

REVIEW

Research perspectives—Pipelines to human tendon transcriptomics

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Abstract

Tendon transcriptomics is a rapidly growing field in musculoskeletal biology. The ultimate aim of many current tendon transcriptomic studies is characterization of in vitro, ex vivo, or in vivo, healthy, and diseased tendon microenvironments to identify the underlying pathways driving human tendon pathology. The transcriptome interfaces between genomic, proteomic, and metabolomic signatures of the tendon cellular niche and the response of this niche to stimuli. Some of the greatest bottlenecks in tendon transcriptomics relate to the availability and quality of human tendon tissue, hence animal tissues are frequently used even though human tissue is most translationally relevant. Here, we review the variability associated with human donor and procurement factors, such as whether the tendon is cadaveric or a clinical remnant, and how these variables affect the quality and relevance of the transcriptomes obtained. Moreover, age, sex, and health demographic variables impact the human tendon transcriptome. Tendons present tissue-specific challenges for cell, nuclei, and RNA extraction that include a dense extracellular matrix, low cellularity, and therefore low RNA yield of variable quality. Consideration of these factors is particularly important for single-cell and single-nuclei resolution transcriptomics due to the necessity for unbiased and representative cell or nuclei populations. Different cell, nuclei, and RNA extraction methods, library preparation, and quality control methods are used by the tendon research community and attention should be paid to these when designing and reporting studies. We discuss the different components and challenges of human tendon transcriptomics, and propose pipelines, quality control, and reporting guidelines for future work in the field.

KEYWORDS

publication guidelines, quality control, sequencing, tendon, tissue source

Lorenzo Ramos-Mucci and Paula Sarmiento contributed as co-first authors.

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1 | INTRODUCTION

Tendon pathologies reduce the quality of life through pain, inflammation, and impaired movement across a clinical spectrum that disproportionately affects both athletes and aging populations. Fundamentally, these pathologies are due to a disruption in tendon metabolism, and range from acute to chronic degeneration, in some cases leading to tears and rupture. Tendon healing, if present, is a slow process that occurs through fibrosis but does not restore full function or normal properties.

The changes that characterize tendinopathies can be used to understand disease progression, establish diagnostics, and develop therapies. Transcriptional differences between states of tendon pathology have uncovered potential targets for the understanding of disease. Yet, the wide variability in reporting of transcriptomic data results in information that cannot be correlated between studies or further analyzed. This lack of standardization is in part due to the current limited literature regarding how the tendon transcriptome changes across the micro-anatomy of the tendon, and in understanding transcriptomic differences between tendons. A major challenge in tendon transcriptomics is the availability of suitable tendon tissue for scientific research.

Animal models offer several advantages, such as greater tissue availability, the ability to induce controlled pathology, to use naturally occurring disease models, and to manipulate factors including genetic background and exercise protocols. However, they also have shortcomings such as tendon size in some rodent models, incomplete annotation of the transcriptome in some species, consideration of the translatability of a tendon disease model to the human condition, and possible orthologous genes.¹

In contrast, human tendon tissues enable direct use of physiologically relevant healthy and diseased tissues. Nevertheless, the use of human tissue requires additional and variable protocols depending on the source of the tissues, consent of the patient or next of kin, and additional protections associated with human subjects research including de-identification of samples of protected health information, and screening for known infectious diseases. Moreover, availability is generally limited, and the quality of extracted RNA from nuclei, cells, or whole tissues is variable depending on the procurement method. Here, we review the current literature on human tendon transcriptomic methodology; we identify different variables and processes that are important to analyze as tendon matricellular integrity and RNA quality after harvesting, and we highlight different considerations for experimental design. Additionally, we suggest protocols and guidelines for future transcriptomic studies to allow for better data integration and analysis, and to facilitate collaborations in the field.

2 | DONOR FACTORS

Here, we review the primary considerations related to donor for procurement of healthy and diseased human tendon.

TABLE 1 Demographic variables in the literature used for human tendon transcriptomic studies

Demographic variable	Description/filtering values	References
Age	Old (69.4 ± 7.3 years old)	2
	Young (9 ± 5.8 years old)	
	Old (63 ± 14 years)	3
	Young (28 ± 5 years)	
	Old (54–70 years)	4
	Young (20–24 years)	
	25.75 ± 5.75 years	5
	20–29 and 53–85 years old	6
Sex	70 ± 11 years old	7
	Male	8,9
Health	Male, female	4–6,10
	Exclusion of previous repairs or procedures and proximal humeral fractures	11

2.1 | Donor inclusion and exclusion criteria

2.1.1 | Demographic factors

Demographic variables are essential to consider since pragmatic donor selection can minimize the confounding factors and covariables that are inherent within the sample pool or that accumulate across lifespan. Existing human transcriptomic studies that specifically evaluate demographic factors are summarized in Table 1. Some of the most common demographic variables considered are as follows.

- Age: different donor ages reflect different states of the tendon lifespan as embryonic (0–8 weeks) and fetal (8–40 weeks) development, maturation (0–20 years), adulthood (starting at 20 years), and natural age-related degeneration (dependent on individual circumstances and systemic health, often after 60 years). Use of embryonic and fetal tissues in research may be subject to additional human subjects' ethical regulations or restrictions, depending on the country and funding source.
- Biological sex: studies show significant differences in metabolic processes between the male and female tendon transcriptome.¹² As a result, we recommend including biological sex as a variable when designing, analyzing, and reporting studies. The influence of medical transition on the tendon transcriptome, and on tendon health for transgender people is completely unknown, and information on the effects of sex hormone supplementation on the tendon transcriptome is similarly scarce.
- Body mass index (BMI): if limited medical history is available, restricting donor inclusion criteria to “normal” BMI can decrease the

likelihood of including patients with comorbidities that impact tendon metabolism. However, where possible, adiposity as a variable should be considered as a specific variable for comparison, or as a variable to be balanced between experimental groups. In contrast, “underweight” BMI (<18.5) donors should be considered carefully for inclusion during experimental design since conditions such as cachexia, sarcopenia, or prolonged limited mobility are common, particularly in cadaveric donors, and these factors impact tendon metabolism and biomechanics.

