

# Development of *COL3A1*-Specific Real-Time PCR Assay for Clinical and Sport Detection Applications

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#### Abstract

PIIINP, whose gene is *COL3A1*, is a protein used as a biomarker, which is altered in various pathologies such as fatty liver of non-alcoholic origin and is also used to detect abuse of human recombinant growth hormone (rhGH) by elite athletes to improve their performance. Therefore, the study of the levels of this protein or its gene could be useful for the diagnosis of these pathologies, in addition to other uses. The objective of this work was the development of a new detection method of the *COL3A1* gene based on the quantification of mRNA in real time. 10 healthy men volunteers were injected intravenously for 3 consecutive days with 0.026 mg<sup>-1</sup>kg<sup>-1</sup>day<sup>-1</sup>person<sup>-1</sup> of rhGH, and serum and blood samples were collected for 7 days at different times. mRNA extraction was performed after lymphocyte purification following standard protocols, and samples were quantified by quantitative real-time PCR using TaqMan probes designed specifically for this gene. The results showed an increase in *COL3A1* expression that was maintained during 7 days of sampling after the last administration, observing more sensitive and robust results when compared to quantification of the PIIINP protein in serum from the same individuals. After the trial set-up, a partial validation was carried out in which parameters such as linearity, inter and intra-assay variation, influence of sex and age, stability of blood samples storage at 4°C and amount of blood sample, were studied.

**Keywords:** *COL3A1*; PIIINP; Collagen; Quantitative real time PCR; Doping abuse

#### Introduction

*COL3A1* gene is located in the autosomic chromosome 2 and encodes the pro-alpha1 chains of type III collagen (PIIINP) protein [1,2]. Collagens are a family of proteins that strengthen and support many tissues in the body and, particularly, PIIINP is a fibrillar collagen that is located in most soft connective tissues along with type I collagen such as skin, lung, uterus, intestine walls and blood vessels walls. Among other functions, it is involved on cortical development regulation, and is the major ligand of ADGRG1 in the developing brain. The C-terminal propeptide, also known as COLFI domain, has crucial roles in tissue growth and repair by controlling both, the intracellular assembly of procollagen molecules and the extracellular assembly of collagen fibrils. It binds a calcium ion which is essential for its function [3].

Several disorders have been described due to mutations in this gen or alterations in the control of its regulation. Some of these are the Ehlers-Danlos syndrome type IV [4], which consists in a connective tissue disorder characterized by hyper extensible skin, atrophic cutaneous scars due to tissue fragility and joint hyperlaxity; tympanosclerosis [5], characterized by the formation of calcium deposits in the middle ear; aortic and arterial aneurysms [6], which are an abnormal dilation or widening of a portion of an artery, due to a weakness of the blood vessel wall; non-alcoholic fatty liver disease [7], that is marked by liver inflammation, which may progress to scarring and irreversible damage. On the other hand, the PIIINP protein levels can be also altered by the treatment with other drugs as the human recombinant growth hormone (rhGH), which is used in children with growth disorders; but it is also used by cheater athletes in the world of professional competition to improve their performance, so some test detections have been developed by the World Anti-Doping Agency (WADA) to detect this sport abuse [8-10].

Currently, there are several validated methods to detect variations in the PIIINP protein levels, but there is no method for the detection of variations of the COL3A1 gene. Among the protein detection methods there are two assays based on radioimmunoassay: the Cisbio RIA-gnost PIIINP and the Orion UniQ" PIIINP RIA; and an assay based on an immunoassay platform: Siemens ADVIA Centaur PIIINP. However, these assays have limitations. Among them it is important to mention its dependence on the gender and age [8,11-14], the fact that only serum (but not plasma) can be used [15], also the faster elimination of the PIIINP and, in case of radioactive methods, the need for high security facilities as well as the risk involved in the handling of these radioactive components. Therefore, the development of new techniques which are reliable, robust and sensitive are still appropriate. Regarding the detection methods of genetic level variation, no validated method for this gene is known. But some non- validated methods based on quantitative PCR have been developed in the study of some pathologies

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related with changes in the COL3A1 RNA levels [5,16,17].

