

# Comprehensive Selection of Reference Genes for Expression Studies in Meniscus Injury Using Quantitative Real-time PCR

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**Abstract.** The meniscus plays critical roles in the knee function. Meniscal tears can lead to knee osteoarthritis. Gene expression analysis may be a useful tool for understanding meniscus tears, and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) has become an effective method for such studies. However, this technique requires the use of suitable reference genes for data normalization. We evaluated the suitability of six reference genes (18S, ACTB, B2M, GAPDH, HPRT1 and TBP) using meniscus samples of (1) 19 patients with isolated meniscal tears, (2) 20 patients with meniscal tears and combined anterior cruciate ligament injury (ACL), and (3) 11 controls without meniscal tears. The stability of the candidate reference genes was determined using the NormFinder, geNorm, BestKeeper DataAssist and RefFinder software packages and comparative  $\Delta$ Ct method. Overall, HPRT1 was the best single reference gene. However, GenEx software demonstrated that two or more reference genes should be used for gene expression normalization, which was confirmed when we evaluated TGF $\beta$ R1 expression using several reference gene combinations. HPRT1 + TBP was the most frequently identified pair from the analysis of samples of (1) meniscal tear samples of patients with a concomitant ACL tears, (2) all meniscal tears, and (3) all samples. HPRT1 + GAPDH was the most frequently identified pair from the analysis of samples of isolated meniscal tear samples and controls. In the analysis involving only controls, GAPDH + 18S was the most frequently identified pair. In the analysis of only isolated meniscal tear samples and in the analysis of meniscal tear samples of patients with concomitant ACL tears and controls, both HPRT1 + TBP and HPRT1 + GAPDH were identified as suitable pairs. If the gene expression study aims to compare non-injured meniscus, isolated meniscal tears and meniscal tears of patients with ACL tears as three independent groups, the trio of HPRT1 + TBP + GAPDH is the most suitable combination of reference genes.

**Keywords:** ACL, anterior cruciate ligament; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MRI, magnetic resonance imaging; TLDA, TaqMan Low-Density Array; AAV, adeno-associated virus; Crt, relative cycle threshold; Ct, cycle threshold; SD, standard deviation; CV, coefficient of variance; RQ, relative quantification; Acc.SD, accumulated standard deviation.

**Keywords:** Knee injury; Meniscal tears; Gene expression; Reverse-transcription quantitative polymerase chain reaction; Expression normalization; Reference genes.

## 1. Introduction

Menisci are important components in joint biomechanics with crucial roles in the knee joint: distributing joint forces, load bearing, and enhancing joint stability (Lee et al., 2014 and Kaleka et al., 2014). Lesion of this structure can cause pain, joint swelling, and osteoarthritis in the long term. Younger people are more likely to have acute lesions due to trauma, whereas older people are more likely to have lesions due to degeneration (Englund et al., 2009, Pauli et al., 2011 and Rai et al., 2013). Patients with traumatic meniscal tears commonly present an associated rupture of the anterior

cruciate ligament (ACL) (Poulsen and Johnson, 2011). Once present, meniscus tears are associated with an accelerated progression of cartilage degeneration in the knee compared with individuals with osteoarthritis but without tears (Biswal et al., 2002 and Hunter et al., 2006).

Recent studies have been performed to understand the gene expression alterations that may have a role in human meniscal tears. In a transcriptome analysis, several genes were identified that were differentially expressed with age and chondrosis in patients with meniscus tears (Rai et al., 2013a). Moreover, Brophy et al. investigated whether the expression of osteoarthritis markers (matrix components, cytokines, chemokines, aggrecanases, metalloproteinases, and transcription factor genes) are age- and sex-related in meniscal tears with and without a concomitant ACL tear (Brophy et al., 2012). The authors demonstrated that the meniscus in younger patients reacts with an intrinsic response and is more prone to inflammatory changes. Conversely, there were no differences in inflammatory cytokines or chemokines in the group of patients over forty years old (Brophy et al., 2012). If confirmed in larger studies, these markers may monitor local events at the surgical sites and detect osteoarthritis progression (Kambic, 2012).