Other demographic factors should be considered and included in a balanced dataset, particularly race and ethnicity, to account for ethnicity-specific expression signatures.¹³ If this and other demographic information is available but not an exclusion factor, we recommend reporting this information in publications and repositories.

2.1.2 | Medical history

A full medical history may not be available for all donors or might be redacted depending on the level of deidentification required for consent and ethics boards, degree of health screening before donation, or the extent of knowledge of medical history obtained from next of kin for a cadaveric donor. Medical history may also be self-reported, without independent verification possible. Yet, medical history is an important factor since many comorbidities, prescription medications, and recreational drugs disrupt tissue metabolism and change transcriptional regulators in tendon.¹⁴⁻¹⁶ Some of the variables to consider and record where possible are as follows.

- Comorbidities: donors with known diseases that directly affect tendon function should be studied separately or excluded. Diseases such as tendinopathy, metabolic diseases (diabetes, dyslipidemia, hypercholesterolemia), multiple sclerosis, muscle dystrophy, arthritis, autoimmune diseases such as lupus, and genetic diseases of connective tissues, and so forth, can directly affect the micro-environment, resulting in changes in tendon performance and metabolism.¹⁴ Similarly, past trauma and fibrotic tendon healing result in different extracellular matrix (ECM) composition and organization and several transcriptional changes.^{11,17}
- Unrelated diseases: diseases that do not directly affect tendon, such as cancer should also be considered for exclusion or reported as confounding factors because of the peripheral effects of their treatment and the potential for systemic abnormalities.¹⁵ For instance, cancer changes the transcriptome of non-tumorous adjacent tissues.¹⁸ Additionally, chemotherapy and radiotherapy can affect the transcription of genes related to cell division, cell death, wound response, stress response, and cell migration.¹⁹ Any disease that results in impaired mobility, or in a protracted bedridden state, could induce sarcopenia or muscle atrophy, therefore, causing disuse-related changes in tendon.²⁰

- Smoking, alcohol, and recreational/illicit drug use: smoking has an antioxidant and xenobiotic effect, leading to increased expression of cell death and inflammation regulating genes. Alcohol and drug use cause changes in metabolism that, if experienced repeatedly, will hinder tendon function.^{16,21}
- Prescription medications: many prescription medications affect tendon metabolism. For instance, corticosteroids affect the composition of the extracellular matrix in the tendon, and aromatase inhibitors may cause a decrease in estrogen levels that can induce other systemic changes like arthralgia.²²

2.2 | Infectious disease status

The use of universal biosafety precautions and personal protective equipment are highly recommended, if not mandatory, when handling human tissues, as is adherence to institutional biosafety committee requirements. However, it is also wise to consider screening potential donors to reduce the risks of working with potentially infectious material. Serological screening occurs most commonly for bloodborne viruses such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), hepatitis B virus (HBV), and any population endemic or regional virus as needed (SARS-CoV-2, Zika, West Nile Virus).

For clinical remnants, infectious disease screening results may already exist in the patient's medical record, or may occur during preoperative preparation, and access to this information should be requested as part of human subjects' research protocol development. For cadaveric tissues, serological infectious disease testing occurs more commonly postmortem than ante mortem, and time (typically 24 h) taken to obtain results may therefore delay tissue availability. One additional precaution in the United States is to avoid the use of tissues from donors designated as “Augmented Donor Risk” by the Centers for Disease Control and Prevention (CDC, www.cdc.gov). “Augmented Donor Risk” identifies behavioral and social exclusion criteria for solid organ donation. History, physical examination, medical records, or autopsy reports may reveal evidence of high-risk behavior or potential exposure to infectious disease that should be avoided. Collection of this information is often performed by the organization supplying the tissue.

3 | TISSUE HARVESTING AND INITIAL PROCESSING

As for all tissues, once tendon is manipulated during surgical (clinical remnant) or biopsy-based intervention and removed from the body, or a potential cadaveric donor enters the agonal period and subsequently dies, metabolic changes begin that trigger RNA degradation.²³ In this regard, there are different considerations and opportunities for intervention to preserve RNA quality based on the tissue source, whether it is from clinical remnants or cadaveric tissues (Figure 1).

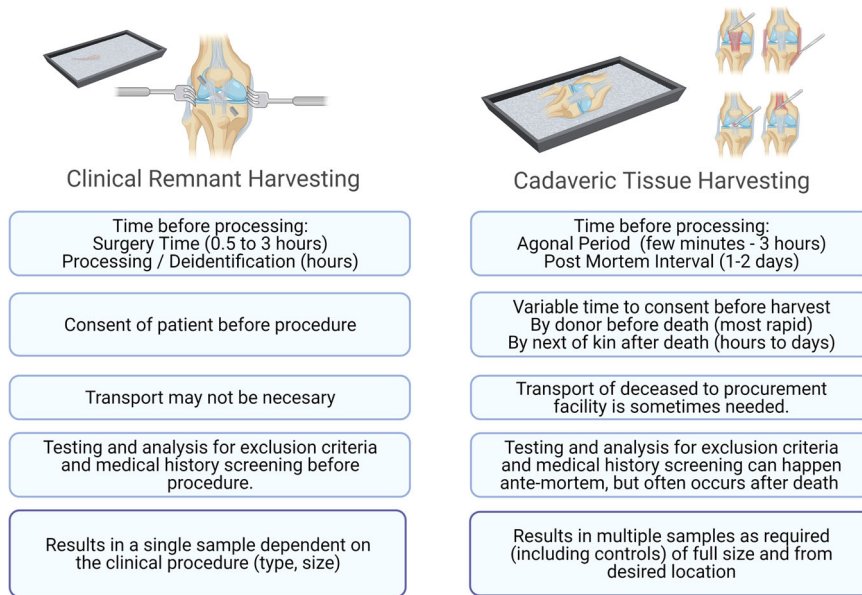


FIGURE 1 Comparison of clinical remnant and cadaveric tissue harvesting and initial processing. Created in Biorender.com [Color figure can be viewed at wileyonlinelibrary.com]

