Professores Titulares: Prof. Dr. Walter Manna Albertoni
Prof. Dr. Flávio Faloppa
Prof. Dr. Akira Ishida
Prof. Dr. Moisés Cohen

Chefê do Departamento: Prof. Dr. Moisés Cohen
GENETIC AND MOLECULAR FACTORS AND ANTERIOR CRUCIATE LIGAMENT INJURIES
INTRODUCTION
✓ Risk factors for ACL Injury:

✓ What about molecular and genetical risks?? Do they really care?

✓ The higher incidence of any specific gene can increase the risk of ACL injury?
CONTRIBUTION

How to determine the influence of a gene in a pathology related to a joint?

BUT...

Contribution of genetic factors is well known in the pathogenesis of various diseases.
INTRODUCTION

2007

ORTHOPAEDICS + GENETICS

REVIEW

Tendon and ligament injuries: the genetic component

Alison V September, Martin P Schwellnus, Malcolm Collins

INTRODUCTION

- **GENE EXPRESSION**: is the process by which information from a gene is used in the synthesis of a functional gene product. These products are often proteins.

- **POLYMORPHISM**: is the occurrence of two or more clearly different morphs or forms, also referred to as alternative phenotypes, in the population of a species.
INTRODUCTION

GENE EXPRESSION \( \times \) POLYMORPHISM

RNA
Ribonucleic acid

DNA
Deoxyribonucleic acid

Each tissue has different expression

Same cell all body
INTRODUCTION

- Detailed analysis of patients with bilateral anterior cruciate ligament injuries
- Genetic risk factors for anterior cruciate ligament ruptures: COL1A1 gene variant
- The COL5A1 gene is associated with increased risk of anterior cruciate ligament ruptures
- Association between matrix metalloproteinase-3 polymorphism and anterior cruciate ligament ruptures
- Matrix metalloproteinase genes on chromosome 11q22 and the risk of anterior cruciate ligament (ACL) rupture
- Gene variants within the COL1A1 gene are associated with reduced anterior cruciate ligament rupture risk
- The association of genes involved in angiogenesis-associated signaling pathway with risk of anterior cruciate ligament rupture
- Interactions between collagen gene variants and risk of anterior cruciate ligament tear
- Is there a genetic predisposition to anterior cruciate ligament tear?
- Changes in transcriptome-wide gene expression of ACL tears
CURRENT CONCEPTS

Genetic and molecular factors and anterior cruciate ligament injuries: current concepts

Diego Costa Astur, João Victor Novaretti, Moises Cohen
GENETIC AND MOLECULAR FACTORS

ACL INJURY RISK FACTORS

ENVIRONMENTAL
ANATOMICAL
GENETIC AND MOLECULAR
BIOMECHANICAL
HORMONAL

COLLAGEN
ANGIOGENESIS-ASSOCIATED SIGNALING PATHWAY
PROTEOGLYCANS
MATRIX METALLOPROTEINASE

COL1A1 COL3A1 COL5A1 COL6A1 COL12A1
AGRECCAN DECORIN MMP-3 MMP-12
EVALUATE EACH DIFFERENT COMPONENT AROUND ANTERIOR CRUCIATE LIGAMENT AND INJURY RISK
HOW TO EVALUATE GENE EXPRESSION?

Reverse Transcription - quantitative polymerase chain reaction (RT-qPCR)

RNA(unstable) → DNA (stable)

Sample extraction  RNA extraction  Reverse Transcription  5 software package to test samples  Gene validation
To know a gene expression we need to determine reference genes.

“Reference genes are the Gold-Standard and Target Genes are the new diagnosis.”

A common method for obtaining reliable data through RT-qPCR is to normalize the Target Gene Expression by using an endogenous Reference Gene.
Assessed the suitability of six reference genes frequently reported in the literature (18S, ACTB, B2M, GAPDH, HPRT1, eTBP), by using ACL injury samples of patients with or without meniscal tears to analyse the gene stability.
- **18S** presenting the highest expression (mean Crt value: 10.85 ± 1.63).

