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Collagen: quantification, biomechanics and role of minor subtypes in cartilage

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Abstract | Collagen is a ubiquitous biomaterial in vertebrate animals. Although each of its 28 subtypes contributes to the functions of many different tissues in the body, most studies on collagen or collagenous tissues have focused on only one or two subtypes. With recent developments in analytical chemistry, especially mass spectrometry, substantial advances have been made towards quantifying the different collagen subtypes in various tissues; however, high-throughput and low-cost methods for collagen-subtype quantification do not yet exist. In this Review, we introduce the roles of collagen subtypes and crosslinks and describe modern assays that enable a deep understanding of tissue physiology and disease states. Using cartilage as a model tissue, we describe the roles of major and minor collagen subtypes in detail, discuss known and unknown structure–function relationships and show how tissue engineers may harness the functional characteristics of collagen to engineer robust neotissues.

Collagens — the most abundant proteins in the body by weight — are the main structural proteins in the extracellular matrix (ECM) of various tissues, including cartilage, bone, blood vessels, skin and other connective tissues (FIG. 1). Collagen has been studied since the 1800s[1]. Since then, it (and its crosslinks) have been shown to be implicated in tissue biomechanics[2–4] and disease states, such as cancer[5,6], arthritis[7,8] and over 40 hereditary diseases[9,10]. Collagen is categorized into 28 subtypes, with types I, II and III making up 80–90% of the collagen in the human body[11]. The so-called 'minor collagens', which do not have a set definition, are present in very low amounts but have vital functional roles[12]. Despite this, most studies in collagen and collagenous tissues have only focused on general collagen (that is, total collagen without subtype specificity) or just one or two collagen subtypes.

In this Review, we first describe the collagen superfamily. We then discuss recent advances in mass spectrometry that allow sensitive quantification of individual collagen subtypes and determination of their role in disease states and tissue biomechanics. Our attention is then turned to the subtypes of cartilage, which have been neglected in the literature. Finally, we describe the need for new studies, in particular, those that leverage high-throughput technologies. We urge tissue engineers to take advantage of bottom-up proteomics techniques to better understand the ECM of engineered tissue, potentially unveiling how to engineer stiffer, stronger and more durable neotissues. A similar approach focusing on all collagen subtypes would lead to a deeper understanding of the roles of minor collagens and may explain the gap in functionality between engineered implants and native tissues. Outside the scope of this Review are collagen assembly, molecular mechanics or in-depth cellular interactions, which have been reviewed in detail elsewhere[13–16].

Collagens and collagen crosslinks

All 28 subtypes of collagen contain specific amino acid sequences that encode one or more triple-helical domains[17]. Triple-helical domains consist of a signature repetition of amino acids G-X-Y, with G always representing glycine, X usually representing proline and Y usually representing hydroxyproline. This repeated sequence creates favourable hydrogen bonding, allowing for three polypeptides, called alpha chains, to be assembled into a triple-helical collagen protein. The 28 types of collagen are divided into groups based on the location, size and distribution of triple-helical domains; these groups are summarized in TABLE 1. Collagens may consist of three of the same alpha chain or of different alpha chains. For example, collagen type I usually consists of two α_1 chains and one α_2 chain[18], collagen type II consists of three α_1 chains[19] and collagen type IX consists of α_1, α_2 and α_3 chains[20]. Even though collagen types I and II are the most abundant, minor collagens, which account for less than 10% of the total collagen content, have important roles in collagenous tissues, as described later.

Collagen types I, II, III, V, XI and IX form covalent enzymatic collagen crosslinks, which strengthen and mature the collagen network[21]. The formation pathway of these collagen crosslinks is displayed in FIG. 2.

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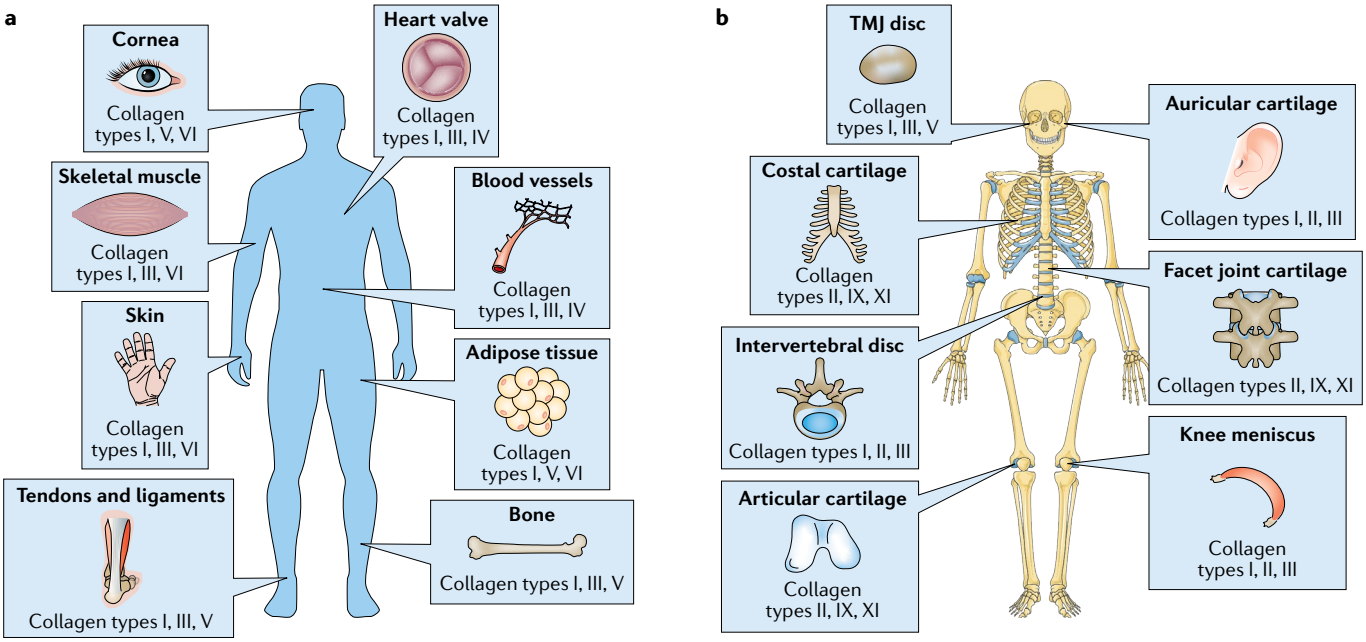


Fig. 1 | **Collagen types of the human body.** Different collagen types are found in various tissues all over the body. Three of the most abundant collagen types are displayed for non-cartilage tissues (panel **a**) and cartilage tissues (panel **b**). TMJ, temporomandibular joint. The skeleton image in panel **b** reprinted from https://smart.servier.com/smart_image/skeleton/ CC BY 3.0.

The first step is catalysed by lysyl oxidase (LOX) and involves deamination of specific hydroxylysines in the collagen amino acid sequence to form hydroxyallysine[22]. Hydroxyallysine reacts with lysine or hydroxylysine in another collagen alpha chain to form the divalent crosslinks dehydro-dihydroxylysinonorleucine (deH-DHLNL) and dehydro-hydroxylysinonorleucine (deH-HLNL). These crosslinks undergo a spontaneous Amadori rearrangement to the more stable hydroxylysinoketonorleucine (HLKNL) and lysinoketonorleucine (LKNL), respectively[23,24]. These molecules are also known as immature, reducible or intermediate crosslinks. Because HLKNL and LKNL are destroyed upon hydrolysis, they are analysed in their borohydride-reduced forms, dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL)[25]. HLKNL and LKNL react with another hydroxyallysine to connect to a third alpha chain of collagen, forming the trivalent crosslinks hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP), respectively[26]. HP and LP, which are also called pyridinoline and deoxypyridinoline, respectively, are known as mature or non-reducible crosslinks. More recently, another ketoimine maturation, arginoline, was found to form when ketoimines undergo oxidation and free arginine addition; arginoline exists in approximately equimolar amounts to HP in articular cartilage[27]. Pyrrole crosslinks, which are created by telopeptide lysines, are discussed in detail elsewhere[21]. There are also non-enzymatic crosslinks, by which lysines or hydroxylysines in helical collagen react with sugars like glucose or ribose to form glucosepane and pentosidine through the Maillard reaction. These crosslinks are known as advanced glycation end products (AGEs) and are associated with ageing and disease states[28].

HP and LP are important for the tensile properties of collagenous tissues, and the amount of HP varies greatly in connective tissues. In the knee, the cruciate ligaments have 3–5 times as much HP per collagen mass than the collateral ligaments, and articular cartilage has about twice the relative HP content than the knee meniscus[3]. In bovine sternomandibularis muscle and nuchal ligament, the relative HP content was shown to increase with animal age[29]; however, the HP content in human intervertebral discs was shown to decrease with age[30]. Moreover, HP and LP are excreted during bone resorption and collagen degradation, and may be used as urinary biomarkers for diseases such as osteoporosis[31] and osteogenesis imperfecta[32], and for cancer metastasis[33].

