defining dynamic processes from  
30 TEM is always difficult, we could observe light undifferentiated histiocytic cells merging  
31 with GCs and syncytial membrane disappearance, suggestive of cellular fusion  
32 (Fig.4e).

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45 Some reactive foam cells plenty of electron lucent vacuoles were also found in these  
46 tumors. They were probably involved in phagocytic processes as they showed  
47 lysosomes with different content (Fig.4f). Few dispersed lymphocytes and granulocytes  
48 were also observed, the latter being involved in macropinocytosis processes.

### 53 54 55 3.3. Primary cilia and Hh signalling presence in GCTB

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57 Immunofluorescent co-localisation of Histone H3.3 mutation G34W (specific for SCs of  
58 GCTB) and Acetylated-tubulin (marker for cilia axoneme) and Arl13b (marker for ciliary  
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3 membrane) showed that stromal cells presented primary cilia (Fig. 5, Suppl. Fig. 1).  
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5 Primary cilia were usually located near nuclei, in agreement with electron microscopy  
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7 findings. Quantitative analyses showed that around 7 mononuclear cells per High  
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9 Power Field (x400) were ciliated, which supposed around 10% of mononuclear cells,  
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11 while no multinucleated giant cell presented cilia (Suppl. Fig.2).

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13 Furthermore, the implications of primary cilia in GCTB were studied by analysis of the  
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15 activation of Hh signalling pathway, a well-known cilia-dependent pathway related to  
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17 tumorigenesis and bone regulation. Immunohistochemical experiments for Hh pathway  
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19 protein Patched, Smo and Gli1 were performed (Suppl. Fig. 3, Fig. 6). Patched (Suppl.  
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21 Fig. 3a) and Smo (Suppl. Fig. 3b) staining were located in paranuclear regions while  
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23 Gli1 labelled subtly but clearly cells nuclei (Fig. 6a-c). Nuclear staining of Gli1 is a sign  
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25 of Hh signalling activation. Thus, these results suggest that Hh signalling pathway is  
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27 present and activated in SCs of GCTB.  
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#### 32 **4. DISCUSSION**

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34 The nature and cellular components of GCTB have constituted the subject of multiple  
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36 previous studies. Most of them wrongly identified all mononuclear cells as stromal cells  
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38 based on conventional microscopy morphology. However, histochemical and electron  
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40 microscopy techniques shed light on cells identification (Hanaoka et al., 1970; Aparisi  
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42 et al., 1977). In this study we have accurately defined ultrastructural features of the two  
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44 different mononuclear cell populations identified by cytoplasm electrondensity and  
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46 nuclear chromatin condensation. Cells showing lesser electrodensity and homogenous  
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48 chromatin are called histiocytic cells here. The round histiocytic cells previously  
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50 described by other authors may correspond to activated histiocytes with phagocytic  
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52 capacity (Hanaoka et al., 1970; Aparisi et al., 1977). Intermediate phenotypes are  
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54 compatible with resting, activated or prior to giant cell formation stages.  
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58 The second mononuclear cell type, stromal cells (SCs), show higher electrondensity in  
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60 their cytoplasm and nuclei with condensed chromatin forming marginal clusters in

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3 contact with nuclear envelope. This phenotype corresponds to stromal cells type 1 of  
4 Aparisi (Aparisi et al., 1977). However, phenotypic variability appears. Probably, those  
5 presenting features suggestive of undifferentiation may suppose cancer stem cells.  
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7 SCs have been defined as immature fibroblasts or primitive osteoblasts. Their close  
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9 histogenic relationship with osteoblasts is based on focal deposition of osteoid seen in  
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11 one third of GCTB (Spjut et al., 1983). Additionally, bone tissue is not only produced by  
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13 reactive osteoblasts but also by SCs (Goldring et al., 1987). According with the results  
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15 provided here, SCs are clearly defined as mesenchymal cells presenting special and  
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17 exclusive ultrastructural features such as presence of large electron-light vacuoles,  
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19 peculiar density of cytoplasm and activation of centrioles. The most novel finding  
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21 provided in the present study is the presence of primary cilia in these cells.  
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27 Primary cilia in osteoblasts and osteocytes contributes to bone formation and  
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29 homeostasis (Mitchison & Valente, 2017). Furthermore, the participation of primary cilia  
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31 in different types of cancer of epithelial and mesenchymal line has been demonstrated.  
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33 Nevertheless, the prevalence of cilia on human tumors remains unclear, and their role  
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35 in cancer is just beginning to be explored (Eguether & Hahne, 2018; Fabbri et al.,  
36  
37 2019). In human osteosarcoma MG63 cell line the number of ciliated cell is significantly  
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39 higher than expected (Kowal & Falk, 2015).  
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42 The cilia-dependant Hh signalling pathway plays a key role in both bone physiology  
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44 and pathology. Thus, it is essential for temporal and spatial regulation of bone  
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46 remodelling (Rodda, 2006; Yang et al., 2015). Moreover, Hh signalling pathway  
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48 (especially through the Ihh ligand) stimulates intramembranous and endochondral  
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50 ossification, bone turnover and remodelling of fractures sites (Yang et al., 2015).  
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53 The deregulation of Hh signalling pathway has been linked to some skeletal  
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55 development diseases (Rodda, 2006; Yang et al., 2015) and tumors (Scales & de  
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57 Sauvage, 2009). Furthermore, Hh signalling pathway in osteosarcoma was related to  
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59 radio-resistance and invasiveness (Qu et al., 2018). Interestingly, the ciliary membrane  
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3 protein Arl13b plays an essential role in tumoral Hh signalling through primary cilia  
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5 (Bay et al., 2018).  
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7 Here, we show Hh pathway activation in some SCs. These results are in accordance  
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9 with Horvai et al. (2012) where 10% of mononucleated cells expressed Ihh (we found a  
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11 similar proportion of ciliated cells, as it is described above). Besides, Horvai et al. also  
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13 showed that GCTB stromal cells expressed Runx2 (Horvai et al., 2012), a well-known  
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15 downstream target of Hh signalling pathway (Rutkovskiy et al., 2016).  
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18 The observation of primary cilia and Hh pathway activation in SCs suggests their role in  
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20 cellular signalling and tumorigenesis. Moreover, some ciliated SCs showed envelope-  
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22 limited chromatin sheets (ELCS), a structure described in cancer and stem cells (Olins  
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24 & Olins, 2009; Cebrián-Silla et al., 2017). These findings suggest that primary cilia may  
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26 play an important role in quiescent cancer stem cells of GCTB. This feature would  
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28 explain why only a specific population of stromal cells show primary cilia and Hh  
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30 activation and they are not constitutively present in every stromal cell. Moreover, the  
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32 dynamic nature of cilia assembly and Hh signalling must be taken into account while  
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34 the techniques performed in this paper are mainly static.  
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36  
37 In conclusion, our study reveals that SCs of GCTB present primary cilia. Moreover, we  
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39 proved that Hh signalling pathway is activated in these cells, showing that primary cilia  
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41 may play an important role in GCTB tumorigenesis and could be consequently used  
42  
43 as a potential therapeutic target.  
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## 46 47 **5. DECLARATIONS**

48  
49 **Funding.** No funding was specifically received for the experiments showed in this  
50  
51 paper.  
52

53  
54 **Competing interests.** The authors declare that they have no conflict of interest.

55  
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57  
58 were approved by the Human Research Ethics Committee “*Comité Ético de*  
59  
60 *Investigación Clínica de Aragón*”.

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2  
3 **Authors contribution.** TC conceived the study, performed ultrastructural experiments  
4 and contributed to write the manuscript, PI performed immunohistochemical and  
5 immunofluorescence studies and contributed to write the manuscript, EM performed  
6 immunohistochemical experiments. MJC and JGV provided the samples. MM  
7 contributed to write the manuscript. CJ conceived and supervised the study, performed  
8 ultrastructural experiments, analysed the results and contributed to write the  
9 manuscript.