- TPB (Crt: 29.96 ± 1.36) and HPRT1 (Crt: 29.69 ± 1.30) had the lowest expression levels.
Reference gene expression stability

All reference genes **STABLE**

**ACTB**: the most suitable reference gene

<table>
<thead>
<tr>
<th>Groups</th>
<th>NormFinder</th>
<th>GeNorm</th>
<th>BestKeeper</th>
<th>DataAssist</th>
<th>ΔCt method</th>
<th>RefFinder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated ACL tear samples</td>
<td>HPRT1</td>
<td>ACTB/18S</td>
<td>TPB</td>
<td>ACTB</td>
<td>HPRT1</td>
<td>HPRT1</td>
</tr>
<tr>
<td>ACL tear samples of patients with a concomitant meniscal tear</td>
<td>ACTB</td>
<td>ACTB/TBP</td>
<td>HPRT1</td>
<td>ACTB</td>
<td>ACTB</td>
<td>ACTB</td>
</tr>
<tr>
<td>ACL controls</td>
<td>ACTB</td>
<td>HPRT1/GAPDH</td>
<td>TPB</td>
<td>ACTB</td>
<td>ACTB</td>
<td>ACTB</td>
</tr>
<tr>
<td>All injured ACL samples</td>
<td>ACTB</td>
<td>ACTB/TBP</td>
<td>HPRT1</td>
<td>ACTB</td>
<td>ACTB</td>
<td>ACTB</td>
</tr>
<tr>
<td>Isolated ACL tear samples and controls</td>
<td>18S*</td>
<td>ACTB/18S</td>
<td>TPB</td>
<td>ACTB</td>
<td>ACTB</td>
<td>ACTB</td>
</tr>
<tr>
<td>ACL tear samples of patients with a concomitant meniscal tear and controls</td>
<td>ACTB</td>
<td>ACTB/18S</td>
<td>HPRT1</td>
<td>ACTB</td>
<td>ACTB</td>
<td>ACTB</td>
</tr>
<tr>
<td>All ACL samples</td>
<td>ACTB</td>
<td>ACTB/18S</td>
<td>HPRT1</td>
<td>ACTB</td>
<td>ACTB</td>
<td>ACTB</td>
</tr>
</tbody>
</table>
Best combinations for reference genes
most frequently identified

Best PAIR
ACTB + 18S
ACTB + TBP

Best TRIO
ACTB + TBP + 18S
ACTB + HPRT1 + 18S
To identify the best combination of reference genes, we also quantified the mRNA expression of target genes: FN1 and PLOD1

- **Fibronectin (FN1):** glycoprotein, promote adhesion, tissue development, and wound healing

- **Lysyl hydroxylases 1** (encoded by PLOD1) promote extracellular matrix structural stability and maturation
GENETIC AND MOLECULAR FACTORS AND ANTERIOR CRUCIATE LIGAMENT INJURIES

GENE EXPRESSION

Effects of reference gene choice

Target gene

FN1
PLOD 1

18S
ACTB
GAPDH

ACTB + 18S
ACTB + TBP
ACTB + TBP + 18S
ACTB + HPRT1 + 18S
ACTB + TBP + 18S + HPRT1

ACTB + HPRT1

TRIO

literature
+ usual

Reference genes TRIO

4 Reference genes

usual Reference genes
FN1 expression was reduced using all six reference genes in ACL injury groups (p < 0.05)