Investigation of gene expression in human meniscal samples may help in improving the understanding of meniscal tears as well as osteoarthritis progression. Moreover, gene expression analysis will be important for guiding patient management and the development of new therapeutic options for these knee afflictions.

Although powerful techniques, including microarrays and high-throughput measurements, have been developed to detect gene expression levels, the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is commonly used in many laboratories (Wang et al., 2015). Moreover, because of its accuracy, sensitivity, and capacity for high-throughput analysis, RT-qPCR is currently considered to be the gold standard technique for evaluation of gene expression (Derveaux et al., 2010). RT-qPCR is one of the most commonly utilized approaches in functional genomics research, and its use in gene expression analysis may become more routine; furthermore, this technique is commonly used to validate data obtained by other methods (Kozera and Rapacz, 2013), including the data of transcriptomic analysis.

To obtain reliable data using RT-qPCR, a common method is to normalize the target gene expression using an endogenous reference gene. Ideally, reference genes should be stably expressed or at least vary only slightly in expression in all tissues or cells under the experiment conditions (Li et al., 2009); therefore, a validation experiment for the evaluation of reference gene expression stability for each target tissue and disease is recommended (Bustin and Mueller, 2005 and Hruz et al., 2011). Normalization with unstable internal controls may result in different values, leading to erroneous results (Yuzbasioglu et al., 2010). However, many authors do not critically evaluate their RT-qPCR experiments; therefore, the experiments are improperly designed and difficult to repeat because of insufficient data quality (Bustin, 2010). Consequently, the use of suitable reference genes with stable expression in the studied tissue (normal and/or injured) is essential for effective data normalization and the acquisition of accurate and meaningful biological data.

Suitability of reference genes has been evaluated in some human musculoskeletal diseases, such as shoulder instability (Leal et al., 2014), rotator cuff tears (Leal et al., 2015a), ACL tears (Leal et al., 2015b), osteoarthritic articular cartilage (hip and knee) (Pombo-Suarez et al., 2008), human lumbar vertebral endplate with modic changes (Zhou et al., 2014), and skeletal muscle with chronic degenerative changes (Yuzbasioglu et al., 2010). To our knowledge, no previous studies have described the best individual or set of reference genes for gene expression analysis in human meniscus samples. A previous study used GAPDH for gene expression normalization in meniscal tear samples of patients with and without a concomitant ACL tear (Brophy et al., 2012).

In this study, we assessed the suitability of six reference genes frequently reported in the literature (18S, ACTB, B2M, GAPDH, HPRT1 and TBP) using meniscus injured samples of patient with or without concomitant ACL tears as well as meniscus non-injured samples by analyzing gene stability with five software packages and comparative delta cycle threshold ( $\Delta C_t$ ) method.

## 2. Materials and methods

## 2.1. Patients

Tissue samples were obtained from 39 patients with medial meniscal tears, including 19 samples of patients with isolated medial meniscal tears and 20 samples of patients with meniscus injury and a concomitant ACL injury. The following inclusion criteria were employed: age between 18 and 50 years old, clinical history compatible to meniscal injury (such as pain, swelling, stiffness, catching and locking), at least one specific physical examination test positive among McMurray (McMurray, 1949), Appley (Apley, 1947) and Steiman (Tria and Klein, 1992) tests that were used to diagnose meniscus injury (Speziali et al., 2015), magnetic resonance imaging (MRI) diagnosis of medial meniscus injury with abnormal signal extending to at least one articular surface involving the posterior horn and or the body of the medial meniscus (Cruess et al., 1987), and arthroscopic confirmation of the medial meniscus lesion involving its posterior horn and or its body. The Lachman test (Torg et al., 1976), Anterior Drawer test (Marshall et al., 1975), and Pivot-Shift tests (Galway et al., 1972) were used to diagnose ACL injury (Astur et al., 2014a and Astur et al., 2014b). Coronal and sagittal MRI view were used to identify meniscal and ACL lesions. All injuries were confirmed during arthroscopic procedure and reclassified if necessary.