Identification and quantification

Methods for identification and quantification of collagen and its subtypes have been in iterative development for over a century[34,35]. Collagen analysis is relevant to fields such as tissue engineering, tissue characterization, drug development and delivery, and biomechanics. Although several methods currently exist for collagen identification and quantification, all lack in sensitivity, specificity and/or cost-effectiveness. Next-generation high-throughput assays may enable the highly sensitive parallel processing of several samples in a short time, with individual subtype specificity. We provide an overview of existing and next-generation methods in this section, covering traditional and antibody-based assays, imaging, mass spectrometry and promising proteomics approaches. A summary of the quantitative assays for collagen subtypes and crosslinks is displayed in FIG. 3.

Table 1 | **Classically defined groups of collagen subtypes**

Group name	Key features	Collagen types
Fibril-forming collagens	Long triple-helical domains for fibril formation	I, II, III, V, XI, XXIV, XXVII
Fibril-associated collagens with interrupted triple helices (FACITs)	Do not form fibrils but associate with fibril surfaces	IX, XII, XIV, XVI, XIX, XX, XXI, XXII
Network-forming collagens	Form repeating patterns	IV, VIII, X
Membrane collagens	Span the cell membrane	XIII, XXIII, XXV
Multiplexins	Have many non-collagenous domains but are not FACITs	XV, XVIII
Others	Do not belong to any of the above categories	VI, VII, XXVIII

Traditional assays

Because hydroxyproline content is highly correlated to the collagen content, the hydroxyproline assay has been frequently used to quantify collagen in biological samples. Since its establishment in the mid-twentieth century, this procedure has been improved and simplified through many iterations[36–38]. This method has been cited as the 'gold-standard' collagen quantification assay[39,40]. Although this assay is relatively simple and low cost, it does not discriminate between collagen types or other non-collagen molecules that contain hydroxyproline, such as elastin, which is present in many collagenous tissues[41]. Moreover, the hydroxyproline content varies among different collagen subtypes; for example, collagen type VI contains less hydroxyproline per chain than fibrillar types[42]. Therefore, although the hydroxyproline assay may be sufficient for estimating overall collagen, additional analysis is needed for highly sensitive or type-specific collagen quantification.

Another assay used to identify collagens is sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), which separates molecules by molecular weight and charge. Because the molecular weights of many collagen alpha chains are documented, they can be identified using a prepared ladder of proteins[43]. Nevertheless, separating the alpha chains is challenging and requires strong denaturants[44]. Semi-quantification of protein in gel bands from SDS–PAGE is possible via densitometric analysis[45]. Although SDS–PAGE alone does not confirm the identity of proteins, they can be identified through antibody binding in a western blot.

High-performance liquid chromatography with fluorescence detection (HPLC-FLD) has been used for the identification and quantification of HP and LP since the mid-1980s. This method uses reverse-phase chromatography with ion-pairing agents[46]. This was extended to quantify the AGE pentosidine and, using post-column derivatization, quantify immature crosslinks HLKNL and LKNL[47]. Although HPLC-FLD has been a gold-standard assay for collagen crosslink quantification for many years, its lengthy chromatography and usage of harsh ion-pairing agents are substantial disadvantages.

◀ Fig. 2 | **Lysyl oxidase pathway. a** | The enzymatic pathway for the formation of pyridinoline and arginoline crosslinks. The initial process of hydroxylysine to hydroxyallysine is catalysed by lysyl oxidase, which is necessary for the cascade of reactions shown in the pathway. **b** | Structural formulas of the immature and mature crosslinks.

Quantitative antibody-based assays

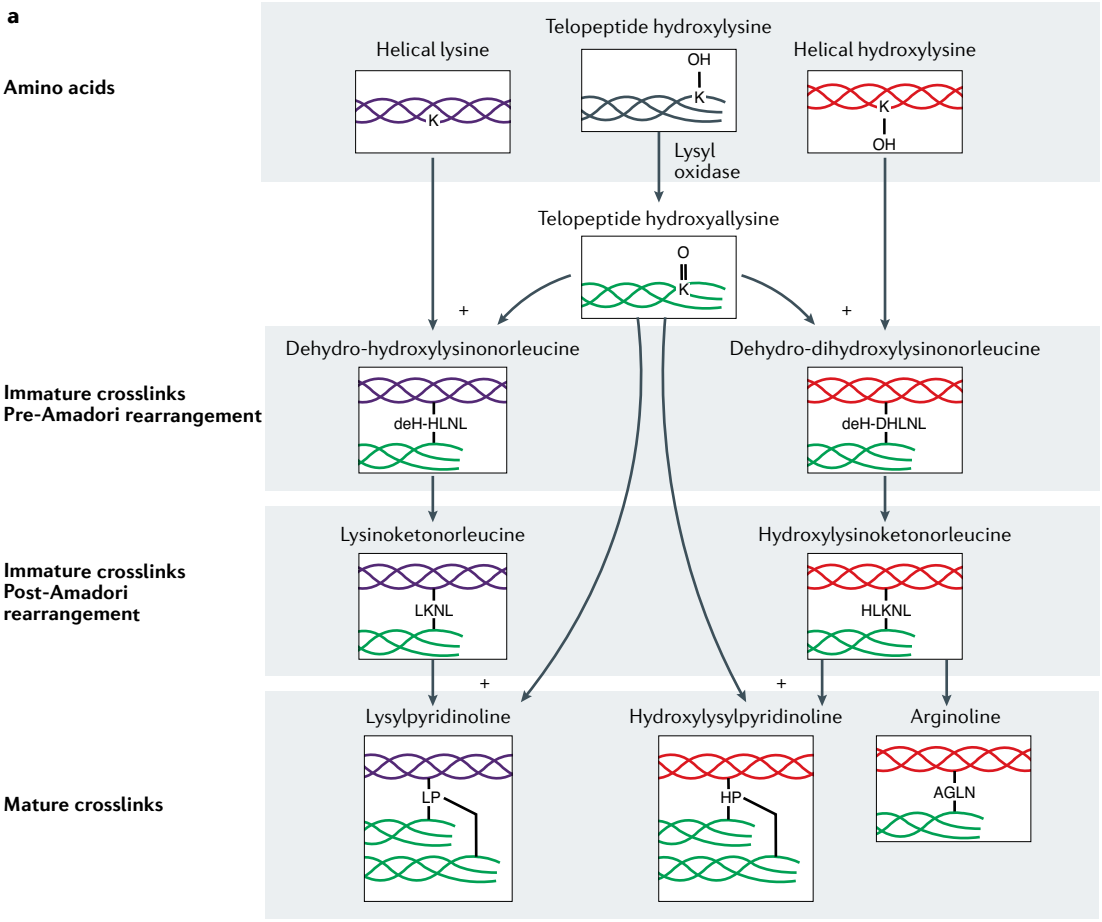
Enzyme-linked immunosorbent assay (ELISA) is a plate-based assay to quantify target molecules based on antigen recognition and, thus, can be used to measure individual collagen subtypes or crosslinks. Commercial ELISA kits exist for several collagen subtypes, and ELISA has been developed for crosslinks such as HP[48], LP[49] and pentosidine[50]. The drawbacks of ELISAs include high cost and requirement for separate runs for different molecules (for example, different collagen types, different collagen crosslinks or collagen from different biological species). Antibodies specific to minor collagen types for many species are not readily available. Identifying or quantifying different collagen types simultaneously in the same sample is not possible, and because different collagen types have a high degree of sequence homology, careful attention is needed when choosing custom epitopes for collagen subtypes.

Protein microarrays are emerging technologies that can characterize proteins in a parallel and high-throughput manner. This technique consists of several parallel, miniaturized assays on a small plate — a concept originally used in the DNA microarray[51]. ECM protein microarrays can be used to profile cell adhesion to different collagen subtypes[52] but have not been used for collagen quantification. Similar immunoassays may also be multiplexed with magnetic-bead-based technologies, which may allow for parallel quantification of different collagen types[53]. Although magnetic-bead-based assays for human collagen types I, II, IV and VI are available, poor concurrence of quantitative values between bead-based assays and ELISAs were demonstrated, and results from different vendors were not consistent[54]. The DNA microarray, in combination with SOMAmers (slow off-rate modified aptamers), can be used to quantify proteins in the SOMAscan assay[55]. This assay can target collagen-subtype fragments in human blood[56] and can be multiplexed, but is not yet fully developed for tissue homogenate.

