

Developmental morphometry of human articular cartilage: a comparative histological study across fetal stages and adult joints

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Abstract

Articular cartilage is a highly specialized connective tissue with limited intrinsic regenerative capacity. Characterizing its developmental architecture is essential for understanding cartilage maturation and informing regenerative approaches. This study conducted histological and quantitative morphometric analyses of human fetal articular cartilage at 23, 27, and 36 weeks of gestation, using adult knee cartilage as a reference. Histological evaluation employed Hematoxylin and Eosin, Safranin O, Alcian Blue, Toluidine Blue, and Masson's trichrome staining, as well as collagen type II immunohistochemistry. Quantitative analysis with FIJI software measured cartilage thickness, area, zonal architecture, chondrocyte density, lacunar size, nuclear morphology, chondron organization, and matrix staining intensity. Results demonstrated progressive structural maturation across gestation, characterized by increased matrix deposition, changes in cellular organization, and emergence of zonal architecture. Fetal cartilage exhibited higher cellularity, larger lacunae, and increased proteoglycan staining, whereas adult cartilage showed reduced cellularity, matrix compaction, and well-defined zonal organisation. The tidemark was absent in fetal samples but clearly present in adult cartilage, indicating development of the calcified interface at later stages. Overall, these findings define the structural transition of human articular cartilage from a highly cellular, developmentally active tissue to a functionally specialized adult form. This developmental framework provides insight into cartilage histogenesis and a basis for designing regenerative strategies that recapitulate fetal-like properties.

Keywords: Articular cartilage, Quantitative morphometric analysis, Immunohistochemistry, Fetal cartilage, Tissue engineering, Histology.

Introduction

Articular cartilage plays a crucial role in enabling smooth movements and load distribution in the joint. Its development involves a progressive transformation from relatively simple tissue at the fetal stage to a highly organised, functionally specialised structure in adulthood (Patel et al., 2023). This process is driven by coordinated interactions between mechanical loading postnatally, growth factor signaling (including TGF- β , BMP, and IGF pathways), transcriptional regulators such as SOX9, and extracellular matrix-mediated feedback mechanisms, which together govern cellular organization, matrix composition, and the establishment of zonal architecture (Buchanan, 2022; Puetzer et al., 2010).

Although adult articular cartilage exhibits a well-defined, arcade-like collagen arrangement with distinct zonal stratification, this organization is largely absent during early life, indicating that postnatal maturation is critical for the establishment of functional architecture (Sophia Fox et al., 2009). The emergence of superficial, middle, and deep zones, along with corresponding variations in chondrocyte morphology and matrix composition, underlies the tissue's ability to withstand compressive and shear forces (Bielajew et al., 2022). Developmentally, articular cartilage undergoes a transition from a relatively thicker, more homogeneous tissue in the fetal stage to a comparatively compact, zonally organized structure in adulthood. This reduction in overall thickness, accompanied by increased structural specialization, reflects maturation of the extracellular matrix and functional adaptation to mechanical demands.

Importantly, fetal cartilage is characterized by a higher cellularity, increased matrix plasticity, and enhanced chondrogenic potential when compared to adult tissue (Choi et al., 2016). Fetal-derived chondrocytes and chondroprogenitors have been shown to exhibit superior proliferative capacity and matrix-forming ability, reflecting a more active state of chondrogenesis (Vinod et al., 2023). These properties are indicative of an early developmental phenotype that supports rapid tissue growth and organization.

Despite these known biological and structural differences, the quantitative characterisation of developing fetal articular cartilage and mature adult articular cartilage remains limited. Most existing studies focus on cellular biology, disease models, or animal systems, with relatively few addressing systematic histomorphometric changes across human developmental stages (Elder and Athanasiou, 2009). To our knowledge, no study has comprehensively evaluated the continuum of morphometric changes from fetal to adult human articular cartilage.

Histomorphometric analysis provides a robust framework to quantify these developmental transitions, including zonal thickness, cellular density, and matrix distribution. FIJI, an open-source image analysis platform based on ImageJ, enables reproducible and semi-automated quantification of histological features, including segmentation of cartilage zones and analysis of stain intensity from dyes such as Safranin O and Masson Trichrome (Harkey et al., 2022; J et al., 2012). These tools facilitate objective assessment of spatial and structural variations in cartilage across developmental stages.

In addition, computational image analysis allows evaluation of matrix organization and cellular morphology, providing insights into the maturation of the extracellular matrix and its relationship to chondrocyte distribution. Such approaches are particularly valuable in developmental studies, where subtle but functionally relevant transitions may not be captured through qualitative assessment alone.

The present study aims to perform a comparative histomorphometric analysis of developing fetal articular cartilage and mature adult articular cartilage across different developmental stages. Specifically, we quantify zonal architecture, cartilage thickness, chondrocyte density, morphology, and matrix distribution using FIJI-based image analysis. This approach enables consistent region-of-interest-based analysis across entire sections, allowing for standardized measurement of cellular and matrix parameters, including cell density,

circularity, zonal proportions, and staining intensity. By establishing developmental benchmarks, this study aims to provide insights into cartilage histogenesis and the structural basis of tissue maturation.

Materials and Methods

Study design

A comparative study using a cross-sectional design was designed to examine the histological and morphometric characteristics of knee joint tissues from different developmental periods (fetus versus adult). Human fetal specimens ($n = 3$) were taken from abortuses at gestation periods of 23 weeks, 27 weeks, and 36 weeks. Human adult samples ($n = 3$) were obtained from non-pathological knee joints. For the fetal specimens, femur and tibia (divided into medial/lateral) and patella and synovial tissue were collected, while femoral and tibial osteochondral units were collected for the adult subjects. All of these tissues were processed through standard histological techniques, including fixation, controlled decalcification of mineralized areas, paraffin embedding, and microtome sectioning at a uniform thickness, and mounting on glass slides.

To assess tissue architecture and extracellular matrix composition, sections were subjected to multiple histological staining protocols. These stains included Hematoxylin and Eosin (to evaluate overall tissue morphology), Safranin O (to evaluate cartilage rich in proteoglycans), Alcian Blue (to evaluate glycosaminoglycan rich cartilage), Toluidine Blue (to evaluate metachromatic matrix), Masson's Trichrome (to evaluate the distribution of collagen within cartilage), and staining for type II collagen in order to measure the amount of extracellular matrix specific to cartilage. Whole slide imaging was conducted on an EVOS Full Scan at $10\times$ magnification, followed by definition of regions of interest from which subsequent quantitative analysis could be made. Morphometric analyses were performed to evaluate total cartilage thickness and thickness in the different zones of the cartilage, along with the calculation of zonal contributions. Chondrocytes were evaluated in a zone-specific manner, and their density was quantified using automated detection of cells. The morphology of chondrocytes and lacunae was measured based upon the area of each cell/lacuna, the degree of circularity of each chondrocyte, and the pattern of chondron clustering. The extracellular matrix was semi-quantitatively evaluated based on the intensity of the staining. For adult joints, further evaluation included the presence and width of the tidemark, evaluation of subchondral regions, and thickness of calcified cartilage. Lastly, the data were combined to allow for a comparison between fetal and adult samples, which will allow for the identification of structural and cellular changes with respect to age within the knee joint (**Figure 1**).

Ethical approval and sample procurement

This study was approved by the Institutional Review Board (IRB) and Institutional Ethics Committee of Christian Medical College, Vellore (IRB Min No. 14498 dated 23.02.2022). All procedures involving human fetal and adult tissue specimens were conducted in accordance with the approved protocol and relevant ethical guidelines. Written informed consent was obtained from all parents, patients, or their legally authorised representatives prior to sample collection.

Human fetal knee joint samples were obtained from medically aborted fetuses aged 23+3 weeks, 27+3 weeks, and 36+4 weeks of gestation. The study cohort consisted of one male fetus and two female fetuses. Immediately post-abortion, the fetal specimens were transported to the laboratory in sterile normal saline (0.9% NaCl), with a mean cold ischemia time of 3–5 hours (range: [e.g., 1.5–6.5 hours]; variability due to differences in procedural timing and logistics across different samples).