The following exclusion criteria were also applied: medial meniscus lesions treated by suture (outside-in, inside-out or all-inside), medial meniscus stable lesions tested by probe palpation such as some longitudinal lesion < 1 cm, radial lesions < 5 mm, partial thickness lesions. The stable, unstable criteria was defined intra-operatively by the surgeon.

Additionally, 11 patients without any history of meniscal tears were included in this study as a control group. These patients were arthroscopically operated for other knee injuries, such as isolated ACL injury. All control patients were physically active. Table 1 displays the main clinical outcomes of the studied cases and controls.

This study was performed with the approval of the Ethics Committee of the Universidade Federal de São Paulo (UNIFESP), Brazil (CEP #51,436). Written informed consent with approval of the ethics committee was obtained from all patients prior to specimen collection.

## 2.2. Tissue samples

To collect tissue samples, patients were prepared in the standard fashion for arthroscopy meniscus surgery. A standard arthroscopic joint evaluation was carried out, confirming the meniscus injury or meniscus and ACL injuries diagnosis. During surgery, about 5 mm<sup>3</sup> samples of the innermost part of the injured area of the posterior horn and the body of the medial meniscus were collected for gene expression analysis.

In the controls, a sample fragment of about 5 mm<sup>3</sup> was resected from the innermost part of the healthy medial meniscus body by arthroscopy.

All tissue specimens were immediately immersed in Allprotect Tissue Reagent (Qiagen, USA) and stored at -20 °C until RNA extraction.

## 2.3. RNA extraction

Total RNA was extracted from 10 to 20 mg of tissue sample using an AllPrep DNA/RNA/miRNA Mini Kit (Qiagen, USA) according to the manufacturer's protocol. The mechanical lysis step was performed using the Tissue Lyser LT equipment (Qiagen, USA). RNA concentration and quality were immediately determined using a Nanodrop ND-1000 (Thermo Scientific, USA) and the integrity of the RNA was verified by gel electrophoresis on a 1% agarose gel. Aliquots of the total RNA were stored at -80 °C until further use.

## 2.4. RT-qPCR

RT-qPCR gene expression quantifications were performed according to MIQE guidelines (Taylor and Mrkusich, 2014). Only RNA samples with the optical density (OD)<sub>260/280</sub> > 1.8 were used, following the MIQE protocol.

First, cDNA was synthesized from 200 ng of RNA using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) according to the manufacturer's protocol.

To detect the range of expression of the six candidate reference genes, reactions were performed with 75 ng of cDNA input using TaqMan Low-Density Array (TLDA) cards (Life Technologies, USA) and ViiA 7 Real-Time PCR System (Life Technologies, USA). Only inventoried TaqMan

Gene Expression Assays (Life Technologies, USA) were chosen for gene expression analysis. The final volume in each TLDA well is approximately 1  $\mu$ l. All reactions were performed in triplicate.

To identify the best combination of reference genes, we also quantified the mRNA expression of target gene, TGF $\beta$ R1, using the candidate reference genes for normalization. TGF $\beta$ R1 is considered one of the main receptor of TGF $\beta$  and has a key role in the canonical TGF $\beta$  signaling pathway (Moore-Smith and Pasche, 2016). Overexpression of TGF $\beta$  via adeno-associated virus (AAV) is capable of modulating the reparative activities of human meniscal cells, allowing for the healing of meniscal lesions (Cucchiariini et al., 2015). Therefore, TGF $\beta$ R1 may also have a role in meniscal tears and healing. Other 9 target genes (TGF $\beta$ 1, GDF5, COMP, TN $\beta$ CTN $\beta$ XB, FN1, LOX, PLOD1 and PLOD2) were also evaluated to identify the best combination of reference genes (data not shown).

For each sample, the candidate reference and target genes were assayed on the same card to exclude technical variations. The 6 reference genes and target genes are summarized in Table 2.