<table>
<thead>
<tr>
<th>Reference genes</th>
<th>ACL-I</th>
<th>ACL-M</th>
<th>p-value</th>
<th>ACL-I</th>
<th>ACL-C</th>
<th>p-value</th>
<th>ACL-M</th>
<th>ACL-C</th>
<th>p-value</th>
<th>ACL-I + ACL-M</th>
<th>ACL-C</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>14.46 ± 1.38</td>
<td>13.89 ± 1.15</td>
<td>0.030*</td>
<td>14.46 ± 1.38</td>
<td>12.96 ± 1.71</td>
<td>&lt;0.001*</td>
<td>13.88 ± 1.15</td>
<td>12.96 ± 1.71</td>
<td>0.223</td>
<td>14.17 ± 1.29</td>
<td>12.96 ± 1.71</td>
<td>0.001*</td>
</tr>
<tr>
<td>ACTB</td>
<td>3.81 ± 1.40</td>
<td>3.41 ± 1.35</td>
<td>0.004</td>
<td>3.81 ± 1.39</td>
<td>2.53 ± 1.82</td>
<td>0.001*</td>
<td>3.51 ± 1.35</td>
<td>2.53 ± 1.82</td>
<td>0.181</td>
<td>3.66 ± 1.37</td>
<td>2.53 ± 1.82</td>
<td>0.017*</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1.83 ± 1.42</td>
<td>1.45 ± 1.21</td>
<td>0.029*</td>
<td>1.84 ± 1.42</td>
<td>0.15 ± 1.61</td>
<td>&lt;0.001*</td>
<td>1.45 ± 1.21</td>
<td>0.15 ± 1.61</td>
<td>0.021*</td>
<td>1.65 ± 1.32</td>
<td>0.15 ± 1.61</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>ACTB + TBP</td>
<td>-0.52 ± 1.42</td>
<td>-0.75 ± 1.38</td>
<td>0.194</td>
<td>-0.52 ± 1.42</td>
<td>-1.83 ± 1.75</td>
<td>0.002*</td>
<td>-0.75 ± 1.38</td>
<td>-1.83 ± 1.75</td>
<td>0.117</td>
<td>-0.63 ± 1.38</td>
<td>-1.83 ± 1.75</td>
<td>0.011*</td>
</tr>
<tr>
<td>ACTB + 18S</td>
<td>9.13 ± 1.38</td>
<td>8.69 ± 1.23</td>
<td>0.049*</td>
<td>9.13 ± 1.38</td>
<td>7.75 ± 1.76</td>
<td>&lt;0.001*</td>
<td>8.69 ± 1.23</td>
<td>7.75 ± 1.76</td>
<td>0.220</td>
<td>8.92 ± 1.31</td>
<td>7.75 ± 1.76</td>
<td>0.016*</td>
</tr>
<tr>
<td>ACTB + TBP + 18S</td>
<td>4.47 ± 1.40</td>
<td>4.13 ± 1.28</td>
<td>0.082</td>
<td>4.47 ± 1.40</td>
<td>3.10 ± 1.72</td>
<td>0.001*</td>
<td>4.13 ± 1.28</td>
<td>3.10 ± 1.73</td>
<td>0.153</td>
<td>4.31 ± 1.34</td>
<td>3.10 ± 1.72</td>
<td>0.011*</td>
</tr>
<tr>
<td>ACTB + HPRT1 + 18S</td>
<td>4.63 ± 1.39</td>
<td>4.22 ± 1.35</td>
<td>0.045*</td>
<td>4.62 ± 1.39</td>
<td>3.13 ± 1.74</td>
<td>&lt;0.001*</td>
<td>4.21 ± 1.35</td>
<td>3.13 ± 1.73</td>
<td>0.066</td>
<td>4.42 ± 1.37</td>
<td>3.13 ± 1.74</td>
<td>0.004*</td>
</tr>
<tr>
<td>ACTB + TBP + HPRT1 + 18S</td>
<td>2.56 ± 1.40</td>
<td>1.91 ± 1.37</td>
<td>0.071</td>
<td>2.26 ± 1.40</td>
<td>1.91 ± 1.37</td>
<td>&lt;0.001*</td>
<td>1.91 ± 1.37</td>
<td>0.81 ± 1.72</td>
<td>0.089</td>
<td>2.09 ± 1.38</td>
<td>0.81 ± 1.72</td>
<td>0.005*</td>
</tr>
</tbody>
</table>
LYSIL HIDROXYLASE (PLOD1)

PLOD1 expression was increased in the ACL injury group using as reference gene:

<table>
<thead>
<tr>
<th>Reference genes</th>
<th>PLOD1 expression (ΔCrt; mean ± SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACL-I</td>
</tr>
<tr>
<td>18S</td>
<td>16.09 ± 0.79</td>
</tr>
<tr>
<td>ACTB</td>
<td>5.46 ± 0.67</td>
</tr>
<tr>
<td>GAPDH</td>
<td>3.48 ± 0.40</td>
</tr>
<tr>
<td>ACTB – TBP</td>
<td>1.12 ± 0.80</td>
</tr>
<tr>
<td>ACTB – 18S</td>
<td>10.77 ± 0.72</td>
</tr>
<tr>
<td>ACTB – TBP + 18S</td>
<td>6.11 ± 0.78</td>
</tr>
<tr>
<td>ACTB – HPRT1 + 18S</td>
<td>6.26 ± 0.69</td>
</tr>
<tr>
<td>ACTB – TBP + HPRT1 + 18S</td>
<td>3.89 ± 0.75</td>
</tr>
</tbody>
</table>
GENETIC AND MOLECULAR FACTORS AND ANTERIOR CRUCIATE LIGAMENT INJURIES