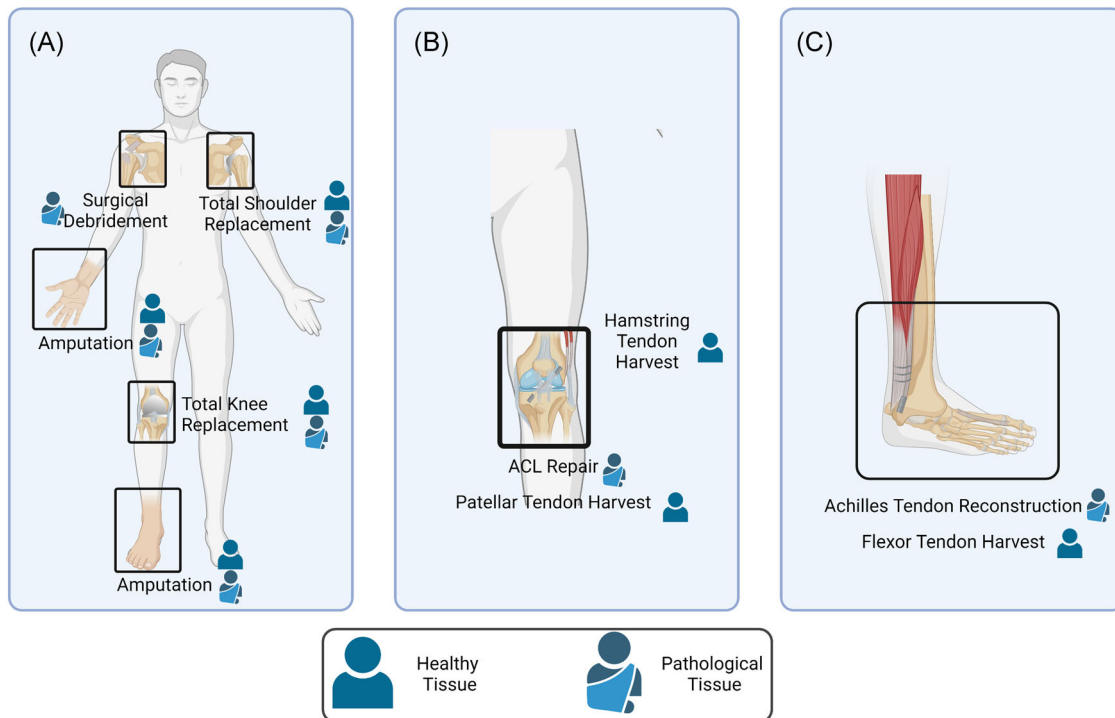


FIGURE 2 Some procedures that allow for tendon harvest as remnant biospecimens. (A) Amputations, arthroplasty, surgical debridement. (B) Tendon repair autografts (anterior cruciate ligament repair with hamstring or patellar tendon, tendon transfer procedures) (C) Tendon repair autografts (Achilles' tendon reconstruction with flexor tendon). Created in Biorender.com [Color figure can be viewed at wileyonlinelibrary.com]

3.1 | Clinical remnant retrieval

Clinical remnant tissues from living patients are typically obtained from surgical procedures (Figure 2) (e.g., residual tendon autografts, amputations), derived from tissue resected to treat either tendon disease, or another disease in the same anatomic location, or from where the clinical standard of care is to also resect the tendon of interest (e.g. long head of biceps tendon in total shoulder

arthroplasty). In many of these scenarios, the tissue is no longer needed and would otherwise be discarded as biohazardous waste after completion of surgery. Additionally, biopsies can be obtained during open or arthroscopic procedures from sites of surgical repair, from adjacent normal tissue, or as a separate procedure under local anesthesia.

All clinical remnants used for research require ethics oversight. Often informed consent is necessary, especially if Protected Health

Information remains associated with the sample, to remain in compliance with the appropriate ethical guidelines of the nation or region where the samples are harvested.²⁴ In the United Kingdom, the Human Tissue Act (2004) makes it necessary to have informed consent for all types of tendon samples, together with specific disclaimers for genetic analysis. However, in the United States, while proposed research using clinical remnants undergoes review by the Institutional Review Board, informed consent of the patient is not required in many circumstances if there is no identifying information (including genetic sequencing) associated with the sample. In other countries (including those within the European Union),²⁵⁻²⁷ informed consent and other biospecimen regulations are derived from specific sections of law in each territory. For instance, India has different types of consent and the adequacy of the informed consent is assessed by an institutional or independent ethics committee registered with the Drugs Controller General of India.²⁸ In China, prior informed consent is necessary for human biospecimens and must be in collaboration with a Chinese research entity and approved by the Office of Human Genetic Resource Administration within the Ministry of Science and Technology.²⁹ In other cases, such as Australia, all states and territories have their own additional laws on human biological materials.²⁷ It is important for researchers to review carefully the applicable legal regulations, abide by regulations and protocols, and maintain required records.

The main advantage of clinical remnants (Figure 1) is that at the time of harvest the tissues are live and the period until storage and protection from RNAses is relatively short. Further, for elective procedures, the patient is generally well-perfused under general anesthesia, and rarely has life-threatening comorbidities. The sample however may be manipulated extensively during surgery and may not be immediately released from the sterile field to the researcher; therefore, surgery time is the main time-limiting factor that can result in drying or contamination of the sample, initiate tissue apoptosis, and metabolic changes associated with hypoxia and ischemia. As a result, we recommend early transfer of specimens to media or solutions or states (e.g. snap freezing) that preserve RNA quality whenever possible. Although the rapid collection of samples is advantageous for live cell isolations or for maintenance of RNA quality, it is important to highlight that the volume of tissue, type of tendon, and health of the tendon available depends on the clinical procedure and may be limited. There is rarely an opportunity to harvest paired controls for comparison, or to harvest both normal and abnormal tendon from the same anatomic location from the same patient.