The relative threshold method (Crt method) was applied, which is a robust method that sets a threshold for each curve individually based on the shape of the amplification curve, regardless of the height or variability of the curve during its early baseline fluorescence. The expression of TGF $\beta$ R1 gene across the samples was calculated using the equation  $\Delta$ Crt, in which [ $\Delta$ Crt = target gene (TGF $\beta$ R1) Crt – the mean of reference genes Crt]. A lower cycle threshold value (Crt) indicates higher gene expression.

#### 2.5. Analysis of reference gene expression stability

We categorized the tissue samples into the following 7 groups: 1) isolated meniscal tear samples (N = 19); 2) meniscal tear samples of patients with a concomitant ACL tear (N = 20); 3) meniscal control samples (N = 11); 4) all injured meniscus (N = 39); 5) isolated meniscal tear samples and controls (N = 30); 6) meniscal tear samples of patients with a concomitant ACL tear and controls (N = 31); 7) all meniscus samples (N = 50). Typically, gene expression studies compare transcript levels between case (i.e., the injured tissue) and control samples, therefore we created the groups #5, #6 and #7. However, some researchers have been investigated a possible association between gene expression and clinical variables (Brophy et al., 2012), therefore we created the groups #1, #2 and #4. In addition, the group composed by only controls (group #3) was created since the understanding of gene expression regulation in non-injured ligaments is still necessary.

For comparisons of candidate reference gene stability we used the software programs NormFinder (<http://123.233.119.36:80/rwt/119/http/P75YPLUNMSXC63DL/publicationsnormfinder.htm>), geNorm (<http://123.233.119.36:80/rwt/119/http/NWTXI35FNZYHK35FN34C6ZUF/~jvdesomp/genorm/>), BestKeeper (<http://123.233.119.36:80/rwt/119/http/P75YPLUHMWYGKLLSPWRX67DJM3SXC7DJN7YC63DF/bestkeeper.html>) and DataAssist (<http://123.233.119.36:80/rwt/119/http/P75YPLUMNFUGK7DFMNVG655MN7UXT3LUF3SX85B/us/en/home/technical-resources/software-downloads/dataassist-software.html>) and the comparative  $\Delta$ Ct method (Silver et al., 2006). We also used the RefFinder software (<http://123.233.119.36:80/rwt/119/http/P75YPLUMMWYX68DJMVYGG55N/referencegene.php>) which integrates the results of geNorm, Normfinder, BestKeeper, and the comparative  $\Delta$ Ct method to compare and rank the tested candidate reference genes.

NormFinder accounts for both intra- and inter-group variations when evaluating the stability of each single reference gene (Andersen et al., 2004). The stability values and standard errors are calculated according to the transcription variation of the reference genes. Stably expressed genes, which have low variation in expression levels, present low stability values. NormFinder analysis also calculated the stability value for two reference genes.

geNorm calculates the expression stability value (M) for each gene based on the average pairwise expression ratio between a particular gene and all other reference genes. geNorm sequentially eliminates the gene that shows the highest variation relative to all the other genes based on paired expression values in all the studied samples. The most stably expressed gene yields the lowest M value, and then the two most stable reference genes are determined by stepwise exclusion of the least

stable gene (Vandesompele et al., 2002). Because of the elimination process, geNorm cannot identify a single suitable reference gene, and ends up by suggesting a pair of genes that shows high correlation and should be suitable for normalization of qPCR studies.

Bestkeeper was used to rank the 6 reference genes based on the standard deviation (SD) and coefficient of variance (CV) expressed as a percentage of the cycle threshold (Ct) level (Pfaffl et al., 2004). The more stable reference gene presents the lowest CV and SD. Bestkeeper also uses a statistical algorithm wherein the Pearson correlation coefficient for each candidate reference gene pair is calculated along with the probability of correlation significance of the pair.

DataAssist software provided a metric to measure reference gene stability based on the geNorm algorithm. In contrast to the other programs, DataAssist uses the relative quantification (RQ) to calculate the stability value of individual candidate reference genes. The lower score represents the more stable the control.

The comparative  $\Delta$ Ct method is based on the comparing relative expression of pairs of possible reference genes within each sample. The stability of the candidate housekeeping genes is ranked according to reproducibility of the gene expression differences among studied samples.