The left knee joint was reserved for chondrocyte isolation, while the right knee joint was used for tissue fixation and subsequent histological processing to obtain sections for morphometric analysis. All handling and processing of fetal specimens were performed following established ethical and biosafety guidelines.

Morphometric analyses were performed on the cartilage lining the developing joint surface (future articular cartilage) of the fetal femoral and tibial epiphyses.

Non-diseased human adult knee joint samples (age ≥ 17 years, $n=1$) were procured from patients undergoing amputation due to traumatic injury or tumor resection involving the knee joint at Christian Medical College, Vellore. Analysis was limited to a single non-diseased adult specimen because of the limited availability of macroscopically intact, non-degenerative human articular cartilage. Accordingly, adult data were interpreted descriptively and used primarily for comparative histological and morphometric assessment. Informed written consent was obtained from all patients or their legal guardians prior to sample collection. Macroscopically normal cartilage regions, confirmed intraoperatively and free from degenerative changes, were harvested immediately post-resection and transported to the laboratory in sterile normal saline (0.9% NaCl), with a mean cold ischemia time of [e.g., 2–4 hours]. The samples were then processed for histological analysis.

For the adult samples, fixing the whole joint was not practically feasible. Thus, osteochondral unit (OCU) plugs (12 mm in diameter) were harvested from the femorotibial compartment of the knee joint, targeting regions with macroscopically intact cartilage as confirmed intraoperatively.

Sample processing

Tissue fixation and decalcification

Samples were fixed in 10% buffered formalin at room temperature for 2 weeks. Following this, the fixed samples were wrapped in gauze, rinsed with Milli-Q water, and decalcified in 10% EDTA (Cat. No. Q12635; Qualigens) for 40 days. Post-decalcification, the samples were given for paraffin embedding. Tissue sections of 4 μm thickness were obtained and mounted on glass slides that were pre-coated with poly-L-lysine.

Histological and immunohistochemical staining

Hematoxylin and eosin staining

Sections were stained with Harris hematoxylin (Cat. No: Q39411, Brand: Qualigens) solution for 8 minutes, dipped twice in 1% acid alcohol, rinsed with tap water, and then immersed in lithium carbonate. Eosin solution was applied for 30 seconds. Subsequently, sections were dehydrated using graded alcohol, cleared with xylene, and mounted with DPX.

Safranin O staining for proteoglycan distribution

Safranin O staining involved sequential treatment with Weigert's Iron Hematoxylin for 5 minutes, 1% acid alcohol 2 dips, and 0.05% fast green solution for 5 minutes, then decolourised with 1% acetic acid for 15 seconds and 1% Safranin O (Cat. No: Q39962, Brand: Qualigens) solution for 30 minutes, then rinsed with 95% alcohol. Subsequently, sections were dehydrated using graded alcohol, cleared with xylene and mounted using DPX.

Alcian Blue staining for glycosaminoglycan content

For Alcian Blue staining, it was preceded first by staining with Alcian Blue solution (pH 2.5, Cat. No. 820D071) for 15 minutes to selectively highlight acid mucins. Following this, the sections were washed in running tap water for 5 minutes, rinsed in distilled water and then counterstained with Neutral Red (Cat. No. 415490250) for 1 minute to visualise the nuclei. As a final step, the sections were quickly dehydrated using graded alcohol, cleared with xylene, and mounted with DPX.

Toluidine Blue staining for metachromatic matrix evaluation

Sections were stained with Toluidine Blue for 5 minutes. Following a brief distilled water wash, differentiation was carried out in 95% alcohol until the mast cell granules appeared distinctly. Following this, the sections were washed with graded alcohols, cleared with xylene and mounted with DPX.

Masson's Trichrome staining for collagen organisation

For Masson Trichrome staining, sections were first stained a minimum of 5 minutes with Harris Hematoxylin (Qualigens Cat. No: Q39411), differentiated with 1% acid-alcohol and stained 5 more minutes in Alcohol PRe-PRePicric solution (Qualigens Cat No: 35973), Further stained in 1% Ponceau Red (HiMedia Cat No: 27195), followed by 1% Phosphomolybdic acid solution (Qualigens Cat No: 13102) applied for 10 min, and finally stained in 2.5% Aniline blue (LEO CHEM Cas No: 42755). After this staining series was completed, the sections were washed in distilled water for 1 min and rinsed with 1% Phosphomolybdic acid for 2 min and finished with an acidic wash of 1% acetic acid (Qualigens Cat No: 21055) for 1 minute. After complete dehydration, they were cleared with xylene and mounted in DPX.

Collagen Type II immunostaining for cartilage-specific ECM

Collagen type II staining, sections underwent pronase (1 mg/mL; Cat. No: 10165913103, Brand: Roche) for 30 minutes at 37°C and hyaluronidase (2.5 mg/mL; Sigma, Cat. No: H3506) for 30 minutes at 37°C. Subsequently, sections were covered with 3% H₂O₂, followed by blocking with a protein block solution (1% BSA + 6% FBS) and incubation for 30 minutes. The sections were then incubated with the primary mouse monoclonal Anti-Collagen type II antibody (5 µg/mL; DSHB Hybridoma Product II-II6B3) at 4°C overnight. Following this, they were treated with a secondary antibody (goat anti-mouse HRP-labelled immunoglobulin, Cat. No: 31430, Pierce) for 30 minutes. Staining was performed with 3,3'-diaminobenzidine (DAB, Sigma, Cat. No: D5637) for 15 minutes, followed by counterstaining with hematoxylin.

Whole-slide imaging and digitalisation

Image acquisition parameters

Histological sections were imaged using the EVOS FL Auto Full Scan Imaging System (Life Technologies) and associated EVOS FL Auto software. Whole-slide scans were obtained at 10X magnification under standardised brightfield illumination and exposure settings to ensure uniform image acquisition across all developmental stages and regions. The captured images were subsequently used for morphometric and staining intensity analyses in FIJI software.

Region of Interest (ROI) selection

ROIs were defined to ensure standardized and reproducible morphometric and staining intensity measurements across samples. For fetal samples, femoral and tibial cartilage sections were harvested, while in adult samples, osteochondral units were drilled from the femorotibial joint, and the cartilage region of the osteochondral unit was selected as the ROI from anatomically comparable femoral and tibial sites. In fetal specimens, measurements were restricted to the cartilage lining the developing joint surface (future articular cartilage). Regions associated with active endochondral ossification, including the primary ossification front and growth plate-like cartilage, were excluded from morphometric analyses wherever identifiable. ROI selections were performed using Fiji software following image calibration. Identical ROI selection criteria were applied uniformly across all developmental stages and specimens.

Morphometric quantification analysis

The morphometric analysis and detailed experimental methodology have already been standardized and reported by our group in a preprint protocol (Jayakumar et al., 2026).

Cartilage length analysis. Using Safranin O-stained sections, cartilage length (μm) was measured as the linear distance from the articular surface to the osteochondral interface (or the osteochondral junction beneath the developing articular cartilage in fetal specimens where a tidemark was absent). Measurements were performed using the calibrated Distance tool in FIJI software.

Cartilage area measurements. Safranin O-stained sections of human fetal articular cartilage at gestational ages 23, 27, and 36 weeks were analysed using FIJI (ImageJ). Non-diseased adult human osteochondral sections were analysed in parallel using the same steps. Femur, tibia (lateral and medial condyles), and femorotibial sections were used to obtain the cumulative cartilage area. For each sample section, the total cartilage area was manually demarcated using the Freehand Selection tool, excluding surrounding non-cartilaginous tissue and bone regions. The measured ROI area (μm^2) was recorded and used for quantitative comparison between fetal and adult samples. All measurements were performed under identical imaging and thresholding conditions to ensure consistency.