3.2 | Cadaveric tissue harvesting

Obtaining tissue from deceased patient donors is a more straightforward process for individual researchers once protocols and agreements are in place since the protocols and tissue procurement are typically managed by a biobank. These organizations coordinate donors, obtain informed consent, deidentify tissues, and prioritize

donors to clinical needs (organ and allograft donation) if the donor is a suitable match, and only after this process to approved research or medical education protocols. Depending on the type of donation and location, these biobanks are regulated by different institutions. Non-Transplant Anatomical Donation Organizations (NADOs) in the United States are required to comply with the Food and Drug Administration regulations, and private organizations such as the American Association of Tissue Banks have optional programs for NADOs to be certified. In the United Kingdom, The Human Tissue Authority licenses and inspects organizations that manage whole body donations. In the European Union, there is not a unique institution that manages these types of organizations and in many cases, they do not require accreditation.²⁵ In China, biobanking requires a separate approval process with the Office of Human Genetic Resource Administration.²⁹

The main difficulty for biobanks with regard to tendon transcriptomic work lies in finding viable tissues.³⁰ Donors typically accepted for research donation are individuals whose underlying health does not allow for organ or allograft donation for clinical purposes. In general, research biobanks are not well known by the general population as an option for donation, and the choice of donating to them is not always presented to the next of kin, or there may not be an organization with local affiliation. Finally, multiple researchers often use the same biobanks, which may result in queued requests for tissues.

One of the main disadvantages of cadaveric tissues with regard to RNA quality is the time to harvest postmortem, known as the postmortem interval. Multiple factors may decrease the quality of the RNA from a transcriptomic perspective. Initially, it is important to consider the underlying cause of death, and whether this was likely associated with protracted duration of poor perfusion or immobility, the duration of the agonal period (Figure 1), which is the period immediately before somatic death. In this period, significant changes precede death and the agonal period can be very short or last a few hours depending on the ultimate cause of death.³¹ Prolonged agonal periods induce significant changes in homeostasis and increase rate of tissue degradation.

The consent process (if not provided ante mortem), screening (social and medical history, infectious disease), and transport of the decedent may require additional time, followed by transport of tissues after procurement to the research site (Figure 1).

Although cadaveric tendon may have a more extended time before harvest that may result in apoptosis and RNA degradation, cadaveric tissues have multiple advantages over clinical remnants. For instance, the tissues can frequently be retrieved in their entirety, making it possible to extract more extensive amounts of RNA, control specific anatomical sections of interest, or perform multiple assays on the same tendon. Additionally, procurement of a variety of within donor control tissues may be possible. For instance, contralateral samples, healthy compared to diseased, or different tendons from the same donor, can be used to analyze differences between specific tendons or disease processes, and help mitigate the biological effects of a longer postmortem interval, and the disadvantages of cadaveric tissue compared to clinical remnants.

3.3 | Postharvest processing

Ideally, once harvested, tissue samples must be frozen or processed for RNA or cell and nuclei extraction as soon as possible, as time between tendon harvest and processing is correlated with RNA integrity³² and long processing times could lead to transcriptional bias.³³ The type of storage available will in part constrain the tissue processing and RNA extraction methods (see Section 4). If possible, the samples should be snap-frozen with liquid nitrogen and stored at temperatures below -20°C while they are processed and readied for shipment. If this is not possible, transporting the tissue with an RNA stabilizer, an RNA protector, an RNase inhibitor, or transport media can preserve the RNA. RNA protectors such as RNAlater (Sigma-Aldrich), or DNA/RNA Shield (Zymo) are used in tendon transcriptomic studies.³⁴ RNAlater enables high RNA yield and quality; however, it can induce transcript bias,³⁵ cell clumping, and protein degradation,³⁶ an important consideration for transcriptomic studies at both bulk and single-cell resolution.

Transport is a process that should be considered carefully; for example frozen tendon samples must be sent on dry ice, if possible, by overnight shipment. Considerations as the thickness of a cooler and quantity of dry ice should be modified according to the time needed for the delivery, ambient temperatures, and time of the year.

An absolute requirement for all human tissue work is the respectful, safe, and correct disposal of any unused and surplus remains. In general, unused or surplus tissues become biohazardous waste. For this process, it is important to carefully review the relevant legislation. In the United States, each state has different requirements for handling of biohazardous material, in addition to requirements associated with national organization accreditation for the procuring organization. In the United Kingdom, the Human Tissue Act established that remains can be incinerated but it is also necessary to respect the wishes of the consenting party. In the European Union, it is necessary to ensure that the remains are treated in an accredited disposal establishment with the treatment the consent allows.

4 | RNA EXTRACTION AND TRANSCRIPTOME SEQUENCING

Tendons are ECM-rich tissues, with low cellular density and consequently low RNA content. The ECM of tendon is also highly aligned with a hierarchal structure, where collagen aggregates to form fibers of increasing size. As a result, both preservation and extraction of RNA are more challenging compared to other tissues with higher cellularity, more accessible cells, and more easily digestible ECM. Proper collection protocols improve RNA quality and quantity and therefore should be planned thoroughly.

Several diverse methodologies for bulk and single-cell RNA-sequencing (RNA-seq) have been developed using human and animal tendons. The method must be chosen based on tissue availability and type of resolution needed; bulk versus single-cell versus

single-nucleus. These methodologies are summarized in the following section along with recommendations.