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## 28 FIGURE LEGENDS

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33 **Fig. 1. Histological diagnosis and preliminary study of GCTB.** a) Hematoxylin-  
34 Eosin staining shows two cellular types in GCTB: mononuclear and multinuclear giant  
35 cells (GC). Some of the mononuclear cells showed a characteristic paranuclear  
36 vacuole (arrow). Scale bar = 50µm b) CD68 is a specific marker for monocyte-  
37 macrophage lineage. Thus, histiocytic mononuclear cells and giant cells (GC)  
38 expressed CD68. Scale bar = 50µm c) Histone H3.3 G34W mutation is a specific  
39 marker of the stromal neoplastic cells of GCTB. According to this, only mononuclear  
40 cells were stained, some of them expressing paranuclear vacuoles (arrow). Scale bar =  
41 50µm d) Immunolabeling for Ki67, marker of cell cycle activation and proliferation,  
42 stained uniquely mononuclear cells. Scale bar = 100µm  
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56 **Fig.2. TEM examination. Ultrastructural features of mononuclear cells of GCTB.**  
57 a) Histiocytic (\* light) and stromal (dark) cells are the main mononuclear components of  
58 GCTB. Scale bar = 5µm b) Stromal cells (SCs) show large paranuclear vacuoles  
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3 (v).Scale bar = 2 $\mu$ m c-d) Centriole activation at different stages. Note the numerous  
4 small vesicles (vs), subdistal appendages (s) and intermediate filaments (if) in relation  
5 with centrioles; features related to centriolar activation. Scale bar = 500nm e) Primary  
6 cilia in a SC. Scale bar = 2 $\mu$ m f) Magnification of squared area in e.Scale bar = 500nm.  
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11 ax: axoneme, bb: basal body,v: vacuoles, vs: vesicles, if: intermediate filaments, mt:  
12 microtubule, s: subdistal appendages, g: Golgi  
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18 **Fig.3. TEM examination. Primary cilia in stromal cells of GCTB.** a) At the beginning  
19 of ciliogenesis, small vesicles derived from Golgi-apparatus fuse in a ciliary vesicle (cv)  
20 which localizes on top of the basal body (bb).Scale bar = 500nm b) Axoneme (ax)  
21 originates from basal body and is surrounded by a cell membrane invagination called  
22 ciliary pocket (cp). Daughter centriole shows pericentriolar satellites (s). Scale bar =  
23 500nm c) Tumoral cells exceptionally showed centriole duplication, with two cilia  
24 emerging. Scale bar = 500nm d) SC shows envelope-limited chromatin sheet (elcs)  
25 and primary cilium.Scale bar = 1 $\mu$ m e) Primary cilium ultrastructural features. Scale bar  
26 = 1 $\mu$ m. mt: microtubule, if: intermediate filaments, pr: polyribosomes, vs: vesicles, sa:  
27 subdistal appendages, s: satellites, arrow: ciliary pocket, black arrowhead: transition  
28 fibres, white arrowhead: coated vesicles, bb: basal body, ax: axoneme.  
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43 **Fig.4. TEM examination. Ultrastructural features of reactive cells: histiocytic light**  
44 **cells and multinucleated giant cells.** a) Histiocytic cells show less electrondense  
45 cytoplasm than SCs and prominent nucleoli (ni). Scale bar = 2 $\mu$ m b) Organelle  
46 distribution of histiocytic cells. Ly: lysosomes. Encircled: intercellular contact.Scale bar  
47 = 1 $\mu$ m c) Detailed area of histiocytic cells organelles showing mitochondria (mt)  
48 surrounded by endoplasmic reticula (er) near a multivesicular body (mvp).Scale bar =  
49 500nm d) Giant Cells show multiple nuclei with prominent nucleoli (ni). Moreover, its  
50 cell membrane shows short prolongations. A stromal cell (SC) appears near the GC.  
51 Scale bar = 5 $\mu$ m e) A histiocytic cell seems to fuse with GC (arrows point cell  
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3 membrane disappearance). Scale bar = 5µm f) Foam cells sometimes show lysosomes  
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5 (ly) containing inclusions of different source. Scale bar = 5µm  
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9 **Fig.5. Stromal cells show primary cilia.** a) Immunofluorescent co-localisation of  
10 Histone H3.3 G34W mutation in green and Arl13b (ciliary membrane) in red showed  
11 that some stromal cells present primary cilia. Scale bar = 20µm b-d) Magnification of  
12 cells showing Histone H3.3 G34W mutation and Arl13b co-localisation.  
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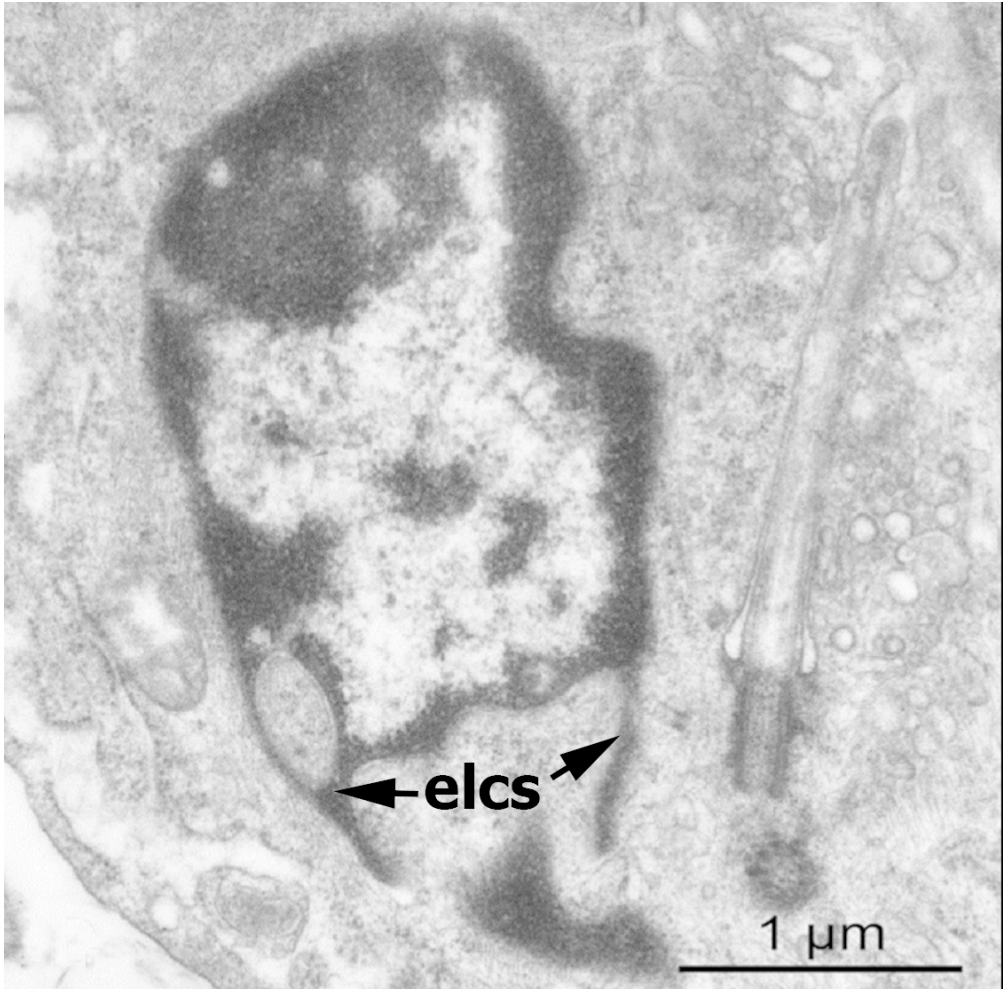
20 **Fig. 6. Immunohistochemical study of Hh pathway activation.** a-c) Nuclear labeling  
21 of Gli1 was present in some mononuclear cells, showing activation of Hh signaling  
22 pathway. GC: Giant Cell. Scale bar = 20µm  
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28 **Supplementary Fig. 1. Immunofluorescence study of primary cilia in SCs GCTB.**  
29 Co-localisation of Acetylated tubulin (red) and H3.3 G34W (green) give supporting  
30 evidence of cilia in SCs of GCTB. Scale bar = 50µm  
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37 **Supplementary Fig. 2. Quantitative analysis of primary cilia presence in GCTB**  
38 **cells.** Number of cells showing primary cilia per High-Power Field (HPF: x400). 18  
39 representative images were analysed in total. In each HPF, between 70 and 100  
40 mononuclear cells were present while the number of giant cells ranges from 0 to 7. No  
41 multinucleated giant cells showed primary cilia, while around 7 to 8 mononucleated  
42 cells (mean of 7,53) per HPF were ciliated (approximately 10% of total mononucleated  
43 cells). Results are expressed as mean ± SEM. Mann-Whitney U Test showed a  
44 statistical significance (p<0,00001).  
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56 **Supplementary Fig. 3. Smo and Patched staining in GCTB.** a) Some mononuclear  
57 cells showed paranuclear staining for Patched. Scale bar = 20µm b) Similarly, only  
58 mononuclear cells showed paranuclear expression of Smo. Scale bar = 20µm.  
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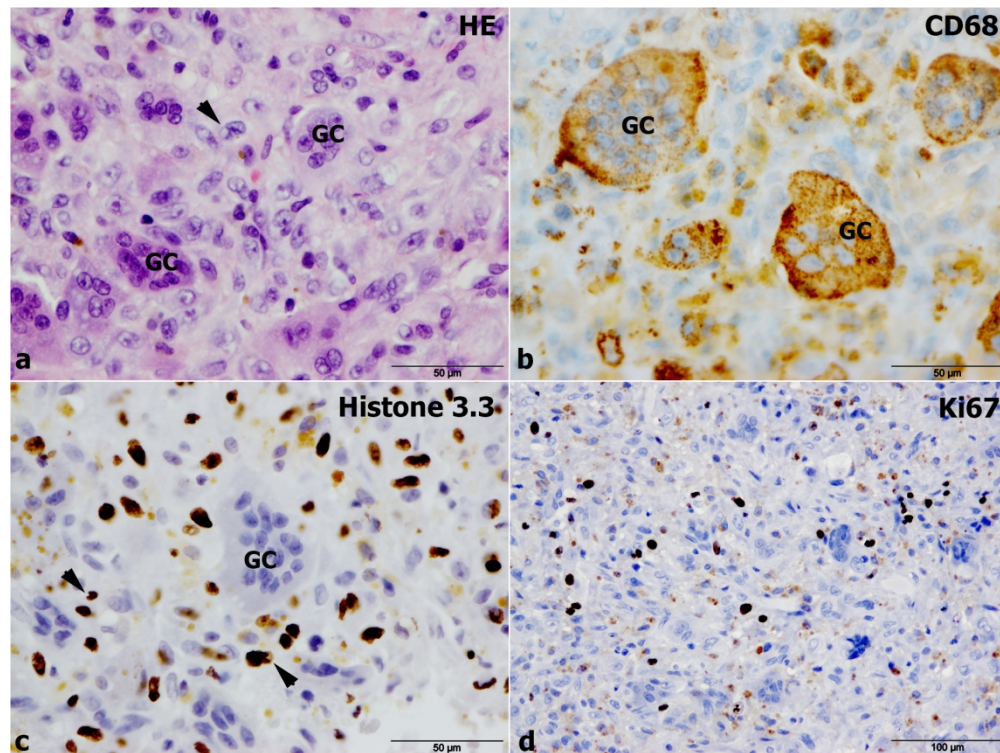