Collagen imaging and spectroscopy

Visible light, fluorescence and many other microscopy techniques allow for visualization of bulk collagen, collagen types and organization parameters, such as fibre or fibril size, and spacing. For light microscopy, histochemical techniques, such as Van Gieson's stain (which was developed in the late nineteenth century), are still commonly used. Collagens are acidophilic and can be stained

with eosin. Other stains include picrosirius red, methyl blue and water blue. Although these stains give appealing visualizations of fibrillar collagen, they have been noted to give inaccurate results based on non-random sampling or sample inhomogeneity. Furthermore, these stains are not collagen-specific[57]; for example, they have been noted to stain other matrix components, such as tenascin[58] and amyloid[59]. Moreover, fibrillar collagen



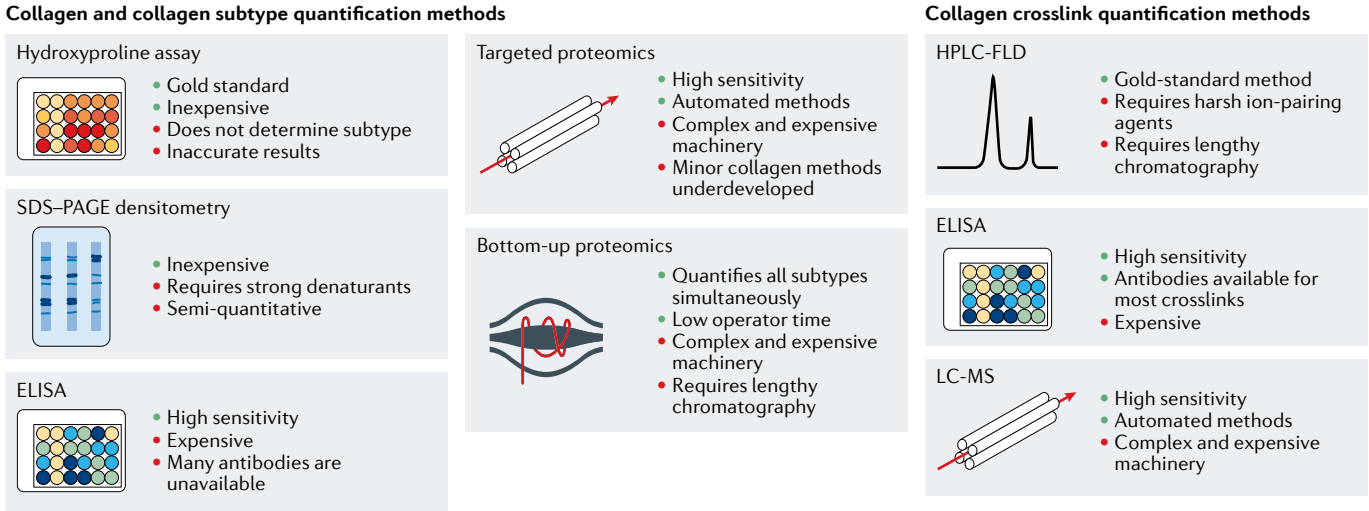


Fig. 3 | **Examples of quantitative assays for collagen and collagen crosslinks.** Several assays for quantification of collagen subtypes and crosslinks are displayed. The advantages and disadvantages of each assay are denoted. ELISA, enzyme-linked immunosorbent assay; HPLC-FLD, high-performance liquid chromatography with fluorescence detection; LC-MS, liquid chromatography-mass spectrometry; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

stains do not differentiate among collagen subtypes[60,61], necessitating the use of additional methods.

Immunohistochemistry (IHC) and immunofluorescence (IF) enable targeted labelling of individual collagen types through antibody binding. As with ELISA, the availability of antibodies for minor collagens or for particular species can pose a challenge, and collagen sequence homology may induce cross-reactivity. Some examples of these techniques are IHC to show the location of collagen type X in avian tissues[62] and IF to show codistribution of collagen types XII and XIV with cartilage oligomeric matrix protein (COMP)[63].

Imaging techniques are particularly useful for examining collagen architecture and organization. Several imaging modalities may be used for quantifying the alignment of fibrillar collagens. These include transmission polarized-light microscopy[64], second-harmonic generation[65] and liquid-crystal-based polarization microscopy[66]. Diffusion tensor imaging, which can measure the differences in water diffusion across collagen fibres, has been used to evaluate the orientation of collagen fibrils in engineered cardiovascular tissues[67]. Electron microscopy techniques, including scanning electron microscopy (SEM), transmission electron microscopy (TEM) and environmental scanning electron microscopy (ESEM), may be used for visualization of collagen fibres and fibrils. For example, TEM has been used to visualize fibril diameter and spacing[68], as well as size and organization[69]; SEM was used to measure the diameter of collagen fibres in normal and degraded articular cartilage[70]; and ESEM has been used to study the morphology and wetting behaviour of sponges made from collagen type I[71].

Some imaging techniques are also useful for discerning among collagen types. For example, immunoelectron microscopy, which uses electron microscopy to label specific proteins, has been used to visualize the location of collagen subtypes, such as types XIII[72], XV[73] and XXVII[74]. Moreover, fluorescence lifetime imaging microscopy (FLIM) can be used to differentiate between collagen types I and III[75]. FLIM has also been used to correlate collagen content to fluorescence lifetime in native cartilage with varying amounts of collagen depletion[76]. This same technique was used in a study of tissue-engineered cartilage to correlate fluorescence lifetime to ultimate tensile strength[77]. A similar FLIM technique was used to quantify crosslinking in collagen hydrogels[78]. Because these FLIM systems can be used in sterile conditions without damaging engineered tissue, they may be used for quality control or release criteria for engineered implants, without the need to manufacture extra implants for destructive analysis.

Spectroscopic techniques, including Fourier-transform infrared (FTIR) spectroscopy, Raman spectroscopy and time-resolved laser-induced fluorescence spectroscopy (TR-LIFS), may be used for analysing collagen or collagen subtypes. In one study, the spectral characteristics of FTIR, such as the amide I band profile and area, were compared among different types of tendon and correlated to pyridinoline crosslinking and fibre organization[79]. In another example of FTIR spectroscopy, highly discriminant absorption bands for individual collagen subtypes were determined[80]. Raman spectroscopy is emerging as a technique to characterize ECM, including the collagen of engineered and native cartilages, both in localized regions and throughout the entire tissue depth[81,82]. Raman spectroscopy has also recently been used to show changes in collagen secondary structure after damage from burning[83] and to discriminate between collagen types I and IV in skin[84]. TR-LIFS has been used to characterize collagen types I, II, III, IV and V in vitro[85] and was used to assess collagen type I in arterial plaques to diagnose atherosclerosis[86].

Quantification with mass spectrometry

Mass spectrometry techniques have been developed for the identification and quantification of proteins, including collagen subtypes. To identify or quantify collagen subtypes with mass spectrometry, collagen

proteins or peptides must be ionized and then analysed in a mass spectrometer. Ionization techniques include matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI). Liquid chromatography (LC) is frequently used in conjunction with ESI to separate analytes out of complex mixtures. After ionization, analytes are separated by their mass-to-charge ratio in time-of-flight (TOF), quadrupole or ion-trap mass spectrometers. Mass spectrometry techniques can be used to quantify overall collagen and collagen crosslinks, individual collagen subtypes with targeted methods or many collagen subtypes at once in bottom-up proteomics methods.

Quantification of overall collagen and collagen crosslinks. LC and multiple reaction monitoring (MRM) — a mass-spectrometry technique that quantifies fragmentation product ions from parent ions — can be used to quantify hydroxyproline in hydrolysed tissues as an estimation of total collagen content[87], similar to the photometric hydroxyproline assay described above, but with higher sensitivity and specificity. MALDI-TOF can also be used to quantify the amount of glycine–proline–hydroxyproline tripeptide as an estimation of the total collagen content[88]. In addition to estimating total collagen, liquid chromatography-mass spectrometry (LC-MS) has been used for measuring collagen crosslinks. In one example, LC-MS MRM was used to quantify HP, LP, DHLNL, HLNL and pentosidine in mouse cervical tissue during pregnancy using a reverse-phase chromatography column and ion-pairing agent[89]. Another technique, using selected ion monitoring rather than MRM, foregoes ion pairing by using a silica hydride column; this method quantified HP and LP in skin and urine samples[90]. LC-MS is more sensitive and accurate than HPLC-FLD for several compounds[91–93], although a thorough comparison between LC-MS and HPLC-FLD for collagen crosslink quantification has not been performed.

Quantification of targeted collagen subtypes. Aside from measuring small molecules, such as hydroxyproline, mass-spectrometry methods can be used to detect intact proteins or specific marker peptides. For example, in an early study, whole fibrils of collagen types I, III and V were identified with MALDI-TOF; however, because enzymatic digestion was not carried out, they formed large polymeric structures, making reproducible quantification difficult[94]. More recently, MRM has been used to quantify peptides resulting from cyanogen bromide and trypsin cleavage of collagen types I, II, III, IV and V in placenta and cartilage samples[95]. MRM has also been used to measure the release of collagen types II and III in human articular cartilage after mechanical injury and cytokine treatment[96] and to identify collagen type I in trypsin digests of leather to determine the animal source[97]. Within mass-spectrometry systems, a number of quantitative approaches exist, including isobaric tags for relative and absolute quantitation (iTRAQ)[98], stable isotope labelling by amino acids in cell culture (SILAC)[99], tandem mass tags (TMTs)[100], oxygen-18 stable isotope labelling[101] and label-free methods based on peak intensity or spectral count[102]. Prior to targeted analysis, operators must determine variables, such as retention time, ionization voltages and the masses of ions, and careful attention must be paid to ensure complete and reproducible enzymatic digestion and chromatography. Although such techniques are promising for quantifying individual collagen subtypes, methods for most minor collagen subtypes are yet to be developed; collagen is part of the insoluble ECM, does not digest easily in trypsin[103] and the low quantity of minor collagens relative to other tissue components render them difficult for targeted analysis.

