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RESEARCH ARTICLE

Morphologic features of the fetal mandibular condyle: Layers, canals and microvascular pattern

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SUMMARY

During organogenesis the mandibular condyle is divided by a fibrovascular septum, the persistence of which in the growing cartilage can lead to a bifid condyle. In this study we have evaluated the morphology of 3rd trimester human fetal temporomandibular (TMJ) specimens in order to determine the pattern of the vascular morphology associated with the layers and vascular canals (VCs) of the developing condyle (covering layers and condyle proper). Eleven human fetuses of 27–38 cm crown-rump length were used for histological (hematoxylin-eosin, Van Gieson stain) and immunohistochemical evaluation (antibodies for bcl2 and CD34) and another two of 24 and 31 cm, for TMJ microvasculature studies after black ink injections. With increasing fetal age, the intermediate loose lamina (LL) of the condylar proliferative layer evolves from a vascular-mesenchymal to a fibrillar pattern, via a transitory stage of a clear space that may be misdiagnosed as lower joint cavity (LJC). Within the condyle proper VCs may be present on its entire sagittal length, deepening variably towards the erosive zone and opened superiorly in the LL loose layer. Vessels of the evolving LL enter the condyle, directly or through the VCs; these vessels retract peripherally with increasing age and the intrinsic vessels of the condyle supplied from the erosive zone become prevalent. Vascular morphogenesis at the level of the LL seems comparable to that at the level of the LJC where characteristic glomeruli regress with increasing age. Lack of vascular regression and closure of central V-shaped defects of the condyle, as observed in 2/22 condyles, may represent a developmental substrate for condylar bifidism or a predisposing condition weakening the condyle, and making it more sensitive to trauma in childhood.

1. Introduction

From an evolutionary point of view, the human temporomandibular joint (TMJ) is a secondary structure that succeeds the primary reptilian joint between Meckel's cartilage and the palatoquadrate bone. In mammals, this primary joint becomes incorporated into the middle ear as the malleus-incus complex (Schroeder, 1991).

A key observation from a study of embryos and fetuses ranging in age from 32 days to 22 weeks was, that each of the TMJs components progressively develops from a common mass of embryonic mesenchyme interposed between the future temporal bone and mandibular regions (Van der Linden et al., 1987).

Apart from Meckel's cartilage, the rest of the temporomandibular joint and its related structures reach their adult shape at 14 weeks (Ogütcen-Toller and Juniper, 1994).

The articular disc (AD) of the TMJ originates as an autonomous formation from dense mesenchyme (we shall term it discal blastema), (Radlanski et al., 1999). As Radlanski et al. (1999) observed, there are also some indications that the AD lifts up from the condylar blastema. As so, Yuodelis (1966a,b) pointed out that the central avascular portion of the adult articular disc may derive from the condylar blastema, and the vascular mesoderm

Abbreviations: AD, articular disc; ADM, actively differentiating mesenchyme; CRL, crown-rump length; CS, clear slit/space replacing the loose lamina of the proliferative layer; CVSD, central V-shaped defect; EVM, erosive vascular mesenchymal layer; FAL, fibrous articular layer; FIL, fibrous intermediate layer; FM, fibrous membrane; FVC, fibrovascular canal; IL, inner cell-rich lamina of the proliferative layer; LJC, lower joint cavity; LL, intermediate loose lamina of the proliferative layer; LPM, lateral pterygoid muscle; OL, outer cell-rich lamina of the proliferative layer; OVCL, outer vascular condylar layer; PL, proliferative layer; TMJ, temporomandibular joint; UJC, upper joint cavity; VC, vascular canal.

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surrounding the interzone may account for the discal anterior rim, retrodiscal pad, capsule, and synovial lining of the joint (Yuodelis, 1966a).

There are differences regarding the terminology of the layers identified in the mandibular condylar cartilage during development (Mérida-Velasco et al., 2009). There is however a general agreement as to the presence of a superficial articular layer covering a proliferative (mesenchymal, progenitor) layer, that, in turn, covers the chondroblastic layer and the layers of hypertrophic chondrocytes (outer, non-mineralized, and inner, mineralized) (Mérida-Velasco et al., 2009). Inward to the hypertrophic chondrocytes an erosive layer is identifiable (Keith, 1982; Morimoto et al., 1987; Linss and Möller, 2007).

In rats, the upper joint cavity (UJC) forms before the lower joint cavity (LJC) (Yamaki et al., 2005; Suzuki et al., 2005); in humans, however, the LJC precedes the appearance of the UJC (Schroeder, 1991). Formation of the LJC commences during the 9th week of development (Mérida-Velasco et al., 2009) when several small blood vessels are identified running postero-anteriorly on the lower surface of the AD, which will eventually turn into the future LJC (Ohnuki, 2000). The mechanisms of UJC and LJC formation differ, only the latter involving local vasculature (Ohnuki, 2000; Suzuki et al., 2005). The only spaces described in the development of the TMJ are the joint spaces.

As Ohnuki (2000) described, when LJC formation begins, it appears to be filled with blood corpuscles and a series of openings can be noted throughout the condyle (Ohnuki, 2000). These radial slits in the condylar cartilage covered by vascularized, dense connective tissue extend from the articular surface to the central part of the mandibular condyle and separate the areas undergoing active endochondral ossification (García Alonso et al., 2006). These authors describe these passages running through the condyle and opening in the LJC, but not within a distinctive condylar layer.

In 1957, Blackwood was the first to report that the condylar cartilage of the mandible is divided by a well-vascularized septum consisting of fibrous tissue during its early stages of development and suggested that its persistence in growing cartilage may lead to an error in development that would give rise to a bifid condition (Matsuda et al., 1997). He has shown that the vascular canals (VCs), as he later renamed those vascularized septa, are present within the cartilage at least until the second-year of life and that they traverse the full thickness of the growing cartilage, reaching the surface articular layer (Blackwood, 1965); according to Blackwood (1965) the true function of VC is to increase the nutrition of the cartilage during periods of rapid growth (Mérida-Velasco et al., 1999, 2009). It was also hypothesized that apoptosis may play an important role in eliminating the vascularized septum of the mandibular condyle during development, and thus may be crucially involved in structural and functional development of the TMJ (Matsuda et al., 1997). Invaginations of vascular mesenchyme in the external portion of the condylar cartilage have also been observed by Mérida-Velasco et al. (2009) who mention that these formations were initially described by Vinogradoff (1910), under the name of "*crampons*" (Mérida-Velasco et al., 1999).

Therefore, there have been studies demonstrating that the outer end of the VCs corresponds to the proliferative layer of the condyle rather than to the articular space:

- Blackwood (1965) observed that via VCs reaching the medullary cavity of the condyle, blood vessels run towards the articular surface; moreover he reported that as the blood vessels approach the articular surface they turn sharply at right angles and run parallel to this surface for some distance—unfortunately, no evidence is offered in that study for the direction of the blood flow or for the exact location of the superficial vessels as referred to the condylar layers;
- Mérida-Velasco et al. (2009) noted that such VCs, postero-lateral, are composed of a central mesenchymatous axis containing vessels, with a superficial layer of cuboidal cells in a pocket-like formation, and associated with the adjacent cartilaginous tissue;
- Linss and Möller (2007) also noticed blood vessels coursing through condylar VCs, but failed to positively identify connections either between these blood vessels and the blood vessels of the articular capsule or between those vessels and the vessels of the condyle.