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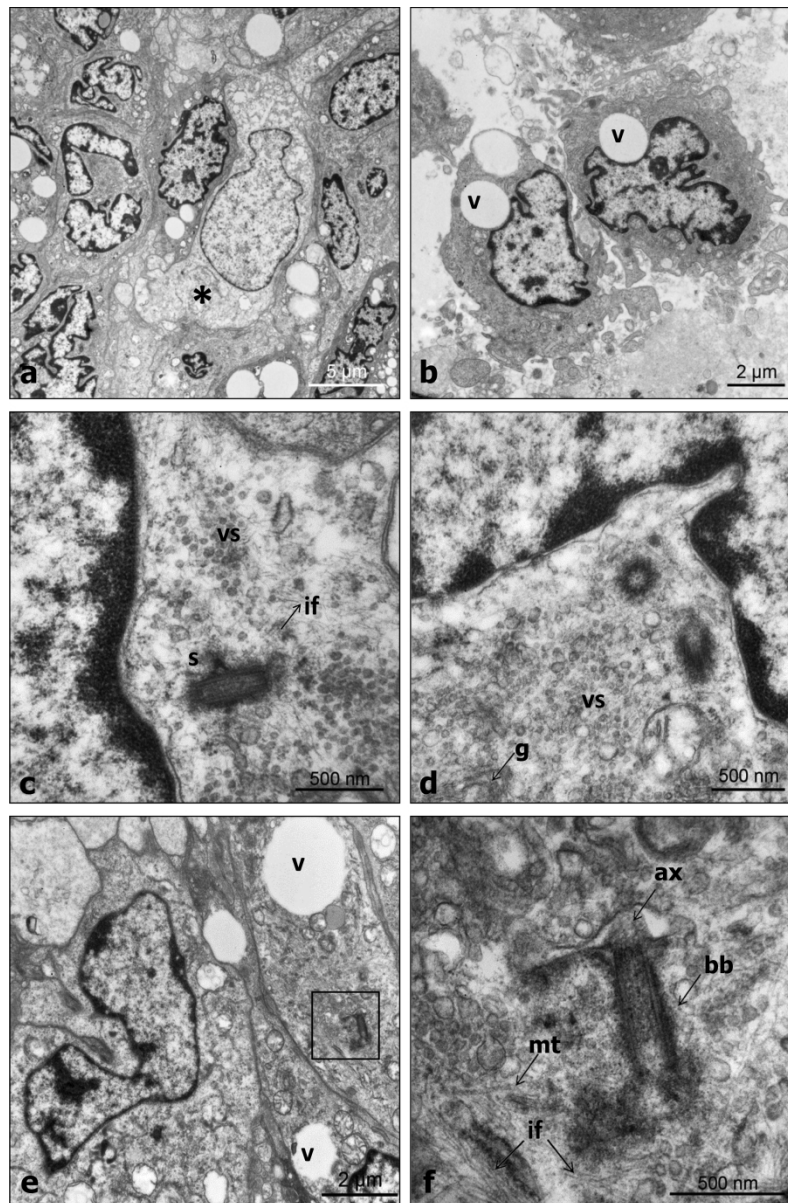


Fig.2. TEM examination. Ultrastructural features of mononuclear cells of GCTB. a) Histiocytic (\* light) and stromal (dark) cells are the main mononuclear components of GCTB. Scale bar = 5 $\mu$ m b) Stromal cells (SCs) show large paranuclear vacuoles (v). Scale bar = 2 $\mu$ m c-d) Centriole activation at different stages. Note the numerous small vesicles (vs), subdistal appendages (s) and intermediate filaments (if) in relation with centrioles; features related to centriolar activation. Scale bar = 500nm e) Primary cilia in a SC. Scale bar = 2 $\mu$ m f) Magnification of squared area in e. Scale bar = 500nm. ax: axoneme, bb: basal body, v: vacuoles, vs: vesicles, if: intermediate filaments, mt: microtubule, s: subdistal appendages, g: Golgi