Bottom-up proteomics approaches. The resolving power of modern ion trap and Orbitrap mass spectrometers allows for quantification of many trypsinized proteins within a single run. After trypsin digestion, bottom-up proteomics analysis, also called shotgun proteomics, may be used for identification and quantification of many different proteins, including collagen subtypes. These proteins may be quantified with the aforementioned tagging methods or with label-free methods, such as intensity-based absolute quantification (iBAQ)[104] or MaxLFQ[105].

Bottom-up approaches are useful for characterizing proteins relevant to disease states, such as cancer, or quantifying ECM components in native tissues. For example, bottom-up proteomics was used to quantify collagen types I, II, III, V, VI, IX, X, XI and XII relative to a reference sample in different types of cartilage[106] and to compare collagen types I, II, III, VI, XI and XII in osteoarthritic and healthy cartilage[107]. Bottom-up approaches also revealed the peptide sequences of collagen types I, III, IV, V and IX in renal cell carcinoma[108] and increased deposition of collagen types X, XII, XIV and XV in colorectal cancer and colorectal liver metastasis compared with healthy tissue[109], which may serve as potential biomarkers. By quantifying native-tissue compositions and determining variations in disease states, new understandings of ECM remodelling through disease progression may be achieved towards developing new treatments. However, although bottom-up proteomics data sets give a great amount of information, they are more costly and slower than most targeted approaches.

Modern assays to quantify collagen subtypes are applicable to all collagen-containing tissues in the body. In the remainder of this Review, cartilage will be used as a model tissue. We describe the role of each collagen subtype in cartilage and outline future directions for collagen-subtype quantification.

Collagens in cartilage

Cartilage is a family of collagenous tissues with major structural and mechanical roles in the body. Depending on the anatomical location, cartilage's functions include bearing load, providing shape, cushioning and lubricating diarthrodial joints. Cartilage contains an ECM rich in glycosaminoglycans (GAGs) and up to 15 types of collagen. Several of these are displayed in FIG. 4, which shows these collagen subtypes' structure, location and roles in cartilage formation. The combination of collagens and other ECM molecules gives cartilage biomechanical properties in compression, shear and tension

that are unlike any other soft tissues. However, because cartilage is largely avascular and aneural, its lack of inherent healing poses substantial medical problems when it is injured or diseased[110]. In this section, we briefly describe the different classifications of cartilage, its degeneration and promising therapies, and the role of each collagen type in cartilage.

Classification of cartilage

Cartilage is categorized into hyaline cartilage, fibrocartilage and elastic cartilage (collectively termed cartilages). This classification is based on the presence of major collagens or elastin in the ECM. For example, the ECM of hyaline cartilage contains mostly collagen type II, whereas the fibrocartilage ECM contains mostly collagen type I. Hyaline cartilage is the most abundant type of cartilage in the body and can be further classified as articular or non-articular, based on whether the cartilage is found on the surface of articulating bones. Articular cartilage distributes forces generated in musculoskeletal movement and provides a low-friction surface to facilitate movement in diarthrodial joints[111,112]. Non-articular hyaline cartilage is found in locations such as the ends of ribs, nose, larynx and in tracheal rings.

Fibrocartilage is distinct from hyaline cartilage, owing to the presence of collagen type I and the lower GAG content. It is also 5–20 times stiffer than hyaline cartilage in tension and at least five times less stiff in compression[113,114]. Fibrocartilage is found in the knee meniscus, the temporomandibular joint (TMJ), intervertebral discs and the pubic symphysis. Some cartilages in articulating joints, such as the mandibular condyle cartilage, are classified as fibrocartilage[115,116]. Fibrocartilage can also be created as repair tissue when hyaline articular cartilage is damaged; however, this repair mechanism is ineffective for long-term replacement[117].

Elastic cartilage is distinct from other cartilages as it contains elastin — a highly crosslinked protein that makes this type of cartilage flexible. Elastic cartilage is primarily found in the ear and epiglottis. Although

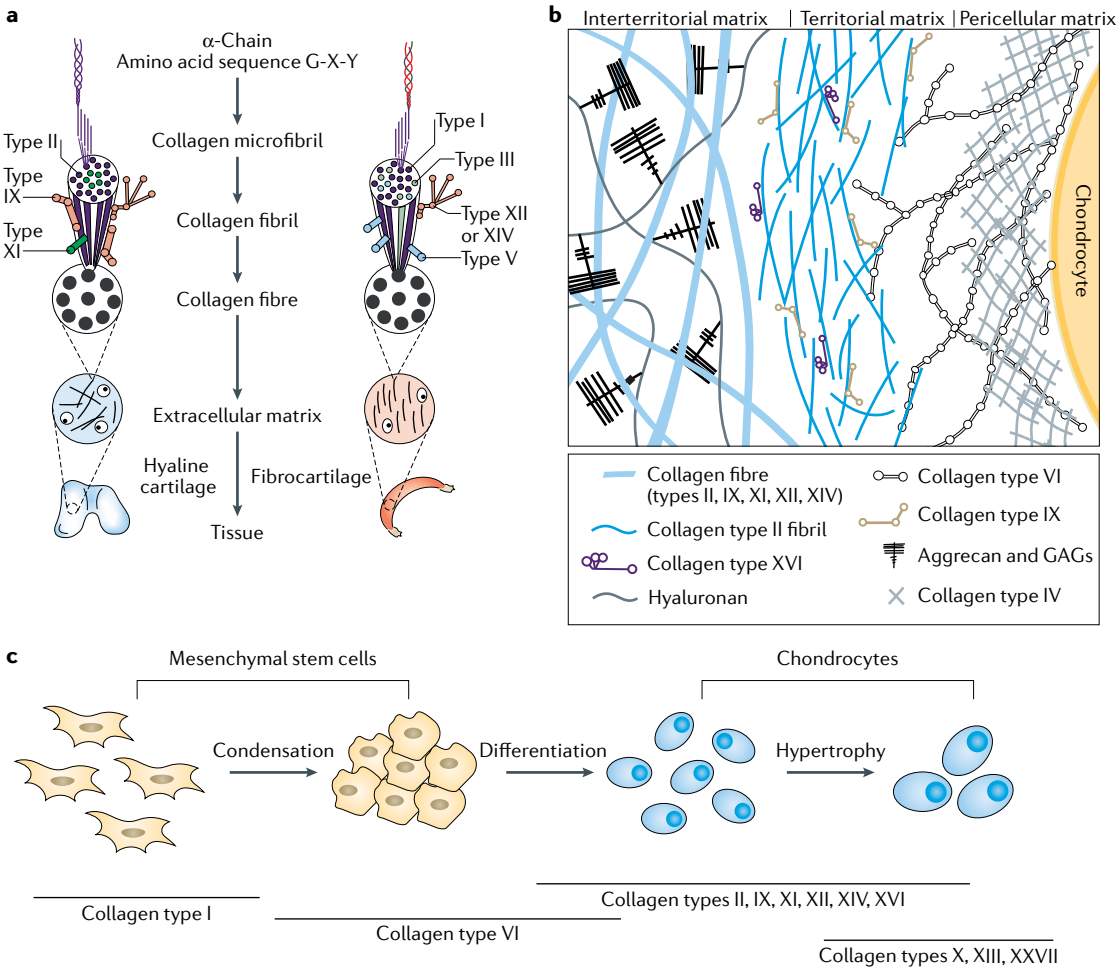


Fig. 4 | **Collagen structure and location in cartilage, and its role in cartilage development. a** | Heterofibrils of different collagen types compose the structure of the cartilage extracellular matrix (ECM). Hyaline and fibrocartilage ECMs are shown as examples. **b** | The locations of different collagen types in hyaline cartilage ECM. Many other ECM molecules are present but not shown. **c** | Different collagens are involved in the process of cartilage formation during embryonic development. Collagen type I is expressed by mesenchymal stem cells. Collagen type VI accumulates during condensation and is then replaced by collagen type II and minor collagens as the cells differentiate and deposit chondrogenic ECM. GAGs, glycosaminoglycans.

septal cartilage of the nose contains elastin, this is categorized as hyaline cartilage[118]. Elastic cartilage is more cellular and has different mechanical properties to hyaline cartilage and fibrocartilage[119]. The main collagen types in elastic cartilage are types I and II[120,121].