Thus, we aimed to evaluate human fetal TMJ specimens of the last trimester and to attempt to pattern the vascular morphology as related to the covering layers of the developing condyle. Also we intended to investigate the morphological characteristics of the VCs and to discuss whether or not such canals, if persisting, can represent morphological precursors of condylar bifidism. We designed the present study as a qualitative one.

We decided to use antibodies for bcl-2 and CD34 in our study as (a) apoptosis regulated by bcl-2, which acts as a suppressor of apoptosis (Wolter et al., 1997) plays an important role in TMJ development (Li et al., 1999; Wu et al., 2008), and (b) the CD34 antibody stains hematopoietic precursors/stem cells and labels vascular endothelial cells, mesenchymal cells and stroma fibrocytes that function as matrix-producing cells (Leong et al., 2003; Pusztaszeri et al., 2006).

2. Materials and methods

This study examined eleven human fetuses obtained following spontaneous abortion in the last trimester of gestation, with crown-rump lengths (CRL) varying from 27 to 38 cm and thus corresponding to 28–40 gestational weeks (Sadler, 1995). Two additional fetal specimens, one of 24 cm CRL (25 weeks) and the other of 31 cm CRL (33 weeks), were injected each with a mixture of black ink and 5% formalin in the ascending aorta. The black ink-formalin injections were performed immediately postmortem, till the skin of the head appeared consistently black. The specimens were further formalin-fixed for ten days after which, samples of TMJs consisting of mandibular condyles and TMJ discs were dissected out. The samples were paraffin embedded and thick microtome sections were cut, at 100 μm each. These were mounted unstained on slides, deparaffinized, and observed at microscope.

All fetuses had a normal craniofacial morphology. The research was approved by our Institutional Bioethics Board.

In order to avoid artificial findings due to artifacts of bad preservation, the fetuses used for histology and immunohistochemistry were formalin-fixed immediately postabortion, TMJ samples were removed immediately after the autopsy, and were also fixed.

TMJs were approached laterally, after the masseter muscles and the parotid glands had been identified and removed. The zygomatic arches were cut out (Fig. 1). Microdissections were carried out using 4.5× magnifying glasses. After identifying the TMJs, samples (n = 20), consisting of the mandibular condyles maintaining attachment to the lateral pterygoid muscles (LPMs) were dissected out en block with the corresponding TMJ discs (except two condyles that were dissected out without discs, 2/20). The temporal component of those TMJs was left in place in all but one specimen.

2.1. Histology

Specimens were fixed in 10% neutral buffered formalin, and decalcified for two weeks with 10% ethylene diamine tetraacetic

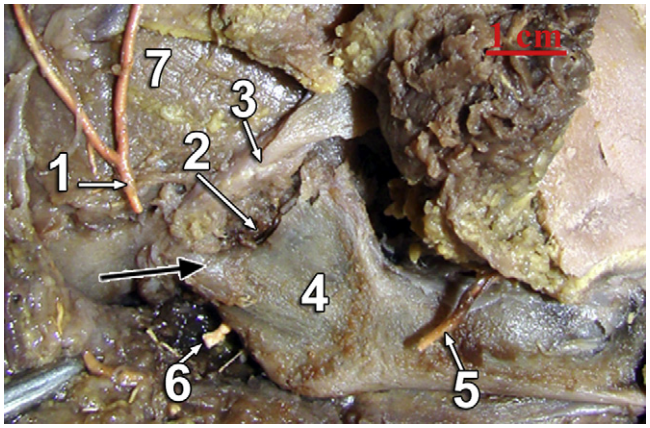

Fig. 1. Human formalin-fixed fetal specimen of 29 cm CRL, left side, lateral view, dissection of the mandibular branch—the masseter muscle and parotid gland were removed and the TMJ and the mandible branch were exposed. (1) Superficial temporal artery; (2) lateral pterygoid muscle; (3) zygomatic arch; (4) mandibular branch; (5) facial artery; (6) maxillary artery; and (7) temporal muscle. The macroscopic and topographic features of the TMJ are comparable to the postnatal ones.

acid (EDTA) in 0.1 M phosphate-buffer solution (PBS, pH 7.4) (Bio-Optica M107, Milan, Italy) prior to standard embedding in paraffin wax. Tissue blocks were oriented, sectioned and histological stains (hematoxylin-eosin or Van Gieson's stain) were applied. Also, 5 μm-thick sagittal sections were cut and processed for immunohistochemical examination.

2.2. Immunohistochemistry

Immunohistochemistry was performed on the remaining sections mounted on silane-coated glass slides (Dako, Glostrup, Denmark). Deparaffinized and re-hydrated sections were incubated for 30 min in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity and then rinsed for 20 min with 0.01 M phosphate-buffered saline (PBS). In order to unmask antigenic sites, sections were heated (3 min × 5 min) in capped polypropylene slide-holders with citrate buffer (pH 6.0) in a microwave oven (750 W). Non-specific binding of antibodies was blocked by incubation with normal horse/goat serum (Sigma–Aldrich, St. Louis, MO) for 30 min, diluted 1:20 in PBS, with 0.1% bovine serum albumin. We used an antibody to the bcl-2 gene product (clone 124, Dako, Glostrup, Denmark) and an antibody to CD34 (Dako, Glostrup, Denmark). Both antibodies were used at a 1:20 working dilution. Each antibody was applied on a section and subsequently incubated overnight at 4 °C in a moist chamber. Immune complexes were then revealed by treatment with a secondary biotinylated rabbit anti-mouse linking antibody and peroxidase-labeled streptavidin, both incubated for 20 min at room temperature (both reagents from LSAB2/HRP kit, DAKO, Glostrup, Denmark, prepared according to manufacturer's instructions). (LSAB+kit, HRP, Dako, Glostrup, Denmark). Immunoreactivity was visualized by development for 2 min with 0.1% 3, 3′-diaminobenzidine and 0.02% hydrogen peroxide (DAB substrate kit, Vector Laboratories, Burlingame, CA, USA). All dilutions ad thorough washes between stages were performed using PBS unless otherwise specified. Sections were subsequently dewaxed in xylene, dehydrated using graded ethanols and counterstained with Mayer's Hematoxylin, mounted with permanent mounting medium (Permount, Histo-Line Laboratories, NJ, USA) and examined using a light microscope (Zeiss) and documented using Zeiss – Axio Imager M1 and its acquisition system and software.

3. Results

Positive and negative controls were performed to test the specific reaction of primary antibody used in this study at a protein level. For positive control testing, the sections from tonsil which express bcl-2 in the germinal center mantle zones and sections from breast cancer which express CD34 were labeled as described in the previous section. Strong positive immunolabeling was both membranous and cytoplasmic. For negative control testing, sections of developing condyle were randomly selected and immunohistochemical labeling performed as described in the previous section but without primary antibodies. These controls resulted in an absence of immunopositivity on the respective slides that further served in histological evaluation of the structures.

3.1. Ink injection findings

Black ink injections (Fig. 2) revealed a series of microvascular features of the TMJ disc–mandibular condyle complex as follows

- the extrinsic vascular pedicles of the TMJ were the vessels of the lateral pterygoid muscle and those within the retrodiscal tissue;
- through discal extremities we found vessels with a segmental distribution, ending in glomerulus-like capillary networks within the LJC;
- blood vessels were identified in all discal regions.