Lastly, RefFinder assigns an appropriate weight to an individual gene and calculated the geometric mean of their weights for the overall final ranking based on the rankings from geNorm, Normfinder, BestKeeper, and the comparative  $\Delta$ Ct.

GenEx software

(<http://123.233.119.36:80/rwt/119/http/M7TX63LZF3UXK5UFFWZYKZLPPSVXMZ5BPSVX85SPNFYGM5D/>) was used to determine the optimal number of reference genes by calculating the accumulated standard deviation (Acc.SD). If larger number of reference genes is used, random variation among the genes' expression partially cancel reducing the SD. A minimum in the Acc.SD plot indicate the number of reference genes that give the lowest SD.

## 2.6. Statistical analysis

To compare TGF $\beta$ R1 expression between the groups, we first verified the distribution of the data using the Shapiro–Wilk normality test for the determination of the appropriate tests for the subsequent statistical comparisons. TGF $\beta$ R1 expression ( $\Delta$ Crt) was not normally distributed. Therefore, the Mann–Whitney test was performed to compare TGF $\beta$ R1 expression between the studied groups. A p-value of < 0.05 was considered statistically significant.

## 3. Results

### 3.1. Reference gene expression levels

The distribution of Crt values for each of the 6 candidate reference genes is shown in Fig. 1. These genes displayed a wide range of expression levels. 18S presented the highest expression level among the candidate reference genes (mean Crt value  $\pm$  SD:  $12.52 \pm 2.34$ ). In contrast, TPB ( $31.16 \pm 1.61$ ) and HPRT1 ( $31.85 \pm 1.79$ ) presented the lowest expression level in meniscal samples.

### 3.2. Reference gene expression stability

Table S1 displays the stability value ranking of single candidate reference genes as determined by the different software packages and comparative  $\Delta$ Ct method. In our analysis, all the reference genes for all analysis groups presented M values less than the geNorm threshold of 1.5, which is recognized as stable (Table S1). However, 18S, ACTB, and B2M presented high SD ( $\pm$  x-fold) of Crt in the analysis involving all samples according to BestKeeper software [SD ( $\pm$  x-fold) = 3.21, 3.64, and 3.41, respectively), whereby any studied gene with SD ( $\pm$  x-fold) > 3 can be considered inconsistent. Furthermore, 18S, ACTB, and B2M presented high SD ( $\pm$  x-fold) in the analysis of (1) all meniscal tear samples, (2) isolated meniscal tears, and (3) isolated meniscal tears and controls. Moreover, ACTB and B2M presented high SD ( $\pm$  x-fold) in the analysis of (1) meniscal tear samples of patients with concomitant ACL tears and (2) meniscal tear samples of patients with concomitant ACL tears and controls. ACTB demonstrated high SD ( $\pm$  x-fold) in the analysis involving only controls.

Although none of the software packages and comparative  $\Delta$ Ct method suggested the same rank of reference genes in the studied sample groups, the methods applied did generate similar rankings of reference gene stability for each analysis group (Table S1).

Table 3 shows the best suitable reference gene by the different methods applied. In the present study, HPRT1 was the most suitable reference gene for the meniscus samples. As previously described, gene expression studies typically compare transcript levels between injured and non-injured tissue samples. When the isolated meniscus tear samples and controls were evaluated together, HPRT1 and GAPDH were the most suitable reference genes. When considering the meniscal tear samples of patients with a concomitant ACL tear and controls, TBP followed by GAPDH was the most stable gene. When considering all injured meniscal tear samples and controls (all samples), HPRT1 followed by TBP was also the most stable gene ( Table 3; Table S1).

When we individually evaluated each group of meniscus samples, we observed that HPRT1 followed by TBP was the most stable gene for the isolated meniscal tear samples as well as for meniscal tear samples of patients with a concomitant ACL tear. In the control group, GAPDH and 18S were the most stable genes. HPRT1 followed by TBP was the most suitable gene in the analysis involving all meniscal tear samples ( Table 3; Table S1).