Cartilage degeneration and therapies

Arthritides are degenerative diseases of cartilage that affect over 30 million US adults, according to the US Centers for Disease Control and Prevention. Osteoarthritis, which is often associated with ageing or injury, leads to cartilage that has different mechanical properties than its healthy counterpart: for example, the tensile modulus can decrease by as much as 90% and the compressive modulus also decreases[122]. Osteoarthritis transforms the collagen in articular cartilage from mostly type II to a mixture of types I, II and III; osteoarthritis can induce a 100-fold upregulation in collagen type I[123], fivefold downregulation of collagen type II[124] and sixfold increased deposition of collagen type III[125]. The collagen type II in articular cartilage has negligible turnover, and the low regenerative capacity of cartilage may partly come from this inability to repair and replace collagen[126].

For treatment of cartilage pathologies, arthroscopic debridement, the surgical removal of damaged cartilage, used to be commonly performed[127] but was shown to not relieve symptoms[128]. Osteochondral autografts and allograft plugs may be used for small chondral defects, although matching the surface shape from the implant to the defect remains a challenge[129]. The dense collagenous matrix in cartilage can impede tissue–tissue integration, hindering the effectiveness of graft approaches[130]. Surgical interventions for cartilage defects also include microfracture and matrix-assisted autologous chondrocyte implantation, although both may lead to fibrocartilage repair tissue and long-term degeneration[131,132]. Microfracture specifically leads to repair tissue that contains collagen type I, unlike healthy articular cartilage[133]. Minor collagens in repair tissue from microfracture or matrix-assisted autologous chondrocyte implantation have not been identified or quantified.

Fibrocartilage disorders, such as those of the knee meniscus, TMJ disc and intervertebral disc, can originate from a variety of aetiologies. Tears in the knee meniscus are common injuries among athletes and often occur alongside knee-ligament injuries, which can severely limit mobility[134]. TMJ pain afflicts approximately 10% of the adult population[135], with a disproportionately high occurrence in premenopausal women[136]. The intervertebral disc shows age-related degeneration earlier than any other connective tissue in the body[137] and is strongly associated with back pain, which has a lifetime prevalence rate of 49–80% (REFS[138,139]). Surgical removal procedures, such as meniscectomy and discectomy, offer short-term relief but lead to worsening of symptoms and eventual fibrocartilage degeneration[140–143].

There are currently no therapies to repair or regenerate either hyaline cartilage or fibrocartilage that are effective in the long term[144]. Because cartilages lack intrinsic healing, tissue-engineering techniques are potential therapies[131]. Tissue-engineered cartilage, or neocartilage, has the potential to fill defects or regenerate damaged tissues to restore function[145,146]. To do this, neocartilage implants must meet the mechanical demands imposed upon the native tissue[147]. A systematic approach to designing neocartilage starts from delineating quantitative design criteria, such as the specific biomechanical and biochemical properties of the native tissue to serve as gold standards[148]. Biomechanical properties include compressive and tensile moduli, and biochemical properties include the quantity of collagen and collagen crosslinks. Although a major effort in the field of cartilage-tissue engineering is to mimic the structure and mechanics of native cartilage, most current attempts to create mimetic neocartilage fall short. Quantification of minor collagens would form the foundation of a systematic approach towards engineering biomimetic cartilages.

Cartilage-tissue engineers frequently use scaffolds, such as collagen or synthetic materials, for cell seeding[149–151]. Collagen scaffolds are naturally occurring biomaterials that support cell adhesion, are degradable and can have a low or pro-healing immune response[152,153]. Collagen can also be blended with natural (for example, silk, hyaluronic acid) or synthetic polymers (for example, poly(lactic acid), poly(L-lactide-co-glycolic acid)) to improve the mechanical properties and support a chondrogenic phenotype in both primary and stem cells[154]. 3D woven composite scaffolds can recapitulate certain biomechanical properties of native cartilage, such as anisotropy, viscoelasticity and tension–compression non-linearity[155]. Although these attributes may help neocartilage formation, collagen and synthetic scaffolds do not recapitulate the variety and distribution of different collagen subtypes and crosslinks of native tissues. It remains unknown whether scaffolds are sufficiently biomimetic to effectively regenerate damaged and diseased tissues.

Collagen subtypes in cartilage

For this Review, we define types I and II as major collagens in cartilages because they account for a majority of the collagen mass (for example, mature articular cartilage collagen is about 90% type II[156] and knee meniscus collagen is about 90% type I[157]). The remaining collagen types (III, IV, V, VI, IX, X, XI, XII, XIII, XIV, XVI, XXII and XXVII) make up the minor collagens in cartilage. It may appear daunting at first for cartilage researchers to consider these 15 collagen types simultaneously. Thus, we recommend organizing collagens not by the classical categorization but by their functions in cartilage, as described in FIG. 5a. For example, in tissue engineering, this system may make it easier to design criteria without an extensive background in collagen biochemistry.

Although the main collagens in cartilage are frequently identified and quantified with histological stains or ELISA methods, minor collagens are rarely identified in engineered cartilage, likely because of their low quantity and lesser known roles; however, these minor collagens are necessary for the function of cartilage[158]. For example, knockouts of many minor collagens result in lethal phenotypes (FIG. 5b). In this section, we describe the function of each major and minor subtype (for example, for supporting collagen fibrils, maintaining chondrocytes and forming interfaces between cartilages and other tissues),

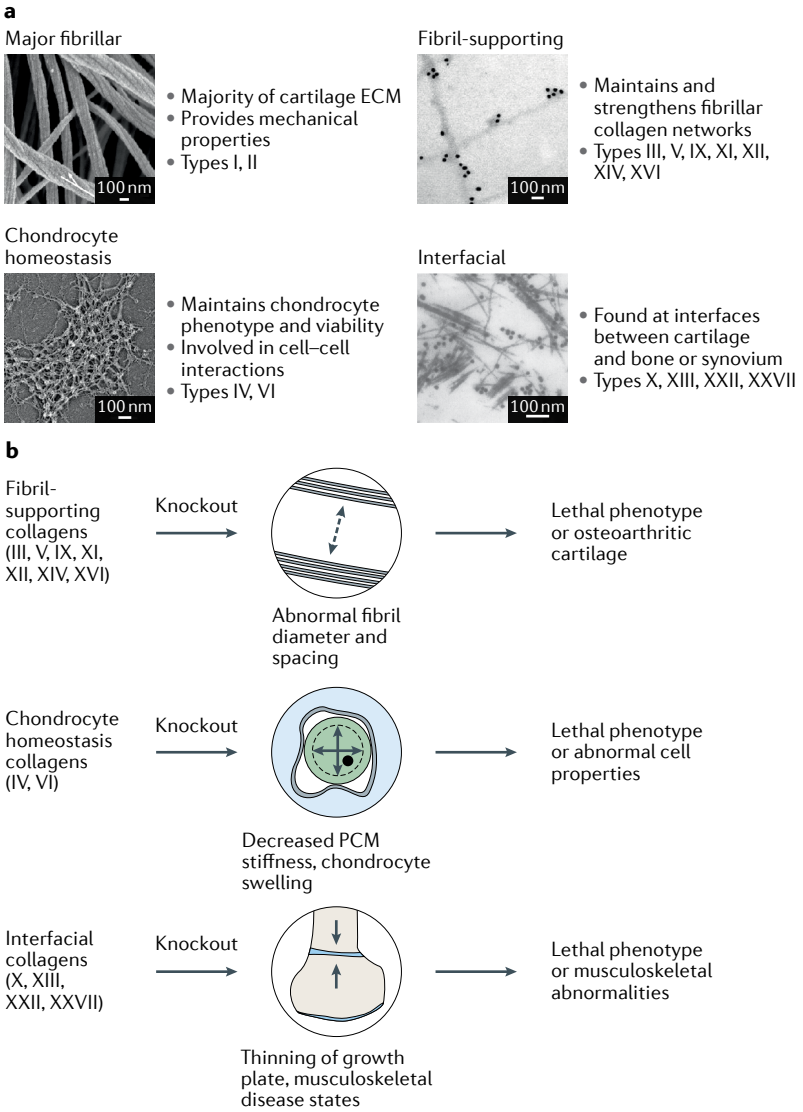


Fig. 5 | **Functional groups of collagen subtypes in cartilage. a** | Scanning electron microscopy image of collagen fibres in knee articular cartilage (the white arrow indicates twisting of fibrils in the axial direction) (top left); immunogold electron microscopy (EM) image of labelled collagen IX (top right); rotary shadowing EM showing a collagen type VI network (bottom left); and immunogold EM image of labelled collagen type X (bottom right). **b** | Knocking out different types of minor collagens can cause lethal or abnormal phenotypes in cartilage and other musculoskeletal tissues. ECM, extracellular matrix; PCM, pericellular matrix. Panel **a**, top left image reprinted from REF.[167], CC BY 4.0; top right image reprinted from *Mol. Cell. Biol.* 2005, **25**, 10465–10478, REF.[248], with permission from American Society for Microbiology; bottom left image reprinted with permission of American Society for Biochemistry and Molecular Biology from REF.[249], Recessive COL6A2 C-globular missense mutations in Ullrich congenital muscular dystrophy: role of the C2a splice variant, Zhang, R.-Z. et al. *J. Biol. Chem.* **285**, 10005–10015 (2010); bottom right image reprinted with permission from REF.[250], Elsevier.

while noting its importance for cartilage-tissue mechanics. A summary of all collagen subtypes in cartilages, including the alpha-chain isoforms, location, description and structure, is provided in TABLE 2.