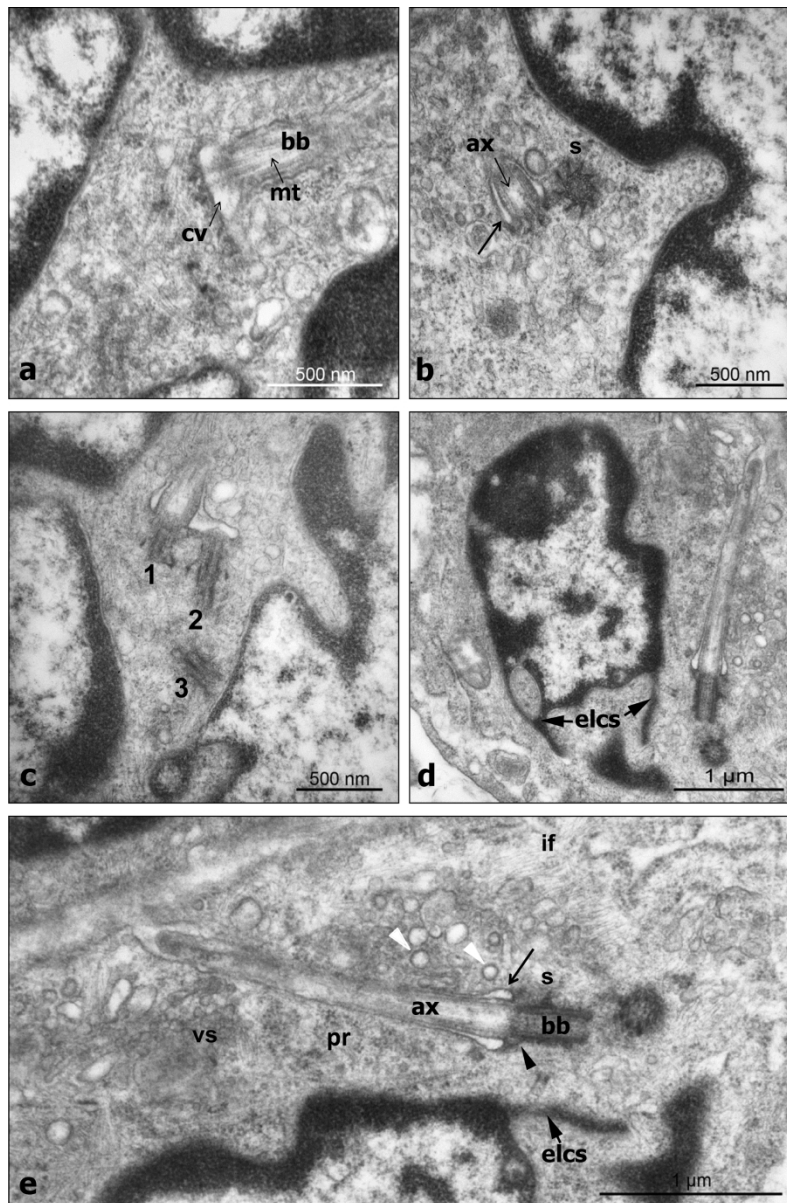


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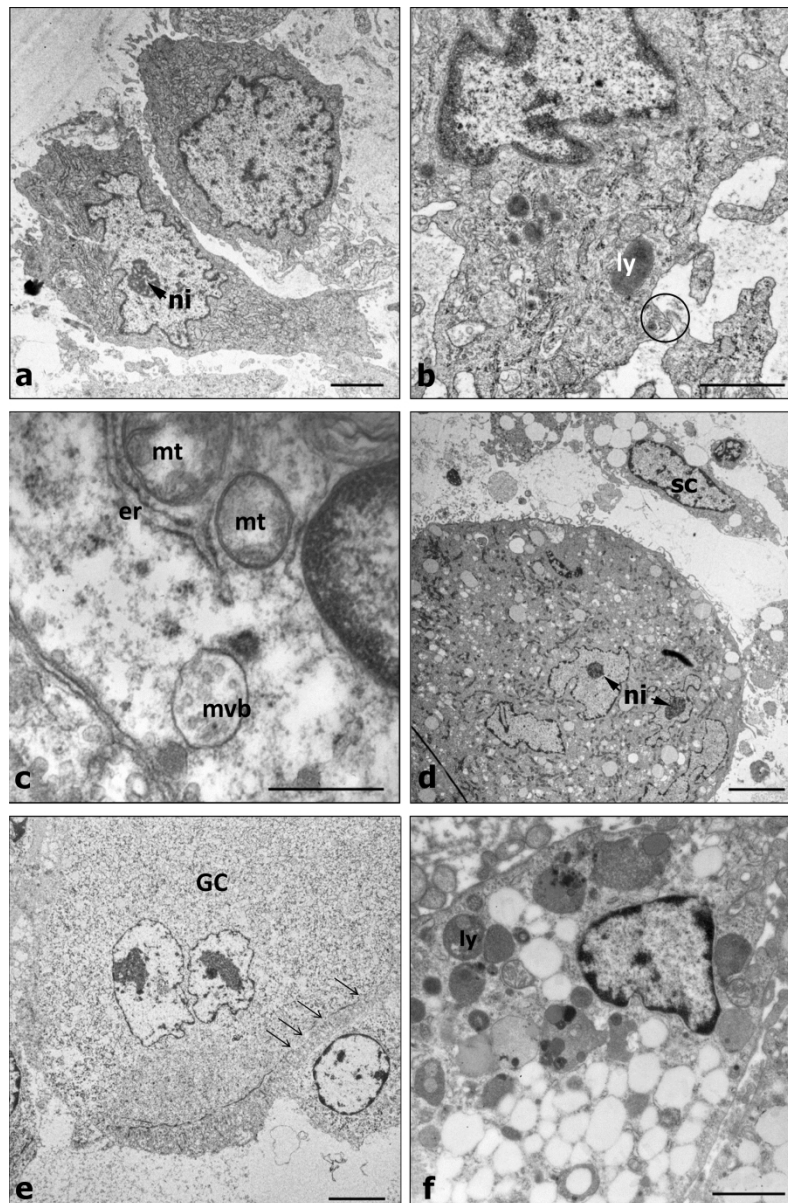


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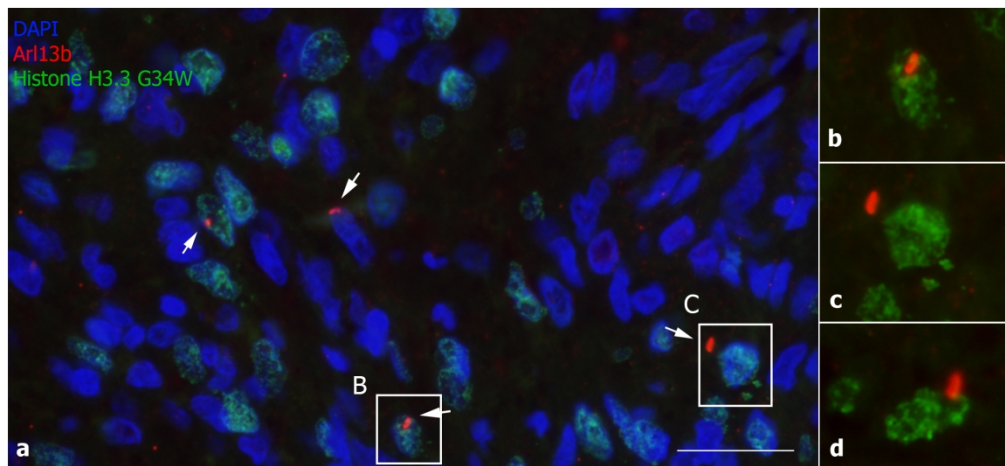