GENE EXPRESSION

SAMPLE

All Protect Tissue Reagent (Qiagen, USA®)
- 20°C for RNA extraction
GENETIC AND MOLECULAR FACTORS AND ANTERIOR CRUCIATE LIGAMENT INJURIES

GENE EXPRESSION

Relationship between 24 involved genes and ACL injury

Mann-Whitney Test

TGFBR1 (ΔCtrl)

p=0.001

ACTB

HPRT1

TBP

18S

COMP (ΔCtrl)

p=0.029

FN1 (ΔCtrl)

p=0.05

TXN (ΔCtrl)

p<0.001

LOX (ΔCtrl)

p=0.0014

ACL INJURY

ACL NON INJURY

Glycoprotein extracellular matrix code GENES
GENETIC AND MOLECULAR FACTORS AND ANTERIOR CRUCIATE LIGAMENT INJURIES

GENE EXPRESSION

24 INVOLVED GENES

- COL1A1
- COL2A1
- COL3A1
- COL5A1
- COL5A2
- COL14A1
- COL12A1
- COL1A2
- GDF7
- THBS4
- COMP
- LOX
- TGBF1
- GDF6
- FN1
- TNXB
- FN1
DNA Polymorphisms

Metalloproteinases

- MMP3
- MMP9

Collagen genes

- COL3A1
- COL5A1

AND +++ Total: 19 genes and 31 SNPs involved

X

RISK OF ACL AND MM TEARS

DNA samples of 238 individuals with ACL tear, 171 individuals with MM tear and 1042 paired sample controls.
POLYMORPHISMS

DNA polymorphisms in Metalloproteinases and Collagen genes

• **A/A** (rs17576/rs17577) haplotype to **MMP9** gene may be associated as a **PROTECTIVE FACTOR** to **ACL tear** \( (p=0.045; \ OR=0.12; \ CI \ 95\%=0.01-0.95) \).

• Additive model to **C** allele in rs522616 (**MMP3**) as a **PROTECTIVE FACTOR** to **MM** injury \( (p=0.0023; \ OR=0.59; \ CI \ 95\%=0.42-0.84) \).

• **GG** genotype in rs3106796 (**COL3A1**) was a **RISK FACTOR** to **MM** injury \( (p=0.021; \ OR=2.47; \ CI \ 95\%=1.13-5.39) \).

• **C/T** (rs3196378/rs12722) haplotype to **COL5A1** gene may be considered as a **PROTECTIVE FACTOR** to **MM** injury \( (p=0.0091; \ OR=0.07; \ CI \ 95\%=0.01-0.50) \).

• **A/C** haplotype to **COL5A1** also may be considered a **PROTECTIVE FACTOR** to this injury \( (p=0.0073; \ OR=0.06; \ CI \ 95\%=0.01-0.46) \).

• The **C/T** (rs679620/rs522616) to **MMP3** gene may be considered a **RISK FACTOR** to **MM tear** \( (p=0.0098; \ OR=1.60; \ CI \ 95\%=1.12-2.29) \).
It is difficult to come to a conclusion.

Results can not be extrapolated to the general population.

More studies are needed in a larger populations of different ethnicities.

More research is needed to support a clear association between ACL rupture and genetic variants.
GENETIC AND MOLECULAR FACTORS AND ANTERIOR CRUCIATE LIGAMENT INJURIES

PERSPECTIVES AND FUTURE DIRECTIONS

What do we think about?

YES

✓ GENE IS A RISK FACTOR FOR ACL INJURY
✓ EXPENSIVE STUDIES, LARGE POPULATIONS
✓ MORE AND MORE STUDIES
GENETIC AND MOLECULAR FACTORS AND ANTERIOR CRUCIATE LIGAMENT INJURIES

SÃO PAULO, BRAZIL

谢谢