4.1 | Bulk transcriptomics

Both snap-frozen and fresh tissue can be used for bulk transcriptomics (Table 2). Snap-frozen bulk transcriptomics have been previously used on human,³⁷ murine,¹⁷ rat,³⁸ and equine³⁴ tendons (Table 2). For frozen samples, usually disruption and homogenization of the tissue is necessary before RNA isolation. The method of homogenization must be reported as it can affect tendon RNA quality.³² Homogenization methods range from dismembrator,² to pestle, and to bead-based homogenization,¹⁷ and should be chosen based on tissue size and laboratory equipment availability. Ideally, tissue homogenization methods should be standardized based on the tendon type, maturity, and species to reduce matrix debris and increase RNA quality. Studies using bead-based homogenization reported the highest RNA integrity number (RIN) values¹⁷ (Table 2); unfortunately, however, RIN values are not consistently reported in all studies.

If performing bulk transcriptomics on fresh tendon tissue, subsequent tissue digestion with a protease is a common method to increase RNA yield. Previous bulk transcriptomic studies on human and murine tendons used the RNeasy Fibrous Tissue Mini Kit (Qiagen) (Table 2) which recommends using a lysis buffer (RLT buffer) with high guanidine-thiocyanate during tissue disruption and proteinase K treatment to digest proteins found in fibrous tissues. Other digestion methods can also be used such as trypsin and EDTA.⁴² The type of tissue disruption, homogenization, and enzymatic digestion used should be reported.

It is important to choose the RNA isolation method or kit based on the scope of the study. Most bulk transcriptomics studies on tendons to date have used the RNeasy kits (Fibrous, Mini, Micro) for RNA extraction (Table 2) which uses a silica-based membrane to bind to guanidine-thiocyanate-containing RNA. RNeasy kits isolate RNA with greater than 200 nucleotides, and therefore exclude shorter RNA transcripts including those for ribosomal RNA (rRNA) and transfer RNA (tRNA). Trizol can also be used for total RNA extraction and offers the additional advantage of deactivating RNases and permits concurrent extraction of RNA, DNA, and protein.⁴³ Trizol can also be combined with other RNA isolation methods, such as the miRNeasy kit,³⁸ which is specifically designed for total RNA extraction (>18 nucleotides) including microRNA (miRNA).

Following RNA isolation, RNA quality should be evaluated. Potential quality control of RNA includes RIN values and NanoDrop Spectrophotometer readings which should be reported. There are several strategies available to increase RNA purity. DNase treatment is common for the removal of trace amounts of genomic DNA (gDNA) and is included in the RNeasy kits previously used in tendon transcriptomics (Table 2). The method for removal of gDNA and DNase inactivation should ideally be reported. Further quality control of contamination can also include femtogram range DNA detection kits to detect remaining gDNA and determine if RNA purity is sufficient.

TABLE 2 Summary of tendon bulk RNA-seq studies and their storage, species, tendon type, tissue processing, and RNA-isolation methods, including the use of RNAlater and tissue processing methods

Tissue storage	Species	Tendon	RNAlater	Tissue processing	RNA Isolation	RNA Quality	RNA Purification	References
Snap-frozen	Human	Achilles	Yes	Pulverized	Trizol and RNeasy spin column (Qiagen)	N/A	rRNA depletion	2
		Rotator cuff	No	N/A	RNeasy Mini kit (Qiagen)	RIN > 7.0	rRNA depletion	37
	Murine	Flexor digitorum longus	No	Bead-based	Trizol and RNeasy Plus Micro Kit (Qiagen)	RIN = 8.4 ± 0.9	Oligo(dT)	17
	Rat	Plantaris	No	Bead-based	Trizol and miRNeasy kit (Qiagen)	RIN > 8.0	No	38
	Equine	Superficial digital flexor	Yes	Pulverized	RNeasy Mini kit (Qiagen)	RIN = 6.8–8.1	rRNA depletion	34
Fresh tissue	Human	Rotator cuff	Yes	RNeasy Fibrous Tissue Mini Kit (Qiagen)		N/A	rRNA depletion	11,39
		Rotator cuff	No	Hybrid-R RNA extraction kit (GeneAll)		N/A	rRNA depletion	40
	Murine	Achilles	No	RNeasy Fibrous Tissue Mini Kit (Qiagen)		N/A	Oligo(dT)	41
		Developing limb	No	Trypsin-EDTA (Invitrogen) and FACS	RNeasy Micro Kit (Qiagen)	N/A	No	42

Note: The precise method of tissue disruption and homogenization as well as RNA quality was not available (N/A) for all studies.

4.2 | Single-cell resolution transcriptomics

Several studies have sequenced at single-cell resolution directly from human and murine tendon tissue (Table 3). The predominant method for cell isolation used in these studies is enzymatic digestion of the tendon ECM (Table 3). The enzymatic mixture, incubation time, and temperature should be reported and have previously consisted of a combination of collagenase, dispase, trypsin, and/or Liberase (Sigma). The main limitations of enzymatic digestion include inducing cellular bias and inducing a stress-response, an important consideration. Stress-response following enzymatic digestion alters the transcriptome of neuronal and glial cells.⁴⁸ Furthermore, the time taken to digest the tissue can also induce a stress response and cause RNA degradation, particularly in tendons.³² Therefore, time is another limiting factor for RNA quality in tendon cell isolation. The cell isolation protocol must be optimized to reduce both stress response and time, while still isolating representative tendon cells. The method should be clearly described in publications; cell viability before encapsulation should also be reported. Furthermore, fluorescence-activated cell sorting (FACS), can be used to isolate viable or specific cells of interest for sequencing.

To date, there are no published studies on tendons using single-nuclei transcriptomics (Table 3). However, single-nuclei transcriptomics has been recently used on myofibers⁴⁹ and could be used for tendons. It is important to consider that snap-frozen tissues are often used for nuclei extraction and require homogenization which can result in a large amount of debris, a potential challenge for single-nuclei encapsulation. Furthermore, both single-cell and single-nuclei isolation can result in cellular bias. Cell populations derived from single-cell versus single-nuclei have similar subpopulations but with varying proportions,⁵⁰ making a cross-platform evaluation of bias difficult. In addition, single-nuclei libraries have been reported to underrepresent immune cells, including lymphocytes derived from human kidney and tumor tissues.⁵¹ Therefore, cellular bias should be carefully examined in tendon single-nuclei and single-cell sequencing applications, cell populations validated using imaging, and benchmarking experiments carried out where possible.