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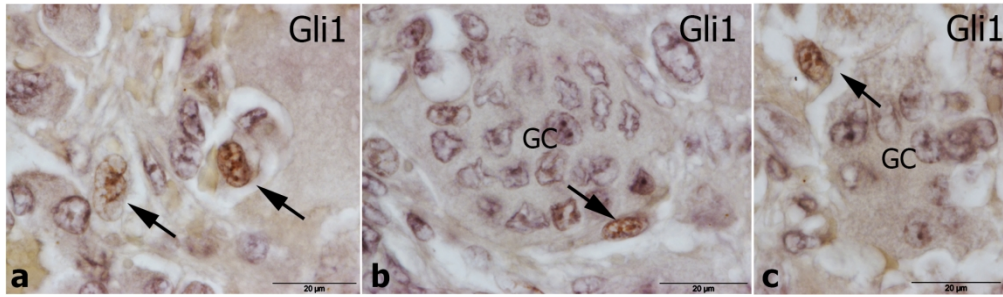


Fig. 6. Immunohistochemical study of Hh pathway activation. a-c) Nuclear labeling of Gli1 was present in some mononuclear cells, showing activation of Hh signaling pathway. GC: Giant Cell. Scale bar = 20µm

**STROMAL CELLS OF GIANT CELL TUMOR OF BONE SHOW PRIMARY CILIA****PRIMARY CILIA IN GIANT CELL TUMOR OF BONE**

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## ABSTRACT

Giant cell tumor of bone (GCTB) is a locally aggressive primary bone neoplasm composed by tumoral stromal cells and a reactive component that consists of monocytic/histiocytic cells that give rise by fusion to osteoclast-like multinucleated cells. Recently, specific histone 3.3 mutations have been demonstrated in stromal cells of GCTB. Many of the pathways related to bone proliferation and regulation depend on the primary cilium, a microtubule-based organelle which protrudes outside the cell and acts as a sensorial antenna. In the present work, we aimed to study the presence and role of primary cilia in GCTB.

Ultrastructural, immunohistochemical and immunofluorescence studies were performed in order to demonstrate, for the first time, that the primary cilium is located in spindle-shaped stromal cells of GCTB. Moreover, we showed Hh signaling pathway activation in these cells. Hence, primary cilia may play a relevant role in GCTB tumorigenesis through Hh signaling activation in stromal cells.

**Keywords:** Giant cell tumor of bone, ultrastructural study, primary cilium, Hh signaling pathway

### Research highlights

- Transmission electron microscopy allows describing and differentiating cellular subpopulations in Giant Cell Tumor of Bone.
- The primary cilium is present in some tumoral Stromal Cells of Giant Cell Tumor of Bone.
- Hedgehog signalling is activated in these cells.

## 1. INTRODUCTION

According to the World Health Organisation (WHO) definition, giant cell tumor of bone (GCTB) is a locally aggressive primary bone neoplasm composed of proliferative mononuclear stromal cells (SCs) and numerous reactive macrophages and large osteoclast-like multinucleated giant cells (GCs) (Athanasou et al., 2013). GCTB usually appears in young adults' metaphysis-epiphysis of long tubular bones. Factors influencing the clinical course and biological aggressiveness of GCTB are unclear. Although malignant transformation in GCTB is uncommon, pulmonary metastasis have been described (Athanasou et al., 2013).

Two mixed mononuclear cell types are present in the GCTB: monocyte-macrophage CD14+/CD68+ cells and SCs (Wülling et al., 2003). Nowadays, it is accepted that SCs represent the proliferative tumoral component of the GCTB while reactive multinucleated GCs are originated from blood monocytes which are recruited into tumoral tissue (Kim et al., 2012; Wülling et al., 2003). Recently, Histone H3 G34W mutation has been proved as a useful diagnostic marker of SC in GCTB (Cleven et al., 2015).

Previous ultrastructural studies dealing with GCTB have been focused on characterization of mononuclear cells and their osteoblastic or osteoclastic differentiation (Anazawa et al., 2006; Aparisi et al., 1977; Garcia et al., 2013). Several reports have also described the ultrastructural similarities of GCTB giant cells with physiological osteoclasts and with multinucleated GC in lesions that mimic GCTB (Anazawa et al., 2006).

The primary cilium is a microtubular non-motile structure composed of a 9+0 axoneme originated from a modified centriole which projects to the extracellular medium (Malicki & Johnson, 2017). Its presence in skeletal cells was detected almost 50 years ago (Tonna & Lampen, 1972). However, the hypothesis that this organelle may play a role in regulating bone development and homeostasis was proposed in the beginning of the

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3 twenty first century (Whitfield, 2003). There is increasing evidence that primary cilia-  
4 dependent signaling pathways, like Hedgehog (Hh) and Wnt, play a crucial role in  
5 regulating mammalian bone development and turnover (Day & Yang, 2008). Thus, Hh  
6 pathway activation is necessary for Runx2 expression, a transcription factor essential  
7 for correct osteoblast differentiation (Rutkovskiy et al., 2016).  
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14 Three Hh ligands have been described: Sonic (Shh), Indian (Ihh) and Desert (Dhh)  
15 (Ramsbottom & Pownall, 2016). Ihh has been mainly implicated in bone formation  
16 (Yang et al., 2015). When these ligands bind the transmembrane receptor Patched  
17 (located in the ciliary membrane) it is removed from primary cilia, allowing Smo  
18 entrance in ciliary axoneme. Then, Gli proteins are activated and translocated into the  
19 cellular nucleus, where they promote target gene transcription (including Runx2)  
20 (Chahal et al., 2018). Hh activation enhances expression of genes related to  
21 proliferation, angiogenesis or epithelial-mesenchymal transition (Chahal et al., 2018).  
22 Thus, excessive Hh signaling activation leads to tumor formation and maintenance of  
23 cancer stem cells (Chahal et al., 2018). Moreover, ciliary proteins, including Arl13b,  
24 have been shown to be essential in Hh signalling and in its oncogenic implications  
25 (Larkins et al., 2011; Bay et al., 2018).  
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40 Here, we show for the first time that SCs of GCTB present primary cilia, which may  
41 have important implications in these tumors through Hh signaling pathway activation.  
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## 48 **2. METHODS**

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50 Six GCTB samples biopsied from patients were included in the present study. All  
51 samples were provided by the Hospital Clínico Lozano Blesa from Zaragoza. All the  
52 protocols developed were approved by the Human Research Ethics Committee of  
53 Aragon (CEICA) and were in accordance with Helsinki Declaration.  
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## 2.1. Electron Microscopy

A piece of every tumor consisting of 2.5x2.5mm was immersed in 2.5% glutaraldehyde for fixation for 4-6h. The samples were washed in PB buffer solution, post-fixed with 2% osmium, rinsed, dehydrated in graded ethanol, stained with 2% uranyl-acetate, cleared in propylene oxide and embedded in araldite (Durcupan, Fluka). Serial semi-thin sections (1.5 $\mu$ m) were cut and lightly stained with 1% toluidine blue. Later, ultrathin (0.08 $\mu$ m) sections were cut with a diamond knife, collected on Formvar coated single-slot grids, counter-stained with 1% uranyl acetate and Reynold's lead citrate for 10minutes. They were examined by a JEOL-JEM-1010 transmission electron microscope (TEM). The images were captured with GatanBioscan Camera with pixel size 6.45 $\mu$ m x 6.45 $\mu$ m and spatial resolution of 1024,1024, 1, 1 (x, y, z, t) and automatic gain.