### 3.3. Analysis of the best combinations of reference genes

Table 4 displays the best combinations of reference genes, as suggested by the software packages, comparative  $\Delta C_t$  method, and by visual inspection of all the ranks generated by these analyses. Overall, HPRT1 + TBP and HPRT1 + GAPDH pairs of genes were the most frequently identified pairs. HPRT1 + TBP was the most frequently identified pair from the analysis of samples from (1) meniscal tear samples of patients with a concomitant ACL tear, (2) all meniscal tears, and (3) all samples. HPRT1 + GAPDH was the most frequently identified pair from the analysis of samples from isolated meniscal tear samples and controls. In the analysis involving only controls, GAPDH + 18S was the most frequently identified pair. In the analysis of only isolated meniscal tear samples and in the analysis of meniscal tear samples of patients with concomitant ACL tears and controls, no more than two methods agreed in the definition of the best pair ( Table 4).

The NormFinder, GeNorm, DataAssist, and BestKeeper software packages only indicated up to 2 genes as the best combination of reference genes. By visual inspection of all the ranks generated, software used, and comparative  $\Delta C_t$  method, we observed that HPRT1 + TBP + GAPDH was more frequently the best trio of reference genes. HPRT1 + TBP + 18S was the best trio only in the analysis of meniscal tear samples from patients with concomitant ACL tears. Additionally, GAPDH + TBP + 18S was the best trio in the analysis involving only control samples.

We used the GenEx software package to determine the appropriate number of reference genes for a reliable normalization. In this analysis, the optimal number of reference genes is indicated by the lowest SD. In all analyses, the Acc.SD of two reference genes did not differ by  $> 0.1$  from the observed metric when using more than two genes (Fig. 2). However, one reference gene is not suitable for gene expression normalization in the analysis involving (1) isolated meniscal tears and controls or (2) meniscal tears of patients with concomitant ACL tears and controls. In these groups of samples, the Acc.SD of one reference gene was  $> 0.1$  from the observed metric when using two or more genes (Fig. 2). Moreover, in the analysis involving all meniscal samples, the Acc.SD of one reference gene was  $> 0.1$  from the observed metric when using four or more genes (Fig. 2). In the analysis of meniscal tears of patients with concomitant ACL tears, the Acc.SD of one reference gene was also  $> 0.1$  from that observed when using more than three genes, which presented the lowest Acc. SD.

### 3.4. Effects of reference gene choice

To evaluate the effect of appropriate reference gene selection, an expression analysis was performed by comparing data from 1) meniscal tears from patients with and without a concomitant ACL tear, 2) isolated meniscal tear samples and controls, 3) meniscal tear samples of patients with a concomitant ACL tear and controls, and 4) injured meniscus samples and controls. This analysis was performed using TGF $\beta$ R1 as the target gene in all the analyses. For reference genes, we used the most frequently identified pairs described above (HPRT1 + TBP, GAPDH + 18S, HPRT1 + GAPDH, and GAPDH + TBP). We also performed collagen genes expression analysis using three reference genes (HPRT1 + TBP + GAPDH, HPRT1 + TBP + 18S, and GAPDH + TBP + 18S), four reference genes (HPRT1 + TBP + GAPDH + 18S), and all studied reference genes. TGF $\beta$ R1 was also normalized by

only 18S, ACTB, GAPDH, and HPRT1, as commonly described in the literature involving joint lesions. B2M + 18S was used as an example of a not suitable reference gene pair for gene expression normalization in meniscus samples.

Although the normalized expression quantities differed between the various combinations of reference genes, the distributions of the target gene expression in the studied samples were similar (Fig. 3).

Table 5 shows TGF $\beta$ R1 expression using the different reference gene combinations for data normalization. TGF $\beta$ R1 expression was significantly increased in the isolated meniscal tear samples compared with the controls when using several reference gene combinations ( $p < 0.05$ ), except for HPRT1 ( $p = 0.121$ ), HPRT1 + TBP ( $p = 0.061$ ), and B2M + 18 ( $p = 0.077$ ).