Zonal division of articular cartilage. Femorotibial sections from both fetal and adult samples stained with Masson's Trichrome were used for zonal analysis. The total articular cartilage thickness was measured using the calibrated straight-line tool in FIJI from the articular surface to the osteochondral interface. Zonal demarcation was standardized across samples using relative percentage-based divisions of the total cartilage thickness: superficial zone (upper 10%), middle zone (50%), and deep zone (40%). Masson's Trichrome staining was used to facilitate visualization of cartilage architecture and orientation; however, zonal boundaries were assigned based on the predefined percentage-based criteria rather than staining intensity. Regions of interest were then generated within each zone for subsequent morphometric analyses.

Chondrocyte evaluation

Zone-wise cell count Femorotibial sections from fetal and adult samples, stained with Masson trichrome, were imaged with 20X magnification for assessment of chondrocyte density in different zones. Regions of interest (ROI) were outlined within each zone using FIJI software. Cell segmentation was achieved by detecting Weigert's iron hematoxylin-stained nuclei, which enabled precise differentiation of chondrocytes from the extracellular matrix. A semi-automated thresholding technique was utilised to segregate nuclei, followed by watershed segmentation to separate clustered or touching nuclei, and subsequent particle analysis for quantification of individual cells. Chondrocyte density was computed as the number of cells per unit area in each zonal ROI. All analyses were performed using consistent imaging and thresholding settings to maintain comparability between gestational and adult specimens.

Nuclear circularity Masson's trichrome-stained sections imaged at 20X magnification were selected for morphometric evaluation of chondrocytes across the different developmental stages. Representative images from each predefined cartilage zone were captured under identical magnification and illumination settings to ensure consistency. Image analysis was performed using FIJI. Prior to quantification, the required parameters, such as area, shape, and the fit ellipse, were selected to obtain the respective values. Circularity values were automatically produced with values ranging from 0 (elongated) to 1 (perfectly circular).

Lacunar area Safranin O sections were imaged at 20X magnification using an Olympus BX43f microscope. For each developmental stage, 15 representative chondrocytes were randomly selected within the deep zone. Lacunar areas were measured by manually tracing the perinuclear lacunar boundaries, calibrated against scale bars. Mean lacunar area (μm^2) and distribution patterns were compared across stages to quantify age-related constriction.

Chondron cluster enumeration. Stained Safranin-O sections imaged at 20X magnification were analysed for chondron density and organisation. Chondrons (chondrocyte clusters with pericellular matrix) were

segmented via thresholding, followed by watershed segmentation to obtain clusters of cells and subsequent particle analysis to quantify density (chondrons/ μm^2). Comparative histograms visualised progressive loosening from dense early fetal packing to sparser adult arrangements.

ECM deposition & stain intensity analysis

Extracellular matrix (ECM) deposition was assessed by determining the stain uptake intensity of Safranin O (for proteoglycan content) and immunohistochemical Collagen II staining (for cartilage-specific collagen) in human fetal (23, 27, and 36 weeks of gestation) and non-diseased adult osteochondral sections. Both stains were imaged at 10X magnification under standardised conditions. Femur, tibia (lateral and medial condyles), and femorotibial sections were analysed to obtain collective ECM intensity data. Staining intensity was quantified by performing colour deconvolution to isolate stain-specific channels, followed by measuring mean grey values within defined ROIs. Background intensity was excluded from the analysis. Mean stain intensity values were used for quantitative comparison across gestational ages and between fetal and adult samples. All measurements were performed using identical thresholding and analysis parameters to ensure consistency across samples.

Tidemark presence identification

Safranin-O-stained cartilage sections were examined at 10X magnification using an Olympus BX43f microscope to identify the presence of tidemark. Fetal and adult cartilage samples were systematically scanned across the full tissue depth. Absence of distinct bands in fetal tissue versus clear demarcation in adults was documented for comparative histological analysis.

Histological evaluation of fetal patella

Patellar samples from fetuses at 23, 27, and 36 weeks of gestation underwent routine Safranin O and Collagen II staining to assess trimester-specific staining patterns. Sections were imaged at 10X magnification and quantified for stain intensity by performing colour deconvolution to isolate stain-specific channels, followed by measurement of mean grey values.

Synovium assessment

Full-thickness fibrous capsule samples (23, 27, and 36 weeks of gestation) were dissected and stained with H&E and Masson's trichrome to study their architecture. In addition, toluidine blue, safranin O and alcian blue staining were employed to analyse ground substance components in the synovial membrane. Collagen II IHC was performed to assess chondrogenesis.

Graphical representation

All quantitative data were analysed and graphed using GraphPad Prism version 8.0.2. Graphical representations, including bar charts with trend lines, were generated directly in GraphPad Prism to visualise developmental courses and zonal specialisation patterns.

Results

Developmental changes in cartilage length

Cartilage length (linear distance from the articular surface to the osteochondral interface) remained relatively stable from 23 to 27 weeks of gestation. This stability postulated the balance between the production and breakdown of the ECM, supported by chondrocyte activity. A small increase at 36 weeks indicates a shift toward more matrix production, which supports late gestational growth. However, in adulthood, thickness dropped significantly. Mean values of femur, tibia (lateral and medial condyles), and femorotibial sections were obtained and presented as final results for fetal samples, and for the femorotibial section in adult cartilage (**Figure 2A**) (**Table S1**). These observations indicate developmental differences in cartilage

morphology across the examined stages; however, the underlying molecular and cellular mechanisms were not investigated in the present study

Developmental changes in cartilage area

Cartilage area (two-dimensional cartilage tissue area) decreased from 23 to 27 weeks. This change reflects the reorganisation of the ECM and structural refinement during early development, without overall tissue growth. The area reached its peak at 36 weeks due to ongoing ECM deposition but saw a sharp decline in adults. Mean values for the femur, tibia (lateral and medial condyles), and femorotibial sections were obtained and presented as final results for fetal samples, and for the femorotibial section in adult cartilage **(Figure 2B) (Table S2)**. This drop is attributed to the reduced chondrocyte activity in building the matrix, faster turnover of the matrix, and long-term loading effects.

These trends show a development focused on ECM buildup rather than chondrocyte growth, transition from a phase of cell multiplication to one of maturation before experiencing age-related decline.

Zonal thickness comparison

Morphometric analysis of zonal areas demonstrated progressive differentiation of superficial, middle, and deep zones during fetal development. In the early gestational ages (23 and 27 weeks), the superficial zone exhibited a relative increase, and the smooth formation of a gliding surface against shear force from fetal movements, while the middle zone decreased, reflecting the compaction and chondrocyte realignment into functional patterns for later stages of development. The deep zone expanded into a structural anchor, which would later become the subchondral bone. At 36 weeks, the growth of superficial zone appeared stabilized and the middle zone re-expanded with increase in proteoglycan production. The deep zone dominated through chondrocyte hypertrophy and matrix production.

In adulthood, the superficial zone remained smallest, serving as dense collagen barrier against friction and shear; the middle zone became dominant, providing bulk compressive resistance; and the deep zone decreased and consolidated through calcification and formation of tidemark **(Figure 2C)**.

Chondrocyte morphometric analysis

Zone-wise chondrocyte count revealed high density in early gestation, which progressively declined through late gestation and into adulthood. Superficial zone count decreased steadily, forming a thin, flattened cellular layer. The middle zone expanded and then stabilised as dominant, establishing an organised collagen-proteoglycan network. The deep zone peaked at 36 weeks before its reduction at the adult period **(Figure 2D)**.

The elevation in circularity at the early gestation reflects rounded isotropic nuclei in rapidly dividing cells under low mechanical load, facilitating uniform matrix synthesis. Progressive decline in the adult group indicated remodelling to elongated, anisotropic nuclear shapes aligned with collagen fibers **(Figure 2E, Figure 3 (A-B))**. This morphological transition from proliferative roundness to load-optimized elongation supports the shift to functional cartilage architecture, enhancing mechanotransduction and metabolic efficiency in mature, ECM-rich matrix.

Measurements revealed relatively larger lacunar spaces in early fetal cartilage and were well appreciated, which reflects active cellular growth under minimal load. This pattern was absent in adult tissue **(Figure 2F, Figure 4B)**. This will increase with maturation and hence lead to a smaller lacunar area and ECM-dominant architecture, optimising mechanical function.