In this context, we have recently established the optimal experimental conditions for the detection of the *COL3A1* gene by quantitative realtime PCR (RTqPCR). For this we were based on the previous knowledge, which indicates that the rhGH treatment produces alterations in the PIIINP protein levels. In this paper, we have focused on develop and assess of this detection method. In this context, a clinical trial with 14 healthy men volunteers that were treated with a low dose (0.026 mg<sup>-1</sup>kg<sup>-1</sup>day<sup>-1</sup>person<sup>-1</sup> s.c.) of rhGH for three consecutive days, blood samples (serum, plasma and PBL) were collected for ten days after the first dose [18]. *COL3A1* gene was evaluate in these samples by RTqPCR approaches and compared with the PIIINP serum levels.

Also, a validation protocol of the method was assessed, and some aspect as the potential influence of gender, age, accuracy, linearity, stability of blood samples storage at 4°C and amount of blood sample were tested, suggesting no influence of these potential confounding factors. Overall, a new validated genetic protocol with high robustness, sensitivity and wide detection time window is proposed for the detection of the COL3A1 gene and could be used to support the diagnosis of diseases related to this gene, and for the detection of rhGH abuse in cheating athletes.

## **Methods and Materials**

### GH treatment in human subjects: Study design

Blood samples from the clinical trial performed at the Barcelona Antidoping laboratory were kindly provided by Professor Jordi Segura. This clinical trial was performed with 14 healthy men volunteers in a randomized and controlled fashion. Recombinant 22 kDa hGH (Genotonorm®, Pfizer, New York, USA) was administered subcutaneously to 10 volunteers, 4 other volunteers were used as controls without drug treatment (blank subjects). The volunteers were recruited applying several selection criteria that included a normal growth and development history, normal analytical values, as well as exploratory information complementary performed prior to enrolling in the trial, 20 to 30 years old and body mass index (BMI) around 22.5  $\pm$  3.5. The protocol was approved by the Ethical Committee of Clinical Research (CEIC) from Parc Salut Mar (nº 2012-003695-38, CEIC-Parc Salut Mar, Barcelona, Spain) and the volunteers were administered daily for 3 days with 0.026 mg<sup>-1</sup>kg<sup>-1</sup>day<sup>-1</sup>person<sup>-1</sup> s.c. after overnight fasting. Since the first administration of GH, blood (EDTA and serum tubes) samples were collected at different time points for 10 days (0 h, 8 h, 24 h, 48 h, 72 h, 96 h, 168 h and 216 h) and processed after blood clotting or immediately for obtaining serum and PBL.

#### Subjects

To validate the results obtained in the clinical trial, lymphocytes samples from 10 sedentary women and 10 sedentary men were collected following the selection criteria established for the volunteers of the clinical trial and stored at -80°C until RNA extraction.

All the enrolled subjects gave their written informed consent obtained from the Andalusian Public Health System Biobank located in Málaga and approved by the Ethics Committee of Clinical Research (CEIC) from IBIMA. The study was carried out in accordance with The Code of Ethics of the WMA (Declaration of Helsinki).

#### Lymphocytes

Blood samples were withdrawn from the antecubital vein. Lymphocytes were obtained from blood treated with tri-potassium

ethylenediaminetetraacetic acid (EDTA- K3). After centrifuged the samples for 10 min at 250 g, plasma was remove and lymphocytes were purified with Histopaque-1077 (Sigma-Aldrich, Spain), following the manufacturer's instructions. The pellet of lymphocytes was stored at -80°C until RNA extraction.

#### RNA isolation, cDNA synthesis and duplex-qPCR

Total RNA was extracted from samples of white blood cells using TRIzol reagent (Invitrogen, Spain), following the manufacturer's instructions. An aliquot containing 0.5  $\mu$ g of total RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen, Spain), 5  $\mu$ M random hexamers and 2 mM dideoxy nucleotides (Roche, Spain) in a total reaction volume of 20  $\mu$ L.