## 2.2. Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed paraffin-embedded sections 4- $\mu$ m thick using EnVision® (Dako) method. The primary antibodies used in this study were: monoclonal rabbit anti-Histone H3.3 G34W (2.5  $\mu$ g/mL, Quimigen S.L. 31-1145-00, Madrid, Spain), polyclonal rabbit anti-Gli1 (1:200, Abcam, ab49314, Cambridge, UK), polyclonal rabbit anti-Smo (1:1000, LS Bioscience, LS-B4911, Seattle, USA) and polyclonal goat anti-Patched (1:100, Santa Cruz, sc-6149, Dallas, USA). Antibodies were diluted with Dako diluents (S2022). The tissue sections were deparaffinize and hydrated using routine methods. Heat-induced antigen retrieval was performed. For Gli1 and Smo, buffer citrate ph6 (Dako S2031) was used and the samples were treated during 6 minutes in an 800-W microwave and at 360 W for 5 additional minutes. For H3.3 G34W and Patched staining, samples were treated with TBS (ph9) for 20 minutes at 96°C. Endogenous peroxidase was blocked using Dako S2001 peroxidase blocking reagent for 30 minutes and sections were washed in distilled water and PBS 3 min, twice.

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3 Afterwards, sections were incubated with primary antibodies at 4°C overnight in a  
4 humidified chamber, washed in PBS three times and incubated with secondary  
5 antibody Rabbit-Mouse Labelled Polymer-DakoEnVision-HRP (K5007) or Polyclonal  
6 Rabbit Anti-Goat Immunoglobulins/HRP (1:200, Dako, P016002-2) for 1 hour. 3,3'  
7 diaminobenzidine was used as chromogene. The samples were washed twice in  
8 distilled water, contrasted with Mayer's hematoxylin for 7 min, washed in tap water for  
9 15 min, dehydrated in a graded series of ethanol, cleared in xylene and coverslipped  
10 with Eukitt.  
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21 Negative controls where based on samples where no primary antibody was added. All  
22 antibodies were commercial and supposedly validated.  
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24 Whole samples were analysed and digital images from representative areas were  
25 captured by Olympus BX53 and Olympus UIS UPLFLN Series objectives. FN: 26.5.  
26 20x AN 0.5 and 40x AN 0.75. Images were captured with Olympus DP72 Digital  
27 Camera. Pixel size 6.45µm x 6.45µm, total number of pixel 1.5 million. Exposition  
28 mode: SFL Auto and spatial resolution of 4140x3096x1x1 (x, y, z, t).  
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### 39 2.3. Double Immunofluorescence Microscopy

40 Formalin-fixed paraffin-embedded tissues were deparaffinized and hydrated and antigen  
41 retrieval was performed using TBS (pH9) at 96 °C for 20 min. Sections were incubated  
42 overnight at 4°C with the following antibodies: mouse-monoclonal anti-Acetylated  
43 tubulin (1:4000, Sigma Aldrich, T7451; St. Louis, MO, USA), mouse monoclonal anti-  
44 Arl13b (1:50, Proteintech, 66739-1-Ig, Manchester, UK) and rabbit-monoclonal anti-  
45 Histone H3.3 G34W (2.5 µg/mL, Quimigen S.L., 31-1145-00, Madrid, Spain). They  
46 were washed in PBS for 5 minutes (three times) and incubated with the respective  
47 secondary antibodies: anti-Mouse AlexaFluor594 (1:1000, ThermoFisher R37115;  
48 Waltham, MA, USA) and anti-Rabbit AlexaFluor488 (1:1000, ThermoFisher A-21206;  
49 Waltham, MA, USA) for 1 hour at RT. Incubations were performed in a dark humidified  
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3 chamber. After washing in PBS, DAPI (1  $\mu\text{g}/\text{mL}$ , Sigma-Aldrich) was added for 1  
4 minute for nuclei counterstaining. Sections were washed in PBS and cover with DAKO  
5 Fluorescence mounting medium.  
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9 Whole samples were analysed and digital images from representative areas were  
10 captured by Olympus BX53 and Olympus UIS UPLFLN Series objectives. FN: 26.5.  
11 20x AN 0.5 and 40x AN 0.75. Images were captured with Olympus DP72 Digital  
12 Camera. Pixel size  $6.45\mu\text{m} \times 6.45\mu\text{m}$ , total number of pixel 1.5 million. Exposition  
13 mode: SFL Auto and spatial resolution of  $4140 \times 3096 \times 1 \times 1$  (x, y, z, t).  
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16 All antibodies were commercial and supposedly validated.  
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18 Images were analysed using the following filters: U-MNUA2 for DAPI, U-MWIABA2 for  
19 Alexa Fluor 488 and U-MWIG2 for Alexa Fluor 594 in a Olympus BX53 microscope and  
20 Olympus UIS UPLFLN Series objectives. FN: 26.5. 20x AN 0.5 and 40x AN 0.75.  
21 Images were captured with Olympus DP72 Digital Camera. Pixel size  $6.45\mu\text{m} \times$   
22  $6.45\mu\text{m}$ , total number of pixel 1.5 million. Exposition mode: Auto and spatial resolution  
23 of  $4080 \times 3072 \times 1 \times 1$  (x, y, z, t).  
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26 Images were analysed with DP Controller Software. Every fluorescent channel was  
27 individually photographed and channels were merged using FIJI Image\_J software,  
28 where contrast and brightness were adjusted (Schindelin et al., 2012).  
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31 Statistical analyses were performed counting ciliated cells per High Power Field (HPF =  
32  $\times 400$ ) and using a non parametric test (Mann-Whitney U). Hence, representative areas  
33 were analysed (total of 18 images). In each HPF, between 70 and 100 mononuclear  
34 cells were present while the number of giant cells ranges from 0 to 7.  
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### 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 **3. RESULTS**

#### 54 55 **3.1. Preliminary histological study.**

56 Hematoxylin-Eosin (HE) and general immunohistochemistry confirmed the diagnosis of  
57 GCTB (Fig.1). Thus, HE revealed the presence of multinuclear giant cells (GCs)  
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3 scattered among mononuclear cells (Fig.1a). CD68 antibody marked mononuclear  
4 histiocytic cells and multinucleated GCs (Fig.1b) while Histone H3.3 mutation G34W  
5 antibody labelled tumoral stromal cells (SCs) (Fig.1c). Ki67 was exclusively expressed  
6 by mononuclear cells (Fig.1d).  
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### 11 12 13 3.2. Ultrastructure of GCTB