In the posterior and anterior parts of the TMJ disc we found glomerulus-like capillary networks which were directed towards the LJC (Fig. 2A–C).

At the level of the anterior side of the mandibular condyle a rich capillary network was identified embedded within the covering layers, and connected both to condylar vessels and to transversal vessels within the LPM, the later displaying a segmental distribution (Fig. 2D and E).

Within the covering layers separating the LPM and the condyle proper, except the capillary network, large dichotomized, muscular-condylar vessels were identified (Fig. 2E).

3.2. Histological findings

In light microscopy, histological and immunohistochemical specimens of the condylar cartilages were found to be hypertrophic at this stage of development. From the inner to the outer layers and the infradiscal space (lower joint cavity, LJC) we identified the following: the erosive zone, layers of chondrocytes, hypertrophic, mineralized and non-mineralized, layer of chondroblasts, a proliferative and a fibrous articular layer (FAL).

In order to better analyze the condylar morphology we defined: (1) the mandibular condyle proper, consisting of the erosive zone, the layers of chondrocytes and the chondroblastic layer and (2) the covering layers of the condyle proper containing the proliferative and the fibrous layers.

The morphology of the examined fetal TMJs had distinct age related characteristics, the covering layers diminishing in thickness and cellular composition.

Beneath the FAL, spindle-like cell-rich, the proliferative layer contained three distinctive cellular laminae: an outer cell-rich lamina (OL), an inner cell-rich lamina (IL), and an intermediate loose lamina (LL) with fewer cells embedded within the extracellular matrix (Fig. 3A). Microvascular elements were found within the LL. In one condyle with a central defect, the LL had an erosive appearance (Fig. 4), being filled not only by vascular elements but also by clustered mesenchymal cells and residues. Therefore the LL appeared in this case as an erosive vascular mesenchymal (EVM) layer separating the OL and IL of the proliferative layer.

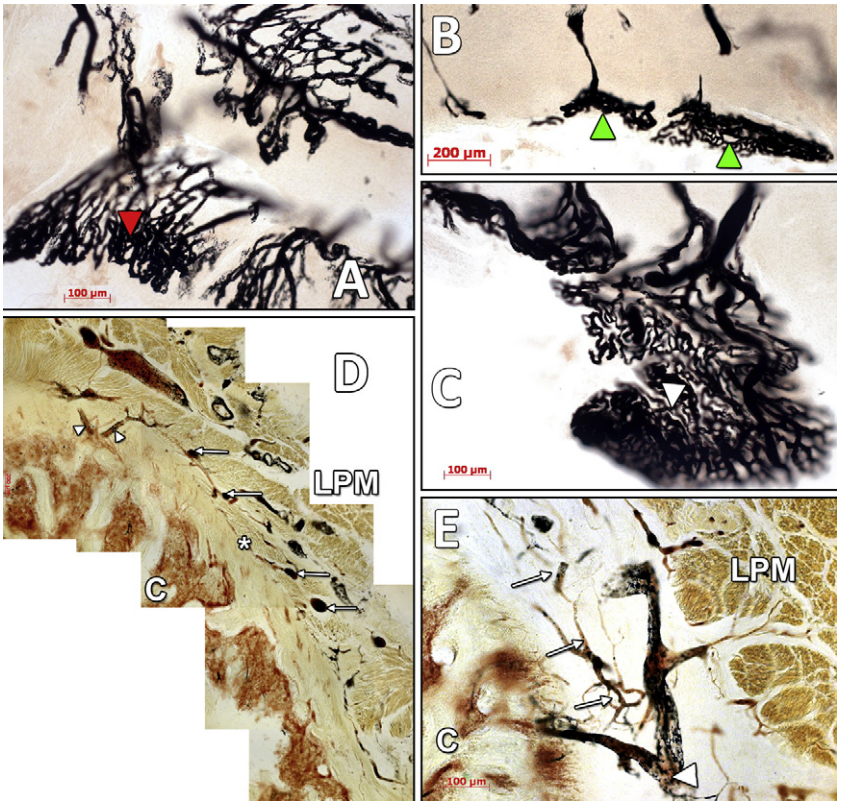

Fig. 2. Sagittal sections of the TMJ disc-mandibular condyle complex, after aortic injections of black ink and diaphanisation. Glomerulus-like capillary networks (arrowheads) of the inferior surface of the TMJ disc are identified: posterior (A) and anterior (B and C). (D) Anterior capillary network (*), condylar vessels (arrowheads) and segmentally arranged vessels (arrows) of the LPM. (E) Capillary network (arrows) within the condylar covering layers, and a large dichotomized, muscular-condylar vessel (arrowhead).

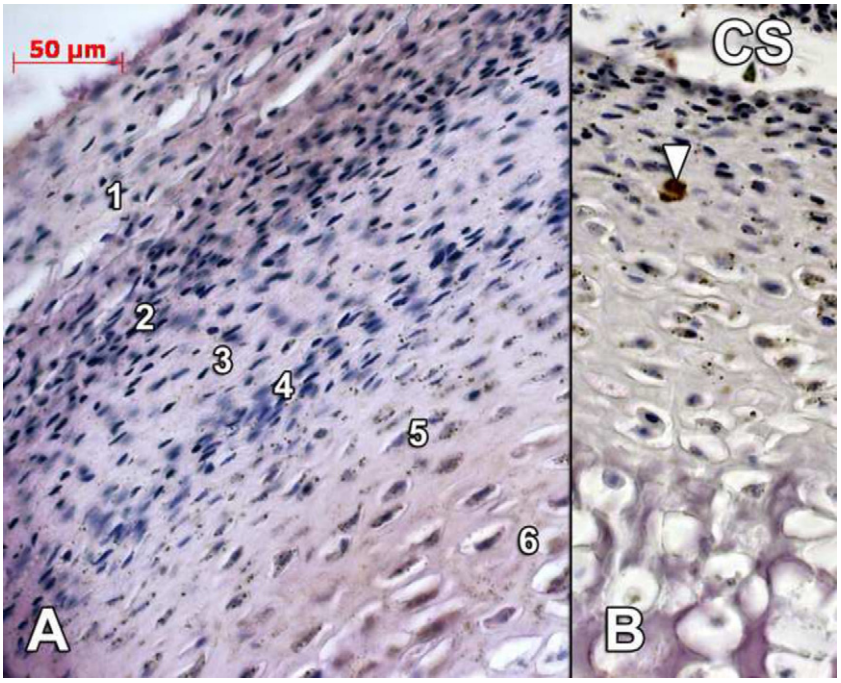

Fig. 3. Sagittal section of a fetal condyle of a 29 cm CRL (A), and of a 31 cm CRL (B) fetus, bcl-2 immunolabeling. (A) The proliferative layer is composed of three laminae, outer (2) and inner (4) cell-rich laminae, separated by a loose, cell-poor lamina (3). The chondroblastic (5) and chondrocyte (6) layers are identified. (B) A bcl-2-positive element is identified (arrowhead) at the transition from the proliferative layer to the chondroblastic one and a clear space (CS) bounded by membranes is identified replacing the loose lamina of the proliferative layer.