Relative levels of human genes TBP and COL3A1 were determined by multiplex (duplex) real-time PCR experiments using TaqMan type probes with HEX and FAM as fluorochromes, BHQ1 as quencher and ROX for background normalization. Probes and oligonucleotide primers were designed using Primer Express 2.0.0 (ABI PRISM, Applied Biosystems) and synthesized by Sigma (Spain), and the selected sequences were the following: for TBP, forward primer 5'-ccgaaacgccgaatataatcc-3', reverse primer 5'-ccgtggttcgtggctctct-3' probe 5'- [FAM] cggtttgctgcggtaatcatgaggat [BHQ1]and 3'; and for COL3A1 forward primer 5'-gtcctattggtcctcctggc- 3', reverse primer 5'- accagggaaaccagcagg -3' and probe 5'-[HEX] ctcgtggtagccctggtgagagaggt [BHQ1]-3. Triplicates of duplex real time PCR mixtures contained 5 µL of TaqMan Gene Expression Master Mix (Applied Byosistem, Spain), 0.2 µM of each primer, 0.20 µM of COL3A1 or IGF-1 probe, 0.25 µM of TBP probe, 1 µL containing cDNA resulting from retro transcription of 25 ng of total RNA and RNase- free water to a final volume of 10  $\mu$ L. The amplification program to quantify each gene was carried out in a PCR Light Cycler 480 II termocycler (Roche) in the Instituto de Investigación Biomedica de Málaga (IBIMA), as follows: 1 cycle at 50°C for 10 min.; 60 cycles with steps at 95°C for 15 s. and at 60°C for 1 min. with simultaneous detection of FAM, HEX, and ROX. Gene expression was normalized to TBP using the mathematic model 2- $\Delta\Delta$ CT described by Livak y Schmittgen [19]. The RTqPCR assay data are reported as mean ± standard deviation and were analyzed with the student's t- test. Differences were considered significant at a p-value less than 0.05.

#### PIIINP ELISA

PIIINP serum levels were measured with an enzymatic method using commercial ELISA kit based on a sandwich ELISA with paramagnetic particles purchased from Siemens and using an ADVIA Centaur CP system (Siemens Healthcare Laboratory Diagnostics, Camberley, UK). Samples were processed following the manufacturer's instructions. The ELISA assay data are reported as mean  $\pm$  SD and were analyzed with the student's t-test. Differences were considered significant at a p-value less than 0.05.

#### Assay quality performance

To determine the suitability of the new *COL3A1* detection assay over a wide range of conditions, we tested the performance of the assay across several criteria: accuracy, linearity, stability of blood samples storage at 4°C, amount of blood sample, gender and age. We designed quality performance experiments based on standardized definitions of these parameters [20]. A representative RNA sample normalized to 25 ng/ $\mu$ L using the Nano Drop 8000 instrument was used to study these parameters and establish a robustness assay.

### **Results and Discussion**

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The development of new detection and quantification methods of the *COL3A1* gene can be very useful in different areas, such as the support of the diagnosis of different pathologies, knowing the patient's status treated with drugs, which produces alterations in this gene, or even for the detection of abuse of drugs by cheater elite athletes. In this work, a quantification method of the *COL3A1* gene was developed. This method allowed assessing variations of this gene and showed a high sensitivity and robustness, as can be observed in the validation process studied.

# Quantitative expression of the COL3A1 gene in PBL after rhGH treatment

After designing the specific primers and probe for the *COL3A1* gene and establishing the TBP protein as housekeeping gen to quantify the expression of this gene in lymphocytes, samples from a rhGH clinical trial were used to test the RTqPCR assay developed in this study. Based on a previous study performed in serum samples in others clinical trials [9] *COL3A1* high levels could be possible at 72 and 96 hours. *COL3A1* and PIIINP levels were tested and compared at all times evaluated in this clinical trial. As displayed in Figure 1, changes in mRNA levels of *COL3A1* were observed. A significant increase of mRNA level was produced after 24 hours of treatment. The maximum expression level was reached at 168 h with  $3.0 \pm 1.10$ -fold increase, though high expression levels were found at all times studied. In comparison with the protein levels, high levels of PIIINP were found at all times tested, but only at 72 and 96 hours results showed significant differences (Figure 1).

#### Linearity

The linearity was tested in the *COL3A1* and TBP gene to establish the range of DNA amounts that enable accurate quantification [20,21]. Such information is valuable for determining the lowest concentration at which reliable genetic altered data can be attained, particularly when DNA is limiting. To study the linearity, a range of concentrations between 1 to 40 ng was studied. All concentrations studied showed that the range of linearity for this assay is greater and lower than the DNA amount tested for both genes with a Pearson coefficient of 0.91 and 0.93 for *COL3A1* and TBP respectively, which indicates that the assay could give reliable data for samples with a limiting DNA concentration.





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#### Assay accuracy

A good parameter to assess an analytical method is the accuracy [20,21]. In this context, to evaluate the intra and inter-assay variability, a cDNA sample of 20 ng was tested six times using the same assay for *COL3A1* and TBP genes. This cDNA sample was tested in six separate experiments. All cDNA samples yielded fairly low coefficients of variation (Table 1) for both genes, which indicates that methodology is very suitable for the detection assay studied.