Alternatively, TGF $\beta$ R1 expression was significantly increased in the meniscal tear samples from patients with concomitant ACL tears compared with the controls only when using GAPDH ( $p = 0.0212$ ) as a reference gene. Moreover, all injured meniscal samples presented increased TGF $\beta$ R1 expression compared with controls only when the target expression was normalized by GAPDH ( $p = 0.008$ ) or GAPDH + 18S ( $p = 0.043$ ).

When we compared isolated meniscus tear samples and meniscal samples from patients with ACL tears, TGF $\beta$ R1 was significantly different between groups when its expression was normalized by B2M + 18S ( $p = 0.049$ ).

#### 4. Discussion

Comparing gene expression in different samples may generate misleading information related to distinctive amounts of material, RNA extraction and efficiency of reverse transcription. To overcome those risk potentials and be accurate, RT-qPCR relies on normalization to an internal control, often referred to as a housekeeping gene. Housekeeping genes are constitutive genes which are transcribed at a relatively constant level. Its products are vital to the basic functions of a cell. It is generally assumed that their expression is minimally affected by experimental conditions. Related to these characteristics, some candidates housekeeping genes are used on various studies to have its stability ranked according to the reproducibility of differences in gene expression among the studied samples. This stability analysis helps to select the most appropriate candidate reference gene (Leal et al., 2014; 2015a; 2015b).

Reference genes have been described for RT-qPCR studies in several diseases and tissues (Yuzbasioglu et al., 2010, Pombo-Suarez et al., 2008, Zhou et al., 2014, Lyng et al., 2008, Rubie et al., 2005, Fu et al., 2010, Wang et al., 2012 and Wisnieski et al., 2013), and our group recently identified the most stable reference genes in the glenohumeral capsule of patients with and without shoulder instability (Leal et al., 2014), in tendon of patients with and without rotator cuff tears (Leal et al., 2015a) and in ACL of patients with or without ACL tears (Leal et al., 2015b). To the best of our knowledge, no prior study has aimed to identify suitable reference genes for gene expression analyses by quantitative approaches in the human meniscus.

In the present study, we used five software packages (NormFinder, geNorm, BestKeeper, DataAssist, and RefFinder) and the comparative  $\Delta$ Ct method to evaluate the stability of reference gene expression. As each analysis uses distinct algorithms, different results can be expected. Therefore, it is important to use more than one software package/method to identify the most suitable reference genes among a set of candidates. Although the distinct analysis differed in the rankings of reference gene stability as well as in the identity of the most suitable pair, at least two programs produced results that agreed for almost all the analyses. Our results demonstrate that the use of 5 statistical tools and comparative  $\Delta$ Ct method aids in the identification of the best reference genes.

Surprisingly, Normfinder, geNorm, and BestKeeper from RefFinder did not lead to the same output as we got from our NormFinder, geNorm, and Bestkeeper interface (data not shown), which is probably due to different versions of the algorithm. This lack of agreement was previously reported in literature (Llanos et al., 2016).

All the reference genes in this study were recognized as stable by geNorm analysis under the different experimental conditions tested. However, 18S, ACTB, and B2M were not suitable by

BestKeeper software in most of the groups of analyses. According to BestKeeper analysis, these reference genes should not be used in studies involving isolated meniscal tears, meniscal tears of patients with and without concomitant ACL injury, as well as in a study involving all types of meniscal samples investigated here.

In the different groups of analyses, HPRT1 appeared to be the most suitable gene overall. GAPDH was also previously used as a reference gene in a gene expression study from human meniscal tears with and without concomitant ACL injury (Brophy et al., 2012); however, this gene was the most stable only in some analyses with meniscal control samples and with isolated meniscal tear samples and controls.

It is increasingly clear that in most situations, a single reference gene is not sufficiently stable (de Jonge et al., 2007). Here, we observed that the use of one reference gene is not appropriate, mainly for the analyses involving meniscal tear samples and controls.