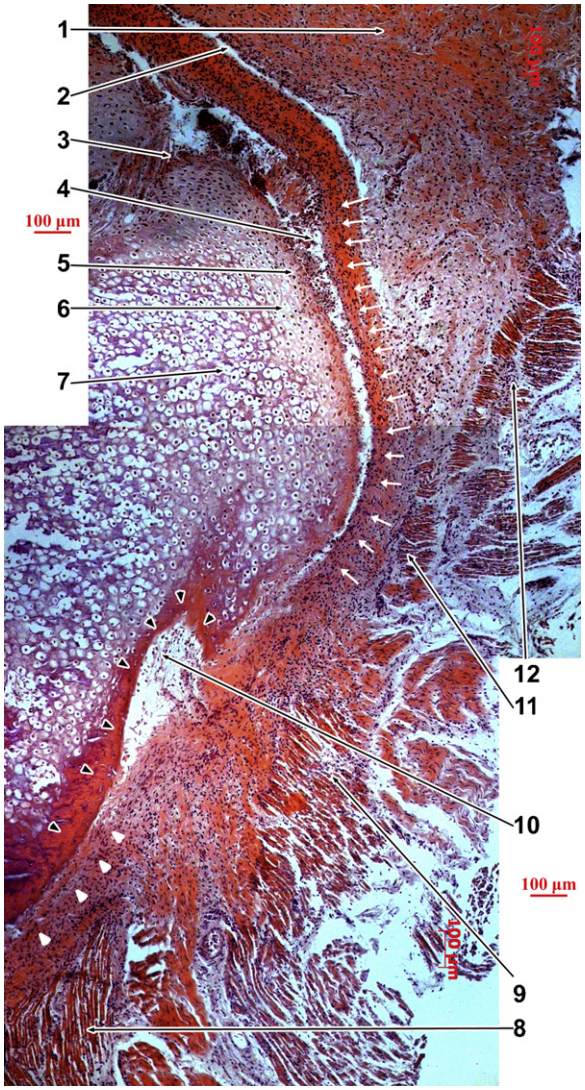

Fig. 4. Sagittal section at the anterior part of the mandibular condyle—the erosive vascular mesenchymal (EVM) layer. (1) AD; (2) LJC; (3) central defect of the condyle proper; (4) EVM layer, beneath the covering layers of the condyle (white arrows); (5) layer of chondroblasts, beneath the inner lamina of the proliferative layer; (6) non-mineralized chondrocytes; (7) mineralized layer of chondrocytes; (8) inferior fascicle of the lateral pterygoid muscle (LPM); (9) intermediate fascicle of the LPM; (10) pterygoid fovea, deeply bordered by intramembranous ossification (black arrowheads); (11) upper fascicle of the LPM.

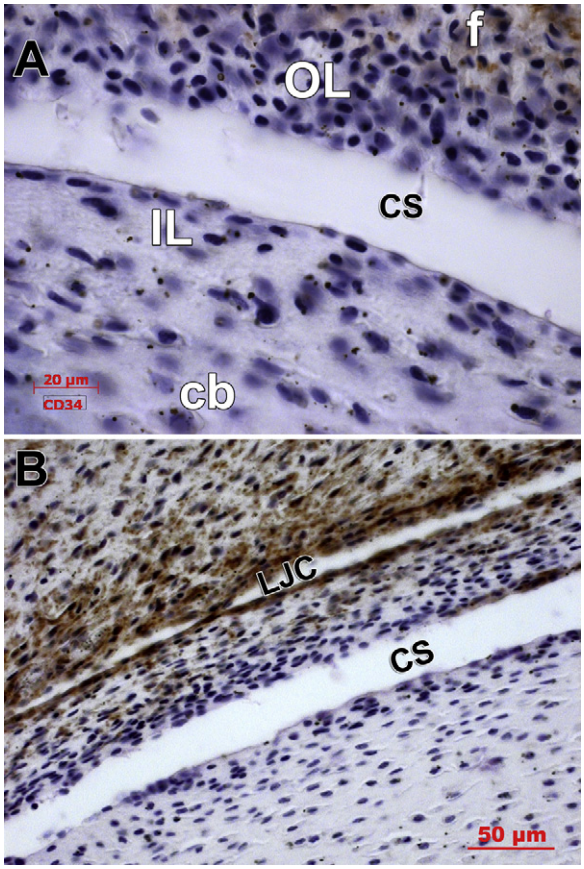

Fig. 5. Sagittal section of a fetal condyle of the 31 cm CRL fetus, CD34 immunolabeling. (A) A clear space (CS), limited by thin membranes separates the outer (OL) and inner (IL) laminae of the proliferative layer; the fibrous (f) and chondroblastic (cb) layers are identified. (B) That CS and the lower joint cavity (LJC) are located on both sides of the covering layers, and the LJC is narrower than the CS.

In a fetus of 31 cm CRL the LL was replaced by a large clear acellular and afibrillar slit/space (CS) bounded by thin membranes (Fig. 5) isolating it from the outer and inner cell-rich laminae. In that specimen we found two concomitant spaces, the LJC and the CS, on both sides of the FAL (Fig. 5B).

With increasing fetal age, the layer of chondroblasts diminished, leading to a chondrocyte dominant morphology of the condyle proper. Also the cell populations of the outer and inner proliferative laminae diminished/disappeared leading to the following layer sequence (Fig. 6):

- the fibrous articular layer (FAL);
- an intermediate loose fibrillar network that we termed fibrous intermediate layer (FIL);
  - in specimens with superficially closed or absent vascular canals (VCs), the FIL uniformly filled the space underneath the FAL (Fig. 7B);
  - considering its histological topography, the FIL seemed to have evolved from the LL/EVM/CS as previously described.
- a fibrous membrane (FM), covering the cartilage, replacing and presumably derived from the fibroblasts of the IL.

In the anterior portion of the condyle the FAL attached distinctive fibrous structures of the TMJ: (a) the mandibular attachments of the AD, (b) the fibrous articular capsule of the TMJ and (c) the inferior fibers of the tendon of the upper head of the LPM. Then, the FAL continued with the periosteum over the membranous bone formed as a shell at the level of the neck of the condyle which in turn attached the inferior LPM (Fig. 6).

The condyle proper was traversed by vascular canals (VCs), with either mesenchymal-vascular content, expanded from the EVM layer, or fibrous content connected to the FAL. As vascular remnants were also identified in some of the canals containing fibrous expansions of the FLA, those canals were termed fibrovascular canals (FVCs).

We identified VCs with mesenchymal-vascular content in 3 condyles and FVCS in 4 different ones, of the total number of examined specimens of 22.