Chondron enumeration showed dynamic changes in chondrocyte organization across gestational stages. Earlier fetal stages exhibited higher chondron density, which gradually decreased with advancing gestation and was lowest in adult cartilage, which had organized lacunar architecture and reduced cellularity **(Figure**

2G, Figure.4B). The shift from closely packed cells to more loosely organized cellular structures appears to be a result of maturation/adaptation.

Tidemark evaluation

In adult cartilage, a distinct band with gradient was identified as the tidemark, clearly demarcation non-calcified from calcified zones. Whereas the same could not be appreciated in fetal stages as well as it was in the adult specimen (**Figure 4A**). This suggests the calcified interface develops late, likely postnatally under mechanical loading.

Quantification of proteoglycan content by measuring stain intensity of Safranin O and Collagen II

Quantitative analysis revealed gestational age-associated changes in ECM composition. Mean values for the femur, tibia (lateral and medial condyles), and femorotibial sections were obtained and presented as final results for fetal samples, and for the femorotibial section in adult cartilage (**Figure 5C**) (**Table S3-S4**). Safranin O mean stain intensity, reflecting proteoglycan content was relatively higher in the 36 weeks sample, and the lowest intensity was observed in non-diseased adult cartilage (**Figure 5A**). Intense staining in fetal stages indicates higher glycosaminoglycan content, which is reduced in adult cartilage. Immunohistochemical Collagen II staining intensity was seen to be higher at 27 weeks, decreased at 36 weeks and lowest in the adult (**Figure 5B**). Its expression is more prominent during fetal stages and becomes more structurally organized but relatively reduced in adult cartilage.

This indicates remodeling of cartilage-specific ECM components during gestation and maturation. These observed trends indicate active reorganization of proteoglycan and collagen II-rich ECM during fetal development, with reduced ECM staining in adult articular cartilage compared to fetal stages.

Staining was also performed using Alcian Blue and Toluidine Blue (**Figure.6A-B**). These yielded results similar to those observed for Safranin O and Collagen II. Patterns showed comparable gestational age-related shifts in ECM components.

Patellar staining patterns

Staining patterns showed similarities across samples, with proportionate increases in stained area from mid- to late trimester (**Figure.7A**). Alcian Blue and Toluidine Blue yielded robust, consistent results, whereas Masson Trichrome exhibited uneven, non-predictive patterns. Meanwhile, Safranin O and Collagen II stain intensities were quantified, revealing a clear decreasing trend toward late gestation (**Figure. 7B**).

Synovium analysis

Analysis of the joint capsule in H&E and Masson's trichrome-stained slides showed a simple squamous to cuboidal epithelial arrangement marking the synovial membrane surrounded by collagen fibres denoting the fibrous capsule. There was no marked difference in staining properties between the 3 groups when stained using Alcian blue, Toluidine blue, and safranin O. Collagen 2 IHC marker was negative in all three groups (**Figure 8**).

Overall, the data demonstrates a transition from a highly cellular, structurally immature fetal cartilage to a compact, zonally organized adult tissue characterized by reduced cellularity and increased matrix specialization.

Discussion

This study demonstrates distinct structural and cellular transitions in human articular cartilage from fetal stages to adulthood, characterised by changes in cartilage dimensions, zonal organisation, cellularity, and ECM composition. Cartilage area and thickness were highest at late gestation (36 weeks), followed by a reduction in adulthood, suggesting a shift from a growth-dominant phenotype to a structurally compact and functionally specialised tissue.

This transition is consistent with known developmental processes, where fetal cartilage exhibits high cellularity, increased proteoglycan content, and a relatively unorganised matrix, whereas adult cartilage is characterised by reduced cellularity, increased matrix density, and well-defined zonal architecture(Decker et al., 2015). The observed reduction in adult cartilage dimensions may reflect structural compaction and matrix reorganisation rather than simple tissue loss, aligning with previous reports of age-related alterations in collagen network organisation and proteoglycan distribution.

Zonal analysis further highlights the progressive establishment of cartilage architecture, with poorly defined zones in early fetal stages, emerging stratification by late gestation, and fully developed zonal organisation in adult tissue(van Turnhout et al., 2011). The absence of a tidemark in fetal samples and its clear presence in adults supports the concept that the calcified cartilage interface develops later, likely in response to postnatal mechanical loading.

At the cellular level, a progressive decline in chondrocyte density, accompanied by changes in nuclear morphology and lacunar size, reflects the transition from a proliferative, developmentally active state to a mature, matrix-dominated phenotype. Fetal cartilage exhibited rounded nuclei and larger lacunae, indicative of high metabolic and proliferative activity, whereas adult cartilage showed more elongated nuclei and reduced lacunar spaces, consistent with structural adaptation and matrix organisation.

These findings align with existing evidence that fetal-derived chondrocytes possess higher chondrogenic potential and matrix-forming capacity, supporting the concept that early developmental stages represent a biologically active phase of cartilage histogenesis(Choi et al., 2016; Vinod et al., 2023). This is further supported by developmental studies demonstrating that articular cartilage originates from specialized interzone progenitor cells, which give rise to distinct joint tissues during early morphogenesis(Decker et al., 2015). The observed increase in ECM staining intensity during fetal stages further reinforces the presence of active-matrix synthesis and remodelling during development.

Together, these findings demonstrate progressive changes in cartilage architecture, cellularity, and extracellular matrix distribution across developmental stages. While previous studies have implicated developmental signalling pathways and mechanical influences in cartilage maturation, the present study did not directly evaluate these mechanisms. Experimental studies have demonstrated that mechanical stimulation is essential for proper joint and cartilage development, with reduced movement leading to impaired zonal organization and joint formation(Jia et al., 2023). Importantly, this study provides quantitative evidence of these transitions in human tissue, addressing a gap in the literature regarding continuous morphometric evaluation from fetal to adult stages.

However, certain limitations must be acknowledged. The limited sample size, particularly the inclusion of only one non-diseased adult specimen, restricts the generalizability of the findings, and adult-fetal comparisons should therefore be interpreted descriptively. Validation in larger cohorts of healthy adult cartilage specimens will be necessary to confirm the observed trends. Additionally, 2D histological analysis may not fully capture the three-dimensional organisation of cartilage, and staining-based quantification may require further validation with biochemical or molecular assays. In addition, body weight and crown-rump length measurements were unavailable for the fetal specimens. Although gestational age was used as the primary developmental reference, these anthropometric parameters would have allowed a more comprehensive assessment of inter-individual developmental variation. Furthermore, the study did not include assessment of cellular proliferation, apoptosis, cartilage resorption, or molecular signalling pathways. Consequently, the observed morphometric changes should be interpreted descriptively, and mechanistic inferences require validation through dedicated molecular and histological investigations.

Despite these limitations, the study establishes a foundational framework for understanding cartilage histogenesis and maturation and identifies key structural transitions that may be relevant in distinguishing normal development from early pathological changes.

Conclusion

The present findings demonstrate that human articular cartilage undergoes a clear transition from a highly cellular, proteoglycan-rich, and structurally immature fetal tissue to a compact, matrix-dominated, and zonally-organised adult structure. This study provides quantitative evidence that human articular cartilage maturation involves a transition from a cell-rich, developmentally-active fetal tissue to a matrix-dense, zonally-organised adult structure with a defined osteochondral interface. By capturing this continuum, the study establishes a structural basis for cartilage histogenesis and underscores the fetal phenotype as a promising template for regenerative approaches aimed at restoring native cartilage architecture and function.