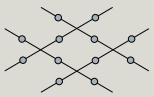
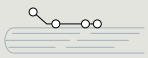
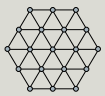
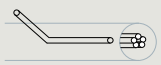
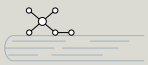
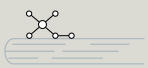
Major fibrillar collagens (I, II). Collagen type I comprises a large portion of the ECM of many connective tissues, lending stiffness to skin, tendon, ligament, bone and fibrocartilage. For example, collagen type I accounts for about 85% of dry weight of the TMJ disc[159]. Its spatial distribution can vary among different cartilages. For example, the collagen type I content in the outer annulus of the intervertebral disc is over 80% by dry weight, but drops to about 70% in the inner annulus[160]. Similarly, in the knee meniscus, the outer red–red zone of the tissue is approximately 80% collagen type I by dry weight; however, in the inner white–white zone, this proportion drops by over half[114]. Collagen type I is also found in elastic cartilage, giving structure and mechanical properties to the tissue, alongside collagen type II and elastin[121]. Collagen type I in hyaline articular cartilage can indicate damage or pathology, and it can be found when ineffective fibrocartilage repair tissue fills a defect, eventually leading to osteoarthritis[110]. Collagen type I is usually found in the heterotrimer $\alpha1_2\alpha2$ (a triple helix of two α1 chains and one α2 chain), but is also found in the $\alpha1_3$ homotrimer (three α1 chains) in fetal tissues, fibrosis and cancer; this isomer is not found in healthy non-fetal tissues[161].

Collagen type II represents 90–95% of the collagen in hyaline cartilage[112]. It is found in the isoform $\alpha1_3$ and, in hyaline cartilage, is interwoven with proteoglycan aggregates consisting of hyaluronic acid, aggrecan core protein and GAGs. The collagen network formed by collagen type II is a crosslinked polymer that also contains collagen types IX, XI, XII and XIV. This collagen network and its crosslinks are highly correlated with tensile stiffness and strength[3], but also have roles in articular cartilage compression[162]. Collagen type II is also present in fibrocartilage and elastic cartilage ECM, making up the majority of the inner white–white zone of the knee meniscus, as well as a portion of the TMJ disc, intervertebral disc and auricular cartilage[114,121,160,163].

The major fibrillar collagens form a hierarchical structure. Collagen molecules, which are about 300 nm long and about 1.5 nm in diameter, are axially staggered by a multiple of approximately 67 nm, which creates a characteristic 'D-period' or 'D-band' (REF.[164]). D-periodic molecular segments pack together in a quasi-hexagonal lattice to form collagen microfibrils[165]. In hyaline cartilage, collagen II microfibrils surround collagen type XI microfibrils to form thin fibrils, with a diameter of about 20 nm (REF.[166]). These fibrils grow laterally, organizing like ropes threaded with thin fibrils, to form fibril bundles or fibres with diameters ranging from 40 to 200 nm (REF.[167]). In fibrocartilage, collagen type I can form fibrils by itself, but it can also form heterofibrils with collagens III and V[168,169]. The fibril size in fibrocartilage can vary greatly by location, from 35-nm fibrils at the meniscus surface to 120-nm fibrils below[170]. Collagen fibrils with diameters in the range 100–150 nm have been observed in the intervertebral disc[171], and thinner fibrils (20–40 nm in diameter) were found in human fetal intervertebral discs[172].

Why collagen type II, not type I, makes up most of the hyaline cartilage ECM, and is almost absent from other parts of the body, remains an important question. There is ample evidence that fibrocartilage, which is abundant in collagen type I, is ineffective at replacing hyaline cartilage as a repair tissue[173,174]. The repaired fibrocartilage is mechanically weaker and more permeable than articular

Table 2 | **The collagen subtypes of cartilage**

Collagen subtype	Cartilage location	Description	Structure	Refs
I	ECM (E, F)	Main fibrillar type of fibrocartilage and elastic cartilages		111,114,121
II	ECM (E, F, H)	Main fibrillar type of hyaline, fibrocartilage and elastic cartilages		3,111,112
III	ECM (E, F, H)	Fibrillar type co-assembling with collagen type I Involved in repair response with collagen type II		124,178,179
IV	PCM (H)	Tetrameric network in PCM of articular cartilage Potential involvement in chondrocyte homeostasis		200–203
V	ECM (E, F)	Fibrillar type co-assembling with collagen type I		180–182
VI	PCM (E, F, H)	Regulation of PCM properties and mechanotransduction		204–206
IX	ECM (E, F, H)	Forms crosslinks with collagen type II; forms collagen type II-IX-XI heterofibrils		183,184
X	Deep zone (H)	Produced by hypertrophic chondrocytes; marker of endochondral ossification		62,207
XI	ECM (E, F, H)	Involved with fibrillogenesis of collagen type II; forms collagen type II-IX-XI heterofibrils		156,188
XII	ECM (E, F, H)	Codistributed with collagen types I and II; may contribute to fibril alignment and stabilization		191–193
XIII	Deep zone (H)	Involved with endochondral ossification		209,210
XIV	ECM (E, F, H)	Involved in fibrillogenesis of collagen types I and II		195,196
XVI	TM (H)	Associates with collagen type II fibrils; may stabilize larger collagen fibrils		197–199
XXII	Synovial junction (H)	Associates with collagen type VI; role in cartilage understudied	Not yet determined	211,212
XXVII	Deep zone (H)	Associated with endochondral ossification; structural role in PCM of growth-plate cartilage	Not yet determined	214–216

Refer to the subsections under 'Collagen subtypes in cartilage' for information about the isoforms. E, elastic; ECM, extracellular matrix; F, fibrocartilage; H, hyaline; PCM, pericellular matrix; TM, territorial matrix.

cartilage, and degrades over two years[110,175]. When articular cartilage is compressed, osmotic swelling pressure from GAGs provides compressive resistance, and this swelling is restrained by the collagen type II network[176]. Collagen type II has a larger molecular spacing than collagen type I due to glycosylated hydroxylysine residues, allowing it to contain 50–100% more water than collagen type I[177]. The ability of collagen type II to hold more water may be important for restraining osmotic swelling and dissipating compressive forces, and, thus, explains why this collagen type is uniquely present in hyaline cartilage[177].

Fibril-supporting collagens (III, V, IX, XI, XII, XIV, XVI). Collagen type III is found in the ECM of various musculoskeletal tissues, organs and skin, and has the isoform $\alpha 1_3$. It is essential for normal collagen type I fibrillogenesis, organizing and regulating the diameter of type I fibrils[178]. Collagen type III is found alongside collagen type I in fibrocartilage, although it accounts for less than 10% of the overall collagen content[157]. In articular cartilage, collagen type III is also crosslinked to collagen type II, which superimposes it onto collagen type II-IX-XI copolymers[179]. Moreover, collagen type III can add additional cohesion or strength when a collagen type II

network is damaged. For example, one study found a sixfold upregulation of collagen type III in osteoarthritic hip cartilage compared with control[125], although this may be due to copolymerization with collagen type I, which is also present in osteoarthritic cartilage[124]. Although global knockout of collagen type III results in perinatal lethality from rupture of major blood vessels[178], cartilage mechanics have not been studied in knockouts.

Collagen type V is found in the ECM of bone, cartilage and corneal stroma. The major isoform of collagen type V is $\alpha1_2\alpha2$, which co-assembles with collagen type I[180], where type I fibrils are copolymerized on a template of collagen type V[181]. Global collagen type V mouse knockouts are embryonic lethal, and a conditional knockout in the cornea showed that collagen type V is necessary for fibrillogenesis; the knockout mice produced normal amounts of collagen type I, but with increased fibril and abnormal structure[182]. This implies that collagen type V increases the mechanical strength in tissues containing collagen type I (for example, fibrocartilage, tendon and skin). However, potentially because of the neonatal lethality of global collagen type V knockouts, the relationship between collagen type V and tissue mechanics has not been determined.

Collagen type IX, which has the isoform $\alpha1\alpha2\alpha3$, is found in articular cartilage, where it forms LOX-mediated crosslinks with collagen type II fibrils to stabilize and strengthen the ECM. Collagen type IX, alongside collagen type XI, is colocalized with collagen type II fibrils[183]. All three alpha chains of collagen type IX contain intermolecular crosslinking sites, where they form covalent bonds with other collagen type IX molecules or with collagen type II[184]. The quantity of collagen type IX decreases with cartilage age, starting at over 10% of the overall collagen content in fetal articular cartilage and dropping to about 1% in adult articular cartilage[156]. This collagen type is also necessary for the maturation of cartilage during fracture repair, as shown in a global knockout study[185]. Knockouts lead to degenerative joint disease resembling osteoarthritis[186]; however, the mechanics of collagen type IX-deficient cartilage have not been tested.