Two of the VCs that we identified were V-shaped (Fig. 7), unilateral, and were noticed in two fetuses, the first one (S1) of 27 cm CRL and the second one (S2) of 33 cm CRL. They both had normal opposite mandibular condyles. The S1 mandibular condyle presented a central V-shaped defect (CVSD1), larger in the superior portion towards the covering layers, and also a VC located on the posterior side of the condylar head. The S2 mandibular condyle presented a

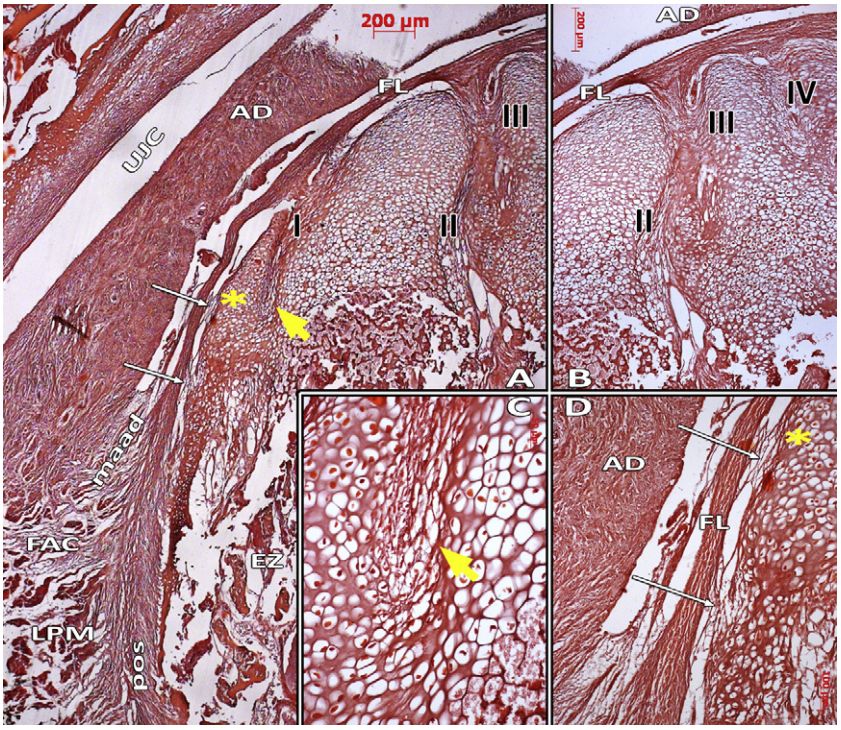

Fig. 6. Sagittal section taken from the anterior part of the fetal TMJ—fetus of 34 cm CRL. (A, detailed in B and C (yellow arrowhead) and D (*)) The temporal component of the TMJ is identified above the upper joint cavity (UJC); beneath the articular disc (AD) the fibrous layer (FL) is identified and evolves antero-inferiorly to split and attach the mandibular attachment of the articular disc (maad), the fibrous articular capsule (FAC), the LPM and then it continues with the periosteum (pos) of the condylar neck. The FL sends fibrovascular septa (I–IV) that penetrate the condyle proper, blind-ended in the chondrocyte layers (I, III and IV) or continued (II) to the erosive zone (EZ). Also, discrete attachments of the FL to the FM covering the cartilage are identified at the antero-superior part of the condyle (arrows, A and D). A vascular element can be identified posterior to the common base of the FVCS II and III. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

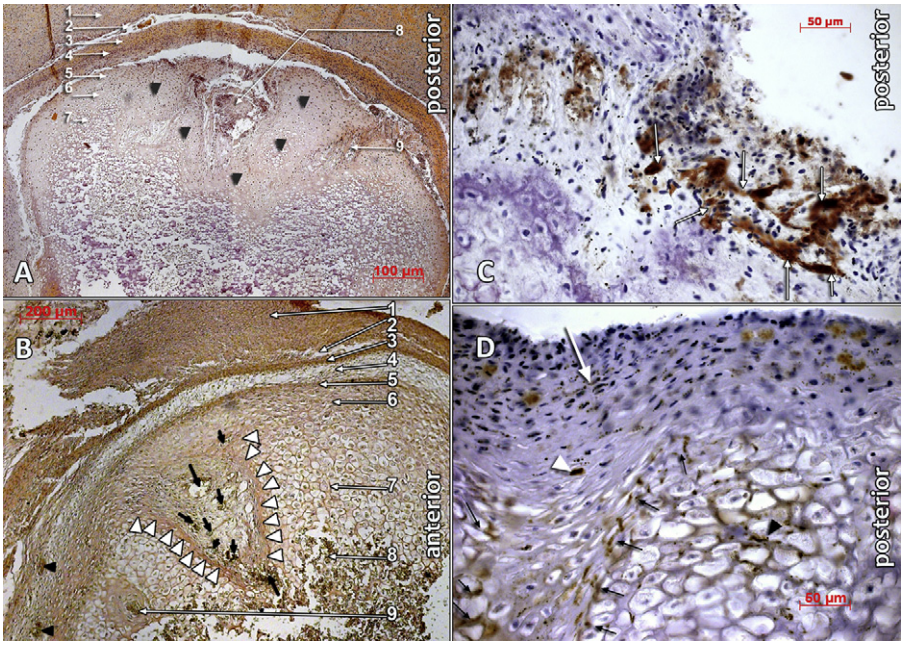

Fig. 7. Sagittal sections of the mandibular condyles in specimens 1 (A, C and D) and 2 (B). Hematoxylin-eosin (A), van Gieson stain (B), CD34 (C), bcl-2 (D). (A) Central CVSD1 (8) and posterior VC (9). (1) AD; (2) LJC; (3) fibrous layer, cell-rich; (4) proliferative layer; (5) layer of chondroblasts; (6) layer of non-mineralized chondrocytes, invaginated to border the CVSD1 (arrowheads); (7) layer of hypertrophic chondrocytes. (B) Central CVSD2, bordered by a condensed extracellular matrix (white arrowheads), with blood vessels (black arrows) connected to those of the erosive zone. (1) AD; (2) infradiscal space, incomplete; (3) FAL; (4) LL, fibrillar, with two blood vessels evidenced in its posterior part (black arrowheads); (5) FM, over the layer of chondroblasts; (6 and 7) layers of chondrocytes, non-mineralized and hypertrophic; (8) erosive zone; (9) condensed extracellular matrix around a blood vessel located within the hypertrophic layer, posterior to the CVSD2. (C) A posterior VC is penetrated by CD-34 positive microvessels (arrows). (D) bcl-2 positive elements are identified within the chondroblastic layer covering the condylar defect (white arrowhead), at the border of that defect (black arrows), and within the hypertrophic layer surrounding it (black arrowhead).

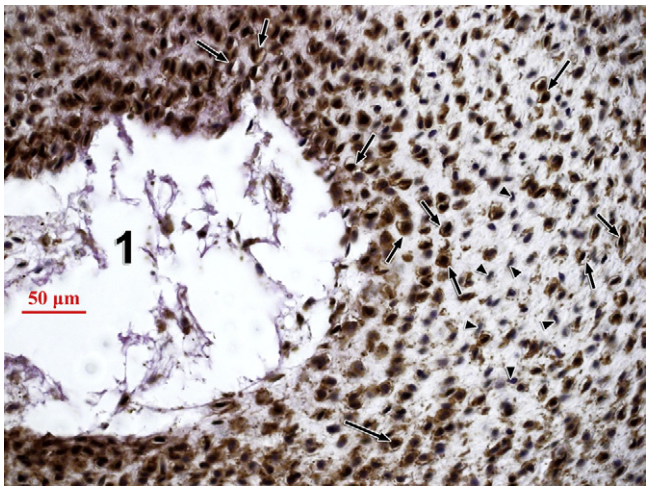

Fig. 8. Sagittal section of the fetal TMJ, immunostained for CD34, fetus of 27 cm CRL. The tissue surrounding the lower joint cavity (LJC) consists of CD34-positive cells (arrows) that differentiate from mesenchyme, and unlabeled spindle-shaped cells (arrowheads); as so, a mesenchymal layer appears interposed between the LJC and the fibrous covering layer of the condyle.

single defect, located centrally and V-shaped (CVSD2). The CVSD1 consisted of a vascular mesenchymal resorption/remodeling large area, clearly dividing the condyle into an anterior and a posterior part, bordered by a distinctive layer of chondrocytes, with chondroblastic septa separating focal areas of chondrocytes. The "roof" of the CVSD1 consisted centrally of a non-homogenous band of mesenchymal-vascular nature extending from the EVM layer and peripherally it consisted of the chondroblastic layer of the condyle proper, thus being completely isolated from the EVM layer. In the CVSD2 case, the defect was enclosed by condensed extracellular matrix, was deeper than the CVSD1 and closed superficially by a continuous, unaltered layer of chondroblasts of that condyle. The apex of the CVSD2 opened into the erosive zone; therefore its vascular content was linked to the vessels of that area.