Other single-cell resolution methods can also be considered for tendon transcriptomics such as MARS-seq, Drop-seq, inDrop, and BD Rhapsody. Currently, only 10X Single-cell methods (Table 3) have been published for tendon transcriptomics, therefore other methods will likely require optimization. 10X offers high cell capture efficiency and high mRNA detection sensitivity,⁵² and therefore is recommended if tissue sample volume is limited.

4.3 | Library preparation

Library preparation is an important step to ensure high library complexity and a library that is tailored to the sequencing platform. Further RNA selection or depletion can be used following initial extraction, depending on the scope of the study, to reduce rRNA

TABLE 3 Summary of methods for single-cell transcriptomics of human and murine tendon tissue

Tissue storage	Species	Tendon	RNAlater	Tissue processing	RNA isolation	RNA quality	References
Fresh Tissue	Human	Multiple	No	Liberase (Sigma), cells frozen (~80) and CITE-seq	Single-cell 3' (10x Genomics)	N/A	44
	Murine	Supraspinatus, hamstring Achilles Patellar	No	Liberase (Sigma) and FACS Collagenase and dispase (Roche) Collagenase and dispase (Roche)	Single-cell 3' (10x Genomics) Single-cell 3' (10x Genomics) Single Cell 3' (10x Genomics)	N/A N/A N/A	45 46 47

Note: Tissue processing, including enzymatic digestion and RNA isolation methods, are listed. RNA quality was not available (N/A) in all studies.

content (Figure 3). For bulk transcriptomics, rRNA depletion kits can be used as well as oligo(dT) primers. rRNA depletion allows for detection of both coding and noncoding RNA, including polyadenylated (poly(A)) and non-poly(A) transcripts. In contrast, oligo(dT) primers hybridize poly(A) tails for first-strand complementary DNA (cDNA) synthesis for library preparation. Poly(A) tails are found in most mRNA, some long noncoding RNAs (lncRNA) and precursor pri-miRNAs⁵³ which does not capture the whole transcriptome. RNA with poly(A) tails have some protection from degradation,⁵⁴ which could be advantageous for tissue with low RNA quality. Similarly, single-cell transcriptomics, 10x Genomics, Drop-seq, and most single-cell methods capture poly(A) transcripts. While this is a potential limitation since no ncRNA effects are studied, poly(A) transcripts are sufficient to determine cell types and subpopulations.

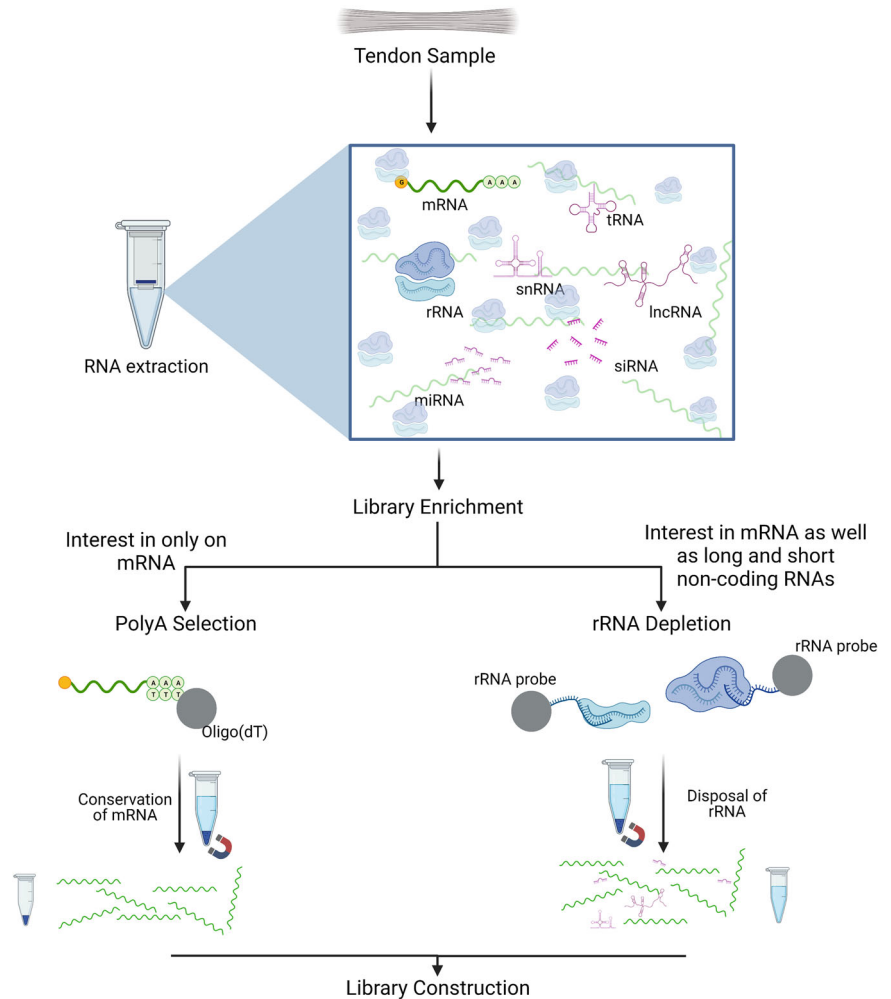
Quality control of library preparation is necessary before sequencing to reduce experimental bias. Ideally, we want to maximize library complexity and minimize amplification-based bias. Batch effect can also be introduced by differences in adaptor ligation efficiencies⁵⁵ and must be evaluated to ensure there is enough DNA ligated to adapters for successful hybridization. Once the library is constructed, the size of the library should similarly be evaluated and reported, as the optimal size will depend on the insert size and next-generation sequencing instrumentation.⁵⁵ Typically, electrophoresis is used to measure library molarity and assess the sizes of the smallest and largest fragments.