Figures

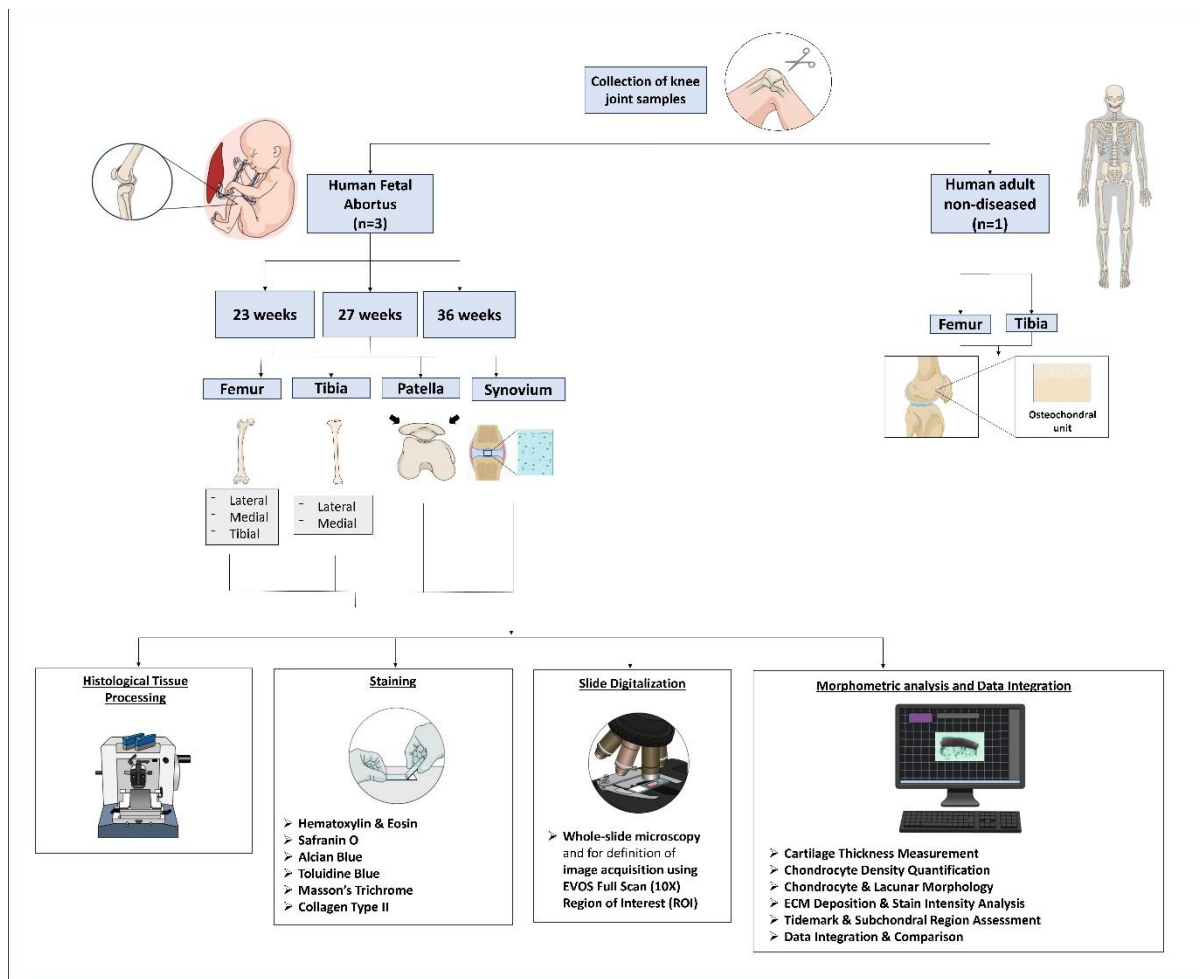


Figure 1. Schematic illustration of histological and morphometric analysis of fetal and adult human articular cartilage. The collection of fetal (23, 27, 36 weeks) and adult knee joint samples (femur, tibia, patella, synovium) was followed by tissue processing, histological staining, Whole Slide Imaging by using an EVOSFLAuto Full Scan microscope, to conducting quantitative morphometric analysis via FIJI software to assess cartilage length and area, lacunar area, chondron organization, zonal cell distribution and architecture. Includes descriptive analysis of tidemark, synovium, and patella regions. In fetal specimens, morphometric analyses were restricted to the cartilage lining the developing joint surface (future articular cartilage). Regions corresponding to the primary ossification front and active endochondral ossification were excluded from quantitative analyses. Representative annotations indicate the anatomical regions selected for measurement.

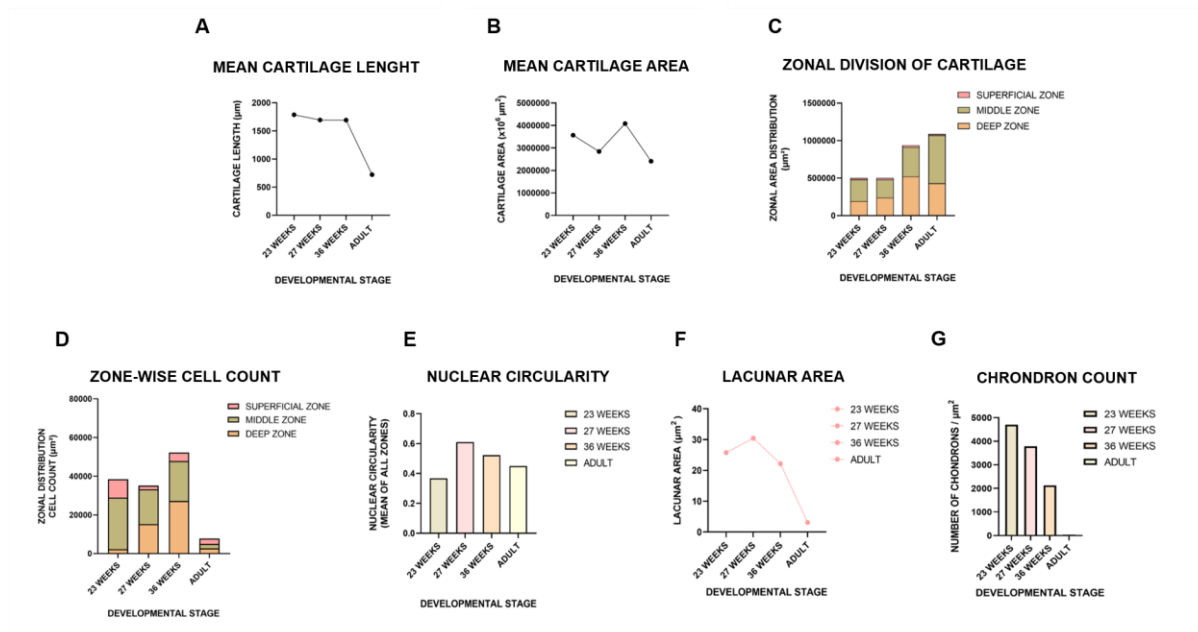


Figure 2. Quantitative morphometric analysis of developing articular cartilage (fetal) and mature articular cartilage (adult). (A) Mean cartilage length (μm) and (B) mean cartilage area (μm^2), representing developmental changes in cartilage dimensions. Measurements were obtained from femoral, tibial (lateral/medial condyles), and femorotibial regions in fetal specimens and the femorotibial region in adult cartilage. (C) Zonal cartilage area distribution across superficial, middle, and deep zones, reflecting maturation-associated architectural organization. (D) Zone-wise cell count, indicating the transition from the highly cellular fetal cartilage to the lower cellularity of mature cartilage. (E) Nuclear circularity, expressed as the average of mean values across the superficial, middle, and deep zones, reflecting changes in chondrocyte morphology during maturation. (F) Mean lacunar area (μm^2), based on 15 representative chondrocytes per sample, reflecting changes in the chondrocyte-matrix microenvironment. (G) Chondron density (chondrons/ mm^2), reflecting developmental changes in chondrocyte-matrix organization. Measurements were performed on cartilage lining the joint surface; in fetal specimens, the primary ossification front and regions of active endochondral ossification were excluded.