In four different fetal specimens over 30 weeks the condyle proper was traversed not by VCs but by FVCs (Fig. 6), anterior, intermediate or posterior, as referred to the sagittal plane. These were evaluated, according to their distal intracondylar end, as: (1) incomplete, blind-ended within the cartilage, and (2) complete, penetrating the erosive zone. Thus, the FAL appeared not only attached to the FM covering the cartilage by the loose FIL but also deeply, by the fibrous cords within the FVCs.

3.3. Immunohistochemical findings

In the younger fetuses examined the lower joint cavity (LJC) was identified within a common mass of actively differentiating mesenchyme (ADM) (Fig. 8) consisting of (a) CD34 immunolabeled cells, rounded or oval, many of which having a noticeable pericellular complete or incomplete halo, and (b) CD34 unlabeled spindle cells, fibroblast-like. Therefore the lower part of that ADM was added over the covering layers of the condylar periphery, while the upper part of the ADM formed the articular disc (AD).

With increase in fetal age, the lower layer of ADM seemed to narrow and to retract peripherally, uncovering the fibrous articular layer.

Also, we identified the developing fibrous articular layer "sandwiched" between two layers of CD34-positive microvascular elements, superficial and deep, the latter seeming to evolve within the proliferative cell layer, towards its inner lamina (Fig. 9); these vascular layers were better and constantly represented at the anterior (Fig. 10) and posterior parts of the condyles (Fig. 9), and were

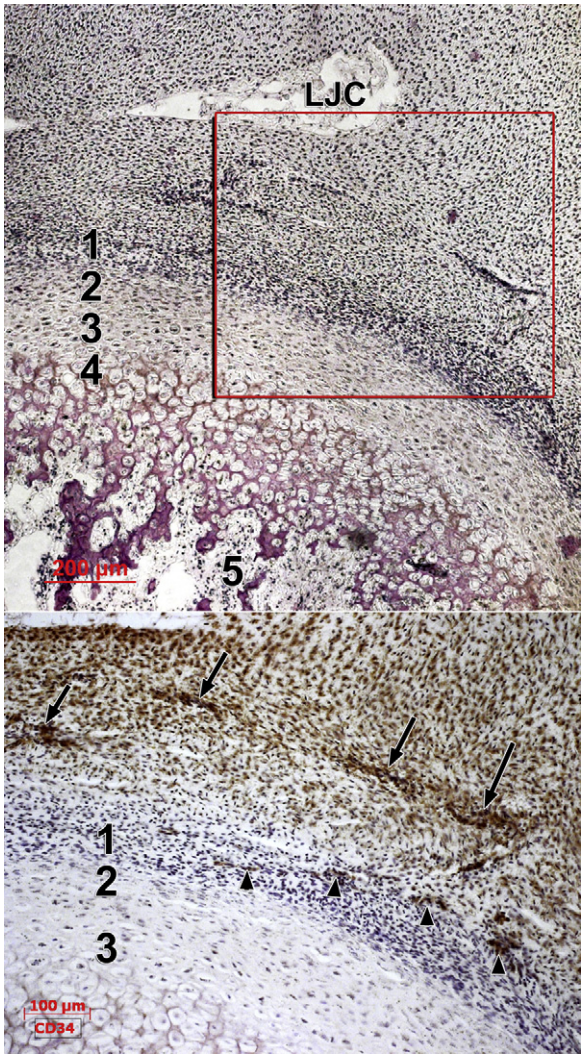

Fig. 9. Sagittal section of the fetal TMJ, unlabeled (upper panel, negative control) and labeled for CD34 (lower panel), fetus of 27 cm CRL. At the posterior part of the TMJ the lower joint cavity (LJC) is located within a mesenchyme-derived layer that covers the condyle; two distinctive vascular layers, upper (arrows) and lower (arrowheads) are identified on the outer and inner sides of the fibrous layer of the condyle, respectively. Proliferative (1), chondroblastic (2), chondrocyte non-mineralized (3) and mineralized (4) layers and the erosive zone (5) are identified.

supplied by vessels extrinsic to the condyle. We noticed that at the posterior part of the neck of the condyle, where bone formation was delayed as compared to the anterior part, the vessels of the fibrous covering layer were also distributed to the erosive zone of the condyle.

Also the CS replacing the LL contained remnants of CD34 positive vascular elements, but only in its extreme periphery.

Positive CD34 labeling also identified in the intrinsic microvessels of the condyle, ascending from the erosive zone to the mineralized chondrocyte layer (Fig. 11).

Taking the results of the ink injections into account, we could therefore define extrinsic and intrinsic condylar vessels (vascular pedicles), of the condyle proper, the extrinsic ones coursing through or beneath the covering layers and connected to anterior and posterior vascular distributors of the TMJ.

We found bcl-2 positive elements scarcely distributed at the outer (Fig. 3B) and inner limits of the chondroblastic layer.

The posterior VC of the S1 condyle was penetrated by CD34-positive microvessels and surrounded by the cartilaginous matrix of the hypertrophic chondrocyte layer (Fig. 7C).

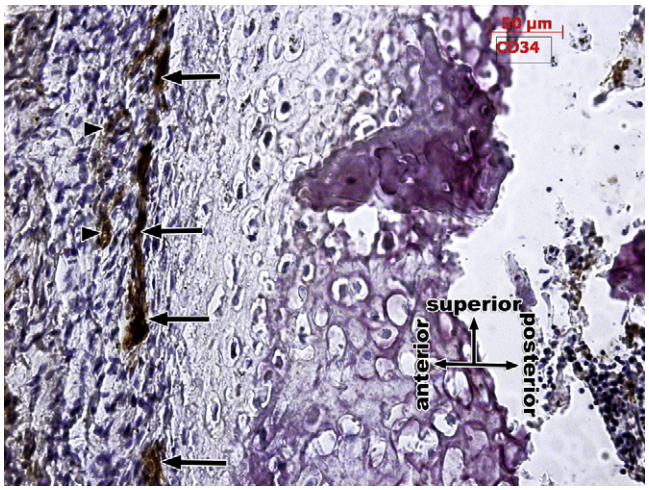

Fig. 10. Sagittal section of the fetal TMJ, immunostained for CD34, fetus of 27 cm CRL. Anterior side of the mandibular condyle: a deep vascular layer (arrows) is identified within the proliferative layer of the condyle, and it receives vessels of a superficial vascular layer (arrowheads).