Collagen type XI has the isoform $\alpha1\alpha2\alpha3$ and is broadly distributed in many tissues. In articular cartilage, it crosslinks with itself in fibrils that also contain collagen types II and IX, and is involved with fibrillogenesis by maintaining the spacing and diameter of collagen type II fibrils[156]. The α3 chain of collagen type XI has the same primary structure as the α1 chain of collagen type II[187]. In articular cartilage, collagen type XI was shown to trimerize with collagen type V in the isoforms $\alpha1(XI)\alpha1(V)\alpha3(XI)$ and $\alpha1(XI)\alpha2(V)_2$ (REF.[188]). This means it would be more accurate to describe collagen types V and XI as a single type, collagen V/XI[189]. A collagen type XI α1 global knockout mouse model was neonatal lethal, with thick banded collagen fibrils in cartilage ECM[190]. Like collagen type IX, collagen type XI decreases with age, accounting for over 10% of overall collagen in fetal articular cartilage and about 3% in adult articular cartilage[156].

Collagen type XII is codistributed with collagen type I in bone, ligament, tendon, fibrocartilage, muscle and skin, and codistributed with collagen type II in articular cartilage[191]. It has the isoform $\alpha1_3$ and is associated with fibril formation, cell adhesion, fibrosis and osteogenesis. One study on passaged chondrocytes showed that collagen type XII is present in cartilage-forming chondrocytes, but not in the other dedifferentiated chondrocytes, indicating that this collagen subtype is necessary to support the formation of hyaline cartilage[192]. It may contribute to fibril organization, alignment and stabilization, helping the matrix bear load[193]. Collagen type XII expression is localized to the growth plate and the surface of articular cartilage, and has been found in juvenile but not embryonic rib hyaline cartilage[193]. Children with collagen type XII mutations show muscle weakness and joint hyperlaxity[194], but cartilage effects have not been reported.

Collagen type XIV has the isoform $\alpha1_3$. A global knockout mouse model of collagen type XIV resulted in deteriorated mechanical properties of skin and tendon, and showed that this collagen type has a role in fibrillogenesis and regulating fibril diameter[195]. In this study, collagen fibrils were thicker and mature fibrils did not form; however, the resulting effects on cartilage mechanics were not tested. Collagen type XIV has also been identified as a binding partner of COMP[63], and mutations of ECM proteins matrilin-3 and COMP decreased the relative amount of collagen type XIV in cartilage, indicating that these proteins stabilize collagen type XIV[196].

Collagen type XVI has the isoform $\alpha1_3$ and is found in the territorial matrix of hyaline cartilage in a population of thin collagen type II fibrils, which also contains collagen type IX (but without colocalization of types IX and XVI)[197]. The role of collagen type XVI is not well understood, but it is hypothesized to stabilize ECM by connecting and organizing large fibrillar networks[198]. It binds to integrins, fibronectin, and fibrillin-1 and fibrillin-2, participating in intermolecular interactions, organization and maintenance[199]. Knockout studies of collagen type XVI have not been performed.

Chondrocyte-homeostasis collagens (IV, VI). Collagen type IV is the main component of the basement membrane in articular cartilage and has the isoform $\alpha1_2\alpha2$. It forms a tetrameric network in the pericellular matrix (PCM) of articular cartilage, where it is involved in maintaining the viability and phenotype of articular chondrocytes[200]. Collagen type IV expression is decreased in damaged cartilage and clinically failed repair tissue[201]. It contains anti-angiogenic domains, suggesting that it may have an important role in cartilage homeostasis by arresting tumour growth in vivo[202]. Owing to its role in the basement membrane, global knockout mouse models are embryonic lethal[203]. Although collagen type IV seems to have an important role in cartilage PCM, the specific mechanisms must be further investigated to understand how this collagen type maintains healthy chondrocyte properties.

Collagen type VI makes up about 1% of total collagen in adult articular cartilage, where it is mainly found in the PCM[156]. It has been hypothesized to have important roles in mediating cell–cell and intermolecular

interactions[204]. It was originally thought to have a single isoform of α1α2α3, but the α3 chain can be switched for an α4, α5 or α6 chain[205]. In a global knockout model, a lack of collagen type VI resulted in swelling cells and decreased stiffness of the PCM, indicating that it regulates PCM and cellular properties[206].

Interfacial collagens (X, XIII, XXII, XXVII). Collagen type X, which has the isoform $\alpha1_3$, creates a hexagonal network that is limited locally to hypertrophic cartilage and close to the calcified zone of the cartilage. Because collagen type X is only found at this boundary between cartilage and bone, we classify it as an interfacial collagen. Although it is not produced by most chondrocytes, about 45% of hypertrophic chondrocyte collagen production is collagen type X[62]. It is highly correlated spatially and temporally with endochondral ossification, making it a biomarker of cartilage hypertrophy[207]. A collagen type X global knockout study showed that, without collagen type X, mice exhibited 14% lethality, whereas the rest had dwarfism and growth-plate compression[208].

Collagen type XIII, which has the isoform $\alpha1_3$, is the only collagen in cartilage that spans the cell membrane. It is observed in the perichondrium, hypertrophic and proliferative cartilage, and in many other tissues[209]. A transgenic mouse overexpressing collagen type XIII showed abnormally high bone mass; collagen type XIII was strongly expressed before mineralization started, suggesting that this collagen type has important roles in endochondral ossification[210].

Collagen type XXII, which has the isoform $\alpha1_3$, is expressed at junctions between many different types of tissues, including the muscle-attachment sites of ribs and the junction of articular cartilage and synovial fluid, where it associates with microfibrils, such as fibrillins, or collagen type VI[211]. It has been shown to bind to collagen-binding integrins in the myotendinous junction, especially α2β1 and α11β1 (REF.[212]); however, its function in articular cartilage remains largely understudied and unknown. Although knockouts in mammals have not been performed, global knockdown of collagen type XXII in zebrafish resulted in muscular dystrophy[213].

Collagen type XXVII, like collagen type X, is found near the osteochondral junction and is associated with endochondral ossification[214]. It has the isoform $\alpha1_3$ and has a structural role in the PCM of growth-plate cartilage[215]. Collagen type XXVII is much less abundant than the other types of fibril-forming collagens and its function is not well understood[216]. Mutations in the collagen type XXVII gene lead to Steel syndrome — a disease involving skeletal abnormalities, such as dislocations, short stature and scoliosis[217].

Collagen and mechanical properties

The structure of collagen subtypes and crosslinks affects the function of the cartilage tissue. For the purpose of this Review, the 'structure' of matrix components refers to measurable physical properties, such as the quantity of collagen, quantity of crosslinks, and the spatial distribution and homogeneity of the collagen network. The function of cartilage refers to the measurable material characteristics of the overall tissue, such as the tensile stiffness, viscoelastic measures (such as the aggregate modulus) and the lubricity of the cartilage surface.

Some structure–function relationships in cartilages have been well characterized, particularly those between the quantity of GAGs and compressive stiffness[218], and between the quantity of collagen crosslinks and tensile stiffness[3,219]. Relationships that correlate collagen content to compressive properties and Poisson's ratio in articular cartilage have also been described[14]. Structure–function relationships of specific collagen subtypes are yet to be explicitly defined, but some may be inferred from previous work; because most of the collagen of fibrocartilage is collagen type I, and most of the collagen of hyaline cartilage is collagen type II, it can be assumed that non-subtype-specific structure–function relationships are mostly due to these two collagen types. Collagen type VI knockout models showed a structure–function relationship between the amount of collagen type VI and PCM stiffness[206]. Because full knockouts of collagen types are often neonatal lethal, and collagens form heterofibrils of more than one collagen type, it may be difficult to determine the influence of individual collagen subtypes on the mechanical properties. Nonetheless, the vital roles of minor collagens (for example, in fibril assembly) suggest that their relative contribution to cartilage biomechanics is far greater than their relative proportion of mass in cartilage ECM.

Because the goal of cartilage-tissue engineering is functional restoration of diseased or damaged cartilage, the engineered tissue must have similar functional properties to native cartilage. With defined structure–function relationships, certain structural measurements can serve as quality-control measures or design criteria for engineering cartilage. For example, specific GAG or measurements of collagen subtype can be used to assess tissue quality. If these structural analyses are non-destructive, they could be used as preliminary benchmarks prior to implantation. For example, native-like amounts of collagen types IV and VI can be indicative of healthy chondrocytes and PCM suitable for implantation. The presence of collagen type II at 50–60% by dry weight and with crosslinks similar to those of native tissue would indicate a tissue with native-like tensile properties. Understanding the structure–function relationships of different collagen subtypes would give insight to further develop the cartilage-tissue-engineering process, and may hold the key to further strengthening neocartilages. For example, quantifying interfacial collagens may indicate how well an osteochondral implant can maintain healthy subchondral bone, and quantification of fibril-supporting collagens can inform tissue engineers whether the ECM is mature and robust.