4.4 | Next-generation sequencing technologies

Transcriptomics is a term typically used for the evaluation of bulk and single-cell transcription using second-generation sequencing technologies (e.g., Illumina, Ion Torrent) which require specific library preparation, amplification, and indexing. These second generation sequencing technologies can provide high throughput (millions of reads) with high accuracy (<1% error) and multiplexing samples can significantly lower costs per sample. Disadvantages of second generation sequencing are the read-length (~150 bp), costs of instruments, and sequencing run-time (>1 day).⁵⁶ Third-generation sequencing technologies are gaining traction and allow for long-read single-molecule sequencing which can be used for de novo transcriptome assembly and transcript isoform identification to map the whole transcriptome. Single-molecule sequencing eliminates the need of library amplification and thus amplification bias and can generate longer transcriptional reads (>135 kb PacBio, >1 Mb Oxford Nanopore) with lower processing times.^{56,57} Disadvantages of third-generation sequencing include error rate (2%–15% error) for Oxford Nanopore, and high costs of instruments (>\$500k) and cost per sample run for PacBio.^{56,57}

An in-depth comparison between these two generations of sequencing technologies has been summarized in other publications.^{56,57} Nevertheless, since third-generation technologies remain inaccessible to most researchers, they are not analyzed separately in this review. Sequencing methodology and read depth should be reported to ensure reproducibility.

FIGURE 3 Procedures for library enrichment. Poly(A) selection allows the conservation of mRNA with poly(A) tails by the hybridization to Oligo(dT) magnetic beads. rRNA depletion allows the conservation of mRNA, and some noncoding RNAs as tRNA, snRNA, lncRNA, miRNA, and siRNA, by the disposal of rRNA bound to magnetic beads [Color figure can be viewed at wileyonlinelibrary.com]



5 | POSTPROCESSING OF TRANSCRIPTOMIC DATA

Although several pipelines and software can be used for the postprocessing of transcriptomic data, there are a few points that should always be consistent for reproducibility.

5.1 | Bioinformatics quality control

In bioinformatics, the first step is to perform quality control of the transcriptome obtained: the total number of reads, GC content, and overall base quality score. Bases with low-quality scores at the end of the reads are trimmed. GC content should be close to the values reported in the human genome with a deviation <10%. GC in humans for the whole genome GC content is 39.3%, for coding RNA is 48.9%, for lncRNA is 39.7%, for rRNA is 50.2%, for miRNA is 51.5%, for tRNA is 55.7%, and for other small RNA (sRNA) is 46.7%, according to the latest human reference genome GRCh38.⁵⁸ Other parameters can be considered for quality control, according to the type of RNA⁵⁸; for instance, incorrect read length distribution can be a sign of overrepresentation for sRNA.

For alignment quality control, capture efficiency and contamination of RNA from an unwanted source should be evaluated. The capture efficiency should range between 50% and 80%. If the capture efficiency is below 50%, it might be necessary to reconstruct the library.⁵⁸ Depending on the library preparation method, the percentage of sense and antisense transcripts can also be used for the analysis of possible DNA contamination. For instance, non-strand-specific protocols should have a 50%/50% distribution, instead strand-specific protocols should have 1%/99% distribution.⁵⁹ Contamination from other sources should be tested by comparing unaligned reads against reference genomes for other species. In addition, for single-cell transcriptomics, cells captured and unique molecular identifiers (UMI) counts per cell should be evaluated, as well as sequencing saturation, and median genes per cell to ensure high library complexity.

The percentage of mitochondrial reads should also be reported, particularly for single-cell RNA-seq applications where mitochondrial reads are used to filter low-quality cells. Mitochondrial reads are tissue-specific and species-dependent with a higher percentage of mitochondrial reads in humans compared to mice.⁶⁰ A filtering standard of mitochondrial reads needs to be properly assessed to ensure accurate attribution of biological significance. Currently in

tendon single-cell studies, mitochondrial read filtering ranges from >20% in mouse Achilles,⁴⁶ to >5% in human tendons.⁴⁴

Several other quality control steps can be conducted during the bioinformatics processing of the raw data. These steps should be considered according to the needs of the study and the type of RNA transcribed. Nevertheless, this information should always be reported at publication.

5.2 | Data integration

Data integration within the final dataset is often necessary in tendon transcriptomic, particularly with human samples which cannot always be prepared simultaneously. As a result, different batches are often analyzed, and batch effects must be accounted for. This process is essential for comparative and differential analysis.

For bulk RNA-seq, batch effects can be addressed by linear batch correction methods such as normalization. Multiple methods exist for bulk data normalization and include data-driven, foreign reference, and all-gene reference and will depend on the scope of the study.^{61,62} Most common is the use of housekeeping genes (data-driven) or spike-ins (foreign reference) and number of reads to normalize data by defining scale factors. For tendon transcriptomics, we recommend using external standard controls instead of relying on housekeeping genes. These normalization methods should be reported since these steps can significantly change the results of differential analysis. Other publications provide more in-depth analysis of the bioinformatics pipeline used for bulk data normalization.^{61,62}

For single-cell RNA-seq, data integration remains a challenge due to the complexity of datasets for which linear integration methods, previously designed for bulk transcriptomics, are inadequate. Most single-cell integration methods rely on nonlinear (or locally linear) strategies.⁶³ Numerous integration methods have been developed (e.g., mutual nearest neighbors, or canonical correlation analysis with Seurat v3).⁶⁴ In human tendon single-cell transcriptomics, several studies have integrated data using negative binomial regression with *sctransform*.^{44,46} Potential limitations in single-cell data integration include overcorrection and removal of biological variability and distortion of gene counts.⁶³ Therefore, choosing the correct integration method is important, and we recommend benchmarking different integration methods if possible. Other publications provide more detailed comparison of integration methods and benchmarking analysis.^{63,64}