In our study, a pair of reference genes seems to be suitable for gene expression normalization in the studied groups. When a larger number of reference genes is used, the SD of the normalization factor (mean of reference gene expression) is reduced, and the random variation among the expression of the tested genes is partially canceled. As the inclusion of additional reference genes increases the time and money required for the analysis, it is important to consider the degree of improvement and overall noise contributed by reference genes when deciding how many reference genes are required. Considering the reproducibility of real-time PCR equipment, we believe that the use of several reference genes does not significantly improve the data quality. However, it is important to highlight that we observed the use of one, two, three, or more reference genes may lead to differences in the statistical analysis result of some group comparisons.

Although different combinations of reference genes were determined as being the most suitable for the various analysis, HPRT1 + TBP and HPRT1 + GAPDH were the most frequently identified pair, and HPRT1 + TBP + GAPDH was the most frequently identified trio. The selection of the appropriate pair or trio should consider the group of meniscus samples that will be investigated. For example, HPRT1 + TBP + 18S was the best trio only in the analysis of meniscal tear samples of patients with concomitant ACL tears, and GAPDH + TBP + 18S was the best trio in the analysis involving only control samples, which is in agreement with the observation that 18S may be stable in these groups according to BestKeeper software.

To identify the best combination of reference genes, we evaluated TGF $\beta$ R1 expression in samples from meniscus tissue of the cases and the controls. Statistical comparison revealed that TGF $\beta$ R1 expression differed between the isolated meniscal tear samples and the controls when using several reference gene combinations ( $p < 0.05$ ), except for HPRT1, HPRT1 + TBP, and B2M + 18. This finding reinforces the use of one reference gene (even the most stable) is not appropriate for gene expression normalization in this group of samples. Moreover, HPRT1 + TBP and B2M + 18 were not the best pair in this group of samples. Only geNorm suggested that HPRT1 + TBP were the most stable genes in the analysis involving isolated meniscal tear samples and controls.

HPRT1 + TBP was the best pair of reference genes in the analysis involving all injured meniscus samples and controls, but not when meniscus tear samples from patients with or without ACL injury were grouped separately with controls. Consequently, if a gene expression study aims to compare non-injured menisci, isolated meniscal tears and meniscal tears of patients with ACL tears as three independent groups of analysis, HPRT1 + TBP + GAPDH seems to be the most suitable combination of reference genes. This trio of reference genes was the most suitable for the different comparisons involving injured and non-injured meniscus samples, as well as in the analysis involving injured meniscus samples of patients with and without ACL tears.

When meniscal tear samples of patients with concomitant ACL tears were compared with the controls, no significant difference was detected, except when TGF $\beta$ R1 expression was normalized only by GAPDH. The use of GAPDH as well as GAPDH + 18S for normalization also lead to the observation that TGF $\beta$ R1 expression differed between meniscal tear samples and controls. As already discussed, these analyses highlight the necessity of the selection of suitable reference genes based on the studied meniscus samples.



During this study, we also evaluated the use of different reference gene combinations in the expression of other nine extracellular matrix genes (data not show). No significance difference was detected between the studied groups using the different reference gene combinations.

Our study presented some limitations. First, we only included a limited number of candidate reference genes, and it is likely that some other genes may also be used as internal references for gene expression studies in meniscus samples from patients with or without history of a meniscal tear. Second, the number of samples available for the Mann–Whitney test was reduced, especially in the control group. However, to the best of our knowledge, no prior study evaluated RNA expression in human non-injured meniscus samples. Nevertheless, it is important to highlight that our results may be relevant to the study of meniscal tears, as well as to the study of normal menisci.

#### 4. Discussion

The use of suitable reference genes for a reliable gene expression evaluation using RT-qPCR should consider the type of meniscus samples investigated (injured or non-injured). HPRT1 was the most suitable reference gene. However, the use of only one reference gene does not seem suitable for gene expression normalization in meniscal tear studies. HPRT1 + TBP and HPRT1 + TBP + GAPDH were the best combination of reference genes for the analysis of involving meniscus samples. However, if the gene expression study aims to compare non-injured menisci, isolated meniscal tears and meniscal tears from patients with ACL tears as three independent groups, HPRT1 + TBP + GAPDH is the most suitable combination of reference genes. The results of this work may benefit future studies of the meniscus that require more accurate gene expression quantification.

The following is the supplementary data related to this article.

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