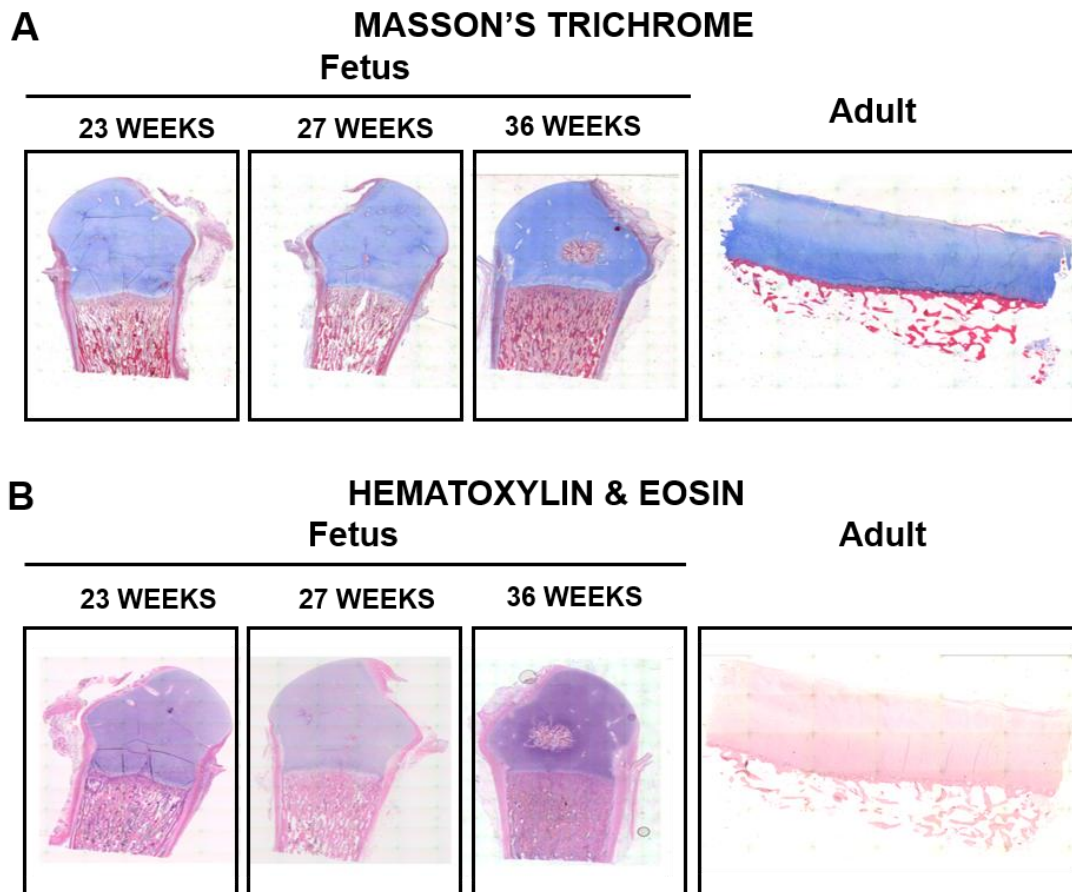


Figure 3. Histological assessment of fetal and adult human femorotibial cartilage samples. (A) Masson's trichrome and (B) hematoxylin and eosin (H&E) staining demonstrate developmental changes in cartilage matrix organization, tissue architecture, and cellular distribution from fetal to adult cartilage. Representative whole-slide images were generated by stitching multiple tiled images acquired at 10× magnification; scale bar, 400 μ m.

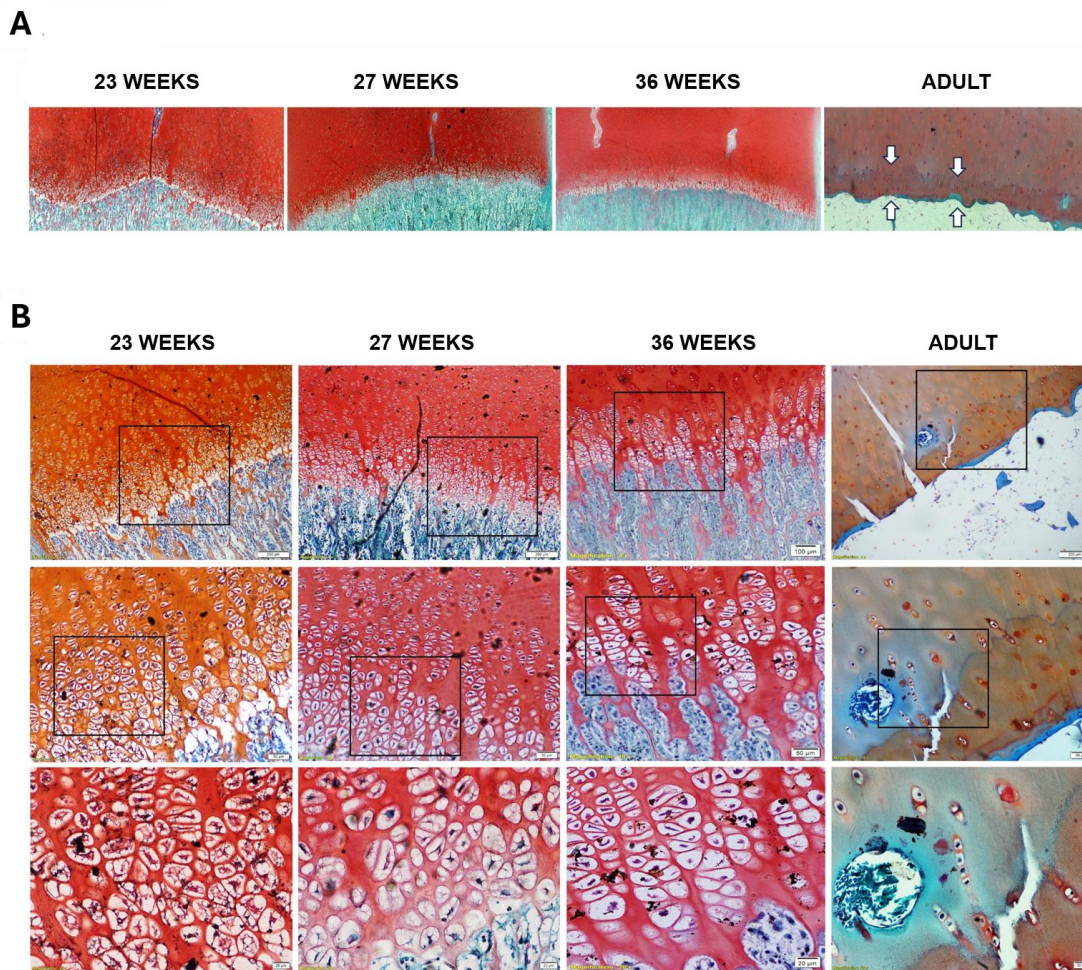


Figure 4. Structural maturation of cartilage using Safranin-O stained cartilage sections: tidemark formation and chondron organization. (A) Representative images depicting tidemark development across fetal and adult cartilage. The tidemark is absent in fetal stages and becomes distinct in adult cartilage (indicated by arrows). The analysed region corresponds to the developing articular cartilage compartment. Areas of active endochondral ossification were excluded from quantitative analyses. (B) Chondron and lacunar organization at multiple magnifications (4X, 10X, and 20X) across fetal developmental stages.