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15 Electron microscopy examination evidenced two well-defined groups: mononuclear and  
16 multinucleated giant cells. In turn, mononuclear cells included two distinguishable  
17 phenotypes based on cytoplasm electrondensity and nuclear chromatin condensation.  
18 Thus, histiocytic cells showed lesser electrondensity and homogenous chromatin, while  
19 SCs cytoplasm were more electrondense and their nuclei showed condensed  
20 chromatin constituting marginal clusters in contact with nuclear envelope (Fig.2a).  
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30 SCs morphology varied from fusiform spindle to oval shapes. They showed fine  
31 membrane prolongations contacting neighbour cells (Fig.2b). They presented  
32 cytoplasmic electron-light no-membranous inclusions conferring a characteristic  
33 vacuolated appearance (Fig.2b). Some mitochondria, scarce granular endoplasmic  
34 reticulum and abundant free ribosomes were observed. Centrioles, located near nuclei,  
35 showed subdistal appendages and they were related to intermediate filaments and  
36 numerous Golgi-derived vesicles (Fig.2c-d). All these findings are suggestive of  
37 centriolar activation.  
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47 Primary cilia were specifically observed in SCs (Fig.2e-f). Primary ciliogenesis  
48 sequentially ordered in Figure 3. Firstly, the ciliary vesicle (constituted by fusion of  
49 Golgi-derived vesicles) localizes on the distal pole of the mother centriole, which forms  
50 cilia basal body (Fig.3a). Then, the 9+0 axoneme grows from the basal body (Fig. 3b).  
51 Occasionally, abnormal duplication of centrioles was evidenced by detection of three  
52 centrioles, two of them originating cilia (Fig.3c). Figures 3d and 3e show a primary  
53 cilium in its maximum length and its ultrastructural features are also showed. Basal  
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3 body presented subdistal appendages and was anchored to cell membrane by  
4 transition fibers. The axoneme was originated from the basal body and it was located in  
5 a cell membrane invagination called ciliary pocket, where coated vesicles are  
6 concentrated as a sign of important molecular exchange (Fig.3e). In some ciliated cells,  
7 nuclei showed characteristic nuclear envelope prolongations (Fig. 3d-e). These nuclear  
8 structures have been previously called envelope-limited chromatin sheets (ELCS). It is  
9 relevant to emphasize that primary cilia in these cells are originated from no-displaced  
10 centrioles and, therefore, goes through the cytoplasm towards the extracellular  
11 medium.  
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24 The mononuclear histiocytic cells showed a less electron dense cytoplasm (Fig.4a),  
25 with abundant mitochondria, granular endoplasmic reticulum and prominent Golgi  
26 apparatus. Although their nuclei showed a diverse morphology, peripheral nucleoli  
27 were always found. According to their histiocytic nature, they presented many  
28 lysosomes and fine phagocytic prolongations (Fig.4b). These cells contact each other  
29 through cytoplasmic prolongations (Fig.4b). A reduced number of these histiocytic cells  
30 showed scarce organelles, abundant ribosomes and multivesicular bodies, features  
31 suggestive of undifferentiated phenotype (Fig. 4c).  
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41 Multinucleated GCs included 10 to 30 nuclei. They showed oval or irregular  
42 morphology and an electron light appearance. They presented a fine marginal  
43 chromatin contacting nuclear membrane and prominent nucleoli (Fig.4d). Their  
44 cytoplasm presented a homogenous distribution of organelles, abundant mitochondria  
45 and lipid inclusions (Fig. 4d). Surrounding multinucleated GCs, both, mononuclear  
46 histiocytic and stromal cells were located. Although defining dynamic processes from  
47 TEM is always difficult, we could observe light undifferentiated histiocytic cells merging  
48 with GCs and syncytial membrane disappearance, suggestive of cellular fusion  
49 (Fig.4e).  
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3 Some reactive foam cells plenty of electron lucent vacuoles were also found in these  
4 tumors. They were probably involved in phagocytic processes as they showed  
5 lysosomes with different content (Fig.4f). Few disperse lymphocytes and granulocytes  
6 were also observed, the latter being involved in macropinocytosis processes.  
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### 11 12 13 14 3.3. Primary cilia and Hedgehog signaling presence in GCTB

15 Immunofluorescent co-localisation of Histone H3.3 mutation G34W (specific for SCs of  
16 GCTB) and Acetylated-tubulin (marker for cilia axoneme) and Arl13b (marker for ciliary  
17 membrane) showed that stromal cells presented primary cilia (Fig. 5, Suppl. Fig. 1).  
18 Primary cilia were usually located near nuclei, in agreement with electron microscopy  
19 findings. A quantitative analyses showed that around 7 mononuclear cells per High  
20 Power Field (x400) were ciliated, which supposed around 10% of mononuclear cells,  
21 while no multinucleated giant cell presented cilia (Suppl. Fig.2).  
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32 Furthermore, we studied the implications of primary cilia in GCTB analysing the  
33 activation of Hedgehog (Hh) signaling pathway, a well-known cilia-dependent pathway  
34 related to tumorigenesis and bone regulation. Immunohistochemical experiments for  
35 Hh pathway protein Patched, Smo and Gli1 were performed (Suppl. Fig. 3, Fig. 6).  
36 Patched (Suppl. Fig. 3a) and Smo (Suppl. Fig. 3b) staining were located in paranuclear  
37 regions while Gli1 labelled subtly but clearly cells nuclei (Fig. 6a-c). Nuclear staining of  
38 Gli1 is an indisputable sign of Hh signaling activation.  
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49 Thus, these results suggest that Hh signaling pathway is present and activated in SCs  
50 of GCTB.  
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## 55 4. DISCUSSION

56 The nature and cellular components of GCTB have been the subject of multiple  
57 previous studies, most of which have wrongly identified all mononuclear cells as  
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3 stromal cells based on conventional microscopy morphology. However, histochemical  
4 and electron microscopy techniques shed light on cells identification (Aparisi et al.,  
5 1977; Hanaoka et al., 1970). In this study we have accurately defined ultrastructural  
6 features of the two different mononuclear cell populations identified by cytoplasm  
7 electron density and nuclear chromatin condensation. Cells showing lesser  
8 electron density and homogenous chromatin are called histiocytic cells here. The round  
9 histiocytic cells previously described by other authors may correspond to activated  
10 histiocytes with phagocytic capacity (Aparisi et al., 1977; Hanaoka et al., 1970).  
11 Intermediate phenotypes are compatible with resting, activated or prior to giant cell  
12 formation stages.

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26 The second mononuclear cell type, stromal cells (SCs), show more electron dense  
27 cytoplasm and nuclei with condensed chromatin forming marginal clusters in contact  
28 with nuclear envelope corresponding to stromal cells type 1 of Aparisi (Aparisi et al.,  
29 1977). However, phenotypic variability appears. Probably, those presenting features  
30 suggestive of undifferentiation may suppose cancer stem cells. SCs have been defined  
31 as immature fibroblasts or primitive osteoblasts. Their close histogenetic relation with  
32 osteoblasts is based on focal deposition of osteoid seen in one third of GCTB (Spjut et  
33 al., 1983). Additionally, bone tissue is not only produced by reactive osteoblasts but  
34 also by SCs (Goldring et al., 1987). According with the results provided here, SCs are  
35 clearly defined as mesenchymal cells presenting special and exclusive ultrastructural  
36 features such as presence of large electron-light vacuoles, peculiar density of  
37 cytoplasm and activation of centrioles. The most novel finding provided in the present  
38 study is the presence of primary cilia in these cells.

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54 Primary cilia in osteoblasts and osteocytes contributes to bone formation and  
55 homeostasis (Mitchison & Valente, 2017). Furthermore, the participation of primary cilia  
56 in different types of cancer of epithelial and mesenchymal line has been demonstrated.  
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3 Nevertheless, the prevalence of cilia on human tumors remains unclear, and their role  
4 in cancer is just beginning to be explored (Eguether & Hahne, 2018; Fabbri et al.,  
5 2019). In human osteosarcoma MG63 cell line the number of ciliated cell is significantly  
6 higher than expected (Kowal & Falk, 2015).  
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13 The cilia-dependant Hh signaling pathway plays a key role in both bone physiology and  
14 pathology. Thus, it is essential for temporal and spatial regulation of bone remodelling  
15 (Rodda, 2006; Yang et al., 2015). Moreover, Hh signaling pathway (especially through  
16 the Ihh ligand) stimulates intramembranous and endochondral ossification, bone  
17 turnover and remodeling of fractures sites (Yang et al., 2015).  
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24 The deregulation of Hh signaling pathway has been linked to some skeletal  
25 development diseases (Rodda, 2006; Yang et al., 2015) and tumors (Scales & de  
26 Sauvage, 2009). Furthermore, Hh signaling pathway in osteosarcoma was related to  
27 radioresistance and invasiveness (Qu et al., 2018). Interestingly, the ciliary membrane  
28 protein Arl13b plays an essential role in tumoral Hh signaling through primary cilia (Bay  
29 et al., 2018).  
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36 Here, we show Hh pathway activation in some SCs. These results are in accordance  
37 with Horvai et al. (Horvai et al., 2012) where 10% of mononucleated cells expressed  
38 Ihh (we found a similar proportion of ciliated cells, as it is described above). Besides,  
39 Horvai et al. also showed that GCTB stromal cells expressed Runx2 (Horvai et al.,  
40 2012) a well-known downstream target of Hh signaling pathway (Rutkovskiy et al.,  
41 2016).  
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51 The observation of primary cilia and Hh pathway activation in SCs suggests their role in  
52 cellular signaling and tumorigenesis. Moreover, some ciliated SCs showed envelope-  
53 limited chromatin sheets (ELCS), a structure described in cancer and stem cells (Olins  
54 & Olins, 2009; Cebrián-Silla et al., 2017). These findings suggest that primary cilia may  
55 play an important role in quiescent cancer stem cells of GCTB. This feature would  
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3 explain why only a specific population of stromal cells show primary cilia and Hh  
4 activation and they are not constitutively present in every stromal cell. Moreover, the  
5 dynamic nature of cilia assembly and Hh signaling must be taken into account while the  
6 techniques performed in this paper are mainly static.  
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13 In conclusion, our study reveals that SCs of GCTB present primary cilia. Moreover, we  
14 proved that Hh signaling pathway is activated in these cells, showing that primary cilia  
15 may play an important role in GCTB tumorigenesis and could be used as a potential  
16 therapeutic target.  
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## 22 23 24 **5. DECLARATIONS**