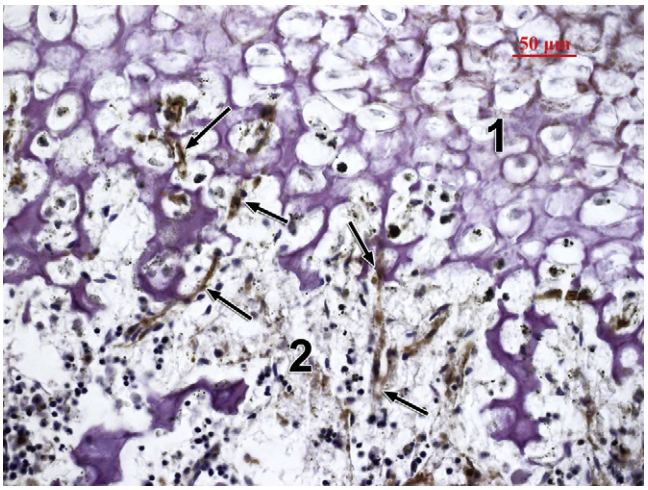

Fig. 11. Sagittal section of the fetal TMJ, immunostained for CD34, fetus of 27 cm CRL. (D) The mineralized chondrocyte layer (1) seems invaded by vessels (arrows) ascending from the erosive zone (2) of the condyle.

The bcl-2 immunolabeling of the CVSD1 found immunopositive elements at the periphery of the CVSD and anterior to it but also within the band enclosing it superficially. Bcl-2-positive cells were also identified within the chondroblastic layer above the defect (Fig. 7D).

4. Discussion

Temporomandibular joint (TMJ) dysfunction has an increasing prevalence, particularly in children and adolescents. Morphological changes of the TMJ during fetal and postnatal development may play a critical role in finalizing the functional structures of the TMJ and therefore may be an important factor in TMJ dysfunction (Matsuda et al., 1997).

However, the literature data on the TMJ morphogenesis in humans is still scarce and this research direction needs a better attention.

4.1. Vascular spaces beneath the articular disk

During the third trimester, important morphological changes occur within the TMJ disc–mandibular condyle complex. In the first part of the third trimester we showed the cellular composition of those structures to be more complex than it is usually considered to be. Our results support the theories of vascular involvement in the formation of the lower joint cavity (LJC) (Ohnuki, 2000; Suzuki et al., 2005) and evaluate the transitory specific glomerular appearance of the capillary networks, on the lower surface of the articular disc (AD). Such blood corpuscles were described by Ohnuki (2000) and also glomerulus-like vascular structures were evidenced within the cartilage canals of the developing bone (Blumer et al., 2008).

As the AD evolves morphologically from a common mass of mesenchyme located between the temporal and mandibular areas, and taking into account that LJCs primary appearance may be viewed as a distinctive vascular layer within that mesenchyme, we considered it to be normal to find an additional layer covering the condyle, structurally similar to the AD. That layer of actively differentiating mesenchyme (ADM) over the covering layers of the condyle proper may leave no derivatives within the condyle; with increasing fetal age it appeared to retract peripherally and it seemed reasonable to us to speculate that it could retract due to the increasing size of the condyle and the mechanical stress and thus it could be incorporated into the structure of the AD (also with an increasing size in that period) as mandibular attachments. However this retraction of the ADM needs better evaluation in further studies performed on human fetuses.

The mesenchymal vascular layer, topographically correlated with the LJC formation, is not the only vascular layer involved in the formation of the TMJ below the disc. A second such vascular mesenchymal layer corresponds to the loose lamina (LL) of the proliferative layer. That outer vascular condylar layer (OVCL) either retracts leaving a clear slit (CS) that will fill with fibrous tissue, or it becomes an erosive vascular mesenchymal (EVM) layer which may also fill the VCs.

The CS succeeding the vascular retraction appears on slides between the covering layers and the condyle proper (covered by the IL of the proliferative layer) and is bounded by thin membranes. As such, it can easy be misdiagnosed as LJC if the latter is not preeminent on the examined slides. This observation raised strong doubts as to the hypothesis of Yuodelis (1966a) that AD may be partly derived from the condylar blastema (see Section 1) and we recommend revaluation of that theory. If the covering layers would be considered as being at least partly derived from discal blastema our results could fit with the observation of Yuodelis.

Mérida-Velasco et al. (2009) identified a vascular invasion layer similar to ours in condyles of 10–11 weeks fetuses but they related it to cartilage destruction. We consider it may not be the case, as it separates the condyle proper from its coverings and it is not located within the condyle proper.

We considered the OCVL supplied by extrinsic vascular pedicles of the condyle but this vascular layer seems important not only as a feeding resource of the condyle but also as a limit between two histologically different structures, the fibrous covering and the condyle proper, each with its pattern of individual histomorphogenesis.

CD31-positive flat capillaries were found in the fibrous layer of the condyle by Suzuki et al. (2005), who considered mechanical stimulus and blood vessels as essential factors to induce cavitation and to make a space, respectively. The microvascular condylar pattern defined by these authors in rats correlates with our evidence in human fetuses.

4.2. The covering layers of the condyle proper

In the third trimester, the covering layers of the condyle proper generally evolve from a predominant cellular organization to a fibrillar one. Seemingly, the outer lamina of the proliferative layer is

related to fibroblast formation and to the morphogenesis of the FAL. The inner lamina of the proliferative layer differentiates not only chondroblasts but also fibroblasts to later form the FM over the cartilage. However, the evidence obtained needs further evaluation, in studies using larger panels of antibodies.

The micrographs and drawings supporting the respective results in the references used in the present paper were carefully scrutinized. In all we identified at least one of the stages of evolution from the LL of the proliferative layer, via EVM or CS, to the FIL we described. So, even though the respective authors made no specific distinction of that layer, they described it, therefore giving consistency to our evidence.

Blackwood (1965) and Thilander et al. (1976) referred to the proliferative layer as being a supplier of cells for the FAL and to the chondroblastic layer respectively. Thilander et al. (1976) described two morphologically different cellular sub-layers of the proliferative layer that only seem to decrease their cell number and increase the amount of intercellular substance after the growth period of the condyle.

As discussed by Mérida-Velasco et al. (2009), authors have suggested that the chondroblastic layer corresponds to cells known as skeletoblasts, as defined by Silbermann et al. (1987) or osteoproliferative cells, as viewed by Geneser in 2000; However, the histomorphology of the fibrous articular layer has not been well documented till now.

The FAL prenatally has/receives well defined distinctive insertions at its extremities as described in Section 3. As one such attachment is that of the lower fascicle of the upper head of the LPM, we consider that from this point the FAL may be viewed as a supracondylar, but infradiscal, continuation of the tendon of the LPM.

The FAL continues with the periosteum over an anterior osseous shell of the condyle resulting from intramembranous ossification. That shell has been shown to respect the dorsal aspects of the condyle and to give the growth process of the condyle specific direction in a superior, lateral and dorsal direction (Yuodelis, 1966b).

4.3. The vascular canals and fibrovascular septa of the condyle proper

The condyle proper may present vascular canals (VCs) and/or fibrovascular canals (FVCs). The supposition that FVCs are a late morphological evolution of the VCs seems reasonable, the fibrous content succeeding the mesenchymal tissue in a normal sequence.