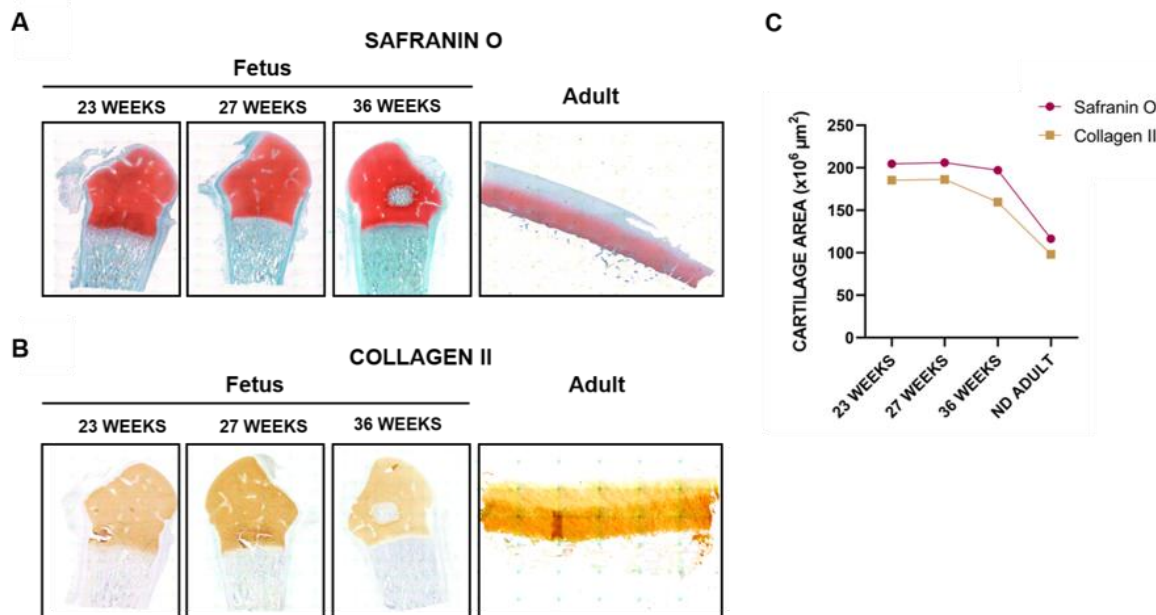


Figure 5. Comparative analysis of proteoglycan and collagen II content using Safranin O (A) and Collagen II staining (B) of femorotibial cartilage sections. The acquired full-scanned images were created by stitching together multiple tiled images of 10x, each representing a 400 μm scale. **(C)** Quantitative staining intensity measurements were performed within the articular cartilage region of interest. In fetal specimens, cartilage associated with active endochondral ossification and the primary ossification front was excluded from analysis.

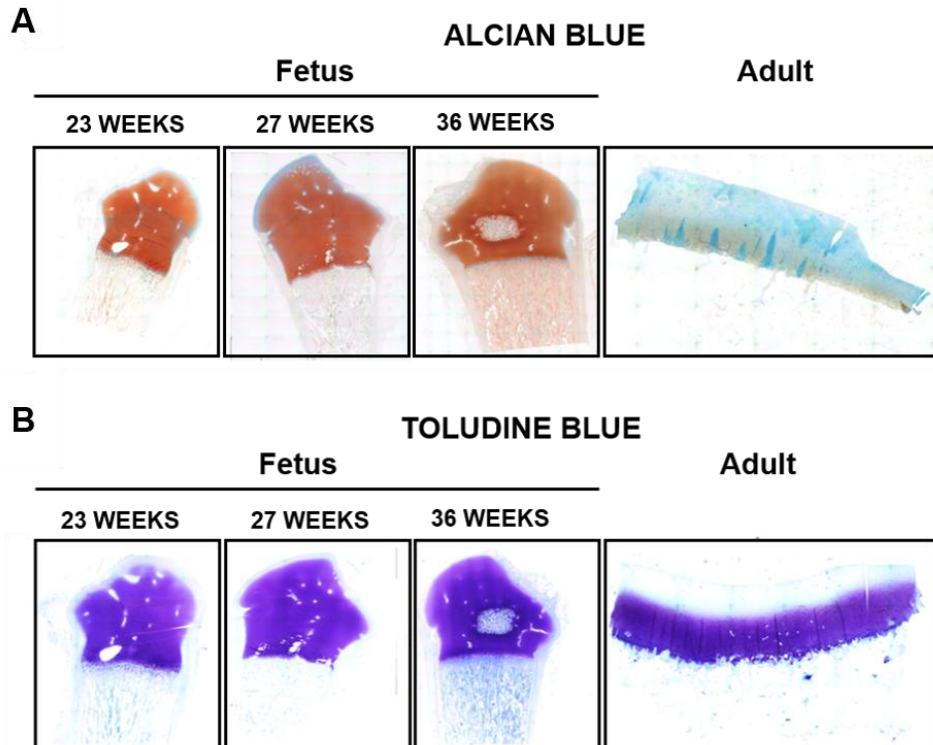


Figure 6. Comparative analysis of glycosaminoglycan content using Alcian Blue and Toluidine Blue staining. (A) Alcian Blue staining (pH 2.5) demonstrates the distribution of acidic glycosaminoglycans within the cartilage matrix; Neutral Red was used as a counterstain. **(B)** Toluidine Blue staining demonstrates metachromatic matrix staining associated with glycosaminoglycan-rich regions. Together, the staining

patterns illustrate developmental changes in cartilage matrix composition and organization. Representative whole-slide images were generated by stitching multiple tiled images acquired at 10× magnification; scale bar, 400 μm.

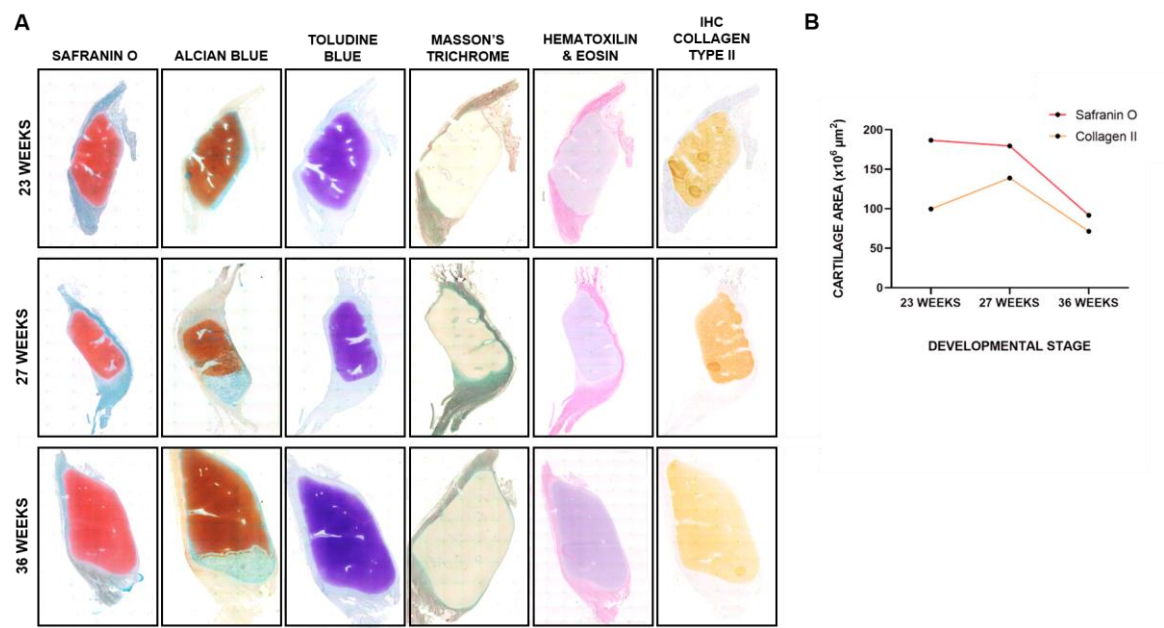


Figure 7. Histological and immunohistochemical characterization of fetal human patellar cartilage and its extracellular matrix composition. (A) Representative histological and immunohistochemical staining of fetal patellar cartilage, demonstrating matrix organization and distribution of cartilage-associated components. Alcian Blue sections were counterstained with Neutral Red. (B) Quantitative mean staining intensity for Safranin O and Collagen II, providing complementary assessment of glycosaminoglycan-rich matrix and cartilage-specific collagen expression. Representative whole-slide images were generated by stitching multiple tiled images acquired at 10× magnification; scale bar, 400 μm.