#### Gender and age effect

Once the functionality of the detection method was confirmed, PBL samples from male and female untreated subjects were collected and tested to study the possible effect of age and gender.

As can be observed in Figure 2, no differences were observed as mean values of  $1 \pm 0.21$  (men, n=10) and  $0.91 \pm 0.16$  (women, n=10) fold mRNA expression were obtained for gender effect, respectively; and homogeneous unified mean values of  $0.93 \pm 0.21$  (n=20) fold mRNA expression were obtained for age effect (from 21 to 30 years, mean 25 years).

Primary study showed that gender and age in the methods developed, affects the quantification of the PIIINP protein levels [8,11-14]. However, the results obtained to test the influence of gender and age with this method suggested that none of these two variables afforded differences when 10 women and 10 men between 21 and 30 years were evaluated. Related with the age effect, more studies with a subgroup

with a larger age range should be studied to verify the non-influence of this parameter.

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#### Volume effects and stability of blood samples storage at 4°C

A limiting aspect in the clinical analysis is the volume of blood needed to perform the different molecular determinations, because a high number of parameters are evaluated in the same sample. Furthermore, the samples must be manipulated following the quality standards [22]. Therefore, it was important to know the minimum volume of blood to obtain an optimal concentration of mRNA to perform the method described in this work. RNA from different blood volumes treated with EDTA-K3 (from 0.5 to 3 mL), as displayed in Figure 3A, was extracted, and the amount of RNA obtained was proportional to the blood volume starting. The minimum blood volume evaluated was 250 µL and the RNA concentration obtained was 28 ng/µL. These results were similar to the obtained previously for PAXgene RNA tubes [23], so this method showed an RNA efficient extraction protocol. After the linearity studies conducted above Figure 4 which showed positive results with 1 ng of cDNA, the results obtained in this experiment show that 250  $\mu$ L of blood would be sufficient to perform the detection of COL3A1 levels with the proposed method, so it could be possible the determination of other molecular or biochemistry parameters in the same sample.

Finally, another parameter to be evaluated was the storage time of the blood collected at 4°C before the RNA extraction, normally in the clinical analysis laboratories samples, it is not processed immediately and takes some time before the sample processing is performed. In this case, PAXgene blood RNA tubes were used to assess the storage



Figure 2: Age and gender effect on COL3A1 gene expression. Left. PBL samples versus age. Right. PBL samples versus gender. Box plot compares median of COL3A1 gene expression and COL3A1 concentrations in women (n=10) and men (N=10). Box plot explanation: upper horizontal line of box, 75<sup>th</sup> percentile; lower horizontal bar within box, median; upper horizontal bar outside box, 90<sup>th</sup> percentile; lower horizontal bar outside box, 10<sup>th</sup> percentile.

	Cts Mean	CV (%)
ТВР		
Intra-assay	23.59	0.91
Inter assay	23.55	1.03
COL3A1		
Intra-assay	25.17	2.71
Inter assay	25.81	1.77

Table 1: Intra and inter assay accuracy.

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effect, which are special for the RNA conservation because in the manufacturer's instructions indicate that the samples can be stored at 4°C for 5 days without RNA degradation process. As displayed in Figure 3B, results obtained for the gene study showed an increase in its expression level of approximately twice when fresh samples were compared with samples stored at 4°C for at least 24 hours. These results remained elevated for 30 days at all times tested, but similar to data obtained from 24 hours, and showed the recommendation of fresh processing to obtain a maximum reliability of the proposed method.

#### Conclusion

A new *COL3A1*-Specific Real-Time PCR Assay has been developed and tested in human samples in a clinical trial after administration of rhGH. This method showed better sensitivity and detection time window in comparison with other assays developed to detect the product of this gene: PIIINP protein. A preliminary validation study was performed on this method and all parameters studied showed a good robustness, although more studies should be performed in this area before its implementation in analysis laboratories. An important aspect of this method was observed when it was tested the samples storage at 4°C, which showed that this method is more reliable in fresh samples.