When present, the VCs may be regarded as intrinsic growth centers of the condyle, separating distinctive areas of cartilage that may have individual patterns of morphogenesis. If we take into account the observation of Blackwood (1965) that the condylar cartilage *in toto* may be evaluated as a growth center for the mandible, fulfilling a function similar to that of the epiphyseal plates of the long bones (Blackwood, 1965) we can consider the VCs to fulfill that function, but strictly in reference to the condyle and not to the mandible. Persistence of these canals after cessation of condylar growth may lead to a bifid condition of the condyle.

We observed that VCs may be found within the condyle at any level of the sagittal plane, and may be classified as anterior, intermediate and posterior. According to Mérida-Velasco et al. (2009) who worked on specimens from 49 human fetuses, these canals appear from mesenchymal invaginations, and here we are in agreement. However, we disagree upon the topographical location of these canals, the respective authors emphasizing that the posteromedial and posterolateral are the only possible VCs locations within the condyle. During late fetal life the number of VCs increases, but in the younger specimens the canals tend to be more numerous in the posterior and lateral aspects of the cartilage (Blackwood, 1965).

We consider that differences related to the frequency and sites of occurrence might be coincidental or due to the number of investigated specimens.

As we observed, the VCs may narrow to FVCs with increasing condylar age. Subsequently, on the one hand, the cartilage decrease leads towards a structural unity of the condyle, and, on the other hand, the fibrous content of these FVCS attaches the FAL within the condyle forming a dynamically solitary unit from these two.

Altered closure of the VCs/FVCS or of large CVSDs may lead to defects in the definitive condylar surface, such as bifidism/trifidism.

Extrinsic vascular pedicles of the condyle use the VCs to enter the cartilage. On the other hand, vessels from the erosive zone ascend into the cartilage as intrinsic vascular pedicles (our term), which were also identified by Blackwood (1965). It may be established that, via the VCs, a collateral path (usually transitory) of condylar vascularization has been observed, but that direct anastomoses of the extrinsic and intrinsic vessels were not observed, either by us or by Blackwood (1965).

The statement of Linss and Möller that the vessels of the condylar channels reach from the trabecular bone to the articular space (Linss and Möller, 2007) is rather unsupported by the evidence they present, that only depict vascular channels through the chondrocyte layers, ending superiorly in communication with a layer above the chondroblastic layer and beneath the fibrous articular covering—the two authors have not defined it, but they have demonstrated the EVM layer. Linss and Möller (2007), referring to the vessels within the VCs, reported that "*connections between these blood vessels and the blood vessels of the articular capsule could not be clearly detected*"; we clearly identified such vascular connections between the vessels of the condyle and those of the fibrous covering layers. The connections of the vessels within the VCs and those of the covering layers were also demonstrated by Blackwood (1965). Linss and Möller (2007) also noticed blood vessels "*in the central part of the discus articularis*" but the figure presented there (Fig. 8) only depicts vessels beneath the disc and not within it. We consider the vessels described by Linss and Möller to be remnants of the vascular layer preceding the LJC, rather than proper discal vessels.

4.4. Morphologic features of the fetal mandibular condyle that may precede a bifid condition

Understanding the physiology, and pathology of the temporomandibular joint is highly dependent upon an accurate morphogenic model (Yuodelis, 1966a). The bifid mandibular condyle (BMC) is a rare anomaly with a prevalence of 0.018% (Menezes et al., 2008). An exhaustive review of the references on the BMC, has recently been presented (Shriki et al., 2005). The etiology of the double headed condyle is uncertain and a large number of hypotheses have been proposed, most of which consider trauma as a potential etiological agent leading to BMC and it is reasonable for us to consider that, with the number and size of the VCs, the condyle is more susceptible to traumatic damage. Other etiologies suggested in the literature are: teratogenic substances, endocrine disorders, nutritional deficiency, infection, irradiation and genetic factors that can alter normal development (Corchero-Martín et al., 2005). A survey of prehistoric and historic skulls found a few BMC; it was presumed that bifid mandibular condyles with anteroposteriorly situated heads are caused by early childhood fractures, whereas those with mediolaterally situated heads are caused by the persistence of connective tissue septa (Szentpétery et al., 1990), but the fractures can be produced at the level of a weak VC and the exposure to trauma to a developing condyle may be directly related to the number and volume of the VCs within it. The developmental origin of BMC is thus related to the persistence of transient developmental structures (Matsuda et al., 1997) and we have to

focus here on a lack of vascular retraction subsequently keeping the VCs opened. Many reports on the BMC underline the persistence of VCC as an etiological factor dividing the mandibular condyle as described by Blackwood in 1957 (Matsuda et al., 1997; Ramos et al., 2006; Shriki et al., 2005).

It should also be considered that mandibular VCCs are very similar in structure and function to the cartilage canals found during the development of several bones (Blumer et al., 2008).

The V-shape of a central condylar defect has not been previously reported, even as a transitory phase in the condylar development. We here describe two cases with unilateral central V-shaped condylar defects but there is no way to prove that these condyles would have been bifid. Given the rare nature of bifid condyles (antero-posteriorly) an incidence of 10% (2 out of the 20 condyles) would suggest that this may just be a transitory phase that does not necessarily proceed to the full manifestation. Anyway, the V-shape of the condylar defects must be kept as a favorable morphology for the eventual development of the bifidity (it is certainly reasonable to speculate that open VCs are more likely to predispose to this condition in the antero-posterior cases) that, in a recent review, was considered to frequently occur unilaterally (Kaneyama et al., 2008), as were also the defects we encountered.

In his review on the developmental abnormalities of the mandibular condyle, Kaneyama et al. (2008) refers to Matsuda et al. (1997) who suggested that apoptosis may be involved in eliminating the condylar septa. As, at that time, Matsuda did not perform a distinctive study on such septa when studying the apoptosis in the development of the temporomandibular joint (in rats), we consider the term "*suggestion*" used by Kaneyama as being justified. Matsuda identified apoptotic cells of two types, A and B, "*in the cellular layers of the condylar subsurface*" corresponding to the proliferative and chondroblastic layers, also identified by Wu in rabbits (Matsuda et al., 1997; Wu et al., 2008). We emphasize that the location of apoptotic type B cells of Matsuda strongly corresponds to our LL/CS space. We found no references on the condylar apoptosis in relation to the condylar defects except the suggestion of Matsuda et al. (1997) that apoptosis seems correlated only with remodeling of the mandibular condyle.

We obtained and presented demonstrations of positive bcl-2 immune reactivity related to condylar defects or located within the proliferative and chondroblastic layers. This reinforces the suggestion of Matsuda et al. (1997) and it also suggests that an imbalance in apoptotic remodeling could predispose to the persistence of condylar defects. It appeared to us that a specific study on the human fetal apoptotic events within the TMJ components must be designed and performed, and that immunohistochemical evaluation with a larger panel of markers of apoptosis, and electron microscopy, could bring additional data.

A persisting condylar defect will weaken the condyle that will become more sensitive to trauma in childhood. Thus, if not a developmental precursor to BMC, the condylar defects can be considered, when persistent, to be morphological factors predisposing to condylar trauma.

Indubitably, more studies have to be performed on human fetal material, to determine a relevant incidence of such defects/septa of the mandibular condyles and also a relevant rate of closure vs. persistence of these transient structures.

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