Outlook

Although bottom-up proteomics allows the simultaneous analysis of many collagen types and targeted MRM techniques offer fast analysis of individual collagen types, no methods exist for high-throughput quantification of

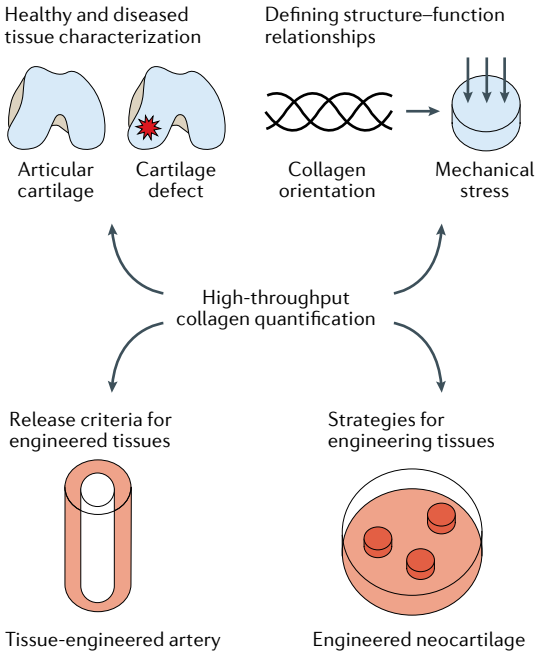


Fig. 6 | **Broad applications for high-throughput, low-cost collagen quantification.** The development of next-generation assays for collagen-subtype quantification will impact many different fields of biomedical engineering. Examples are shown for healthy and diseased tissues, structure–function relationships, tissue-engineering release criteria and tissue-engineering strategies.

both major and minor collagen subtypes. The development of such a method would be an important milestone for understanding the distributions and roles in all tissue types of minor collagens. Ideally, this would be low cost and allow for processing of many samples quickly, while keeping operator time to a minimum. Because collagen has roles throughout the body, characterization studies on a multitude of tissues will not only deepen our understanding of ECM composition, tissue disease states and structure–function relationships but also lead to design criteria for engineered tissues (FIG. 6).

Characterization in disease states

Although shotgun proteomics has increased our understanding of the collagen profile of cartilages and other musculoskeletal tissues[220–222], sufficiently powered studies to characterize the collagen subtypes of these native tissues must be performed. If highly sensitive targeted or bottom-up LC-MS methods for all minor collagens can be performed in a short (for example, 5-min or 10-min) run, this would allow the analysis of collagens in tissues from different ages, sexes and species to provide a more comprehensive understanding of the biochemical makeup of healthy native tissues. Once these values are defined, screening efforts can show how cellular phenotype and tissue proteome change through disease or injury. For example, MRM was recently used to show that collagen types II, III and VI were deposited in significant amounts in osteoarthritic articular cartilage compared with the control articular cartilage[223], and label-free, bottom-up proteomics was used in an in vitro lung-fibrosis model to quantify the relative amounts of collagen types I–VIII, XII, XV, XVI and XVIII[224]. In recent years, machine learning has become increasingly important for analysing large data sets in biomedicine[225–227]. Proteomic data sets with thousands of quantified peptides are excellent candidates for machine-learning algorithms, which may lead to new discoveries of biomarkers for prediction or early detection of many different diseases. In the coming years, quantitative assessments of collagen subtypes in healthy and diseased tissues will be of the utmost importance in understanding the ECM of native tissue and how it remodels through degeneration and disease.

Biomechanical relationships

Several structure–function relationships between biochemical and biomechanical properties are yet to be defined for cartilages, particularly those of minor collagens. Knockout or mutation mouse studies have been completed for collagen types IX[228], XI[229] and XIV[195], but only one study has tested the resultant effects on cartilage mechanics; this knockout study of collagen type VI found a significantly reduced Young's modulus of the PCM but not of the bulk cartilage[230]. The quantity, spatial distribution, homogeneity and assembly of other minor collagens have yet to be tied to the functional properties of cartilage.

Defined structure–function relationships are largely limited to compressive and tensile moduli, which do not constitute the full picture. Cartilages are viscoelastic and lubricious, making it necessary to understand correlations with functional properties such as instantaneous and relaxation moduli, permeability and tribological properties. Once high-throughput quantification methods for minor collagens are developed, it may be possible to draw correlations between specific collagen subtypes and these and other additional functional properties. For example, well-defined correlations between tensile stiffness and major fibrillar collagens exist but are not yet complemented by studies on fibril-supporting collagens. Collagen type VI has been shown to correlate to PCM stiffness, but collagen type IV has not. It is not known whether the presence of interfacial collagens could be correlated with integration strength or if collagen type XXII at the interface with synovial fluid could have a role in cartilage lubricity. These relationships, if determined experimentally, would further help to elucidate the roles of minor collagens in different tissues.

Screening for engineered therapeutics

Minor collagens and collagen-subtype quantification are absent from tissue engineering, perhaps owing to the lack of robust and high-throughput methods for collagen-subtype quantification. Although the hydroxyproline assay is frequently used to estimate overall collagen, individual collagen subtypes are rarely quantified and relatively little attention has been paid to minor collagens in engineered tissues. For example, collagen type XIV is only mentioned in two tissue-engineering studies: one identified mRNA[231] and the other identified the protein with LC-MS[232]. Collagen-subtype identification is present in some tissue-engineering studies.

For example, IF has been used to identify collagen types I and III in cultured fibroblasts for engineering ligaments[233]; IHC has been used to visualize collagen types I and II in mesenchymal-stem-cell-seeded scaffolds for engineering of intervertebral discs[234]; collagen type III mRNA has been identified in engineered arterial grafts[235]; and TR-LIFS has been used to assess the relative expression of collagen types I, III, IV and V in osteogenic ECM from adipose-derived stem cells[236]. However, the lack of subtype specificity for collagen histological stains and the non-quantitative nature of collagen IHC and IF techniques inhibit a deep understanding of the role of minor collagens in engineered tissues. Ideally, engineered tissues are fully biomimetic, exhibiting the same quantities of all collagen subtypes in native tissues. With the advent of new high-throughput collagen-quantification technologies, all tissue engineers will be able to measure the biomimicry of their tissues on a collagen-subtype level; the quality of engineered cartilages, bones, heart valves, ligaments, tendons, blood vessels and skin will particularly depend on their major and minor collagen content.

Collagens for engineering neocartilages

Tissue engineers have put much effort into harnessing major fibrillar collagens to improve the mechanical properties of neocartilage, for example, by using tensile stimulation to increase the alignment of collagen fibrils[237]. Spectroscopic techniques are promising for quantitative and non-destructive measurement of collagen in cartilage-tissue engineering. For example, TR-LIFS was used to measure collagen type II[238] and diffuse fibre-optic Raman spectroscopy with hydroxyproline assay was used to assess collagen deposition[239] in engineered cartilage.

Compared with the tissue-engineering research on total or major collagens, relatively little has focused specifically on minor collagens. Some tissue-engineering studies using primary chondrocytes or stem cells quantified collagen type IX with ELISA[240] and identified collagen type XI[241] and types VI, IX, XI, XII and XIV with mass spectrometry[232]. However, no studies have determined if specific collagen types in engineered cartilage ECM are similar to those in native tissues. Similarly, there has been no attempt to promote deposition of minor collagens for engineered cartilages. We suggest that, because different collagen types are expressed by mesenchymal stem cells and differentiated chondrocytes during fetal development, tissue engineers could use these collagen types as markers of neocartilage development. For example, the self-assembling process in cartilage-tissue engineering is reminiscent of mesenchymal condensation[242] and follows the pattern of collagen type VI being remodelled and replaced by collagen type II[243].

Collagen crosslinks have been the subject of some cartilage-tissue-engineering studies and could be used to better characterize engineered tissues. For example, the deposition of HP crosslinks in engineered cartilage has been enhanced via hypoxia[244], endogenous LOX[245] and mechanical stimulation with centrifugation[246]. HP is the only crosslink identified in engineered cartilages. A study on the presence of HLKNL, arginoline or AGEs would better describe the quantity of different crosslinks in engineered cartilages.

The need for different collagen subtypes in engineered cartilages is indicated by their functional roles. The fibril-supporting collagens maintain and regulate fibrils of collagen types I and II, and, thus, should be used to assemble fibrils with correct spacing and diameter. Although chondrocyte-homeostasis collagens do not directly affect the strength of cartilage ECM, they interact with chondrocytes and maintain healthy tissue. Interfacial collagens are particularly important for osteochondral implants. Endochondral ossification continues until the ages of about 18 years for females and about 21 years for males[247]; thus, these collagens are needed to produce implants that ossify and grow with the patient. A lack of minor collagens accounts for neonatal lethality or inferior cartilage tissue. Therefore, native-like amounts of all minor collagens are necessary in engineered cartilages. A better understanding and new methods for identifying novel biochemical and biophysical stimuli to enhance the deposition of collagen crosslinks and specific collagen subtypes are the next key steps towards strong, durable and biomimetic cartilages.

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