5.3 | Cell annotation

For downstream analysis of single-cell and single-nuclei tendon transcriptomics, methods for cell cluster annotation need to be standardized, and consensus-reached in nomenclature, particularly for tenocytes (tendon fibroblast populations). Previously, studies on human tendons have annotated tenocytes based on COL1A1/2

expression⁴⁴ and on COL3A1 and DCN expression.⁴⁵ Further annotation of the tenocyte subpopulations remains a challenge. Previously, on multiple human tendons, five tenocyte subpopulations were annotated as: PTX3 + tenocytes, SCX + POSTN + tenocytes, ITGA7 + tenocytes, CXCL14 + tenocytes, and MGP + tenocytes.⁴⁴ Meanwhile, in the human hamstring tendon, two normal tenocytes and one mural tenocyte subpopulation were identified and annotated as: POSTN + GSN + CXCL14 + tenocytes, SCX + TNMD + FBLN1 + tenocytes, and NOTCH3 + ACTA2 + mural tenocytes.⁴⁵ Both datasets used SCX, POSTN, and CXCL14 to annotate tenocytes but these genes were found in different combinations within the subpopulations. In addition, the human tendons dataset identified the ITGA7 + tenocytes to be perivascular and also expressed ACTA2,⁴⁴ similar to the mural tenocytes identified in the hamstring tendon.⁴⁵ Standardizing annotation will facilitate the comparison of datasets and ensure that each tenocyte subpopulation is properly identified and characterized. The same is also true for mouse tendon datasets where Col1a1 has been used to annotate tenocytes, and Col22a1 has been used to annotate junctional fibroblasts.⁴⁶

5.4 | Reproducibility

To test reproducibility between samples several different approaches can be taken. For instance, rerunning samples and comparing the transcript counts is an easy way to evaluate them. A microarray can evaluate specific known proteins and compare their transcription.

For bulk transcriptomics, spike-in standards can also be used to assure reproducibility. This mechanism uses a commercial set of unlabeled, polyadenylated transcripts. As a result, these can measure the effectiveness of the process without the influence of processing factors as starting RNA yield and quality. Similarly, these standards can also be used to standardize samples between different studies conducted on multiple platforms.⁶⁵

For single-cell transcriptomics, reproducibility can be ensured with high cell numbers, replicates, and a combination of transcriptomics with other "omics" techniques such as proteomics and metabolomics. Multiomic datasets can provide a more significant amount of information about the processes triggered by transcriptomic changes, but provide further data integration challenges.⁶³

Additionally, multiplexing methods can decrease discrepancies between batch effects and lower costs per sample. These techniques allow to add barcodes to multiple samples and then allow analysis as a single mixed sample. These methods can be used on both bulk⁶⁶ and single-cell⁶⁷ transcriptomics.

6 | SCIENTIFIC PAPER REPORTING GUIDELINES

Reporting all the factors considered in tissue procurement, tissue processing, RNA extraction, and postprocessing of data will help standardize the tendon transcriptomics field and improve the utility

of data to be translated toward improved biological understanding and clinical outcomes. Therefore, we suggest the following reporting guidelines.

1. Inclusion and exclusion criteria for the study should be listed, including if none were used, or if donors were deidentified and information was not available. Similarly, if available, the demographics of donors should also be available, including, as a minimum, sex, age, BMI, and race.
2. Quality control methods should be reported following the best practices for RNA-seq. We recommend reporting the RIN number of initial RNA extractions, library coverage, and depth, total reads or reads/cell, and mitochondrial reads as the minimum standard. Similarly, for bulk transcriptomic, we suggest using spike-in standards and the normalization of the data to this specific standard and including the process followed. Finally, we would like to encourage the use of multi-omics techniques to improve data dimensionality.
3. Deposition of datasets in open repositories is often required by funding agencies and publishers. Possible repositories include Gene Expression Omnibus (GEO), a public functional genomics data repository supporting MIAME-compliant data submissions, and the Human Cell Atlas (HCA) data portal for single-cell and multi-omics datasets. Alternative repositories may be more appropriate if transcriptomic datasets are accompanied by genomic or methylomic datasets. In the metadata, we encourage submitters to add deidentified donor information to the information for each sample (i.e. age, sex, BMI, relevant medical history, PMI if applicable), and this should be approved by ethics boards before the start of the study. Additionally, the anatomic site and health status of the tendon studied, and basic quality control measurements (i.e., RIN number, total number of sequences, and their respective percentages of aligned and assigned) should all be reported.

7 | CONCLUSIONS

This paper discusses the considerations, difficulties, and guidelines that can enhance tendon transcriptomics through RNA quality improvement, protocol definition, and standardized points of comparison between studies. Standardization of transcriptomic datasets of healthy and pathological tendons will help ensure attribution of true biological significance and provide proper comparator groups for evaluating tendon disease.

As discussed, the greatest challenge in human tendon transcriptomics remains the availability of healthy human tendon tissue. Yet, the available data can be further analyzed if relevant donor factors are considered through the experimental design stage, and metadata is evaluated for confounding factors. Additionally, we recognize that tendon tissue harvest has a big impact on RNA quality, and it is necessary to improve efficiency to avoid RNA degradation. Another challenge in tendon transcriptomics lies in the standardization of RNA

extraction methods, post-processing, and quality control measurements. Due to the range of RNA extraction methods used in human and animal tendon studies, we recommend to review and compare relevant differences in tissue storage and pretreatment methods (snap-freezing, homogenization, and enzymatic digestion). These factors are increasingly important for single-cell and single-nuclei resolution transcriptomics. Use of repositories and reporting guidelines for library preparation and bioinformatics quality control parameters will also allow the field to obtain more significant insight by sharing data.

Ultimately, a community-based approach with clear reporting, sharing of protocols, and standardization of cellular annotation is needed to ensure the power of transcriptomic studies to improve treatment and understanding of human tendon disease is realized.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Lorenzo Ramos-Mucci and Paula Sarmiento conducted the literature review and drafted the manuscript. Sarah Snelling and Dianne Little edited and critically revised the manuscript.

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