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#### References

- Solomon E, Hiorns LR, Spurr N, Kurkinen M, Barlow D, et al. (1985) Chromosomal assignments of the genes coding for human types II, III, and IV collagen: a dispersed gene family. Proc Natl Acad Sci 82: 3330-3334.
- Huerre-Jeanpierre C, Henry I, Bernard M, Gallano P, Weil D, et al. (1986) The pro alpha 2(V) collagen gene (COL5A2) maps to 2q14----2q32, syntenic to the pro alpha 1 (III) collagen locus (COL3A1). Hum Genet 73: 64-67.
- 3. Van der RM, Garrone R (1991) Collagen family of proteins. FASEB J 5: 2814-2823.
- Smith LT, Schwarze U, Goldstein J, Byers PH (1997) Mutations in the COL3A1 gene result in the Ehlers-Danlos syndrome type IV and alterations in the size and distribution of the major collagen fibrils of the dermis. J Invest Dermatol 108: 241-247.
- Sakowicz BM, Kuczkowski J, Przybyła T, Grden M, Starzynska A, et al. (2017) Gene expression profile of collagen types, osteopontin in the tympanic membrane of patients with tympanosclerosis. Adv Clin Exp Med 26: 961-966.
- 6. Lee ST, Kim JA, Jang SY, Kim DK, Kim JW, et al. (2008) A novel COL3A1 gene mutation in patient with aortic dissected aneurysm and cervical artery

dissections. Heart Vessels 23: 144-148.

 Dyson JK, Anstee QM, McPherson S (2014) Non-alcoholic fatty liver disease: a practical approach to diagnosis and staging. Frontline Gastroenterol 5: 211-218.

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- Powrie JK, Bassett EE, Rosen T, Jørgensen JO, Napoli R, et al. (2007) Detection of growth hormone abuse in sport. Growth Horm IGF Res 17: 220-226.
- Bosch J, Such-Sanmartín G, Segura J, Gutiérrez-Gallego R (2012) Tracking growth hormone abuse in sport: performance of marker proteins in a controlled setting. Anal Chim Acta 745: 118-123.
- Holt RI (2013) Detecting growth hormone misuse in athletes. Indian J Endocrinol Metab 17: S18-S22.
- Mesotten D, Van den Berghe G (2006) Changes within the GH/IGF-I/IGFBP axis in critical illness. Crit Care Clin 22: 17-28.
- Niemela O (1994) Collagen breakdown products as markers of fibrosis and cirrhosis. Alcohol Suppl 2: 345-352.
- Saggese G, Baroncelli GI, Federico G, Bertelloni S (1995) Effects of growth hormone on phosphocalcium homeostasis and bone metabolism. Horm Res 44 Suppl 3: 55-63.
- 14. Kurdy NM (2000) Serology of abnormal fracture healing: the role of PIIINP, PICP, and BsALP. J Orthop Trauma 14: 48-53.
- Stoffel K, Engler H, Kuster M, Riesen W (2007) Changes in biochemical markers after lower limb fractures. Clin Chem 53: 131-134.
- Belangero PS, Leal MF, Figueiredo EA, Cohen C, Pochini Ade C, et al. (2014) Gene expression analysis in patients with traumatic anterior shoulder instability suggests deregulation of collagen genes. J Orthop Res 32: 1311-1316.
- 17. Klatte SF, Schmidt T, Uckert M, Scheffler S, Kalus U, et al. (2018) Comparative Analysis of Different Platelet Lysates and Platelet Rich Preparations to Stimulate Tendon Cell Biology: An In Vitro Study. Int J Mol Sci 19: E212.
- Ferro P, Ventura R, Perez-Mana C, Farre M, Segura J (2016) Genetic and protein biomarkers in blood for the improved detection of GH abuse. J Pharm Biomed Anal 128: 111-118.
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.
- Price EP, Dale JL, Cook JM, Sarovich DS, Seymour ML (2012) Development and validation of Burkholderia pseudomallei-specific real-time PCR assays for clinical, environmental or forensic detection applications. PLoS ONE 7: e37723.
- Araujo P (2009) Key aspects of analytical method validation and linearity evaluation. J Chromatogr B Analyt Technol Biomed Life Sci 877: 2224-2234.
- Ezzelle J, Rodriguez-Chavez IR, Darden JM, Stirewalt M, Kunwar N, et al. (2007) Guidelines on Good Clinical Laboratory Practice. Bridging Operations between Research and Clinical Research Laboratories. J Pharm Biomed Anal 46: 18-29.
- Krawiec JA, Chen H, Alom-RS, Jaye M (2009) Modified PAXgeneTM method allows for isolation of high-integrity total RNA from microlitre volumes of mouse whole blood. Lab Anim 43: 394 -398.