25  
26 **Funding.** No funding was specifically received for the experiments showed in this  
27 paper.  
28

29  
30 **Competing interests.** The authors declare that they have no conflict of interest.  
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33 **Ethics approval and consent declarations.** All protocols and consents developed  
34 were approved by the Human Research Ethics Committee "Comité Ético de  
35 Investigación Clínica de Aragón".  
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39 **Authors contribution.** TC conceived the study, performed ultrastructural experiments  
40 and contributed to write the manuscript, PI performed immunohistochemical and  
41 immunofluorescence studies and contributed to write the manuscript, EM performed  
42 immunohistochemical experiments. MJC and JGV provided the samples. MM  
43 contributed to write the manuscript. CJ conceived and supervised the study, performed  
44 ultrastructural experiments, analysed the results and contributed to write the  
45 manuscript.  
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## FIGURE LEGENDS

**Fig. 1. Histological diagnosis and preliminary study of GCTB.** a) Hematoxylin-Eosin staining shows two cellular types in GCTB: mononuclear and multinuclear giant cells (GC). Some of the mononuclear cells showed a characteristic paranuclear vacuole (arrow). Scale bar = 50 $\mu$ m b) CD68 is a specific marker for monocyte-macrophage lineage. Thus, histiocytic mononuclear cells and giant cells (GC) expressed CD68. Scale bar = 50 $\mu$ m c) Histone H3.3 G34W mutation is a specific marker of the stromal neoplastic cells of GCTB. According to this, only mononuclear cells were stained, some of them expressing paranuclear vacuoles (arrow). Scale bar = 50 $\mu$ m d) Immunolabeling for Ki67, marker of cell cycle activation and proliferation, stained uniquely mononuclear cells. Scale bar = 100 $\mu$ m

## **Fig.2. TEM examination. Ultrastructural features of mononuclear cells of GCTB.**

a) Histiocytic (\* light) and stromal (dark) cells are the main mononuclear components of GCTB. Scale bar = 5 $\mu$ m b) Stromal cells (SCs) show large paranuclear vacuoles (v). Scale bar = 2 $\mu$ m c-d) Centriole activation at different stages. Note the numerous small vesicles (vs), subdistal appendages (s) and intermediate filaments (if) in relation with centrioles; features related to centriolar activation. Scale bar = 500nm e) Primary cilia in a SC. Scale bar = 2 $\mu$ m f) Magnification of squared area in e. Scale bar = 500nm. ax: axoneme, bb: basal body, v: vacuoles, vs: vesicles, if: intermediate filaments, mt: microtubule, s: subdistal appendages, g: Golgi

**Fig.3. TEM examination. Primary cilia in stromal cells of GCTB.** a) At the beginning of ciliogenesis, small vesicles derived from Golgi-apparatus fuse in a ciliary vesicle (cv) which localizes on top of the basal body (bb). Scale bar = 500nm b) Axoneme (ax) originates from basal body and is surrounded by a cell membrane invagination called

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3 ciliary pocket (cp). Daughter centriole shows pericentriolar satellites (s). Scale bar =  
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5 500nm c) Tumoral cells exceptionally showed centriole duplication, with two cilia  
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7 emerging. Scale bar = 500nm d) SC shows envelope-limited chromatin sheet (elcs)  
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9 and primary cilium. Scale bar = 1 $\mu$ m e) Primary cilium ultrastructural features. Scale  
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11 bar = 1 $\mu$ m. mt: microtubule, if: intermediate filaments, pr: polyribosomes, vs: vesicles,  
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13 sa: subdistal appendages, s: satellites, arrow: ciliary pocket, black arrowhead:  
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15 transition fibers, white arrowhead: coated vesicles, bb: basal body, ax: axoneme.  
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20 **Fig.4. TEM examination. Ultrastructural features of reactive cells: histiocytic light**  
21 **cells and multinucleated giant cells.** a) Histiocytic cells show less electron dense  
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23 cytoplasm than SCs and prominent nucleoli (ni). Scale bar = 2 $\mu$ m b) Organelle  
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25 distribution of histiocytic cells. Ly: lysosomes. Encircled: intercellular contact. Scale bar  
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27 = 1 $\mu$ m c) Detailed area of histiocytic cells organelles showing mitochondria (mt)  
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29 surrounded by endoplasmic reticula (er) near a multivesicular body (mvb). Scale bar =  
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31 500nm d) Giant Cells show multiple nuclei with prominent nucleoli (ni). Moreover, its  
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33 cell membrane shows short prolongations. A stromal cell (SC) appears near the GC.  
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35 Scale bar = 5 $\mu$ m e) A histiocytic cell seems to fuse with GC (arrows point cell  
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37 membrane disappearance e). Scale bar = 5 $\mu$ m f) Foam cells sometimes show  
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39 lysosomes (ly) containing inclusions of different source. Scale bar = 5 $\mu$ m  
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45 **Fig.5. Stromal cells show primary cilia.** a) Immunofluorescent co-localisation of  
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47 Histone H3.3 G34W mutation in green and Arl13b (ciliary membrane) in red showed  
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49 that some stromal cells present primary cilia. Scale bar = 20 $\mu$ m b-d) Magnification of  
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51 cells showing Histone H3.3 G34W mutation and Arl13b co-localisation.  
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56 **Fig. 6. Immunohistochemical study of Hh pathway activation.** a) ~~Some~~  
57 ~~mononuclear cells showed paranuclear staining for Patched~~ b) ~~Similarly, only~~  
58 ~~mononuclear cells showed paranuclear expression of Smo~~ a-c) Nuclear labeling of Gli1  
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3 was present in some mononuclear cells, showing activation of Hh signaling pathway.  
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5 GC: Giant Cell. Scale bar = 20 $\mu$ m  
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9 **Supplementary Fig. 1. Immunofluorescence study of primary cilia in SCs GCTB.**

10 Co-localisation of Acetylated tubulin (red) and H3.3 G34W (green) give supporting  
11 evidence of cilia in SCs of GCTB. Scale bar = 50 $\mu$ m  
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17 **Supplementary Fig. 2. Quantitative analysis of primary cilia presence in GCTB cells.**

18 Number of cells showing primary cilia per High-Power Field (HPF: x400). 18  
19 representative images were analysed in total. In each HPF, between 70 and 100  
20 mononuclear cells were present while the number of giant cells ranges from 0 to 7. No  
21 multinucleated giant cells showed primary cilia, while around 7 to 8 mononucleated  
22 cells (mean of 7,53) per HPF were ciliated (approximately 10% of total mononucleated  
23 cells). Results are expressed as mean  $\pm$  SEM. Mann-Whitney U Test showed a  
24 statistical significance ( $p < 0,00001$ ).  
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37 **Supplementary Fig. 3. Smo and Patched staining in GCTB.** a) Some mononuclear

38 cells showed paranuclear staining for Patched. Scale bar = 20 $\mu$ m b) Similarly, only  
39 mononuclear cells showed paranuclear expression of Smo. Scale bar = 20 $\mu$ m.  
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