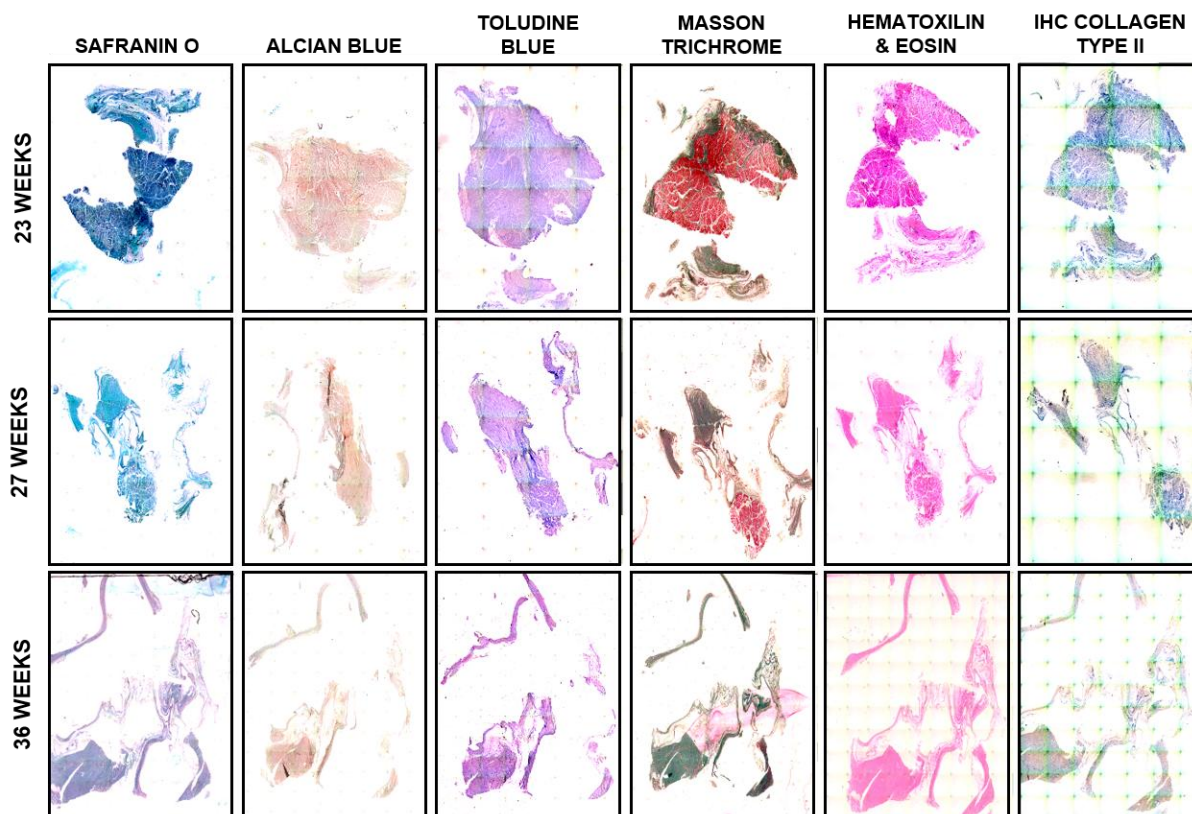


Figure 8. Histological and immunohistochemical characterization of fetal human synovium. Representative staining demonstrates the cellular organization and extracellular matrix characteristics of the developing fetal synovium. Whole-slide images were generated by stitching multiple tiled images acquired at 10× magnification; scale bar, 400 μ m.

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J.A.J. carried out the computational histological work, and G.P. was responsible for staining and obtaining the samples. S.R., S.M.A., S.*, D.V.F., and E.V. performed the experiments and collected data. All authors were involved in data analysis and interpretation. E.V and S.M.A provided financial support. All authors were involved in preparing the final version of the manuscript. We acknowledge Ms Esther Rani and Mr. Ashok Kumar for technical support, and the Centre for Stem Cell Research (a unit of BRIC-inStem Bengaluru) and Christian Medical College, Vellore, for infrastructural support.

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References

- Bielajew, B.J., Donahue, R.P., Lamkin, E.K., Hu, J.C., Hascall, V.C., Athanasiou, K.A., 2022. Proteomic, mechanical, and biochemical characterization of cartilage development. *Acta Biomater* 143, 52–62. <https://doi.org/10.1016/j.actbio.2022.02.037>
- Buchanan, J.L., 2022. Types of Fibrocartilage. *Clinics in Podiatric Medicine and Surgery, Update on Fibro-Cartilaginous Disease* 39, 357–361. <https://doi.org/10.1016/j.cpm.2022.02.001>
- Choi, W.H., Kim, H.R., Lee, S.J., Jeong, N., Park, S.R., Choi, B.H., Min, B.-H., 2016. Fetal Cartilage-Derived Cells Have Stem Cell Properties and Are a Highly Potent Cell Source for Cartilage Regeneration. *Cell Transplant* 25, 449–461. <https://doi.org/10.3727/096368915X688641>
- Decker, R.S., Koyama, E., Pacifici, M., 2015. Articular cartilage: structural and developmental intricacies and questions. *Curr Osteoporos Rep* 13, 407–414. <https://doi.org/10.1007/s11914-015-0290-z>
- Elder, B.D., Athanasiou, K.A., 2009. Systematic Assessment of Growth Factor Treatment on Biochemical and Biomechanical Properties of Engineered Articular Cartilage Constructs. *Osteoarthritis Cartilage* 17, 114–123. <https://doi.org/10.1016/j.joca.2008.05.006>
- Harkey, M.S., Michel, N., Kuenze, C., Fajardo, R., Salzler, M., Driban, J.B., Hacihaliloglu, I., 2022. Validating a Semi-Automated Technique for Segmenting Femoral Articular Cartilage on Ultrasound Images. *Cartilage* 13, 19476035221093069. <https://doi.org/10.1177/19476035221093069>
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9, 676–682. <https://doi.org/10.1038/nmeth.2019>
- Jayakumar, J A, Rani, B, Parasuraman, G, Srinivasaiah, S B and Vinod, E(2026). A Reproducible FIJI-Based Workflow for Quantitative Analysis of Cartilage Histomorphometry . *Bio-protocol Preprint*. 10.21769/p2939.
- Jia, Y., Le, H., Wang, X., Zhang, J., Liu, Y., Ding, J., Zheng, C., Chang, F., 2023. Double-edged role of mechanical stimuli and underlying mechanisms in cartilage tissue engineering. *Front Bioeng Biotechnol* 11, 1271762. <https://doi.org/10.3389/fbioe.2023.1271762>
- Patel, J., Chen, S., Katzmeyer, T., Pei, Y.A., Pei, M., 2023. Sex-dependent variation in cartilage adaptation: from degeneration to regeneration. *Biol Sex Differ* 14, 17. <https://doi.org/10.1186/s13293-023-00500-3>
- Puetzer, J.L., Petite, J.N., Lobo, E.G., 2010. Comparative review of growth factors for induction of three-dimensional in vitro chondrogenesis in human mesenchymal stem cells isolated from bone marrow and adipose tissue. *Tissue Eng Part B Rev* 16, 435–444. <https://doi.org/10.1089/ten.TEB.2009.0705>
- Sophia Fox, A.J., Bedi, A., Rodeo, S.A., 2009. The Basic Science of Articular Cartilage: Structure, Composition, and Function. *Sports Health: A Multidisciplinary Approach* 1, 461–468. <https://doi.org/10.1177/1941738109350438>

van Turnhout, M.C., Kranenbarg, S., van Leeuwen, J.L., 2011. Contribution of postnatal collagen reorientation to depth-dependent mechanical properties of articular cartilage. *Biomech Model Mechanobiol* 10, 269–279. <https://doi.org/10.1007/s10237-010-0233-7>

Vinod, E., Parasuraman, G., Lisha J, J., Amirtham, S.M., Livingston, A., Varghese, J.J., Rani, S., Francis, D.V., Rebekah, G., Daniel, A.J., Ramasamy, B., Sathishkumar, S., 2023. Human fetal cartilage-derived chondrocytes and chondroprogenitors display a greater commitment to chondrogenesis than adult cartilage resident cells. *PLoS One* 18, e0285106. <https://doi.org/10.1371/journal.pone.0285106>